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Process development for hospital wastewater treatment by Trametes versicolor

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Process development for hospital wastewater treatment by *Trametes versicolor*

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CERTIFIQUEM:

Que el llicenciat en Biotecnologia Josep Anton Mir Tutusaus ha realitzat sota la nostra direcció, en els laboratoris del Departament d'Enginyeria Química, Biològica i Ambiental el treball que amb el títol ***Process development for hospital wastewater treatment by* Trametes versicolor**, es presenta en aquesta memòria, la qual constitueix la seva Tesi per optar al Grau de Doctor per la Universitat Autònoma de Barcelona.

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Bellaterra, setembre de 2017

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Abstract

Micropollutants are a wide group of organic compounds that are detected at most compartments of the environment. Their environmental concentration is usually in the range of few $ng \cdot L^{-1}$ to $\mu g \cdot L^{-1}$, but remain biologically active even at such low concentrations, may be accumulated through the food chain and pose a threat to the environment, fauna and human health. Among micropollutants, pharmaceutical active compounds (PhACs) are of special concern. It is accepted that the main sources of PhACs to the environment are effluents from wastewater treatment plants (WWTPs), where conventional activated sludge processes are not able to degrade most of them. Answering to these concerns, the scientific community has devoted extensive research into mechanisms to degrade, transform and /or remove micropollutants from hospital wastewater, where PhACs are present at higher concentrations. Among the possible treatments, white-rot fungi (WRF) are regarded as a cost-effective possibility due to their relatively low cost in comparison with physical and chemical treatments and their capacity to transform most of the compounds thanks to their versatile enzymatic machinery. WRF have been studied for the removal of pharmaceuticals in wastewater, but contamination by wastewater-native microorganisms has produced very short-term reactor operations.

The present thesis tackles this problem and proposes several strategies to lengthen the fungal treatment. It also serves as proof of concept of a long-term white-rot fungal operation treating non-sterile real hospital wastewater.

First of all, several air-pulsed fluidized bed bioreactors were set up in order to study the effect of the addition of a coagulation-flocculation pretreatment, of the addition of a UV pretreatment and the effect of operating the reactors as a sequencing batch reactor (SBR) or in a continuous fashion on the length of operation. The chosen alternative was a continuous reactor with a coagulation-flocculation pretreatment. This treatment train was then evaluated in a non-spiked hospital wastewater treatment with partial biomass restoration, leading to a two-month operation.

Additionally, several process variables, namely, pellet size, aeration and

carbon-to-nitrogen ratio were studied and the values that led to a longer operation were selected. Those previous studies enabled for the first time a long-term operation of a fungal fluidized bed bioreactor treating real non-sterile wastewater.

The importance of conjugation and deconjugation processes is highlighted in this thesis. Conjugated microcontaminants are not usually detected by the current analytical techniques, thus undervaluing the concentration of the pollutant studied. Therefore, an effort should be made to analyze conjugated forms of compounds. If successful, it could be a breakthrough that greatly facilitates the study of removal of micropollutants in real wastewater.

Molecular biology analyses such as denaturing gradient gel electrophoresis (DGGE), DNA sequencing and real-time PCR (qPCR) were performed in the non-spiked experiments to give insight on the microbiological communities arisen during the reactor treatment and to confirm the presence of *T. versicolor* throughout the operation. Results suggested that the fungus was active even when no laccase activity was detected.

Resum

Els microcontaminants són un ampli grup de compostos orgànics que s'han detectat a la majoria de compartiments del medi ambient. La seva concentració ambiental està compresa entre pocs $ng \cdot L^{-1}$ fins a $\mu g \cdot L^{-1}$, però es mantenen biològicament actius fins i tot a concentracions tan baixes, poden ser acumulats a través de la cadena tròfica i suposen una amenaça per al medi ambient, la fauna i la salut humana. Entre els microcontaminants, els fàrmacs (PhACs) generen una especial preocupació. És acceptat que la principal font d'entrada de fàrmacs al medi ambient és via efluents de les estacions depuradores d'aigües residuals (WWTP), on els mecanismes convencionals de llots activats no són suficients per degradar-ne la majoria. En resposta a aquestes preocupacions, la comunitat científica ha destinat molta recerca a mètodes per a la degradació, transformació i/o eliminació de microcontaminants d'aigües residuals d'hospital, on els fàrmacs estan presents a major concentració. D'entre els tractaments possibles, els fongs de podridura blanca (WRF) es presenten com una possibilitat atractiva gràcies al seu baix cost en comparació amb tractaments físics i químics i la seva capacitat de transformar la majoria de compostos gràcies a la seva versàtil maquinària enzimàtica. Els WRF han estat estudiats per a la degradació de fàrmacs en aigües residuals, però la contaminació per microorganismes presents a l'aigua residual ha produït que les operacions en reactor fossin molt curtes.

Aquesta tesi aborda aquest problema i proposa diverses estratègies per allargar el tractament. També serveix com a prova que una operació prolongada amb WRF tractant aigua residual d'hospital no estèril és possible.

Primer de tot, diversos reactors de llit fluïditzat per polsos d'aire es van operar per estudiar l'efecte que tenien l'addició d'un pretractament de coagulació-floculació, l'addició d'un pretractament amb llum UV i l'efecte de l'operació com a batch seqüencial (SBR) i en continu en la llargada del tractament. La millor alternativa va ser un reactor en continu amb un pretractament de coagulació-floculació. Aquest tren de tractament va ser evaluat en un tractament d'aigua residual d'hospital no dopada amb renovació parcial de la biomassa, i va permetre una operació de dos mesos

de durada.

A més, diverses variables de procés, a saber, mida del pèl·let, aeració i la ràtio carboni-nitrogen es van estudiar i els valors que van suposar una operació més llarga van ser seleccionats. Aquests estudis previs van permetre per primera vegada una operació prolongada d'un reactor fúngic de llit fluïditzat tractant aigua residual d'hospital no estèril.

Aquesta tesi també remarca la importància dels processos de conjugació i desconjugació. Les tècniques analítiques actuals no solen detectar els microcontaminants conjugats, i això impedeix una precisa mesura de la concentració del contaminant estudiat. Per tant, s'haurien de destinar esforços a l'anàlisi de les formes conjugades de compostos. Si s'aconsegueix, podria significar un gran avenç que faciliti l'estudi de l'eliminació de microcontaminants en aigües reals.

Les anàlisis de biologia molecular com gel d'electroforesi en gradient desnaturalitzant (DGGE), seqüenciació d'ADN i PCR quantitativa (qPCR) es van aplicar els experiments no dopats per donar informació sobre les comunitats microbiològiques formades durant els tractaments en reactor i per confirmar la presència de *T. versicolor* durant l'operació. Els resultats suggereixen que el fong es mantenia actiu fins i tot quan l'activitat de l'enzim lacasa no es detectava.

Resumen

Los microcontaminantes son un amplio grupo de compuestos orgánicos que se han detectado a la mayoría de compartimentes del medio ambiente. Su concentración ambiental está entre pocos $ng \cdot L^{-1}$ hasta $\mu g \cdot L^{-1}$, pero se mantienen biológicamente activos incluso a concentraciones muy bajas, pueden ser acumulados a través de la red trófica y suponen una amenaza para el medio ambiente, la fauna y la salud humana. De entre los microcontaminantes, los fármacos (PhACs) generan una especial preocupación. Es aceptado que la principal fuente de entrada de fármacos al medio ambiente es vía efluentes de las estaciones depuradoras de aguas residuales (WWTP), dónde los mecanismos convencionales de lodos activados no son suficientes para degradarlos. En respuesta a estas preocupaciones, la comunidad científica ha destinado mucha investigación a métodos para la degradación, transformación y/o eliminación de microcontaminantes de aguas residuales de hospital, dónde los fármacos están en mayor concentración. De entre los tratamientos posibles, los hongos de podredumbre blanca (WRF) se presentan como una posibilidad atractiva gracias a su bajo coste en comparación con tratamientos físicos y químicos y a su capacidad de transformar la mayoría de compuestos gracias a su versátil maquinaria enzimática. Los WRF han sido estudiados para la degradación de fármacos en aguas residuales, pero la contaminación debida a microorganismos presentes en las aguas residuales ha producido que las operaciones en reactor fueran muy cortas.

Esta tesis aborda este problema y propone varias estrategias para alargar el tratamiento. También sirve como prueba que una operación prolongada con WRF tratando aguas residuales no estériles de hospital es posible.

Primeramente, varios reactores de lecho fluidizado por pulsos de aire se operaron para estudiar el efecto que tenían la adición de un pretratamiento de coagulación-floculación, la adición de un pretratamiento con luz UV y el efecto de la operación como batch secuencial (SBR) y en continuo en la duración del tratamiento. La mejor alternativa fue un reactor en continuo con un pretratamiento de coagulación-floculación. Este tren de tratamiento fue evaluado en un tratamiento de agua residual de hospital no dopada con

renovación parcial de biomasa, y permitió una operación de dos meses de duración.

Además, varias variables de proceso, a saber, tamaño de pellet, aeración y la ratio carbono-nitrógeno se estudiaron y los valores que dieron una operación más larga fueron seleccionados. Estos estudios previos permitieron por primera vez una operación prolongada de un reactor fúngico de lecho fluidizado tratando agua de residual no estéril de hospital.

Esta tesis también remarca la importancia de los procesos de conjugación y desconjugación. Las técnicas analíticas actuales no suelen detectar los microcontaminantes conjugados, y ello impide una precisa medida de la concentración del contaminante estudiado. Por lo tanto, se deberían destinar esfuerzos en el análisis de las formas conjugadas de los compuestos. Si se consigue, ello significaría un avance que facilite el estudio de la eliminación de microcontaminantes en aguas reales.

Los análisis de biología molecular como gel de electroforesis en gradiente desnaturalizante (DGGE), secuenciación de ADN y PCR cuantitativa (qPCR) se aplicaron en los experimentos no dopados para dar información sobre las comunidades microbiológicas desarrolladas durante los tratamientos en reactor y para confirmar la presencia de T. versicolor durante la operación. Los resultados sugieren que el hongo se mantuvo activo incluso cuando la actividad de la enzima lacasa no fue detectada.

List of abbreviations

ABT	1-aminobenzotriazole
ABTS	2,2-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt
AOP	Advanced oxidation processes
AS	Activated sludge
BLD	Below limit of detection
BLT	Bacterial luminescence toxicity
BLQ	Below limit of quantification
BPA	Bisphenol A
CAS	Conventional activated sludge
CBZ	Carbamazepine
CBZE	epoxycarbamazepine
COD	Chemical oxygen demand
CRT	Cellular residence time
DC	Dissolved carbon
DCF	Diclofenac
DCF	Diclofenac
DCW	Dry cell weight
DEET	N,N-Diethyl-meta-toluamide
DGGE	Denaturing gradient gel electrophoresis
DIC	Disolved inorganic carbon
DM	Defined medium
DNA	Deoxyribonucleic acid
DOC	Disolved organic carbon
DW	Dry weight
E1	Estrone
E2	17β-estradiol
E3	Estriol
EE2	17α-ethynyl-estradiol (EE2)
EC_{50}	Half maximal effective concentration
EDC	Endocrine disrupting compounds
FBR	Fluidized bed reactor
HOBT	1-hydroxy-benzotriazole
HPLC	High performance liquid chromatograpy

HRT	Hydraulic residence time
HWW	Hospital wastewater
IBP	Ibuprofen
ITS	Internal transcribed spacer
KTP	Ketoprofen
LiP	Lignin peroxidase
LME	Lignin modifying enzyme
LOD	Limit of detection
LOQ	Limit of quantification
MBR	Membrane bioreactor
MEB	Malt extract broth
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids
MnP	Manganese peroxidase
ND	Non-detected
NDV	N-desmethylvenlafaxine
NP	Naproxen
NSAID	Non-steroidal anti-inflammatory drug
OD	Optical density
ODV	O-desmethylvenlafaxine
OXB	Oxybenzone
PAH	Polycyclic aromatic hydrocarbons
PCP	Personal care products
PCR	Polymerase Chain Reaction
PhAC	Pharmaceutically active compound
PPCP	Pharmaceuticals and personal care products
qPCR	Real time PCR or quantitative PCR
rDNA	Ribosomal DNA
SBR	Sequencing batch reactor
SD	Standard deviation
SMX	Sulfamethoxazole
SPE	Solid phase extraction
SRT	Solids retention time
TC	Total carbon
TIC	Total inorganic carbon
TN	Total nitrogen

TOC	Total organic carbon
TP	Transformation product
TSS	Total suspended solids
TU	Toxicity units
UV	Ultraviolet
VA	Violuric acid
VEN	Venlafaxine
VP	Versatile peroxidase
WRF	White-rot fungi
WW	Wastewater
WWTP	Wastewater treatment plant

Contents

1

General introduction

Continuous wastewater treatment for organic micropollutants removal: background, bioremediation capabilities and limitations of white-rot fungal based systems

Abstract

1.1 Overview

Micropollutants can be defined as substances whose bioaccumulative, persistent and toxic properties may have a negative effect on the environment and/or human health, even at trace concentrations. This diverse group contains, but is not restricted to: pharmaceutically active compounds (PhACs), personal care products, endocrine disruptors, pesticides and industrial chemicals. Several authors have referred to them also as emerging contaminants, preferably termed contaminants of emerging concern (Sauvé and Desrosiers, 2014). Most of them are unregulated pollutants, although future regulation might be written depending on research (Verlicchi et al., 2010). These contaminants remain biologically active even at concentrations of few ng·L-1, may be accumulated through the food chain and can have negative effects on the environment, fauna and human health. The World Health Organization (2016), for example, raised concern on the development of antibiotic resistance on target bacteria due to exposure to non-lethal concentrations of antibiotics.

The origin of these pollutants is diverse: from industrial waste streams to human-excreted metabolized and non-metabolized medicaments. Typically such compounds enter the environment through municipal or industrial effluent, but they are not completely removed in wastewater treatment plants (WWTPs), which are mainly

designed for removing organic matter and suspended solids (Evgenidou et al., 2015; Frédéric and Yves, 2014; Kaiser et al., 2014). In fact, micropollutants have been found in surface water, groundwater, drinking water and sewage (Dai et al., 2015).

Answering to these concerns, the scientific community has devoted extensive research into mechanisms to degrade, transform and /or remove micropollutants from wastewater. Among the possible treatments, white-rot fungi (WRF) are regarded as a cost-effective possibility due to their relatively low cost in comparison with physical and chemical treatments and their capacity to transform most of the compounds studied so far thanks to their versatile enzymatic machinery.

This chapter reviews the bioremediation capabilities of WRF and the success examples of application with different types of micropollutants, primarily focusing on continuous treatments. Some drawbacks of the technology, largely related to the non-sterility of wastewater, are analyzed and solutions discussed.

1.2 Bioremediation capabilities of white-rot fungi

1.2.1 White-rot fungi and their enzymatic machinery

The term white-rot fungi is not a taxonomical grouping but rather a collection of fungal species that are able to degrade lignin, which is a heterogeneous polyphenolic polymer that forms a structural part of higher plants –e.g. trees. The name white-rot refers to the bleached aspect of the lignocellulosic substrate –e.g. wood– after being attacked by such fungi: the lignin is the major contributor to the color of bark. WRF are mainly basidiomycetes and some relevant species include *Pleurotus ostreatus, Phanerochaete chrysosporium, Trametes versicolor, Ganoderma lucidum* and *Irpex lacteus*.

Lignin is very recalcitrant to biodegradation and white-rot fungi are the main group of organisms that take advantage of this ecological niche (Dashtban et al., 2010). The lignin composition is highly variable and links both hemicellulose and cellulose, creating a barrier that prevents penetration of enzymes into the lignocellulosic structure. In the environment, WRF efficiently break down lignin to release the more easily metabolized carbohydrates hemicellulose and cellulose –oxidation of lignin yields no net energy gain (Leonowicz et al., 1999).To do so, they rely on a combination of extracellular ligninolytic enzymes, organic acids, mediators and accessory enzymes. A bold feature of this enzymatic machinery is its non-specificity, due to its action via the generation of radicals. This property makes the extracellular white-rot fungal enzymes capable of transforming a wide range of organic molecules, including micropollutants.

White-rot fungi secrete lignin modifying enzymes (LMEs) and other compounds for lignin degradation. LMEs include laccase, lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP). The main difference between laccases and peroxidases is that the former uses molecular oxygen whilst the others use hydrogen peroxide (H_2O_2) as electron acceptor.

Laccases (EC 1.10.3.2) are copper-containing enzymes that oxidize a variety of substrates through a radical-catalyzed mechanism. Laccases can be found in fungi, a number of plants and several bacterial species. They have low substrate specificity and are part of the family of multicopper oxidases. Briefly, the mechanism relies on the four-electron reduction of molecular oxygen to water, resulting in the oxidation of a range of substrates, typically phenols and amines (Jones and Solomon, 2015). Laccases have been largely studied for biotechnological applications such as industrial catalysts, detoxification of paper and pulp, textile and petrochemical effluents and bioremediation (Rodríguez Couto et al., 2006). Lignin peroxidase (EC 1.11.1.14) is a fungal hemoprotein similar to the plant peroxidases that synthesize lignin. LiP oxidizes veratryl alcohol to radical cations that mediate in the oxidation of lignin (Harvey et al., 1992). Similarly to laccase, LiP was able to degrade recalcitrant aromatic compounds such as polycyclic aromatic and phenolic compounds (Reddy, 1995). Manganese peroxidase (EC 1.11.1.13) is the most common lignin-modifying enzyme of white-rot and litter-decomposing fungi (Järvinen et al., 2012). MnP is a glycosylated heme enzyme that oxidizes the Mn^{2+}in a highly specific binding site to Mn^{3+}, a highly reactive species (Hofrichter, 2002). The manganese (III) ion is then chelated by carboxylic acids, which then act as redox-mediators. MnP has also been studied for biotechnological applications including pulp bleaching, dye decolorization and was also reported to degrade humic substances (Hofrichter et al., 1997), polycyclic aromatic hydrocarbons (PAH) (Bogan and Lamar, 1996) and chlorophenols (Hofrichter et al., 1998).Versatile peroxidase (EC 1.11.1.16) is also a heme peroxidase present in some species of WRF. Similarly to MnP, VP has a Mn^{2+} binding site, but additionally, it has other multiple active sites and exhibits a broad range of enzymatic activities. Therefore, it can oxidize substrates in Mn^{2+}-sufficient or Mn^{2+}-deficient media (Camarero et al., 1999; Ruiz-Duenas et al., 1999).

All species of WRF don't necessarily produce all LMEs. In fact, production and secretion of these enzymes differ greatly between species. Some authors proposed a classification of WRF in terms of LME secretion (Hatakka, 1994), but even if one species could theoretically produce a particular subset of LMEs, a particular strain of that species might not. In addition, composition of the growth medium and culture conditions highly condition the production of ligninolytic enzymes (Nerud and

Misurcova, 1996). For example, *Trametes* sp. has been shown to produce LiP, MnP and laccase (Poojary et al., 2012), but the strain *T. versicolor* ATCC42530 produced only laccase at Font et al. (2003) growth conditions.

In addition to LMEs, WRF can also produce and secrete redox mediators that act as vehicles for electron transfer and further expand the range of substrate for the ligninolytic enzymes (Cañas and Camarero, 2010). Moreover, low molecular-weight LME substrates, or even by-products of LME catalysis may result in such mediators (Marco-Urrea et al., 2010c; Morozova et al., 2007; Pointing, 2001). Some well described synthetic mediators include ABTS [2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)] and HBT (1-hydroxybenzotriazole); some less studied, natural laccase mediators comprise phenol, aniline, 4-hydroxybenzoic acid, and 4-hydroxybenzyl alcohol (Johannes and Majcherczyk, 2000). In spite of the extraordinary extracellular enzymatic system of WRF, it is not the only responsible of microcontaminant degradation.

P450s are a superfamily of intracellular heme-containing monooxygenases ubiquitous in all biological kingdoms. In fungi, they play a role in housekeeping biochemical reactions, detoxification of xenobiotics and adaptation to hostile ecological niches (Durairaj et al., 2016). P450 is an important part of fungal metabolism: this enzymatic system comprises as much as 150 genes in *P. chrysosporium*, most of them with unknown function, that express distinctively in response to different xenobiotics and carbon sources (Yadav et al., 2006). The involvement of P450 in degradation of several micropollutants has been largely described: TNT (Spiker et al., 1992), PAH (Yadav and Reddy, 1993), the dye malachite green (Cha et al., 2001), the organochlorine compounds PCDDs and DDT (Kamei and Kondo, 2005; Xiao et al., 2011), carbamazepine and clofibric acid (Marco-Urrea et al., 2009), ketoprofen (Marco-Urrea et al., 2010b), the UV filter 4-MBC (Badia-Fabregat et al., 2012) and the agrochemicals imiprothrin, oxytetracycline and carbofuran (Mir-Tutusaus et al., 2014).

Fungal P450 shares some similarities with its mammalian and human counterparts. In fact, human liver cytochrome P450 is involved in the metabolism and detoxification of xenobiotics, as is the fungal P450 (Michaels and Wang, 2014). These similarities include the capacity of forming glucuronides and conjugates in general (Bezalel et al., 1996). In humans, conjugation increases water solubility of xenobiotics so they can be excreted via urine (Dalgaard and Larsen, 1999; Lynn et al., 1978). WRF, however, have been consistently reported to reverse such modifications and deconjugate human conjugates (Badia-Fabregat et al., 2015a; Mir-Tutusaus et al., 2017a).

1.2.2 Advantages over bacterial treatment

The fungal enzymatic systems are an important capability that supports WRF's suitability for bioremediation of micropollutants from wastewater, but it is not the only one.

Micropollutants are typically found in wastewater streams at trace concentrations. This fact poses a difficulty for bacterial degradation as bacteria typically use the contaminants as growth substrates. If the pollutant is present at a low concentration, the bacterial species that is supposedly able to degrade it will not be able to colonize the matrix (Harms et al., 2011). Degradation of organic pollutants in white-rot fungi, on the other hand, is part of the secondary metabolism. In other words, fungi need a carbon source other than the contaminant to grow, meaning that WRF transform micropollutants co-metabolically (Wen et al., 2011). This does not mean that WRF cannot metabolize the micropollutant: *T. versicolor* could metabolize, mineralize, and incorporate into amino acids some micropollutants such as diclofenac and benzophenone-3 (Marco-Urrea et al., 2010c,b; Badia-Fabregat et al., 2014). However, the concentration of micropollutants is insufficient to maintain fungal growth and a secondary carbon source is therefore needed. This feature enables WRF to attack the micropollutants present in the wastewater even at minute concentrations.

Municipal and municipal-like wastewater commonly contains a mixture of a wide range of trace organic pollutants: from caffeine and insect repellents such as DEET to sunscreens, preservatives, antibiotics, hormones and other pharmaceutically active compounds (Wang et al., 2014; Yang et al., 2017). It is noteworthy that although they are found at trace concentrations, they retain high biological activities. Bacteria are usually less versatile when treating combinations of pollutants: a specific bacterial species can be a good degrader of a single or a small subset of similar micropollutants, but bacteria in general have difficulties when removing mixtures of contaminants. Conventional activated sludge, for instance, does no degrade most of pharmaceuticals and personal care products in municipal wastewater (Verlicchi et al., 2012).White-rot fungi's non-specific enzymatic machinery, on the other hand, is especially well suited for coping with this scenario, as their ability to degrade mixtures of several contaminants has been widely demonstrated (Mir-Tutusaus et al., 2014; Shreve et al., 2016; Valentín et al., 2007).

Waste effluents with concentrated pollutants pose a problem for conventional wastewater treatment processes: pulp and paper bleach industry effluent contains chlorinated and phenolic compounds; olive oil mill effluent is acidic and contains toxic phenols; textile and dyestuff industry effluents contain structurally distinct dyes; pharmaceutical industry effluent might contain residues of the active compound

produced (Harms et al., 2011). White-rot fungal processes, on the other hand, have been reported to survive these conditions and degrade the pollutants in such waste streams (Nogueira et al., 2015; Ntougias et al., 2015; Van Driessel and Christov, 2001; Zhuo et al., 2011). Therefore, white-rot fungal based treatments are regarded as a good option for on-site treatment of these wastewaters.

1.3 White-rot fungi and continuous wastewater treatment for micropollutants removal

In this section several continuous fungal operations treating a variety of micropollutants are reviewed (Table 1.1). Special interest is invested in works carried out in non-sterile conditions.

1.3.1 Pharmaceutically active compounds

Pharmaceutically active compounds, or PhACs, are molecules that enter the environment and remain active, either as unmetabolized parent compounds or as pharmaceutically active metabolites (also referred as transformation products, or TPs). Drugs are administered to humans or animals and reach the environment via excretory systems in an unmodified, partially metabolized or completely metabolized state (Ebele et al., 2017). These molecules can promote drug tolerance or resistance to the original target organisms (e.g. antibiotic resistance in bacteria, or analgesic tolerance in humans) and unwanted effects in non-target organisms (e.g. alteration of sex ratio and decreased fertility) (Annamalai and Namasivayam, 2015; Jorgensen and Halling-Sorensen, 2000) even at a very low concentration. The intended biological activity allowed scientists to categorize several compounds into families: analgesics and anti-inflammatories, antibiotics, psychiatric drugs, beta-blockers or lipid regulators, among many others. In this section continuous PhAC removal is reviewed, opening with sterile and defined matrices and moving on to non-sterile and complex matrices such as wastewater.

Although several fungal species have been found to have PhAC degradation capabilities and showed promising results (Castellet-Rovira et al., 2017), continuous bioreactor treatments focused mainly on *Trametes versicolor* and *Phanerochaete chrysosporium*. *P. chrysosporium* was investigated in several operation modes and reactor configurations for the continuous removal of analgesics and anti-inflammatories diclofenac (DCF), ibuprofen (IBP) and naproxen (NPX), and psychiatric drugs carbamazepine (CBZ) and diazepam in sterile defined media. Nearly complete removal

of DCF, IBP and NPX was achieved when biomass was auto-immobilized in the form of pellets and stirred tanks were used with an HRT of 1 d (Rodarte-Morales et al., 2011; Rodarte-morales et al., 2012). The fungus was not able to remove diazepam and an unstable CBZ removal of 0-63% was achieved when spiking at 0.5 $mg{\cdot}L^{-1}$. Similar results were achieved when operating a fixed bed reactor, even in a 100-day long operation: complete removal of analgesics and anti-inflammatories and limited and unstable removal of diazepam (0-30%) and CBZ (0-40%) (Rodarte-Morales et al., 2012). These series of studies exemplified a general trend in fungal PhAC degradation: analgesics and anti-inflammatories are usually well removed whilst the psychiatric drugs family is more recalcitrant. The possibility of CBZ and the sulfonamide antibiotics sulfamethazine (SMT), sulfathiazole (STZ) and sulfapyridine (SPY) removal by *T. versicolor* pellets was investigated in a sterile fluidized bed bioreactor treating defined media. Jelic et al. (2012) and Rodríguez-Rodríguez et al. (2012) obtained a 54% removal of CBZ when spiking with 200 $\mu g{\cdot}L^{-1}$ and >94% removal of the sulfonamides spiked at 5 $mg{\cdot}L^{-1}$.

Some studies used non-sterile defined media, sometimes referred as non-sterile synthetic wastewater, as an approach to real application. Nguyen et al. (2013) and Yang et al. (2013a) used this approach to study the behavior of a membrane bioreactor (MBR) inoculated with *T. versicolor* lumps with an HRT of 2 d. Again, analgesics and anti-inflammatories were highly removed (salicylic acid, ketoprofen, ibuprofen, naproxen), with the exception of diclofenac, with an unstable removal of 0-60%. CBZ and the antibiotic metronidazole were poorly removed at 21 and 38% removal, respectively. Psychiatric drugs amitriptyline and primidone were also well removed. Long-term operations of 165 d and 160 d were achieved by Li et al. (2015; 2016) using immobilized *P. chrysosporium* in a countercurrent seepage bioreactor and a rotating suspension cartridge reactor treating NPX and CBZ spiked non-sterile defined media. The operations removed up to 70-90% of carbamazepine, value not achieved in any other study reviewed. A similar non-sterile media was compared with the use of non-sterile spiked municipal wastewater in a plate bioreactor described in Zhang and Geißen (2012). Immobilized *P. chrysosporium* removed in that operation an 80 and 60% of CBZ in the defined media and wastewater, respectively.

In order to shed light on the effect of sterility, Gros et al. (2014) operated the same 10 L fluidized bed reactor with sterile and non-sterile hospital wastewater (two wastewaters were collected on different days) inoculated with *T. versicolor*. The X-ray contrast agent iopromide and the antibiotic ofloxacin were removed up to 87 and 98.5%, respectively, in the sterile reactor and 65.4 and 99%, respectively, in the non-sterile reactor. Further approaching real-life application, several studies were carried out using real wastewater.

Badia-Fabregat et al. (2015b) operated a fluidized bed reactor with *T. versicolor* pellets treating non-spiked, non-sterile veterinary hospital wastewater. Some compounds in the analgesics and anti-inflammatory family were well removed, but some exhibited an increase in their concentration (ketoprofen, piroxicam, diclofenac, indomethacine). An impressive 83% removal was obtained for diazepam and complete removal of ranitidine, clopidrogel and the antibiotic ciprofloxacin were achieved, but other pharmaceuticals were poorly removed. In a hospital wastewater spiked with ketoprofen and ibuprofen, 80 and 100% removal values were achieved using a similar fungal system (Mir-Tutusaus et al., 2016). Comparing both studies, it is interesting to note that ketoprofen was well removed in the spiked matrix, but its concentration rose when the matrix was not spiked. This is related to conjugation/deconjugation processes that hamper PhAC analysis and is briefly discussed in Section 1.5. A similar non-spiked study used non-sterile flocculated hospital wastewater to feed a similar fluidized bed bioreactor inoculated with *T. versicolor* pellets (Mir-Tutusaus et al., 2017a). The reactor was operated for 56 d and nearly complete removal was achieved for the analgesics and anti-inflammatories family with the exception of ketoprofen, whose concentration rose, which was in accordance to Badia-Fabregat et al. (2015b). Around 60% of antibiotics were removed and psychiatric drugs were well removed overall.

1.3.2 Endocrine disruptors

Among the various types of micropollutants, endocrine disruptors are receiving increasing attention as they are widespread and can pose serious risks to the environment and public health, even at low concentrations (Auriol et al., 2006). Indeed, these chemicals interfere with the hormone systems and produce adverse developmental, reproductive, neurological, and immunological effects in mammals. These compounds can be found in many products including plastic bottles, metal food cans, detergents, flame retardants, food, toys, cosmetics, and pesticides (Yang et al., 2017). Previous studies have confirmed significant removal of various trace organic contaminants, including endocrine disruptor compounds (EDCs), by white-rot fungal cultures under sterile batch test conditions. However, little is known about EDCs removal in continuous flow fungal reactors.

Estrogen compounds including natural ones, estrone (E1), 17β-estradiol (E2), estriol (E3), and synthetic 17α-ethinylestradiol (EE2) are commonly detected in sewage effluents and considered to be significant contributors to the estrogenic activity of wastewaters due to their high endocrine disruptor activity even at extremely low

concentrations (Cabana et al., 2007; Shreve et al., 2016). Removal of these compounds in continuous mode using white-rot-fungi has been reported by some authors. Blánquez and Guieysse (2008) explored the potential of the white-rot fungi *Trametes versicolor* to biodegrade 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) in a fluidized bed bioreactor operated during 26 days at a hydraulic retention time of 120 h. The results showed that E2 and EE2 were completely removed at volumetric removal rates of 0.16 and 0.09 $mg{\cdot}l^{-1}{\cdot}h^{-1}$, respectively, when fed at 18.8 and 7.3 $mg{\cdot}l^{-1}$, respectively. Shreve et al. (2016) explored the potential of the same fungus *T. versicolor* using the strain NRRL 66313 to remove continuously E1, E2 and EE2 from a mixture of nine trace organic contaminants with 350 $\mu g{\cdot}L^{-1}$ concentration each and during 8 days. The results showed that *T. versicolor* was able to decrease the estrogenic activity of the mixture and especially of the target contaminants (more than 71%) with the following trend E2 > E1 > EE2. Nguyen et al. (2013) studied the continuous removal of 30 trace organic contaminants, E1, E2 EE2, E3 and 17-b-estradiol-17-acetate among them in a fungus-augmented bioreactor. The reactor contained the white-rot fungus *T. versicolor* and activated sludge and was operated for 110 d. It was fed continuously with synthetic wastewater spiked with the selected contaminants each with a concentration of approximately 5 $\mu{\cdot}l^{-1}$. Data from this study highlighted the high removal of these compounds (> 90%) by the fungus-augmented bioreactor. The degradation of the same endocrine disrupting compounds, except 17-b-estradiol-17-acetate, was also recently investigated by Kresinová et al. (2017) using *Pleurotus ostreatus* HK 35. The strain was, first, tested in a laboratory-scale continuous-flow reactor and then in a scaled-up bioreactor under non-sterile conditions. Results revealed that the EDCs degradation in the trickle-bed bioreactor containing the mixed culture of the fungus and wastewater-autochthonous bacteria was very efficient in both cases. In the same work, the authors investigated also the bioreactor inoculated with the same strain as a tertiary treatment step to remove EDCs, among them E1 and EE2, from effluent of secondary treatment. Results also showed the potential of *P. ostreatus* HK 35 to remove these compounds and that 100 and 71% of E1 and EE2 were removed, respectively, within 24 hours.

Phenolic compounds, mainly, bisphenol A (2,2-bis (4-hydroxyphenol) propane), nonylphenol (4-nonylphenol), and triclosan (5-chloro-2(2,4-dichloro-phenoxy)phenol) are xenobiotic compounds frequently detected in receiving waters downstream of areas of intense urbanization (Boyd et al., 2003; Kolpin et al., 2002). These chemicals are classified as endocrine disruptors since they can mimic or interfere with the hormonal system of different organisms (Cabana et al., 2009; Naylor, 1995). Although they have lower estrogenic activity than natural or synthetic estrogens, their elevated

concentration in wastewater draw attention to these EDCs. Bisphenol A is used as raw material for the production of polycarbonates and epoxy resins; nonylphenol mainly originates from the degradation of nonylphenol polyethoxylates, a widely used industrial surfactant and triclosan is widely used in soaps, mouthwashes, toothpastes and other products in household personal care and hospital applications. The application of white-rot fungi in continuous mode for the treatment of these phenolic compounds has been scarcely described. Continuous removal of bisphenol A was studied by Yang et al. (2013a) in a membrane bioreactor (MBR) inoculated with *T. versicolor* and operated in non-sterile conditions for three months. Results showed that the performance of the fungal MBR was dependent on trace organic contaminants loading. Indeed, 80 to 90% were removed at an HRT of two days and bisphenol A loading of 475 $mg{\cdot}L^{-1}d^{-1}$. Continuous removal of bisphenol A was also reported in other studies and reached 75% in a fungus-augmented bioreactor operated during 110 d (Nguyen et al., 2013) and 61.9 % in the conditions of the study described previously by Shreve et al. (2016).

Regarding the antibacterial agent triclosan, it has been reported to be well removed (>95%) in continuous mode using *T. versicolor* at an initial concentration of 5 $\mu g{\cdot}L^{-1}$ in synthetic medium (Nguyen et al., 2013). However, low (34%) or no removal was observed using the strains *T. versicolor* NRRL 66313 and *P. ostreatus* HK 35 in an effluent from secondary treatment (Kresinová et al., 2017; Shreve et al., 2016). Nguyen et al. (2013) also reported the removal of benzophenone, octocrylene and oxybenzone (three UV filters) with values of 68, 90 and 96%, respectively. However, Shreve et al. (2016) observed no removal of oxybenzone.

1.3.3 Pesticides

Few studies have investigated pesticide removal in continuous mode using different white-rot fungi and conditions. The potential of the white-rot fungi *Bjerkandera adusta* for the degradation of the insecticide hexachlorocyclohexane (HCH) in a spiked soil in a slurry system was investigated by Quintero et al. (2007). The bioremediation studies in the reactor were performed for 30 d and the operational conditions tested were solid load (10% and 30%) and concentration of pollutants in the soil (25 and 100 $mg{\cdot}kg^{-1}$). The results showed that higher degradation percentages were obtained for a solid concentration of 10% and a concentration for each isomer of 25 $mg{\cdot}kg^{-1}$ and were of 94.5%, 94.5%, 78.5% and 66.1%, for α-, γ-, δ- and β-HCH isomers, respectively.

The performance of a continuous packed bed bioreactor degrading the organophosphorus insecticide chlorpyrifos by the fungus *Aspergillus* sp. was studied at

varying insecticide loading rates by Yadav et al. (2015). *Aspergillus* sp. was found to be quite efficient in the biodegradation of chlorpyrifos and its removal efficiency varied from 68 to 89% with the flow rate ranging from 10 to 40 $mL{\cdot}h^{-1}$ and the HRT from 24 to 100 h. Results also showed that the continuous packed bed bioreactor was able to regain its performance quickly after the perturbation in the flow rate. The potential of the same filamentous fungus *Aspergillus niger* to degrade continuously an herbicide, atrazine, in wastewater was evaluated by Marinho et al. (2017).

T. versicolor showed potential in the biodegradation of clofibric acid in a fluidized bed bioreactor. The study operated for 24 d a continuous reactor with an HRT of 4 days and achieved a 80% removal (Cruz-Morató et al., 2013b). Interestingly, the identification of transformation products and a toxicity assessment showed that the treated effluent was more toxic than the initial feed, probably due to the presence of hydroxyl-clofibric acid. Continuous removal of six pesticides, namely, atrazine, propoxur, fenoprop, ametryn, clofibric acid and pentachlorophenol, was investigated by Nguyen et al. (2013) with the same fungus in a MBR treating synthetic medium. The main results showed that fungus-augmented reactor achieved good removal of fenoprop (57%), clofibric acid (65%) and pentachlorophenol (92%) compared to conventional MBR. Toxicity assays were not performed in this case. The effect of a continuous dosing of a mediator (1-hydroxy benzotriazole, HBT) to the fungus-augmented MBR was also investigated during the last 30 days of operation. The results showed no significant difference in removal of atrazine and ametryn by the MBR, even after doubling the mediator dose to 10 µM. Shreve et al. (2016) observed no removal of atrazine and N,N-diethyl-3-methylbenzamide (DEET) within a mixture of nine contaminants spiked on sterile WWTP effluent.

1.3.4 Industrial chemicals

Continuous treatment of chemical industrial has been also examined by few researchers. Palli et al. (2016) investigated the biodegradation of 2-naphthalensulfonic acid polymers (NSAP) in a wastewater in a continuous packed bed bioreactor working for three months under non-sterile conditions. The bioreactors were inoculated by *B. adusta* and *P. ostreatus* immobilized on straw. The results showed that the fungus *B. adusta* exhibited a limited enzymatic activity and was not able to remove the tested contaminant. However, the reactor inoculated with *P. ostreatus* showed a stable laccase activity during the whole experiment and noticeable NSAP biodegradation was achieved after two weeks of work and remained until the end of the experiment (30 to 60 %). In another study, high

removal (> 95%) of two industrial chemicals, namely 4-tert-Butylphenol and 4-tert-Octylphenol, among thirty contaminants, was observed in an augmented fungal MBR (Nguyen et al., 2013).

1.4 Limitations of fungal based systems and how to overcome them

Despite all the potentialities of WRF, and the high amount of interesting studies about fungi being used for micropollutant removal, fungal systems for wastewater treatment are not being commonly applied at industrial scale. In this section, we review the main drawbacks of the technology and how can they be overcome.

1.4.1 Need for nutrient addition

As discussed in Section 1.2.2, although organic micropollutants contain carbon, some WRF need an additional assimilable carbon source for growth and survival. Wastewater usually contains organic carbon and nitrogen (Verlicchi et al., 2010), both needed for microbial growth, and bacteria are perfectly capable of assimilating both. In the case of WRF, most experiments used glucose-based or malt extract-based spiked media (a.k.a. synthetic wastewater) and few studies can be found using real wastewater. The need for nutrient addition in real wastewater treatments by WRF was identified only after using real wastewater. Cruz-Morató et al. (2013a) and Badia-Fabregat et al. (2015a) highlighted the need of glucose and ammonium tartrate addition for maintaining pelleted *T. versicolor* biological activity and enzymatic production in a fluidized bed bioreactor treating wastewater. Zhang and Geißen (2012) also found that glucose and ammonium tartrate addition were required for carbamazepine removal in a plate bioreactor inoculated with polyether foam-immobilized *P. chrysosporium* treating WWTP effluent. Other studies using fluidized bed bioreactors and treating flocculated wastewater obtained similar results when adding ammonium chloride instead of ammonium tartrate (Mir-Tutusaus et al., 2016, 2017a). However, some WRF were able to assimilate COD from wastewater: Palli et al. (2017) operated a fluidized bed reactor with *Pleurotus ostreatus* and observed significant growth of the fungus and reduction in the COD. In those cases where a fungal species able to assimilate wastewater COD is used, there is no need for nutrient addition. This in turn could reduce bacterial growth, but overgrown

Table 1.1: Removal efficiencies of fungal systems for different micropollutants.

Family	Compound	Fungus	Duration of the treatment	Reactor	HRT	Matrix	Temperature (ºC)	pH	Sterility	Initial concentration
Analgesics	Acetaminophen	*T. versicolor*	56 d	FBR	3 d	HWW (flocculated)	No	> 20000	> 99.3	mir-tutusaus2017
and anti-	Diclofenac	*P. chrysosporium*	30 d	stirred tank	24 h	Kirk medium	Yes	1	100	rodarte-morales2011
inflammatories		*P. chrysosporium*	50 d	stirred tank	24 h	Kirk medium	Yes	1	>93	rodarte-morales2012
		P. chrysosporium	100 d	fixed bed	24 h	Kirk medium	Yes	1	100	rodarte-morales2012
		P. chrysosporium	70 d	stirred tank	-	-	Yes	0.9-1.7	34-90	rodarte-morales2012b
		T. versicolor	90 d	MBR	48 h	Malt extract-based	No	300-1500	0-60	yang2013
		T. versicolor	110 d	MBR	2 d	Malt extract-based	No	5	50	nguyen2013
		T. versicolor	26 d	FBR	3.3 d	Veterinary HWW	No	123	-177	badia-fabregat2015b
		T. versicolor	56 d	FBR	3 d	HWW (flocculated)	No	951	99.8	mir-tutusaus2017
	Ibuprofen	*P. chrysosporium*	30 d	stirred tank	24 h	Kirk medium	Yes	1	100	rodarte-morales2011
		P. chrysosporium	50 d	stirred tank	24 h	Kirk medium	Yes	1	>93	rodarte-morales2012
		P. chrysosporium	100 d	fixed bed	24 h	Kirk medium	Yes	1	100	rodarte-morales2012
		P. chrysosporium	70 d	stirred tank	-	-	Yes	0.8-1.2	65-95	rodarte-morales2012b
		T. versicolor	110 d	MBR	2 d	Malt extract-based	No	5	>95	nguyen2013
		T. versicolor	26 d	FBR	3.3 d	Veterinary HWW	No	212	30	badia-fabregat2015b
		T. versicolor	28 d	FBR	3 d	HWW (flocculated)	No	20	100	mir-tutusaus2016
		T. versicolor	56 d	FBR	3 d	HWW (flocculated)	No	>20000	> 85.5	mir-tutusaus2017
	Indomethacine	*T. versicolor*	26 d	FBR	3.3 d	Veterinary HWW	No	34	-79	badia-fabregat2015b

Table 1.1: Removal efficiencies of fungal systems for different micropollutants.

Family	Compound	Fungus	Duration of the treatment	Reactor	HRT	Matrix	Temperature (ºC)	pH	Sterility	Initial concentration
	Ketoprofen	*T. versicolor*	110 d	MBR	2 d	Malt extract-based	No	5	94	nguyen2013
		T. versicolor	26 d	FBR	3.3 d	Veterinary HWW	No	320	-57	badia-fabregat2015b
		T. versicolor	28 d	FBR	3 d	HWW (flocculated)	No	20	80	mir-tutusaus2016
		T. versicolor	56 d	FBR	3 d	HWW (flocculated)	No	5109	-3.6	mir-tutusaus2017
	Naproxen	*P. chrysosporium*	30 d	stirred tank	24 h	Kirk medium	Yes	1	83	rodarte-morales2011
		P. chrysosporium	50 d	stirred tank	24 h	Kirk medium	Yes	1	0-92	rodarte-morales2012
		P. chrysosporium	100 d	fixed bed	24 h	Kirk medium	Yes	1	90	rodarte-morales2012
		P. chrysosporium	70 d	stirred tank	-	-	Yes	0.9-1.3	0-94	rodarte-morales2012b
		T. versicolor	110 d	MBR	2 d	Malt extract-based	No	5	>99	nguyen2013
		T. versicolor	26 d	FBR	3.3 d	Veterinary HWW	No	85	71	badia-fabregat2015b
		P. chrysosporium	165 d	seepage reactor	2 d	Kirk medium	No	1	100	li2015
	Piroxicam	*T. versicolor*	26 d	FBR	3.3 d	Veterinary HWW	No	136	-59	badia-fabregat2015b
	Salicyclic acid	*T. versicolor*	110 d	MBR	2 d	Malt extract-based	No	5	90	nguyen2013
		T. versicolor	26 d	FBR	3.3 d	Veterinary HWW	No	3730	81	badia-fabregat2015b
Anthelmintics	Albendazole	*T. versicolor*	26 d	FBR	3.3 d	Veterinary HWW	No	8	45	badia-fabregat2015b
	Thiabendazole	*T. versicolor*	56 d	FBR	3 d	HWW (flocculated)	No	blq	70.0	mir-tutusaus2017
Antibiotics	Ciprofloxacin	*T. versicolor*	56 d	FBR	3 d	HWW (flocculated)	No	366	47.1	mir-tutusaus2017
		T. versicolor	26 d	FBR	3.3 d	Veterinary HWW	No	42	100	badia-fabregat2015b

Table 1.1: Removal efficiencies of fungal systems for different micropollutants.

Family	Compound	Fungus	Duration of the treatment	Reactor	HRT	Matrix	Temperature (ºC)	pH	Sterility	Initial concentration
	Metronidazole	*T. versicolor*	110 d	MBR	2 d	Malt extract-based	No	5	38	nguyen2013
		T. versicolor	26 d	FBR	3.3 d	Veterinary HWW	No	1736	40	badia-fabregat2015b
	Ofloxacin	*T. versicolor*	8 d	FBR	batch	HWW	No	202	99	gros2014
		T. versicolor	8 d	FBR	batch	HWW	Yes	32	98.5	gros2014
		T. versicolor	56 d	FBR	3 d	HWW (flocculated)	No	2537	71.1	mir-tutusaus2017
	Ronidazole	*T. versicolor*	56 d	FBR	3 d	HWW (flocculated)	No	bld	-7745.2	mir-tutusaus2017
	Sulfamethazine	*T. versicolor*	26 d	FBR	3 d	Defined medium	Yes	5	>94	rodriguez-rodriguez2012
	Sulfamethoxazole	*T. versicolor*	56 d	FBR	3 d	HWW (flocculated)	No	1130	78.2	mir-tutusaus2017
	Sulfapyridine	*T. versicolor*	26 d	FBR	3 d	Defined medium	Yes	5	>99	rodriguez-rodriguez2012
	Sulfathiazole	*T. versicolor*	26 d	FBR	3 d	Defined medium	Yes	5	>95	rodriguez-rodriguez2012
	Trimethoprim	*T. versicolor*	56 d	FBR	3 d	HWW (flocculated)	No	748	52.3	mir-tutusaus2017
Anticoagulants	Warfarin	*T. versicolor*	56 d	FBR	3 d	HWW (flocculated)	No	10	94.8	mir-tutusaus2017
Antihypertensives	Valsartan	*T. versicolor*	56 d	FBR	3 d	HWW (flocculated)	No	113	34.2	mir-tutusaus2017
Antiplatelet agents	Clopidrogel	*T. versicolor*	26 d	FBR	3.3 d	Veterinary HWW	No	247	100	badia-fabregat2015b
Diuretics	Furosemide	*T. versicolor*	26 d	FBR	3.3 d	Veterinary HWW	No	462	83	badia-fabregat2015b
		T. versicolor	56 d	FBR	3 d	HWW (flocculated)	No	bld	-664.4	mir-tutusaus2017

Table 1.1: Removal efficiencies of fungal systems for different micropollutants.

Family	Compound	Fungus	Duration of the treatment	Reactor	HRT	Matrix	Temperature (ºC)	pH	Sterility	Initial concentration
	Hydrochloro-thiazide	*T. versicolor*	56 d	FBR	3 d	HWW (flocculated)	No	408	58.4	mir-tutusaus2017
H1 and H2 receptor antagonists	Loratadine	*T. versicolor*	56 d	FBR	3 d	HWW (flocculated)	No	1	46.9	mir-tutusaus2017
	Ranitidine	*T. versicolor*	56 d	FBR	3 d	HWW (flocculated)	No	1830	96.7	mir-tutusaus2017
		T. versicolor	26 d	FBR	3.3 d	Veterinary HWW	No	219	100	badia-fabregat2015b
Lipid regulators	Atorvastatin	*T. versicolor*	26 d	FBR	3.3 d	Veterinary HWW	No	30	-8	badia-fabregat2015b
		T. versicolor	56 d	FBR	3 d	HWW (flocculated)	No	15	94.9	mir-tutusaus2017
	Fluvastatin	*T. versicolor*	26 d	FBR	3.3 d	Veterinary HWW	No	44	-64	badia-fabregat2015b
	Gemfibrozil	*T. versicolor*	110 d	MBR	2 d	Malt extract-based	No	5	>95	nguyen2013
		T. versicolor	26 d	FBR	3.3 d	Veterinary HWW	No	35	55	badia-fabregat2015b
		T. versicolor	56 d	FBR	3 d	HWW (flocculated)	No	6364	100.0	mir-tutusaus2017
Psychiatric drugs	10.11-epoxyCBZ	*T. versicolor*	56 d	FBR	3 d	HWW (flocculated)	No	673	43.6	mir-tutusaus2017
	2-hydroxyCBZ	*T. versicolor*	56 d	FBR	3 d	HWW (flocculated)	No	1661	99.9	mir-tutusaus2017
	Acridone	*T. versicolor*	56 d	FBR	3 d	HWW (flocculated)	No	126	-183.5	mir-tutusaus2017
	Amitriptyline	*T. versicolor*	110 d	MBR	2 d	Malt extract-based	No	5	86	nguyen2013
	Carbamazepine	*P. chrysosporium*	50 d	stirred tank	24 h	Kirk medium	Yes	0.5	0-63	rodarte-morales2012
		P. chrysosporium	100 d	fixed bed	24 h	Kirk medium	Yes	0.5	0-40	rodarte-morales2012

Table 1.1: Removal efficiencies of fungal systems for different micropollutants.

Family	Compound	Fungus	Duration of the treatment	Reactor	HRT	Matrix	Temperature (ºC)	pH	Sterility	Initial concentration
		P. chrysosporium	70 d	stirred tank	-	-	Yes	2.2-1.0	5-90	rodarte-morales2012b
		T. versicolor	25 d	FBR	3 d	Defined medium	Yes	200	54	jelic2012
		P. chrysosporium	100 d	plate reactor	36 h	Modified Kirk medium	No	1	80	zhang2012
		P. chrysosporium	100 d	plate reactor	36 h	Municipal wastewater	No	1	60	zhang2012
		T. versicolor	110 d	MBR	2 d	Malt extract-based	No	5	21	nguyen2013
		P. chrysosporium	165 d	seepage reactor	2 d	Kirk medium	No	1	80	li2015
		P. chrysosporium	160 d	rotating cartridge	3 d	Modified Kirk medium	No	1	70-90	li2016
		T. versicolor	12 h	bottle reactor	batch	Effluent from WWTP	Yes	350	0	shreve2016
		T. versicolor	56 d	FBR	3 d	HWW (flocculated)	No	251	61.0	mir-tutusaus2017
	Citalopram	*T. versicolor*	56 d	FBR	3 d	HWW (flocculated)	No	297	39.0	mir-tutusaus2017
	Diazepam	*P. chrysosporium*	50 d	stirred tank	24 h	Kirk medium	Yes	0.25-0.5	0	rodarte-morales2012
		P. chrysosporium	100 d	fixed bed	24 h	Kirk medium	Yes	0.25-0.5	0-30	rodarte-morales2012
		T. versicolor	26 d	FBR	3.3 d	Veterinary HWW	No	236	83	badia-fabregat2015b
		T. versicolor	56 d	FBR	3 d	HWW (flocculated)	No	bld	-58.3	mir-tutusaus2017
	Norfluoxetine	*T. versicolor*	56 d	FBR	3 d	HWW (flocculated)	No	13	90.1	mir-tutusaus2017
	Olanzapine	*T. versicolor*	56 d	FBR	3 d	HWW (flocculated)	No	132	98.7	mir-tutusaus2017
	Primidone	*T. versicolor*	110 d	MBR	2 d	Malt extract-based	No	5	95	nguyen2013
	Razodone	*T. versicolor*	56 d	FBR	3 d	HWW (flocculated)	No	36	98.0	mir-tutusaus2017

Table 1.1: Removal efficiencies of fungal systems for different micropollutants.

Family	Compound	Fungus	Duration of the treatment	Reactor	HRT	Matrix	Temperature (ºC)	pH	Sterility	Initial concentration
	Setraline	*T. versicolor*	56 d	FBR	3 d	HWW (flocculated)	No	99	98.3	mir-tutusaus2017
	Venlafaxine	*T. versicolor*	56 d	FBR	3 d	HWW (flocculated)	No	495	41.0	mir-tutusaus2017
Glucocorticoid	Dexamethasone	*T. versicolor*	56 d	FBR	3 d	HWW (flocculated)	No	122	77.2	mir-tutusaus2017
Contrast agent	Iopromide	*T. versicolor*	8 d	FBR	batch	HWW	No	419.7	65.4	gros2014
		T. versicolor	8 d	FBR	batch	HWW	Yes	105	87	gros2014
β-blockers	Atenolol	*T. versicolor*	56 d	FBR	3 d	HWW (flocculated)	No	59	14.3	mir-tutusaus2017
	Sotalol	*T. versicolor*	56 d	FBR	3 d	HWW (flocculated)	No	252	-151.9	mir-tutusaus2017
Endocrine disruptors	17α-ethynyl-estradiol (EE2)	*T. versicolor*	26 d	FBR	120 h	Defined medium	Yes	7.3	>97	blanquez2008
		T. versicolor	110 d	MBR	2 d	Malt extract-based	No	5	90	nguyen2013
		T. versicolor	12 h	bottle reactor	batch	Effluent from WWTP	Yes	350	71.3	shreve2016
		P. ostreatus	28 d	trickle bed	46 h - 8 h	Effluent from WWTP	No	10	50	kresinova2017
	17β-estradiol (E2)	*T. versicolor*	26 d	FBR	120 h	Defined medium	Yes	3-18.8	>99	blanquez2008
		T. versicolor	110 d	MBR	2 d	Malt extract-based	No	5	>99	nguyen2013
		T. versicolor	12 h	bottle reactor	batch	Effluent from WWTP	Yes	350	>99	shreve2016
	17β-estradiol-17-acetate	*T. versicolor*	110 d	MBR	2 d	Malt extract-based	No	5	>95	nguyen2013
	4-n-nonylphenol	*P. ostreatus*	28 d	trickle bed	46 h - 8 h	Effluent from WWTP	No	10	50	kresinova2017

Table 1.1: Removal efficiencies of fungal systems for different micropollutants.

Family	Compound	Fungus	Duration of the treatment	Reactor	HRT	Matrix	Temperature (ºC)	pH	Sterility	Initial concentration
	Bishpenol A	*T. versicolor*	90 d	MBR	48 h	Malt extract-based	No	300-1500	40-80	yang2013
		T. versicolor	110 d	MBR	2 d	Malt extract-based	No	5	75	nguyen2013
		T. versicolor	12 h	bottle reactor	batch	Effluent from WWTP	Yes	350	61.9	shreve2016
		P. ostreatus	28 d	trickle bed	46 h - 8 h	Effluent from WWTP	No	20	80	kresinova2017
	Estriol (E3)	*T. versicolor*	110 d	MBR	2 d	Malt extract-based	No	5	95	nguyen2013
	Estrone (E1)	*T. versicolor*	110 d	MBR	2 d	Malt extract-based	No	5	94	nguyen2013
		T. versicolor	12 h	bottle reactor	batch	Effluent from WWTP	Yes	350	83.5	shreve2016
		P. ostreatus	28 d	trickle bed	46 h - 8 h	Effluent from WWTP	No	45	>99	kresinova2017
	Triclosan	*T. versicolor*	110 d	MBR	2 d	Malt extract-based	No	5	>95	nguyen2013
		T. versicolor	12 h	bottle reactor	batch	Effluent from WWTP	Yes	350	0	shreve2016
		P. ostreatus	28 d	trickle bed	46 h - 8 h	Effluent from WWTP	No	25	34	kresinova2017
UV filters	Benzophenone	*T. versicolor*	110 d	MBR	2 d	Malt extract-based	No	5	85	nguyen2013
	Octocrylene	*T. versicolor*	110 d	MBR	2 d	Malt extract-based	No	5	90	nguyen2013
	Oxybenzone	*T. versicolor*	110 d	MBR	2 d	Malt extract-based	No	5	96	nguyen2013
		T. versicolor	12 h	bottle reactor	batch	Effluent from WWTP	Yes	350	0	shreve2016
Pesticides	Ametryn	*T. versicolor*	110 d	MBR	2 d	Malt extract-based	No	5	29	nguyen2013
	Atrazine	*T. versicolor*	110 d	MBR	2 d	Malt extract-based	No	5	11	nguyen2013

Table 1.1: Removal efficiencies of fungal systems for different micropollutants.

Family	Compound	Fungus	Duration of the treatment	Reactor	HRT	Matrix	Temperature (ºC)	pH	Sterility	Initial concentration
		T. versicolor	12 h	bottle reactor	batch	Effluent from WWTP	Yes	350	0	shreve2016
		Aspergillus sp.	8 d	bottle reactor	batch	Vishniac solution	Yes	30	72	marinho2017
	Chlorpyrifos	*Aspergillus* sp.	45 d	packed bed	-	-	Yes	180-250	90	yadav2014
	Clofibric acid	*T. versicolor*	110 d	MBR	2 d	Malt extract-based	No	5	65	nguyen2013
		T. versicolor	24 d	FBR	4 d	Defined medium	Yes	160	80	cruz-morato2013
	Fenoprop	*T. versicolor*	110 d	MBR	2 d	Malt extract-based	No	5	57	nguyen2013
	DEET	*T. versicolor*	12 h	bottle reactor	batch	Effluent from WWTP	Yes	350	0	shreve2016
	Pentachlorophenol	*T. versicolor*	110 d	MBR	2 d	Malt extract-based	No	5	92	nguyen2013
	Propoxur	*T. versicolor*	110 d	MBR	2 d	Malt extract-based	No	5	20	nguyen2013
	α/β/γ/δ-Hexa-	*B. adusta*	30 d	slurry reactor	batch	Slurry with 10% solids	Yes	25	95	quintero2007
	chloro-	*B. adusta*	30 d	slurry reactor	batch	Slurry with 10% solids	Yes	25	66	quintero2007
	cyclohexane	*B. adusta*	30 d	slurry reactor	batch	Slurry with 10% solids	Yes	25	95	quintero2007
		B. adusta	30 d	slurry reactor	batch	Slurry with 10% solids	Yes	25	79	quintero2007
Industrial chemicals	2-naphthalen-	*B. adusta*	30 d	packed bed	3 d	Filtered	No	3167-	0	palli2016
	sulfonic acid	*P. ostreatus*	90 d	packed bed	3 d	petrochemical WW	No	-3445	30-60	palli2016
	4-tert-Butylphenol	*T. versicolor*	110 d	MBR	2 d	Malt extract-based	No	5	>95	nguyen2013

fungal biomass should then be purged regularly. Nevertheless, it can be fairly accepted that nutrient addition can be needed to operate a white-rot fungal reactor for the treatment of wastewater. This poses a problem to full scale application, as the cost of glucose and nitrogen addition would be enormous, especially taking into account the large volumes of wastewater treated in WWTPs, and potentially increase the COD and nitrogen load.

This limitation could be partially overcome (i) by optimizing the nutrient addition, because when nutrients are added at consumption rate lower quantities are needed and nutrients' concentration in the effluent equals zero, therefore not increasing COD or nitrogen load. This in turn prevents overgrowth of fungal biomass; (ii) by replacing the glucose and ammonium tartrate/chloride by similarly constituted, cheaper sub-products; or (iii) by reimagining the use of the technology: white-rot fungal systems could be viable, even taking into account the costs of nutrient addition, when smaller volumes of micropollutant-contaminated wastewater are treated. This is the case, for example, of hospital wastewater, veterinary hospital wastewater and several industrial wastewaters (Verlicchi et al., 2010). A fourth answer to this limitation is (iv) the immobilization of fungal biomass onto lignocellulosic materials. These substrates act also as carbon and nitrogen sources for WRF, thus avoiding the need of nutrient addition (Ehlers and Rose, 2005; Lu et al., 2009; Torán et al., 2017). Moreover, lignocellulosic materials are very abundant and are usually byproducts of other industries, reducing their price (Dashtban et al., 2010; Leonowicz et al., 1999).

1.4.2 Immobilization of fungal biomass

Fungal dispersed mycelium usually causes bioreactor operation difficulties such as growth on the reactor walls and agitators, foaming and increased need of mixing and oxygen supply. The immobilization of fungal biomass overcomes most of these difficulties –or reduces them.

The immobilization can be accomplished by the growth of the fungus in form of pellets (auto-immobilization). This is usually accomplished by growing the fungus in Erlenmeyers with liquid media in shaking conditions. Quite a few studies have dealt with the pelletization of different fungal species, the optimal pellet diameter and the study of mass and oxygen transfer into the pelleted biomass (Borràs et al., 2008; Casas López et al., 2005; Feng et al., 2004; Leštan and Lamar, 1999; Sharma and Padwal-Desai, 1985; Sitanggang et al., 2010; Wittier et al., 1986). Some studies have reported successful pellet production in a fluidized bed reactor, even in a pilot-scale bioreactor, thus enabling the

upscaling of the technology (Borràs et al., 2008; Mir-Tutusaus et al., 2016, 2017a). Additionally, Espinosa-Ortiz et al. (2015) reviewed several fungal pelleted reactor configurations with the perspective of treating wastewater.

The immobilization can also be carried out by growing the fungus onto a carrier. Some studies have done so using inert carriers such as polyurethane foam cubes (Li et al., 2016; Yadav et al., 2015). Gao et al. (2008) listed amongst the advantages of immobilizing *P. chrysosporium* in polyurethane foam the improved survival and increased enzymatic activity of the fungus in non-sterile cultures. But taking into account WRF's ability of degrading lignin, cellulose and hemicellulose, several other authors have looked into the immobilization onto non-inert carriers such as wood chips, serving both as support and carbon source(Li et al., 2015; Pedroza-Rodríguez and Rodríguez-Vázquez, 2013; Rodarte-Morales et al., 2012; Sirtori et al., 2009). Interestingly, when Ehlers and Rose (2005) immobilized several WRF in pine chips, fungi were shown to penetrate the wood, possibly using the cellulose and hemicelluloses as carbon source. In this case, WRF not only benefited from the immobilization, but bacteria were not able to use the carbon source, hence avoiding substrate competition. Recent studies have also reported improved micropollutant degradation and fungal survival with *T. versicolor* immobilized in wood chips, even when treating real wastewater (Torán et al., 2017).

In general, immobilization and auto-immobilization led to more robust operations in non-sterile conditions (Hai et al., 2013; Leidig et al., 1999; Nilsson et al., 2006; Tang et al., 2011). Experiments with immobilized biomass tend to use fixed-bed column reactors rather than the stirred-tank or fluidized bed reactors usually used with pelleted biomass.

1.4.3 Competition with autochthonous microorganisms

The decline in micropollutant removal observed in several studies has been largely attributed to bacterial contamination and it has been identified as the main bottleneck of the technology (Espinosa-Ortiz et al., 2015; Gao et al., 2008; Hai et al., 2008, 2013; Libra et al., 2003). Indeed, bacteria has been shown to exert competitive pressure for the substrate, thus leading to the loss of fungal biomass, and to destabilize fungal enzymes (Hai et al., 2008; Libra et al., 2003). For that reason, researchers have since proposed a wide range of alternatives for dealing with this limitation.

1.4.3.1 Favoring fungal growth

Favoring fungal growth usually involves supplying the conditions that distinctively favor WRF over bacteria. These strategies include operation at optimal fungal pH,

immobilization of fungal biomass, periodical biomass renewal and optimizing the carbon-to-nitrogen ratio (C/N ratio) of the nutrients supplied.

- Operation at optimal fungal pH. Most white-rot fungi's optimal pH is acidic; not surprisingly, lignin modifying enzymes' optimal pH is also acidic (Pazarlioglu et al., 2005). Although a specific bacterial species might find it difficult to grow at acidic pH, bacteria is a diverse domain and acidic pH does not suppress bacterial growth. However, pH too acidic ceased enzyme production of *T. versicolor* and led to the loss of pelleted morphology in a fluidized-bed reactor (Borràs et al., 2008; Libra et al., 2003). Therefore, acidic pH does not distinctively favor fungi over bacteria, but it does improve the viability and activity of WRF.

- Partial biomass renovation. Initially, partial biomass regeneration was developed as a strategy for stabilizing the age of fungal biomass in a sterile treatment when nutrients were added at growth-limiting conditions, thus extending the operational time (Blánquez et al., 2006). They purged 1/3 of the fungal biomass in the reactor and added the same amount of fresh biomass every week, obtaining a solids residence time of 21 d. The work concluded that partial biomass renovation helped in maintaining a young and active culture in the bioreactor. The strategy was continued in several sterile operations (Blánquez et al., 2008; Blánquez and Guieysse, 2008) and in a non-sterile treatment of wastewater by Badia-Fabregat et al. (2015b) in an attempt to improve the enzymatic production and integrity of pellets. It was also successfully applied in a non-sterile operation of flocculated wastewater, allowing for a 56-day treatment (Mir-Tutusaus et al., 2017a). In summary, the substitution of old biomass by fresh fungus allowed for a more stable fungal population in the reactors, in turn maintaining enzymatic activity for a longer period of time and favoring white-rot fungal colonization.

- Carbon-to-nitrogen ratio. In systems where nutrient addition is needed –i.e. where biomass is not immobilized in lignocellulosic substrates–, the ratio between carbon and nitrogen may play a role in favoring fungal over bacterial populations. On the one hand, high C/N ratios mimic ligninolytic conditions (wood has a high C/N ratio), increasing white-rot fungal production of lignin modifying enzymes (Eggert et al., 1996); and not contrarily, limiting conditions of carbon or nitrogen have also been reported to enhance LME production (Viswanath et al., 2014). On the other hand, lower carbon-to-nitrogen ratios favor fungal growth over bacterial growth (Demoling et al., 2007; Rousk and Bååth, 2007). It is important to notice

that lower ratios do not favor white-rot fungi exclusively, but rather the growth of fungal species in general. Therefore, a compromise must be found between favoring fungal growth over bacteria and favoring LME production. However, one should take into account that LME production has rarely been linked to an increase of micropollutant removal. For example, in a recent publication treating flocculated wastewater in a fluidized bed bioreactor, a rather low C/N ratio of 7.5 was chosen in terms of PhAC degradation and biomass integrity (Mir-Tutusaus et al., 2017b).

- Immobilization. In addition to the advantages of immobilization discussed in Section 1.4.2, auto-immobilization of WRF in the form of pellets allows a high concentration of fungus inside the reactor, thus hindering bacterial colonization. If the immobilization is carried out on lignocellulosic carriers the fungal concentration tends to be lower, but most bacterial species find it difficult to grow on lignocellulosic substrates.

1.4.3.2 Washing out bacteria

Another strategy for overcoming the competition with native microorganisms is by means of decoupling the hydraulic retention time (HRT) and solids retention time (SRT), sometimes also referred as cellular retention time (CRT). The purpose of these strategies is to keep the fungal biomass in the reactor while washing out the bacteria and other microorganisms, therefore increasing the retention time of WRF while keeping an HRT able to wash out the other microorganisms.

In order to achieve this decoupling, some authors auto-immobilized the WRF, typically in the form of pellets while others immobilized the fungi on inert carriers or lignocellulosic substrates, as discussed in Section 1.4.2. A third option for decoupling HRT and fungal retention time is by the use of membrane technology. Membranes are widely used and can be found at industrial scale in several WWTPs (Joss et al., 2006; Rubirola et al., 2014). They allow for a higher SRT and have been successfully applied with fungal biomass for the removal of organic micropollutants at laboratory scale (Hai et al., 2009; Nguyen et al., 2013; Yang et al., 2013a).van Leeuwen et al. (2003) described a technology using 100 µm microscreens that allowed for Rhizopus production under non-aseptic conditions thanks to the manipulation of HRT and SRT.

These four approaches allow for the retention of fungal biomass inside the reactor, therefore permitting the decrease of the HRT without affecting the SRT. A lower HRT leads to the washout of non-attached microorganisms, and bacterial concentration has

been linked with the loss of degradation capacity, enzymatic production and viability of WRF (Blánquez et al., 2008; Hai et al., 2013, 2009; Mir-Tutusaus et al., 2016). Therefore, lower HRT favor white-rot fungal viability by washing out non-attached microorganisms. However, it is noteworthy that bacteria can attach to virtually everything, including pellets, immobilized fungal biomass, inert carriers and reactor surface (Fletcher, 1994). While fungal survival might be improved, lower HRTs often meant lower degradation of several contaminants by WRF: for example, batch tests required up to 8 days to remove the more recalcitrant compounds such as carbamazepine, ciprofloxacin or acridone in wastewater (Cruz-Morató et al., 2013a; Jelic et al., 2012) and Blánquez et al. (2007) reported reduced decolorization of a textile dye when lower HRTs were applied, similarly to Asses et al. (2009). Moreover, washing out of bacteria comes inevitably with the washout of extracellular enzymes and mediators produced by the fungus (Badia-Fabregat et al., 2017; Nguyen et al., 2013). However, as reviewed in Section 1.2.1, not only extracellular enzymes play a role in microcontaminant degradation. In fact, several authors reported concentration of LMEs not being crucial to maintain good removal percentages (Anastasi et al., 2010; Blánquez et al., 2004; Yang et al., 2013a). In spite of that, maintaining a sufficient concentration of LMEs in the reactor is desirable for compounds whose biotransformation is LME-dependent.

In summary, both HRT and SRT must be optimized in order to achieve a compromise between bacteria-and-enzyme washout, micropollutant removal and fungal survival.

1.4.3.3 Suppressing bacteria

Another strategy for assisting fungi in the competition with autochthonous microorganisms is the direct suppression of bacteria. This could obviously be achieved by sterilization, but it is not feasible in the wastewater treatment industry. Regardless, two approaches have been studied in order to reduce the bacterial count.

Sankaran et al. (2008)suggested the use of ozone (O_3) as a selective disinfectant in order to decrease bacterial contamination in a non-sterile continuous fungal cultivation on corn-processing wastewater. The aim of the work was the production of fungal biomass, rather than COD or micropollutant removal; that is why the researchers used very high dosages of ozone (57 $mg{\cdot}L^{-1}$), while ozone doses in full scale WWTPs range between 5 and 15 $mg{\cdot}L^{-1}$ (Ternes et al., 2003; Verlicchi et al., 2010). Ozonation behaves similarly to acidic pH in the sense that it favors most fungal species over bacteria. In fact, Sankaran et al. (2008) inoculated the reactor with *R. oligosporus* but the fungal population was replaced by a wastewater-native fungus. Cheng et al. (2013) used ozone

as a bactericide in a white-rot fungal dye-decolorization continuous operation, thus maintaining the bacterial concentration at around 10^5 CFU·mL^{-1}. The study reported a 99.4% inhibition of contaminating bacteria and the involvement of ozone in the degradation of the Acid Blue 45 dye. Indeed, ozone has been reported to improve biodegradability of refractory organic matter and to degrade several micropollutants (Contreras et al., 2003; Fujioka et al., 2014; Gomes et al., 2017; Kusvuran and Yildirim, 2013; Ternes et al., 2003; Yang et al., 2016). Therefore, care must be taken when using ozonation as a disinfectant in assigning removal efficiency to the WRF and to the ozonation itself.

The addition of pretreatments can potentially reduce the inlet concentration of bacteria. A recent study successfully extended the operation of a *T. versicolor* fluidized bed reactor treating hospital wastewater from 10 to 28 days (Mir-Tutusaus et al., 2016). Specifically, a coagulation-flocculation pretreatment reduced the bacterial count of the influent wastewater from 10^7-10^8 to 10^3-10^5 CFU·mL^{-1}, allowing for a longer-term operation. Coagulation and flocculation processes have been largely applied in WWTPs and are regarded as cost-effective (Liu et al., 2012; López-Maldonado et al., 2014). Therefore, the addition of this and other pretreatments might be a noteworthy strategy that enables WRF to operate with urban-like wastewaters.

1.4.3.4 A final note on non-sterility

Some studies in non-sterile conditions have been reviewed in this section. However, two groups can be distinguished: studies operating in non-sterile conditions with defined medium or tap water and studies using wastewater. The studies using defined medium or tap water usually rely on contamination by air-borne microorganisms and microorganisms present in non-sterile surfaces. Such contamination could be regarded as mild and operations tend to be longer. The other group, using wastewater, deals with the contamination due to growth of native wastewater microorganisms. Bacterial count in those cases tends to be very high, the contamination could be regarded as heavy and the reactor operations tend to be shorter. The latter studies should be encouraged, because in addition to be a more reliable representation of real conditions, consortia formed in those operations could play a role in degradation of micropollutants and fungal metabolism intermediate products.

1.4.4 Fungal treatments require high HRTs

As discussed in section Section 1.4.3.2, low hydraulic retention times produced lower degradation for some micropollutants and higher loss of extracellular enzymes. Fungal treatments usually require a HRT of around 1-3 days for the removal of microcontaminants (Blánquez et al., 2007, 2008; Hai et al., 2008, 2009). In fact, micropollutant removal is usually improved by increasing fungal treatment HRT when toxic compounds are not accumulated. In general, then, WRF require higher HRTs to remove micropollutants than bacteria to remove organic matter. This adds a difficulty on combining a fungal treatment step on a conventional WWTP, reinforcing the idea of using white-rot fungal operations as on-site treatments in specific contaminated streams (enumerated in Section 1.2.2 and Section 1.4.1). In those streams, the fungal treatment would be a treatment to decrease micropollutant concentration prior to discharge to the WWTP. If a fungal treatment were to be included in a conventional WWTP, some options could be considered: first, the increase of SRT or fungal concentration in the reactor could be optimized in order to allow higher removal efficiencies, thus enabling the coupling; second, low hydraulic retention times, between 6 to 12 h, are enough to remove several families of compounds such as analgesics, anti-inflammatories (Marco-Urrea et al., 2009, 2010b) and endocrine disruptors (Kresinová et al., 2017; Shreve et al., 2016), although enzyme washout should be taken into consideration. Therefore, wastewaters containing mainly these families of pollutants could be treated with fungal systems at low HRTs.

1.5 Future work

Most research articles focused on biodegradation of numerous micropollutants by several WRF in sterile conditions. This was needed to understand the factors controlling the biodegradation of the compounds, their mineralization, and their degradation pathways (Golan-rozen et al., 2011; Marco-Urrea et al., 2010c; Mir-Tutusaus et al., 2014). Such studies in sterile conditions are still required, for example, to understand which compounds can be biotransformed by LMEs, by intracellular P450 system or by both, and which require the presence of LME mediators.

But in order to advance the technology towards industrial scale, sterility must be discarded. Wastewater sterilization is not feasible from an economic and ecological point of view, so fungal research in applied science should focus on non-sterile conditions. The difficulty of non-sterile fungal operations has been discussed, and it

greatly shifts the focus on the research field: from establishing WRF's biodegradation capabilities to guaranteeing the fungus' survival and activity during the fungal operation. Besides favoring fungal survival over other microorganisms, some studies have concentrated in the microbiological community evolution during fungal treatments. Such studies should be encouraged, as bacterial and fungal interspecies interactions and its consequences in micropollutant removal are not fully understood. Some interesting interactions have already been found: the growth in *T. versicolor*-inoculated reactors of other fungi capable of degrading micropollutants, the inhibition of *T. versicolor* by the yeast *Candida*, and the increased laccase production of WRF in contact with non-sterile soil or with soil fungi, bacteria and yeasts (Badia-Fabregat et al., 2015a; Baldrian, 2004; Mir-Tutusaus et al., 2017a). Such findings can greatly affect the applicability of the technology and the operation of industrial scale reactors.

Similarly, the journey towards full scale operation requires the use of real, non-spiked matrices. Well understood, defined media can help in offering insight into the intricacies of a specific setup and they are needed in order to optimize several factors prior to, for example, upscaling the system. However, real matrices –e.g. wastewater– should be preferred: although real, non-spiked matrices are more difficult to extract conclusions from, they resemble the most a full scale application, thus building confidence on the technology. Moreover, results vary greatly between defined and real matrices, making it difficult to extrapolate conclusions drawn from the former to the latter. This should be no surprise, as the complexity of a real matrix –microbial diversity, chemical composition, trace contaminants, etc. – is impossible to replicate in a defined medium. In addition, as discussed in Section 1.4.3.4, the bacterial contamination problems when using real wastewater will be more difficult to deal with, but they will be more similar to a real operation. Lastly, because real matrices are a source of variability, successful fungal operations using real wastewater greatly increase the systems' robustness.

The use of non-spiked real matrices, however, poses a big pressure on analytical techniques. Not only are micropollutants found at a very low concentration, but they are also commonly found in the form of glucuronides and other conjugated forms (Dalgaard and Larsen, 1999; Kuehl et al., 2006; Lynn et al., 1978; Sanchez and Kauffman, 2010).Conjugated microcontaminants are not usually detected by the current analytical techniques, thus undervaluing the concentration of the pollutant studied. This in turn underestimates the removal capacity of WRF, as they have been consistently described to deconjugate such compounds (Badia-Fabregat et al., 2015a; Mir-Tutusaus et al., 2017a).Therefore, an effort should be made to analyze conjugated forms of compounds. If successful, it could be a breakthrough that greatly facilitates the study of removal of

micropollutants in real wastewater.

Fungal systems in full scale industrial operations have a long history: fungal biotechnology is well established in the food and beverage industry, in the pharmaceutical industry for the production of several drugs and in the enzyme production industry, for example (Adrio and Demain, 2003). But, in regards to wastewater treatment, only a few pilot scale examples can be found, and, to the best of author's knowledge, only one focusing on micropollutants removal (Djelal and Amrane, 2013; Jin et al., 2002; Kresinová et al., 2017). However, the technology is mature enough for different pilot scale reactor setups to flourish in the near future, and such studies should be encouraged.

2

Objectives

The main aim of this thesis is to develop a long-term process based on the white-rot fungus *Trametes versicolor* to remove pharmaceutically active compounds from hospital wastewater. In order to increase the operation time of such process, the work has been divided into specific goals:

- To assess the effect of operational strategies and the addition of pretreatments on the length of operation.
- To optimize several process variables to imporove fungal stability.
- To determine the applicability of a fungal membrane bioreactor for the removal of micropollutants.
- To assess the feasibility of coupling a UV/H_2O_2 process with biological treatments for the removal of pharmaceutically active compounds in hospital wastewater.
- To study the microbial communities and its evolution in non-sterile reactors using DGGE, sequencing and qPCR.
- To prove the concept of a long-term fungal operation for the removal of micropollutants from hospital wastewater.

3

Materials and methods

3.1 Reagents, fungus and wastewater

Ketoprofen (KTP), ibuprofen (IBP) and naproxen (NP) used for the spiked experiments in fluidized bed reactors (FBR) were of HPLC purity grade and were purchased from Sigma-Aldrich (Barcelona, Spain). Carbamazepine (CBZ), sulfamethoxazole (SMX), oxybenzone (OXB), diclofenac (DCF) and bisphenol A (BPA) were of HPLC purity grade and were purchased from Sigma-Aldrich (Australia). Thiamine hydrochloride, peptone, yeast extract, malt extract, glucose, ammonium chloride and other chemicals were purchased from Sigma-Aldrich (Spain, or Australia for chapter 8). All other chemicals used were of analytical grade. All the pharmaceutical and the corresponding isotopically labelled standards used in chapter 5 and chapter 7 analysis were of high purity grade (>90%) and they were purchased from Sigma–Aldrich (Steinheim, Germany), US Pharmacopeia USP (MD, USA), Europea Pharmacopoeia EP (Strasbourg, France), Toronto Research Chemicals TRC (Ontario, Canada) and CDN isotopes (Quebec, Canada). Individual as well as isotopically labelled standard solutions were prepared according to Gros et al. (2012). Coagulants Hypol DW217 and Hyfloc AC50 and flocculants Himoloc JO2030 and Himoloc DR3000 were kindly provided by Derypol, S.A. (Barcelona, Spain).

T. versicolor (ATCC#42530 and ATCC#7731 for chapter 8) was maintained on 2% malt agar slants at 25°C until use. Subcultures were routinely made. A mycelial suspension of *T. versicolor* was obtained with the traditional method, previously described (Font et al., 2003). Briefly, four 0.5 cm^3cubes of fungal growing area of Petri plates were cut and inoculated in a 500 mL Erlenmeyer with 150 mL of 20 $g{\cdot}L^{-1}$ malt extract adjusted at pH 4.5. After 5-6 days orbital shaking at 135 rpm (r = 25 mm) at 25ºC the mycelium was obtained. It was separated from the media by means of a strainer and homogenized with a homogenizer Ystral GmgH X/10/20 in a 0.8% v/v NaCl solution at a relation 1:1 v/v. The resultant suspension could be immediately used for the formation of pellets or stored at 4ºC. In chapter 6 and chapter 7 the mycelial suspension was produced by the novel

Table 3.1: Hospital wastewater information.

HWW ID	Date of collection	Hospital name (location)	Experiment	Storage (ºC)	Spiking	Physicochemical characteristics
HWW1 HWW2 HWW3	22/01/2015 04/03/2015 12/06/2015	Josep Trueta (Girona, Catalonia)	chapter 4	4	20 mg·L^{-1} of KTP and IBP	Table 4.1
HWW4 HWW5	14/09/2015 26/10/2015	Sant Joan de Déu (Barcelona, Catalonia)	chapter 5	4	-	Table 5.1
HWW6 HWW7	04/02/2016 07/04/2016		chapter 6	4	10 mg·L^{-1} of KTP, IBP and NP	Table 6.1
HWW8	05/09/2016		chapter 7	-20	-	Table 7.1
HWW9	30/03/2016		chapter 9	-20	-	-

method of homogenization of pelleted biomass. All manipulations were conducted under sterile conditions.

Hospital wastewater was freshly collected directly from the sewer manifold prior to every experiment. HWW date of collection, location, and other information is summarized in Table 3.1.

In chapter 8 a synthetic wastewater was used as described by Yang et al. (2013a) and contained, per liter: 0.5 g·L^{-1} malt extract broth (MEB), 0.2 g·L^{-1} KH_2PO_4, 0.05 g·L^{-1} $MgSO_4$ and 0.01 g·L^{-1} $FeSO_4$; the pH was adjusted to 4.5 with HCl and micropollutants CBZ, SMX, OXB, DCF and BPA were added to the wastewater at an initial concentration of 500 µg·L^{-1} each.

3.2 Medium and pellet formation

Fungal pellets were obtained by inoculating 2.7 mL L^{-1} (chapter 4), 4 mL·L^{-1} (chapter 5), 4 mL·L^{-1} or 20 mL·L^{-1} (chapter 6) and 20 mL·L^{-1}(chapter 7) of the mycelial suspension in a 2 L of defined medium adjusted to pH 4.5 (Table 3.2) in a sterile glass air-pulsed fluidized bioreactor. The pH was controlled at 4.5 by adding HCl 1 M or NaOH 1 M and the O_2 was measured to ensure proper aeration. Fluidized conditions in the reactors were maintained by using 1 s air pulse every 4 s. The aeration rate was 0.8 L min^{-1} and the temperature was maintained at 25°C.

In chapter 8 and chapter 9 pellets were obtained as previously described (Font et al., 2003): 1 mL of mycelial suspension was inoculated in a 1 L Erlenmeyer flask with 250 mL of 20 g·L^{-1} malt extract adjusted at pH 4.5. The flasks were maintained for 5-6 days under orbital agitation (135 rpm, r = 25 mm) at 25ºC, to finally obtain the pellets. Pellets were separated from the media with a strainer, washed with MilliQ water and resuspended in 0.8% v/v NaCl solution at a relation 1:1 v/v. The resultant pellets could be immediately

Table 3.2: Composition of the defined medium.

Component	Concentration
Glucose ($g{\cdot}L^{-1}$)	7
NH_4Cl ($g{\cdot}L^{-1}$)	2.1
Thiamine ($mg{\cdot}L^{-1}$)	10
Macronutrients ($mL{\cdot}L^{-1}$)	100
Micronutrients ($mL{\cdot}L^{-1}$)	10

Table 3.3: Composition of macronutrients and micronutrients of the defined medium.

Micronutrients	Concentration ($g{\cdot}L^{-1}$)	Macronutirents	Concentration ($g{\cdot}L^{-1}$)
Nitrile triacetic acidic	1.5	KH_2PO_4	20
$MgSO_4{\cdot}7H_2O$	3.0	$MgSO_4{\cdot}7H_2O$	5
$MnSO_4{\cdot}H_2O$	0.5	$CaCl_2$	1
NaCl	1.0		
$FeSO_4{\cdot}7H_2O$	0.1		
$CoSO_4{\cdot}7H_2O$	0.2		
$ZnSO_4{\cdot}7H_2O$	0.1		
$CuSO_4{\cdot}5H_2O$	0.01		
$AlK(SO_4)_2{\cdot}12H_2O$	0.01		
H_3BO_3	0.01		
Na_2MoO_4	0.01		

used or stored at 4ºC. All manipulations were conducted under sterile conditions.

3.3 Wastewater treatments

3.3.1 Coagulation-flocculation preliminary experiments

The coagulation-flocculation experiments in chapter 4 were carried out following ASTM D2035-13 guidelines (2013) in a jar-test apparatus (Flocculator SW1 from Stuart Scientific, Staffordshire, UK). Coagulants Hypol DW217 and Hyfloc AC50 and flocculants Himoloc JO2030 and Himoloc DR3000 were tested in jar tests which involved 2 min of coagulation at 200 rpm, 15 min of flocculation at 20 rpm and 30 min of settling.

3.3.2 UV-C irradiation preliminary experiments

In chapter 4 the HWW pretreated by coagulation-flocculation was stored at room temperature in a 2 L sterile glass bottle and stirred magnetically. This pretreated HWW was then pumped, using a 0.5 $L{\cdot}d^{-1}$ flow rate, into a Jebo UV-H9 UV-C sterilizer unit (9 W,

Table 3.4: Coagulant and flocculant concentrations used in the pretreatments.

HWW ID	Coagulant concentration (mg·L^{-1})	Flocculant concentration (mg·L^{-1})	Experiment
HWW1	150	15	chapter 4
HWW2	95	11	
HWW3	37	4.5	
HWW4	95	10	chapter 5
HWW5	190	20	
HWW6	85	10	chapter 6
HWW7	63	6	
HWW8	38	3.3	chapter 7
HWW9	43	4.8	chapter 9

mercury-arc low pressure lamp, 254 nm wavelength, JEBO, United States) with a residence time of 9.6 h. The effluent was collected in a sterile bottle until the volume required to fill the bioreactor was reached (2 L). Samples for the heterotrophic plate count and absorbance measurement at 650 nm were taken every 24 h.

3.3.3 Coagulation-flocculation pretreatment

Hospital wastewater was pretreated with a coagulation-flocculation process as described above (Section 3.3.1). Coagulant Hyfloc AC50 and flocculant Himoloc DR3000 were used in the concentrations summarized in Table 3.4.

3.3.4 Fungal treatments in fluidized bed reactor (FBR)

The fluidized bed bioreactors were used for the production of pelleted biomass and for the fungal wastewater treatments in FBR. Reactors employed were air-pulsed fluidised bed glass bioreactors with a working volume of 2 L (Figure 3.1). Temperature was maintained at 25ºC and pH was controlled at 4.5 by HCl 1M or NaOH 1M addition. Fungal pellets were kept inside the bioreactor by means of a mesh.

After the pellet growth phase in the reactor, the pellets were retained in the reactor, the medium was withdrawn and the reactor filled with the corresponding HWW. Before placement in the bioreactor, the influent was spiked or not according to Table 3.1. In chapter 4 two bioreactors were run in parallel, one operating continuously with a hydraulic residence time (HRT) of 3 days and the other operating as an SBR with a cycle of 3 d. The first experiment used raw HWW2, the second experiment used HWW1 with a coagulation-flocculation pretreatment, and the third experiment used HWW3 with a

coagulation-flocculation process followed by a UV irradiation step, as summarized in Figure 4.1. In chapter 5 two bioreactors were run in parallel, one inoculated with *T. versicolor* (RA) and one uninoculated control (RB), both operating continuously with a hydraulic residence time (HRT) of 3 days. HWW4 was used for the startup and during the first 29 days of operation of both RA (inoculated with *T. versicolor*) and RB (uninoculated control) bioreactors, whereas HWW5 was used for the following 27 days in both reactors. A partial biomass renovation strategy was carried out in the fungal bioreactor, as described by Blánquez et al. (2006), with 1/3 of biomass renovated every 7 days, which produced a cellular retention time (CRT) of 21 d. In chapter 6 the bioreactors operated continuously with an HRT of 3 days. Fluidized conditions in the reactors were maintained by using 1s air pulse every 4s or 0.5s air pulse every 4s, depending on the experiment, resulting in an aeration rate of 0.8 $L \cdot min^{-1}$ or 0.4 $L \cdot min^{-1}$, respectively. Carbon-to-nitrogen ratio was set to 7.5:1, 20:1 or 30:1 (mol carbon/mol nitrogen), depending on the experiment. Carbon source (glucose, $C_6H_{12}O_6$), and nitrogen source (ammonium chloride, NH_4Cl), were mixed in concentrated stock solutions to achieve the C:N ratios studied. In chapter 7 a single reactor was operated continuously with a hydraulic residence time (HRT) of 3 days. A partial biomass renovation strategy produced a cellular retention time (CRT) of 21 d (Blánquez et al., 2006). In chapter 9 the reactors were operated as a batch, per duplicate, for 7 d. When not specified, fluidized conditions in the reactors were maintained by using 1s air pulse every 4s, resulting in an aeration rate of 0.8 $L \cdot min^{-1}$. When not specified, nutrients for maintenance (glucose as a carbon source and and NH_4Cl as a nitrogen source) were added to the reactors with a molar C/N ratio of 7.5 at consumption rate (1200 mg glucose·g Fungal $DCW^{-1} \cdot d^{-1}$). The wastewater in the feed storage tank was replaced every 2-3 days by fresh wastewater.

3.3.5 Fungal treatments in membrane bioreactor (MBR)

Two identical MBR systems were operated for 70 days: one inoculated with the fungus and with partial biomass renovation (Fungal, F); and one inoculated with the fungus, with the same partial biomass renovation strategy and partial ozonation (Fungal-ozone, FO_3). The FO_3 was tested under two different ozonation intervals (twice a week or three times a week).

The partial biomass renovation strategy was based on Blánquez et al. (2006): one third of the reactor supernatant was withdrawn. The withdrawn media was centrifuged and the precipitated biomass was discarded, while fresh fungal biomass was mixed with the

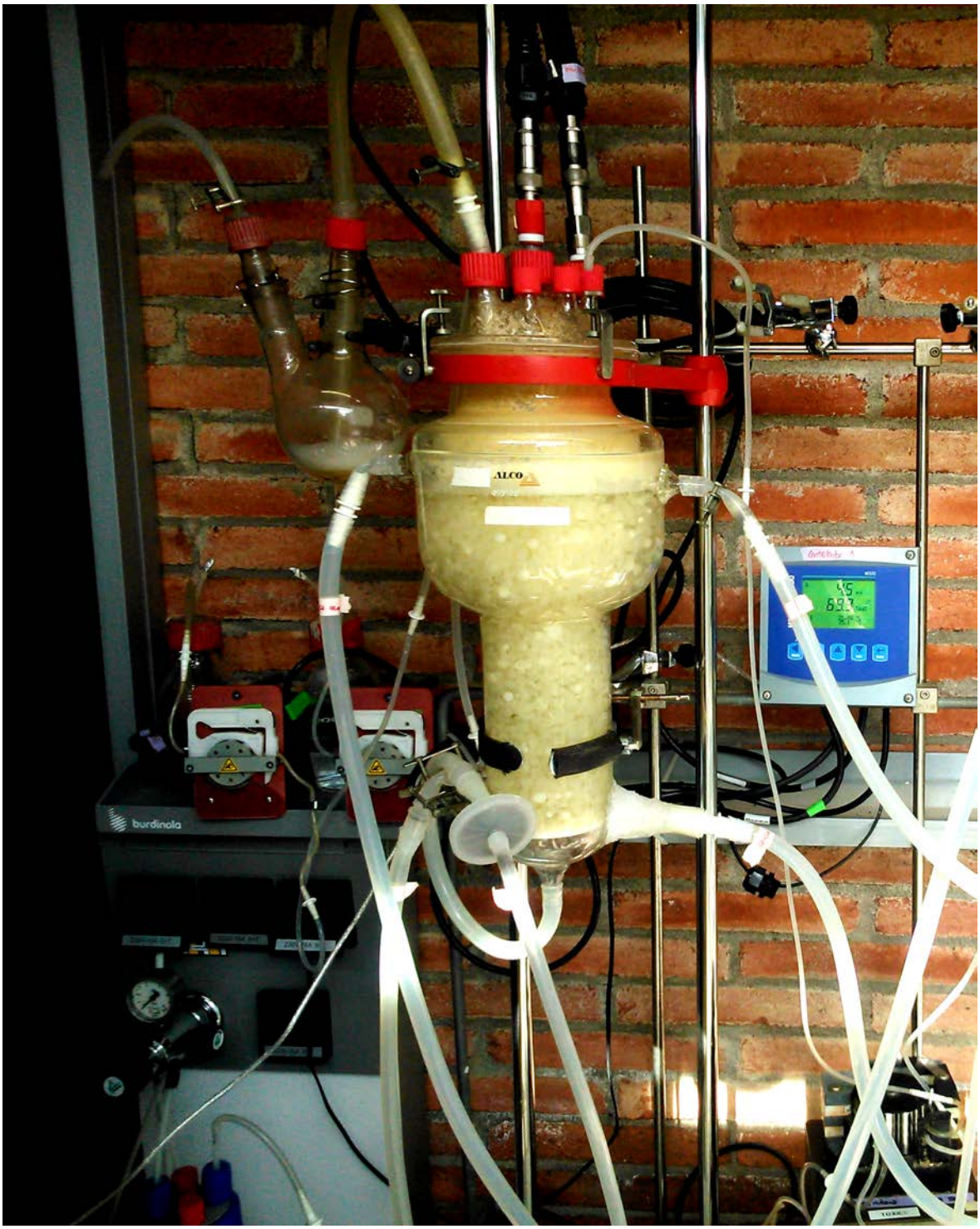

Figure 3.1: One of the 2 L air-pulsed bioreactors used in this thesis.

supernatant and added to MBR. This resulted in an SRT of 21 d. In the case of the FO_3 MBR, the partial ozonation was carried out as follows: 33% of the liquid media was ozonated for 5 min. with 10 mg $O_3 \cdot L^{-1}$ twice a week (first 35 days, corresponding to a 5% of weekly ozonated supernatant) or 50% of the liquid matrix three times a week (days 35-70, corresponding to a 10% of weekly ozonated supernatant). The ozone generator (CD10/AD, ClearWater Tech, USA) and ozone monitor (OM-1000, Biotek ozone, Taiwan) are described elsewhere (Fujioka et al., 2014).

The MBRs consisted of a glass reactor with a working volume of 1 L. The MBRs were maintained at 25ºC under non-sterile conditions. The fungal initial concentration in the inoculated reactors was 3 $g \cdot L^{-1}$. A membrane module with a coarse-pore membrane of pore size of around 100 µm was submerged in the reactors. The influent and effluent pumps were operated on on/off cycles of 8 min. and 2 min. resulting in an HRT of 12h. An aeration pump (ACO-002, Zhenjiang Sensen Industry Co. Ltd, China) supplied air and provided mixing of the reactor media. Samples were taken twice a week from the feed, the supernatant and from the permeate. In the case of the ozonated reactor, samples for HPLC analysis were also taken before and after the ozonation.

3.3.6 Activated sludge treatment

All the biodegradation tests with activated sludge were performed using a 1 L lab-scale Applikon stirred tank reactor coupled with a proportional-integral-derivative (PID) controller. The biomass originated from Celrà WWTP (Catalonia, Spain, 20,000 equivalent inhabitants, 2,100 $m^3 \cdot d^{-1}$), with a hydraulic retention time (HRT) of 48 h and a sludge retention time (SRT) of 20-22 days) (Collado et al., 2014). The biomass concentration during the experiments was 3 $gTSS \cdot L^{-1}$ and aerobic conditions (>2.5 mg $O_2 \cdot L^{-1}$) were achieved with a continuous air supply. pH was controlled at 7.5 and the temperature maintained at 25°C. Organic solution (sodium acetate, propionate and yeast extract), phosphate buffer, trace and inorganic solution were added as described elsewhere (Collado et al., 2013).

3.3.7 UV/H_2O_2 treatment

Photo-oxidation was carried out in a UV Laboratory Reactor System from UV-Consulting Peschl® which consists of an immersion-type photo-reactor with a working volume of approximately 550 mL. The UV lamp used was a 15 W Heraeus Noblelight TNN 15/32 low-pressure mercury vapor lamp emitting at 254 nm. The photo-reactor was covered with aluminum foil to minimize loss of UV light and avoid any reflections, and

magnetically stirred. Potassium ferrioxalate actionometry (Hatchard and Parker, 1956) was used to characterize the intensity of the light, resulting in an irradiance of 0.049 W cm^{-2} (Benito et al., 2017). The experiments were carried out with 500 mL of wastewater, 15 mg·L^{-1} of H_2O_2 and a reaction time of 10 minutes that corresponds to an irradiance of 29.4 J cm^{-2}. H_2O_2 concentration was analyzed by a spectrophotometric method using titanium (IV) oxysulfate (Shahbazi et al., 2014).

3.4 Degradation experiments

3.4.1 Batch fungal degradation experiments

In chapter 6: *T. versicolor* pellets (4 g wet weight) were inoculated in 250 mL Erlenmeyer flasks containing 50 mL of medium to give a dry cell weight concentration of 2 gDCW·L^{-1}. Flasks were incubated at 25ºC under orbital shaken conditions (135 rpm). The degradation medium contained, per liter: 0.8 g glucose, 0.19 g NH_4Cl, 1.168 g 2,2-dimethylsuccinate, 10 mL micronutrients and 100 mL macronutrients solution (Borràs et al., 2008). KTP, IBP and NP were added into the flasks at a final concentration of 10 mg·L^{-1} each. Experiments were conducted in dark, sterile conditions and per triplicate.

3.4.2 Batch ozonation tests

In chapter 8: 500 mL of MilliQ water and synthetic wastewater were spiked with 500 µg·L^{-1} of the cocktail of micropollutants. The media were ozonated for 5 min. with 10 mg O_3·L^{-1} ozone, conditions analogous to the ones used with the ozonation of the FO_3 reactor. Samples for micropollutant analysis were taken at the start and at the end of every batch. The tests were conducted in duplicate.

3.5 Analysis of pharmaceutically active compounds

3.5.1 Spiked wastewater

When spiked wastewater was used (namely, in chapter 4 and chapter 6, samples were filtered through a Millipore Millex-GV PVDF 0.22 µm membrane and placed in amber vials to avoid photodegradation. Analyses were carried out using a Dionex Ultimate 3000 HPLC system equipped with a UV detector. The separation was achieved on a GraceSmart RP 18 column (250mm x 4.6mm, particle size 5µm). In chapter 4 the mobile

phase consisted of 0.01 M K_2HPO_4 containing 2 g L^{-1} methane sulphonic acid adjusted to pH 6.5 (Pump A) and acetonitrile (Pump B). The flow rate was 1.5 mL min^{-1} and the eluent gradient started at 15% B and increased to 50% from 0 to 15 min; the gradient decreased to 15% B from 15 to 16 min and remained at 15% B from 16 to 20 min. In chapter 6 the mobile phase was MQ water adjusted at pH 3.5 with methane sulphonic acid (Pump A) and acetonitrile (Pump B). The flow rate was 1.5 mL·min^{-1} and the eluent gradient started at 20% B and increased to 30% up to 20 min; the gradient increased to 50% B from 20 to 28 min and decreased to 20% B from 28 to 30 min. In both methods a sample volume of 20 µL was injected from a Dionex autosampler and the detection was carried out at 210 nm. All determinations were performed at 30°C.

3.5.2 Synthetic wastewater

When synthetic wastewater was used (namely, chapter 8), micropollutant concentration in the fungal membrane bioreactor and permeate was measured at different time intervals using HPLC (Shimadzu, Kyoto, Japan) at the detection wavelength of 280 nm, as previously described by Asif et al. (2017). The HPLC system was equipped with a UV-Vis detector and C-18 column (300×4.6 mm) having a pore size of 5 mm (Supelco Drug Discovery, Sigma Aldrich, Australia). Milli-Q water buffered with 25 mM KH_2PO_4 and HPLC grade acetonitrile were used as the mobile phase for micropollutant quantification. Two eluents, namely eluent A (20% acetonitrile + 80% buffer, v/v) and eluent B (80% acetonitrile + 20% buffer, v/v), were passed through the C-18 column at a flow rate of 0.7 mL/min for 30 min in time dependent gradients as follows: [Time (min), A (%)]: [0, 85], [8, 40], [10, 0], [22, 0], [24, 85]. The limit of detection (LOD) for this method was approximately 10 µg·L^{-1}. Before micropollutants analysis, known standards of each micropollutant were analyzed to determine the time at which the peak of specific micropollutant appeared. After that, standards prepared from stock solution containing the mixture of selected micropollutants were analyzed to prepare the calibration curve (peak area vs. concentration). Coefficient of determination (R^2) for all the calibration curves was greater than 0.99.

3.5.3 Non-spiked wastewater

When non-spiked wastewater was used (namely, in chapter 5, chapter 7 and chapter 9), samples collected from the experiments were filtered through 0.45 µm PVDF filters (Millipore, Barcelona, Spain) and kept in PETcontainers at -20°C until PhAC analysis. The analytical procedure performed is based on Gros et al. (2012). Briefly, samples were

filtered through 1 μm glass fibber followed by 0.45 μm PVDF membrane filters (Millipore; Billerica, MA, USA) and an appropriate volume of Na_2EDTA was added to obtain a final concentration of 0.1% (w/w). Then, 25, 10 and 25 mL of sample (in chapter 5, chapter 7 and chapter 9, respectively) for raw HWW and 50, 25 and 50 mL (in chapter 5, chapter 7 and chapter 9, respectively) for treated wastewater were pre-concentrated by SPE (Solid Phase Extraction) using Oasis HLB (3 cc, 60 mg) cartridges (Waters Corp. Mildford, MA, USA), which were previously conditioned with 5 mL of methanol and 5 mL of HPLC grade water. Elution was done with 6 mL of pure methanol. The extracts were evaporated under nitrogen stream and reconstituted with 1 mL of methanol-water (10:90 v/v). 10 μL of internal standards mix at 1 $ng{\cdot}\mu L^{-1}$ in methanol were added in the extracts for internal standard calibration. Chromatographic separation was carried out with an Ultra-Performance liquid chromatography (UPLC) system (Waters Corp. Mildford, MA, USA), equipped with an Acquity HSS T3 column (50 mm x 2.1 mm i.d. 1.7 μm particle size) for the compounds analyzed under positive electrospray ionization (PI) and an Acquity BEH C18 column (50 mm × 2.1 mm i.d., 1.7μm particle size) for the ones analyzed under negative electrospray ionization (NI), both from Waters Corporation. The UPLC instrument was coupled to 5500 QqLit, triple quadrupole–linear ion trap mass spectrometer (5500 QTRAP, Applied Biosystems, Foster City, CA, USA) with a Turbo V ion spray source. Two MRM transitions per compound were recorded by using the Scheduled MRMTM algorithm and the data were acquired and processed using Analyst 2.1 software.

3.6 Microbial community analysis

Liquid samples from the reactors were filtered through 0.22 μm GV Durapore membrane filters (Merck Millipore, USA) and filters were stored at -80ºC. Pelleted biomass samples were centrifuged at 14,000 rpm and liquid fraction was discarded before cold-storage at -80ºC. Total DNA extraction was conducted using the PowerWater and PowerSoil DNA Isolation Kits (MoBio Laboratories, USA) for filters and pellets, respectively. 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 primer 341f. Final concentrations of the PCR reactions consisted of 1x PCR buffer, 2 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 consisted of: 94ºC for 5 min; 20 cycles of 94ºC for 1 min, 65ºC for 1 min (-0.5ºC/cycle), 72ºC for 3 min; 15 cycles of 94ºC for 1 min, 55ºC for 1 min,

72ºC for 3 min; and a single final extension of 72ºC for 7 min. Fungal DNA was amplified using a nested approach over a ~400 bp fragment from the internal transcribed spacer (ITS) of fungal ribosomal RNA gene. 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' side of primer ITS1f and PCR reactions had the same final concentrations except for $MgCl_2$ (1.5 mM). PCR program for fungi was identical for both amplification rounds and consisted of: 95ºC for 5 min; 35 cycles of 94ºC for 30 s, 55ºC for 30 s, 72ºC for 30 s; and a single final extension 72 ºC for 5 min.

Denaturing gradient gel electrophoresis (DGGE) was performed using the Dcode 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 30-70% 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 60ºC, stained with 1 µg/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 analysed 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 using micropipette tips, placed inside Eppendorf tubes containing 15 µl of MQ water and stored for 24 hours at 4ºC to allow DNA diffusion. Amplified DNA fragments from recovered gel bands were placed in 96-well plates and sequenced by Macrogen (South Korea). Obtained sequences were trimmed with FinchTV software and checked for chimeras using Mothur(Schloss et al., 2009). Each 16S rRNA sequence was assigned to its closest neighbor according to the Basic Local Alignment Search Tool (BLAST) results (Altschul et al., 1997).

3.7 Toxicity tests

Two types of toxicity tests were carried out in different experiments. The toxicity test used in chapter 5 was a Microtox acute toxicity bioassay kit from Azur Environmental (Carlsbad, US). Briefly, the test is based on the diminution of bacterial bioluminescence of *Allivibrio fischeri* (formerly known as *Vibrio fischeri*) after 5 and 15 min of exposure to dilutions of the samples (pH 7). Toxicity was expressed as toxicity units (TU) and the samples were collected weekly from the bioreactors.

The toxicity test used in chapter 8 was the bacterial luminescence toxicity screen (BLT-Screen) method described by van de Merwe and Leusch (2015). The method is based on measuring bioluminescence inhibition in *Photobacterium leiognathi*. In brief, lyophilized bacteria were rehydrated in hydration buffer and incubated at 4°C for 24 h prior to use. Five microliters of the luminescent bacteria was then added to each well of a 96-well plate containing samples serially diluted with buffered saline assay media using a multi-channel pipette. After 30 min the luminescence of each well was measured on a Fluostar plate reader (BMG Labtech, Germany). Serially diluted standard curves of pentachlorophenol (which was used as the reference compound) and Milli-Q water (negative control) in phosphate buffered saline assay media were included in duplicate for quality control. The inhibition of luminescence relative to controls (regarded as *toxicity*) was plotted against sample concentration, and the IC_{20} (concentration that causes 20% inhibition of bacterial luminescence) was calculated from the straight line regression. In the BLT-Screen, a reduction in bacterial luminescence >20%, expressed as $TU_{IC(REF)_{20}}$ higher than 1, indicates toxicity. MBR feed and permeate samples were tested in duplicate for toxicity.

3.8 Other analyses

Glucose concentration was measured per triplicate with an YSI 2700 SELECT enzymatic analyzer (Yellow Spring Instruments). Laccase activity was measured per triplicate using a modified version of the method for the determination of manganese peroxidase where 2,6-dimetoxyphenol (DMP) is oxidized by laccase in the absence of a cofactor (Kaal et al., 1993). Changes in the absorbance at 468 nm were monitored for 2 min on a Varian Cary 3 UV/Vis spectrophotometer at 30°C. Activity units per liter (U L^{-1}) are defined as the micromoles per liter of DMP oxidized per minute. The molar extinction coefficient of DMP was 24.8 mM^{-1} cm^{-1} (Wariishi et al., 1992). In chapter 8 laccase activity was measured as described in (Hai et al., 2009): briefly, activity was measured by monitoring the change in absorbance at 468 nm due to the oxidation of 2,6-dimethoxy phenol (DMP) at room temperature over 2 minutes using a spectrophotometer (PharmaSpec UV-1700, Shimadzu, Kyoto, Japan). Mycelium bound laccase activity was determined as in Yang et al. (2013a) and presented as U·mg $MLSS^{-1}$.

The conductivity was determined by a CRISON MicroCM 2100 conductometer, and the absorbance at 650 nm was monitored by a UNICAM 8625 UV/VIS spectrometer. Heterotrophic plate count (HPC) was analyzed per triplicate according to APHA-AWWA-WEF (1995). Chloride, sulfate, nitrite, nitrate and phosphate anions were

quantified by a Dionex ICS-2000 ionic chromatograph. The total suspended solids (TSS), dissolved inorganic carbon (DIC) and dissolved organic carbon (DOC) were determined according to APHA-AWWA-WEF (1995). In chapter 8, DOC and total nitrogen (TN) were analysed simultaneously by a Shimadzu TOC/TN-VCSH analyser (Japan). Mixed liquor suspended solids (MLSS) concentration and mixed liquor volatile suspended solids (MLVSS) concentration were determined following APHA-AWWA-WEF (1995) guidelines. The N-NH_4^+ concentration and chemical oxygen demand (COD) were analyzed by using commercial kits LCH303 and LCK114 or LCK314, respectively (Hach Lange, Germany). Pellet size was determined with a Zeiss Axioskop microscope (Oberkochen, Germany). Transmembrane pressure (TMP) was monitored with a vacuum gauge (Model 840064, Sper Scientific Ltd. USA).

Soluble microbial product (SMP) and extracellular polymeric substances (EPS) samples were taken weekly, extracted according to (Zhang et al., 1999) and were normalized as the sum of protein and polysaccharide contents. Protein content was determined by the Folin method using bovine serum albumin as standard and polysaccharide content was measured based on the phenol-sulphuric acid method with glucose as the standard.

3.9 Statistical analysis

As recommended by Bolks et al. (2014), a robust Regression on Order Statistics (ROS) approach was used for dealing with left-censored data, namely: below limit of detection (BLD) and below limit of quantification (BLQ) values. When ROS was not possible, values BLD and BLQ were considered to have a concentration half of the limit of detection and half of the limit of quantification, respectively (EPA, 2000). In chapter 7, when ROS was not possible, values BLD and BLQ were considered to have a concentration BLD/sqrt(2) and BLQ/sqrt(2), respectively (Tekindal et al., 2017). ROS analysis was performed using the R package NADA (Lee, 2013). Data analysis and calculations like summary statistics, pharmaceutical removals and one-factor analysis of variance (ANOVA) were performed with R: A language and environment for statistical computing (R Core Team, 2015). Differences were considered as significant at $p < 0.05$.

4

Pretreatments and operational strategies

Continuous treatment of non-sterile hospital wastewater by *Trametes versicolor*: How to increase fungal viability by means of operational strategies and pretreatments

Based on the homonymous article Mir-Tutusaus, J.A., Sarrà, M., Caminal, G., 2016. Journal of Hazardous Materials 318, 561–570. doi:10.1016/j.jhazmat.2016.07.036

Abstract

Hospital wastewaters have a high load of pharmaceutical active compounds (PhACs). Fungal treatments could be appropriate for source treatment of such effluents but the transition to non-sterile conditions proved to be difficult due to competition with indigenous microorganisms, resulting in very short-duration operations. In this article, coagulation-flocculation and UV-radiation processes were studied as pretreatments to a fungal reactor treating non-sterile hospital wastewater in sequential batch operation and continuous operation modes.The influent was spiked with ibuprofen and ketoprofen, and both compounds were successfully degraded by over 80%. UV pretreatment did not extent the fungal activity after coagulation-flocculation measured as laccase production and pellet integrity. Sequential batch operation did not reduce bacteria competition during fungal treatment. The best strategy was the addition of a coagulation-flocculation pretreatment to a continuous reactor, which led to an operation of 28 days without biomass renovation.

4.1 Introduction

There is a growing concern among regulatory agencies and in the scientific community about pharmaceutical active compounds (PhACs) occurring in environmental water bodies. Although healthcare waste management is imperative, hospital wastewater (HWW), with similar pollutant load as urban wastewater but much higher concentrations of PhACs, is still commonly discharged into public sewage systems. HWW constitutes the main source of PhACs in the influent of wastewater treatment plants (WWTP), which are not designed to remove these pollutants (Verlicchi et al., 2015; Frédéric and Yves, 2014). Consequently, these effluents could be a vector to introduce PhACs in the environment through wastewater reuse purposes such as irrigation, landscape and surface or groundwater replenishment (Fatta-Kassinos et al., 2011). Therefore, a specific HWW treatment step before the HWW is mixed with urban wastewater would prevent PhAC contamination of the WWTP influent. Among the potential technologies for removing recalcitrant organic pollutants in wastewater, fungal treatment is a particularly promising strategy to biodegrade those pollutants due to their unspecific enzymatic systems. Several studies have reported the capacity of white rot fungi (and particularly *T. versicolor*) in degrading a wide range of emerging pollutants including PhACs (Marco-Urrea et al., 2009; Nguyen et al., 2014). These studies have been carried out at different scales, from within Erlenmeyer flasks to bench bioreactors, and they have been conducted mainly in sterile conditions to best monitor the fungal degradation during the batch process. The studied pollutant concentrations are on the order of a few mg L^{-1}, but the typical concentrations in wastewater are on the order of 1 ng L^{-1} (Badia-Fabregat et al., 2015b; Cruz-Morató et al., 2014).

Moreover, the long-term operation of fungal biodegradation processes during a continuous fungal treatment of a synthetic textile wastewater, in sterile conditions, was demonstrated with a cellular retention time (CRT) of 21 days (Blánquez et al., 2006). The biomass was retained in the reactor but periodic partial biomass removals were performed to limit the aging of the biomass and consequently to guarantee that metabolic activity occurred under growth-limiting conditions. However, the partial biomass renovation strategy was not enough to maintain fungus viability during the treatment of HWW under non-sterile conditions (Badia-Fabregat et al., 2015a). Few other references can be found that investigate the treatment of non-sterile wastewater by fungi in a continuous mode. The non-favorable competition between the inoculated fungus and the microorganisms in the real wastewater demonstrated the difficulty of developing an efficient treatment on an industrial scale due to the relatively short fungal

viability time (Badia-Fabregat et al., 2015a; Cruz-Morató et al., 2013a; Yang et al., 2013a; Zhang and Geißen, 2012).

A novel strategy to extend the fungal viability period has not yet been established and is essential to guarantee the long-term operation for the continuous treatment of HWW, which may be achieved through a continuous pumping of the influent or by sequential batch reactor (SBR) operation. In both strategies the biomass is retained in the bioreactor. In addition, a pretreatment for the HWW may reduce the bacteria level in the influent of the fungal bioreactor and consequently extend its viability. These processes include coagulation-flocculation and UV radiation, both methods widely used in WWTP (López-Maldonado et al., 2014; Tzfati et al., 2011). Their technology can be readily applied, but none of the methods have been used as pretreatments before.

Therefore, the main objective of this study is to increase the *T. versicolor* viability inside the reactor during a continuous treatment of non-sterile HWW. To achieve this, the following two approaches have been examined: first, the addition of two pretreatments, a coagulation-flocculation step and a UV irradiation step, and second, the continuous operation of the reactor and a SBR. The influence of both strategies on the viability period has been studied. The HWW was spiked with ibuprofen (IBP) and ketoprofen (KTP) for analytical purposes.

4.2 Results and discussion

Three hospital wastewaters, HWW1 through HWW3, were characterized physically, chemically and biologically (Table 4.1). Most of the parameters are in the same range as other hospital effluents with the exception of conductivity and chloride concentration, which were higher than those reported (Cruz-Morató et al., 2014; Verlicchi et al., 2015). Although their concentrations were not uncommonly high, the COD and TSS concentrations in HWW3 were twice that in HWW1 and HWW2, while the ammonia concentration was very low. HWW3 also had the highest chloride concentration (15,723 mg Cl L^{-1}). However, HWW1 had the greatest HPC, $4.3 \cdot 10^9$ cfu mL^{-1}, while HWW2 and HWW3 had counts in the 10^7 cfu mL^{-1} range.

Table 4.1: Physical, chemical and biological characterization of hospital wastewater.

Sampling date		22/01/2015		04/03/2015			12/06/2015
Experiment		HWW1		HWW2			HWW3
	Raw	Flocculated	Raw	Flocculated	Raw	Flocculated	Flocculated & UV
pH	6.84	8.12	7.07	7.08	7.2	7.3	7.93
Conductivity (mS·cm^{-1})	1.23	1.16	1.72	1.82	4.69	4.76	4.46
Absorbance at 650 nm	0.189	0.000	0.154	0.012	0.353	0.006	0.009
HPC (cfu·mL^{-1})	$4.3{\cdot}10^{9} \pm 3.5{\cdot}10^{8}$	$7.5{\cdot}10^{5} \pm 2.2{\cdot}10^{5}$	$7.4{\cdot}10^{7} \pm 1.7{\cdot}10^{7}$	$5.4{\cdot}10^{4} \pm 7.0{\cdot}10^{3}$	$1.9{\cdot}10^{7} \pm 6.1{\cdot}10^{6}$	$1.8{\cdot}10^{6} \pm 5.2{\cdot}10^{5}$	$4.0{\cdot}10^{5} \pm 2.9{\cdot}10^{5}$
Chloride (mg Cl·L^{-1})	1603.8	1810.6	1874.0	2073.4	15723.7	16125.9	15372.9
Sulfate (mg S·L^{-1})	31.6	25.6	42.0	37.5	19.8	25.9	23.4
Nitrate (mg N·L^{-1})	170.6	190.1	239.9	223.6	236.7	287.3	282.8
Phosphate (mg P·L^{-1})	0.2	0.5	0.1	0.1	0.7	0.0	0.2
Ammonia (mg N · L^{-1})	15.5	8.0	24.4	8.5	0.2	1.8	0.1
TSS (mg·L^{-1})	150	30	145	55	350	105	55
COD (mg O2·L^{-1})	343 ± 13	108 ± 4	293 ± 15	297 ± 1	614 ± 20	313 ± 2	44 ± 3
DIC (mg·L^{-1})	77 ± 3	63 ± 3	117 ± 3	95 ± 1	76 ± 3	73 ± 2	80 ± 2
DOC (mg·L^{-1})	49 ± 7	47 ± 3	56 ± 3	87 ± 2	99 ± 9	90 ± 6	21 ± 4

4.2.1 Study of the pretreatments

4.2.1.1 Coagulation-flocculation

The type and dosage of the coagulants are two of the most important parameters to consider when optimizing this process (Saritha et al., 2015). Hypol DW217 and Hyfloc AC50 were evaluated as coagulants at concentrations of 1 – 5 mg L^{-1} and 150 – 300 mg L^{-1}, respectively, and Himoloc JO2030 and Himoloc DR3000 were evaluated as flocculants at a concentration of 15 – 30 mg L^{-1}, as recommended by the company who provided these products. Table 4.2 shows the experimental design and the obtained results. Coagulant Hypol DW217 produced consistently low absorbance reductions, independently of the flocculant type and concentration used, while coagulant Hyfloc AC50 achieved zero absorbance values with both flocculants and at almost all tested concentrations. Consequently, Hyfloc AC50 was considered as a suitable coagulant for reducing the absorbance of HWW. The results were consistent regardless of the flocculant used, even when taking into account that Himoloc DR3000 is a high molecular weight flocculant with medium cationicity and Himoloc JO2030 is a very high molecular weight flocculant with high anionicity. Experiment 15, nonetheless, showed a typical coagulant overdose behavior visible only with DR3000 (López-Maldonado et al., 2014; Saritha et al., 2015; Zhao et al., 2012). This result suggests that the different characteristics of the two flocculants did indeed play a distinct role in the coagulation-flocculation process. Finally, products Hyfloc AC50 and Himoloc DR3000 were chosen as the coagulant and flocculant, respectively.

The final concentrations of the coagulant and flocculant had to be adjusted for every HWW. Table 4.3 summarizes the doses used in each pretreatment, while Table 4.1 shows the physical, chemical and biological characterization of the three raw and pretreated wastewaters used. In general, pretreated samples have similar or lower parameters than

Table 4.2: Absorbance reduction of different coagulant-flocculant mixtures. Absorbance of the initial raw wastewater was 0.189.

Experiment ID	Coagulant concentration (mg·L^{-1})		Flocculant concentration (mg·L^{-1})		Final absorbance at 650 nm
1	Hypol DW217	1	Himoloc JO2030	15	0.155
2		1		30	0.174
3		5		15	0.144
4		5		30	0.141
5	Hyfloc AC50	150	Himoloc JO2030	15	0.008
6		150		30	0.000
7		300		15	0.000
8		300		30	0.000
9	Hypol DW217	1	Himoloc DR3000	15	0.145
10		1		30	0.133
11		5		15	0.078
12		5		30	0.131
13	**Hyfloc AC50**	**150**	**Himoloc DR3000**	**15**	**0.000**
14		150		30	0.000
15		300		15	0.129
16		300		30	0.000

Table 4.3: Absorbance reduction of different coagulant-flocculant mixtures. Absorbance of the initial raw wastewater was 0.189.

HWW	Coagulant concentration (mg·L^{-1})	Flocculant concentration (mg·L^{-1})
1	150	15
2	95	11
3	37	4.5

the raw wastewaters (Liu et al., 2012). The HPC reduction achieved after the coagulation-flocculation pretreatment is very important, with reductions by factors of 10 to 10^4. Additionally, a substantial reduction in TSS is observed in all 3 cases, although a reduction in COD is only observed in 2 out of 3 HWWs tested.

4.2.1.2 UV-C irradiation

UV disinfection is a widely known technology used as a tertiary treatment in WWTP as an attractive alternative to chlorination/dechlorination processes because it is a chemical-free process, reduces the presence of potential chlorinated hydrocarbons and also deactivates chlorine-resistant microorganisms (Hijnen et al., 2006; Tapas K. Das, 2001). However, its use as a pretreatment is much less studied. The experiments were planned as preliminary work to determine whether UV-C irradiation could be a suitable pretreatment for the bioreactor influent.

Several studies highlight the role of the concentration and size of the suspended solids in UV disinfection (Hijnen et al., 2006; Wang et al., 2006). Particles provide shelter to microorganisms against UV by absorbing or reflecting the UV. This can be partially

overcome by using higher intensities and contact times, although the improvement is still limited. Das (2001) proposed 5–10 mg L^{-1} of TSS as an acceptable range in which UV disinfection is effective. The TSS of the raw HWW used in the study ranged from 145 – 350 mg L^{-1}, which is much higher than the maximum of that acceptable range and could thus explain the ineffectiveness of the UV treatment. However, the flocculated HWWs had a TSS level of 30 – 105 mg L^{-1}, still above the recommended range. Nevertheless, a 10 to 100 – fold diminution of HPC was obtained. This may be explained by the long contact time used in the experiments. No significant reduction in absorbance was found, due to the flocculated HWW having values very close to zero. Therefore, UV-C irradiation was applied as a pretreatment only after a coagulation-flocculation process.

4.2.2 Bioreactor operation

The testing of both the HWW pretreatment and the operation mode was performed to find approaches that increase the duration of *T. versicolor* viability in the bioreactor. Although widely used in wastewater and drinking water treatment plants, coagulation-flocculation and UV-radiation had not been studied as pretreatments for a fungal operation. As summarized in Figure 4.1, three experiments were conducted with (A) no pretreatment, (B) coagulation-flocculation pretreatment and (C) coagulation-flocculation followed by a UV-radiation pretreatment. In each experiment, two reactors were operated simultaneously: one in continuous mode (1) and one in SBR mode (2). As reviewed elsewhere, the SBR operation would act as selective disinfection, namely, the elimination of undesired microorganisms while retaining the fungal biomass (Espinosa-Ortiz et al., 2015). Both continuous and SBR modes had an HRT of 3 d, as the processes of pellet settling and liquid phase draining and refilling in the SBR took only 15 min and 95% of the liquid could be removed. Glucose and NH_4Cl were added at their consumption rates to keep the fungus in growth-limiting conditions and to prevent COD increases in the effluent. A low carbon-to-nitrogen ratio of 7.5 was chosen to enhance the fungus survival over the native bacteria (Rousk and Bååth, 2007).

HPC, laccase activity, and IBP and KTP removal were monitored; the results are presented in Figure 4.2, Figure 4.3 and Figure 4.4, respectively. The operation was stopped after repeated measures of the IBP and KTP degradation percentage dropped below 80%.

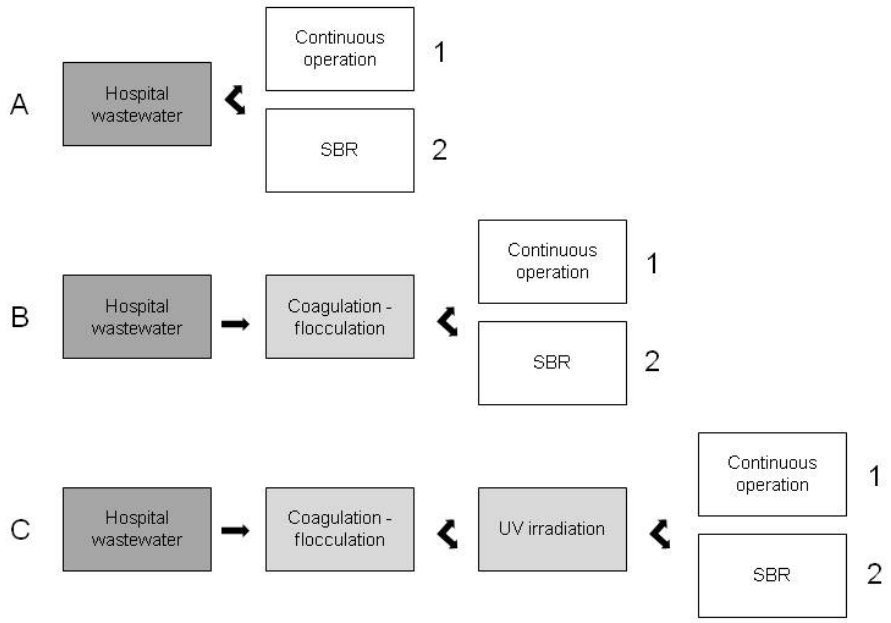

Figure 4.1: Bioreactors experiments diagram. A, B and C are the experiment ID; 1 and 2 represent the mode of operation. Grey boxes represent the pretreatment applied to the HWW.

4.2.2.1 Heterotrophic plate count evolution

Most of the studies found in the literature focus on degradation of selected compounds in sterile conditions or non-sterile tap water (Marco-Urrea et al., 2009; Mir-Tutusaus et al., 2014; Nguyen et al., 2013; Yang et al., 2013a). However, Espinosa-Ortiz et al. (2015) highlighted the necessity of working in non-sterile matrices. In real non-sterile hospital wastewater, concentration of microorganisms, expressed as HPC, is especially relevant. Figure 4.2 shows the HPC results for all the experiments. In experiment A, with no pretreatment, both the continuous operation and the SBR exhibited a 100-fold increase in the initial concentration of heterotrophs after 3 d of treatment, with an HPC of approximately 10^8 cfu·mL^{-1} after 6 d operation. Experiment B, with coagulation-flocculation pretreatment, exhibited a slight increase in the continuous treatment during the 27 d of treatment, which led to a 100-fold HPC increase by the end of the process. However, increases by factors of 10-1000 were observed in each 3 d SBR cycle. The dramatic change in HPC can be explained by the native HWW microorganisms consuming the glucose and NH_4Cl added in addition to the nutrients in

the wastewater itself. The resulting heterotroph population could be adhered to the fungal biomass and suspended solids. The final HPC was approximately 10^7 cfu·mL^{-1} for the continuous treatment and approximately 10^8 cfu·mL^{-1} for the SBR. In experiment C (Figure 4.2), with a coagulation-flocculation pretreatment followed by a UV radiation step, the initial concentration of heterotrophic microorganisms was lower than the other two experiments (approximately 10^4 cfu·mL^{-1}); however, a very rapid 1000-fold increase was detected within the first 10 days of operation in both treatment strategies. This low initial HPC was due to the UV-radiation step, while the fast increase could be either due to the regrowth of microorganisms sheltered by suspended solids or to growth of resistant bacteria due to decreased competition with other bacteria, many of which were eliminated by the UV radiation (Lee et al., 2015). The HPC increased until it reached a concentration of 10^8 cfu mL^{-1} in the 10th day of continuous operation but stabilized at approximately 10^6 cfu mL^{-1}. The HPC in the SBR peaked at 10^9 cfu mL^{-1} in the 4th cycle and remained at 10^8 cfu mL^{-1} in the last batch. There seems to be a threshold HPC of approximately 10^8 cfu mL^{-1}, beyond which the operation could not continue, regardless of the pretreatment or operation mode. In addition, the continuous mode exhibited a consistently lower HPC than that of the SBR mode.

4.2.2.2 Laccase activity profile

Figure 4.3 shows the laccase profiles measured in all three experiments. Experiment A exhibited a maximum activity of approximately 50 UA·L^{-1} at Day 4 for both operation modes. The laccase profile diminished from that day up to undetectable levels after 9 d. In experiment B, laccase peaked at the end of the first cycle but then dropped significantly after the second cycle. In subsequent cycles, the laccase production was recovered up to the initial cycle level during the 5th cycle; afterward, the laccase activity showed an important reduction following each additional cycle. However, low laccase production was observed in the continuous reactor. Even when the measured laccase activity decreased, it is evident that *T. versicolor* was able to produce enzymes because the laccase profile stayed above the theoretical laccase activity assuming zero production, shown as dotted lines in the stages in Figure 4.3. Finally, in experiment C, the laccase profiles showed a maximum peak at Day 6 for the continuous treatment or 2nd batch for the SBR. Afterward, the laccase production decreased dramatically, and no laccase activity was detected after the 4th batch or in the 14th day of continuous operation. It is noteworthy that in experiment C, the theoretical laccase activity assuming no production was higher than the actual activity in the reactor, indicating

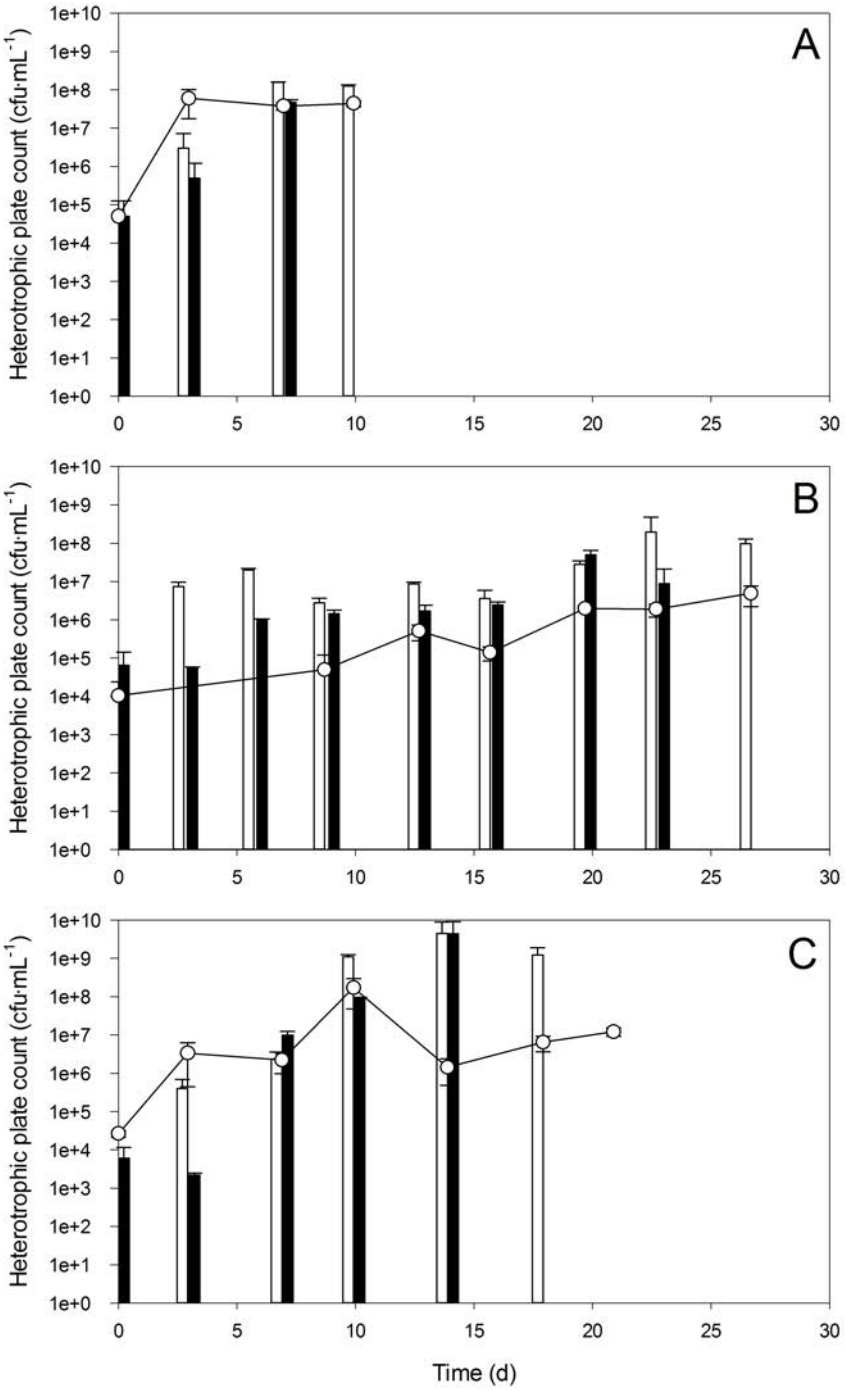

Figure 4.2: Heterotrophic plate count profile in the reactors with no pretreatment (A); with a flocculation step (B); with a flocculation and a UV-radiation step (C). Legend: black bars represent the initial concentration in the SBR treatment; white bars, the final concentration in the SBR treatment; white circles, the concentration profile in the continuous treatment.

deactivation of the enzyme. This may be explained as an effect of bacterial competition, which has been reported to inhibit fungal enzyme secretion capacity and to inactivate the secreted enzyme (Heinfling et al., 1998; Libra et al., 2003).

Although laccase can be produced by both fungi and bacteria (Gianfreda et al., 1999), the control experiments with HWW demonstrated that the production of laccase by its native bacterial microorganisms was not significant (data not shown). Consequently, the laccase production may be confidently linked to fungus activity. However, low or nonexistent laccase activity cannot be the sole indicator of *T. versicolor* inactivity. Previous studies have detected the presence of the fungus with denaturing gradient gel electrophoresis (DGGE) in continuous treatments where laccase activity was very low and HWW could inhibit the laccase activity assay (Badia-Fabregat et al., 2015b). Several other continuous-mode fungal water treatment operations have reported very low or no extracellular enzymatic activity (Gao et al., 2008; Yang et al., 2013a).

4.2.2.3 Ibuprofen and ketoprofen degradation

Ibuprofen and ketoprofen were the PhACs selected to spike the HWW. Both pharmaceuticals are among the most widely used anti-inflammatory drugs, and both can be degraded by *T. versicolor* (Marco-Urrea et al., 2009, 2010b; Tran et al., 2010).

The results for the rates of IBP and KTP degradation are presented in Figure 4.4. In experiment A, in the continuous treatment, 90% of ibuprofen was degraded, while ketoprofen degradation rates were below 70% after day 5. In the SBR mode, both IBP and KTP had degradation rates of 80% and above for the first and third batches. The experiment was finally stopped at day 12 when the threshold degradation was not reached, and the breakdown of the fungal pellets into free mycelium was observed. In experiment B, the elimination rates of both pharmaceuticals stayed above 80% for the majority of the continuous operation. During the SBR operation, KTP degradation was approximately 90% during the first 5 cycles but decreased significantly thereafter. However, IBP was completely degraded during 7 cycles and only was detected in the last cycle. Both reactors were operated for 28 days until the degradation rates fell below 80%. Other signals of low fungal activity were also observed, including low laccase production and the loss of original biomass pellet shape. In experiment C, the degradation of IBP remained constant at 78% during the first 14 days of continuous treatment but then increased up to 95%. KTP degradation, however, was higher than 80% until Day 11 when it quickly dropped to degradation rates lower than 40%. During SBR operation, KTP degradation was higher than 80% during all cycles, but the removal of KTP removal was

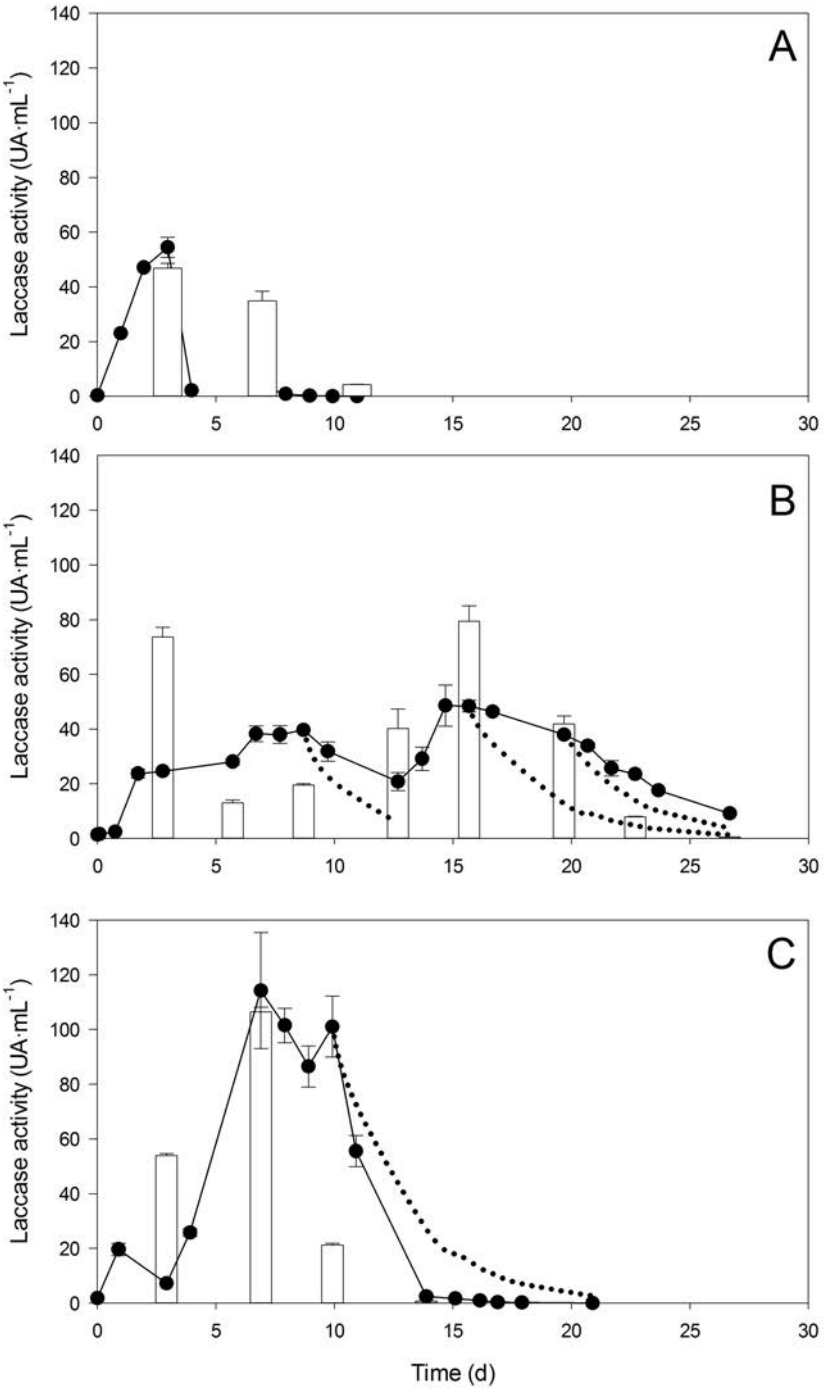

Figure 4.3: Laccase activity profile with no pretreatment (A); with a flocculation step (B); with a flocculation and a UV-radiation step (C). Legend: bars represent the final concentration in the SBR treatment; circles, the activity profile in the continuous treatment; dotted lines represent the theoretical concentration of laccase if no generation is assumed.

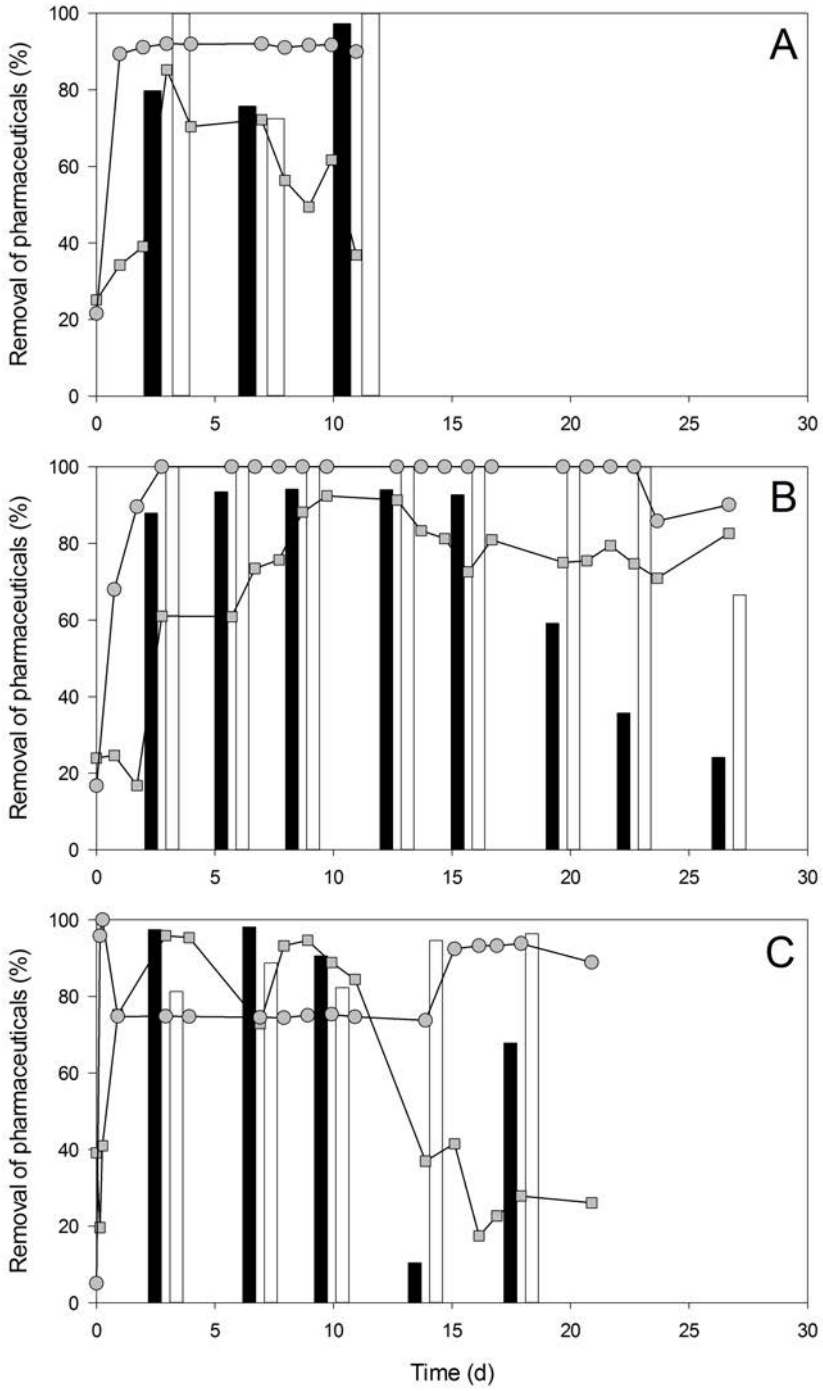

Figure 4.4: Removals of ketoprofen and ibuprofen with no pretreatment (A); with a flocculation step (B); with a flocculation and a UV-radiation step (C). Legend: black bars and white bars represent the ketoprofen and ibuprofen degradation, respectively, in the SBR; grey squares and grey circles represent the ketoprofen and ibuprofen degradation, respectively, in the continuous treatment.

high only in the three first cycles. At the end of fourth cycle, the degradation was minimal although it recovered to approximately 65% in the fifth cycle, when the experiment was stopped due to low fungal activity. Few references can be found regarding IBP and KTP removal in wastewaters. Badia-Fabregat et al. (2015a) reported a 67% and 46% removal of IBP and KTP, respectively in sterile non-spiked urban wastewater, lower than the 80% threshold used in the present article. Although in sterile conditions, Nguyen et al. (2013) reported a very high 90% removal of ketoprofen in a fungus-augmented membrane bioreactor.

The results of experiments A and B indicated that ibuprofen was eliminated faster than ketoprofen during long-term operation in both continuous and SBR strategies, although the results of experiment C are slightly different. These results agree with previous reports (Badia-Fabregat et al., 2015a; Nguyen et al., 2014). The pathway of fungal degradation of IBP remains unknown, but it does not involve laccase. This result is in agreement with the present results, as IBP was removed when no laccase activity was detected. This pathway does not involve the cytochrome P450 system either. The UV pretreatment seemed to decrease the removal of IBP during the early reactor operation, which is possibly related to its distinct degradation pathway. Ketoprofen was eliminated in both reactors; this is consistent with previous reports, which state that KTP is degraded by *T. versicolor*, with the P450 system involved in the degradation and the laccase system playing only a minor role Marco-Urrea et al. (2010b). As a result, ketoprofen was degraded even when no laccase activity was detected in the bioreactor. To conclude, IBP and KTP have been effectively eliminated in both reactors when treating non-sterile HWW with long-duration treatments.

Experiment B, which used a coagulation-flocculation pretreatment before the fungal reactor, was selected as the best strategy because it exhibited the longest viability period of 28 d. This viability period could be further extended by implementing a partial biomass renovation strategy to overcome the biomass aging process. The sequential batch reactor operations showed better IBP and KTP reductions than continuous operations in experiments A and C but not in experiment B, where the continuous operation maintained elimination between 70 – 90% for a long period of time. As a result, a second step in the pretreatment of HWW effluent did not improve the fungal bioreactor. In addition, the use of the continuous operation mode was chosen as the best strategy for long-term fungal degradation with fungal biomass retained in the bioreactor. This strategy has operational advantages, such as requiring less labor because it is easy to automate. To the best of our knowledge, this is the first time that an air-pulsed fungal reactor without biomass renovation treated non-sterile HWW with high elimination

yields for 28 days.

4.3 Conclusions

Pretreating HWW with a coagulation-flocculation process can reduce the initial HPC level up to 10^4 cfu mL^{-1}, which extended the fungal viability during the continuous treatment of a non-sterile real effluent. The addition of a UV pretreatment did not lead to better performance of the fungal bioreactor in terms of higher PhAC reductions or longer operation time. Testing of the operation modes indicated that continuous operation is preferred over SBR because the reduction capacity of *T. versicolor* was maintained for a longer period. The optimal treatment setup, a coagulation-flocculation pretreatment coupled to a fungal treatment in an air-pulsed fluidized bioreactor with retained biomass in pellet form, maintained fungal activity for 28 days without purging or biomass renovation. Consequently, a length of operation similar to the operation in sterile conditions was achieved. Future work should consider partial biomass renovation to further extend the viable duration of fungal degradation and to overcome the biomass aging process, which are essential requirements to guarantee longer-term continuous HWW treatment.

5

Validation of pretreatment

Pharmaceuticals removal and microbial community assessment in a continuous fungal treatment of non-sterile real hospital wastewater after a coagulation-flocculation pretreatment

Based on the homonymous article Mir-Tutusaus, J.A., Parladé, E., Llorca, M., Villagrasa, M., Barceló, D., Rodriguez-Mozaz, S., Martinez-Alonso, M., Gaju, N., Caminal, G., Sarrà, M., 2017. Water Research 116, 65–75. doi:10.1016/j.watres.2017.03.005

Abstract

Hospital wastewaters are a main source of pharmaceutical active compounds, which are usually highly recalcitrant and can accumulate in surface and groundwater bodies. Fungal treatments can remove these contaminants prior to discharge, but real wastewater poses a problem to fungal survival due to bacterial competition. This study successfully treated real non-spiked, non-sterile wastewater in a continuous fungal fluidized bed bioreactor coupled to a coagulation-flocculation pretreatment for 56 days. A control bioreactor without the fungus was also operated and the results were compared. A denaturing gradient gel electrophoresis (DGGE) and sequencing approach was used to study the microbial community arisen in both reactors and as a result some bacterial degraders are proposed. The fungal operation successfully removed analgesics and anti-inflammatories, and even the most recalcitrant pharmaceutical families such as antibiotics and psychiatric drugs.

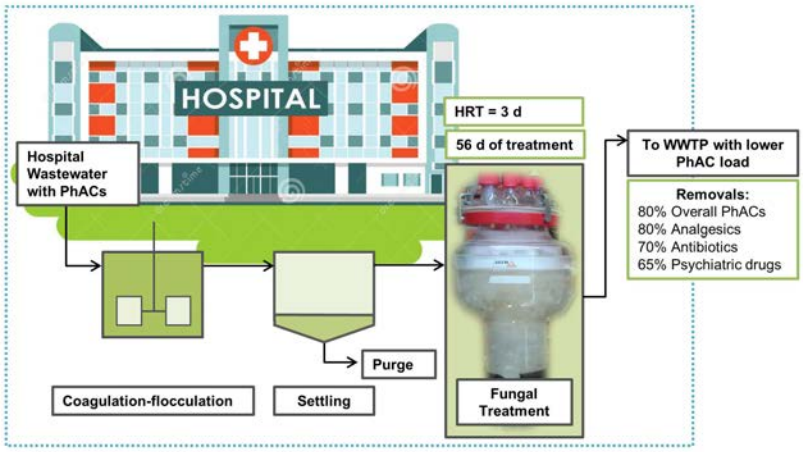

Figure 5.1: Graphical abstract.

5.1 Introduction

Pharmaceutical active compounds (PhACs) have been found in water bodies at significant concentrations (Gros et al., 2012). The primary source of these contaminants in the environment is known to be through wastewater treatment plant (WWTP) effluents, not designed to remove these emerging pollutants(Deblonde and Hartemann, 2013). PhACs are found at especially high concentrations (up to $\mu g \cdot L^{-1}$ and $mg \cdot L^{-1}$) in hospital wastewater (HWW), which is typically discharged untreated into the sewer network. In consequence, the on-site treatment of these hospital effluents prior to discharge has arisen as a promising possibility (Verlicchi et al., 2010). These recalcitrant compounds would be total or partially degraded and transformed into more degradable compounds for further downstream treatment.

White-rot fungi (WRF) have demonstrated the capability to degrade several PhACs and consequently, a fungal approach to treat on-site hospital effluents emerges as an attractive perspective. First studies on fungal treatment performance concerning pharmaceutical removal were carried out in sterile conditions and with single-spiked pollutants (Marco-Urrea et al., 2009, 2010b; Jelic et al., 2012). Studies in non-sterile, more complex matrices are scarcer but have demonstrated the ability of fungi to transform and/or remove PhACs from non-sterile HWW(Cruz-Morató et al., 2013a, 2014). One of the drawbacks of the technology in non-sterile conditions is the difficulty in maintaining the fungal activity for a long period of time since bacteria exert competitive pressure in fungal survival. The implementation of a coagulation-floculation step before the fungal treatment of spiked HWW reduced the microbial load of the influent thus allowing the maintenance of fungal activity for 28 days (Mir-Tutusaus et al., 2016). Furthermore, a

partial biomass renovation, previously described by Blánquez et al. (2006), could extend the treatment by overcoming the biomass aging process. This approach has been implemented and is discussed in the present manuscript. To approach a real application, a non-spiked matrix is preferred.

Additionally, despite some studies have investigated the bacterial and fungal communities in fungal bioreactors treating wastewater (Badia-Fabregat et al., 2015a), it still remains unclear which microorganisms are responsible for the PhACs elimination. The assessment of microbial assemblage would enhance the knowledge about this type of systems and help in the design of future treatments.

This study provides the validation of previous work in spiked HWW (Mir-Tutusaus et al., 2016), while approaching real application. The main focus of the manuscript has been the discussion of PhACs removal and its relation to microbial community evolution. Moreover, a long operation of this kind of reactors in non-sterile HWW has never been achieved before and it would signify a promising step in the maturation of fungal technology in wastewater treatment. The objectives of the study are thus to test the ability of WRF *Trametes versicolor* to treat real non-sterile HWW after a coagulation-flocculation pretreatment for a long period of time, to evaluate the bacterial and fungal communities arisen during the treatment and to assess the removal efficiency for PhACs.

5.2 Results

The results of HWWs characterization (Table 5.1) show that the measured physicochemical parameters were in the same range as other HWW. Pharmaceuticals concentrations, presented in Table 5.2 ranged from ng·L^{-1} to few µg·L^{-1}, results also in agreement with previous studies (Badia-Fabregat et al., 2015a; Cruz-Morató et al., 2014). The pretreatment diminished the absorbance at 650 nm from 0.215 and 0.265 to values very close to zero and the COD from 633 and 1012 mg O_2·L^{-1} to 215 and 300 mg O_2·L^{-1}, respectively. This reduction is in accordance with previous experiments (Mir-Tutusaus et al., 2016).

As stated before, in the case of RA 1/3 of biomass was purged and renovated with fresh pellets every 7 days as an approach to maintain a stable and active culture in the bioreactor. The control reactor RB was not inoculated. A fungal control reactor without biomass renovation was not operated, so the impact of biomass replacement in length of

Table 5.1: Physicochemical characterization of the hospital wastewaters.

Sampling date	14/09/2015		26/10/2015	
	HWW4		HWW5	
	Non flocculated	Flocculated	Non flocculated	Flocculated
pH	7.8	8.1	8.7	8.0
Conductivity ($mS \cdot cm^{-1}$)	1.99	1.98	1.69	1.29
Absorbance at 650 nm	0.215	0.000	0.265	0.012
Chloride ($mg\ Cl \cdot L^{-1}$)	284.3	315.0	271.3	216.5
Sulfate ($mg\ S \cdot L^{-1}$)	67.1	73.1	59.9	43.6
Nitrate ($mg\ N \cdot L^{-1}$)	0.0	0.1	4.1	2.7
Phosphate ($mg\ P \cdot L^{-1}$)	1.3	2.0	0.5	0.2
Ammonia ($mg\ N \cdot L^{-1}$)	36.0	35.6	9.9	9.7
TSS ($mg \cdot L^{-1}$)	284	58	193	93
COD ($mg\ O_2 \cdot L^{-1}$)	633	215	1012	300
DIC ($mg \cdot L^{-1}$)	94 ± 1	71 ± 1	49 ± 1	39 ± 1
DOC ($mg \cdot L^{-1}$)	50 ± 4	53 ± 4	211 ± 11	56 ± 5

operation will not be included in the RA/RB comparison. A complete study on biomass renovation can be found elsewhere in sterile conditions (Blánquez et al., 2006). In addition, present results can be compared to those previously described in the treatment of HWW in non-sterile conditions without biomass renovation (Mir-Tutusaus et al., 2016). The fungal reactor (RA) underwent an incident where pH was sustained below 3 for several hours. pH is critical in fungal systems, as supported by Borràs et al. (2008), and this low pH period led to a substantial loss of pelleted morphology. This incident took place during the change of HWW, circumstance that hampered the interpretation of results. The reactor recovered pelleted morphology and laccase production through weekly biomass renovation but behaved differently apropos of PhACs removal. The main conclusions of the treatment could be drawn from the first 28 days of operation; however, understanding the second period and the recovery from a low pH period could expand the knowledge of the system and give insight towards real application, hence all the results are presented.

5.2.1 Monitoring of bioreactors

The lack of on-line monitoring variables usually difficults the evaluation of the bioreactor performance. In this operation laccase activity and glucose concentration were monitored during the treatments (Figure 5.2). Laccase activity was not detected in

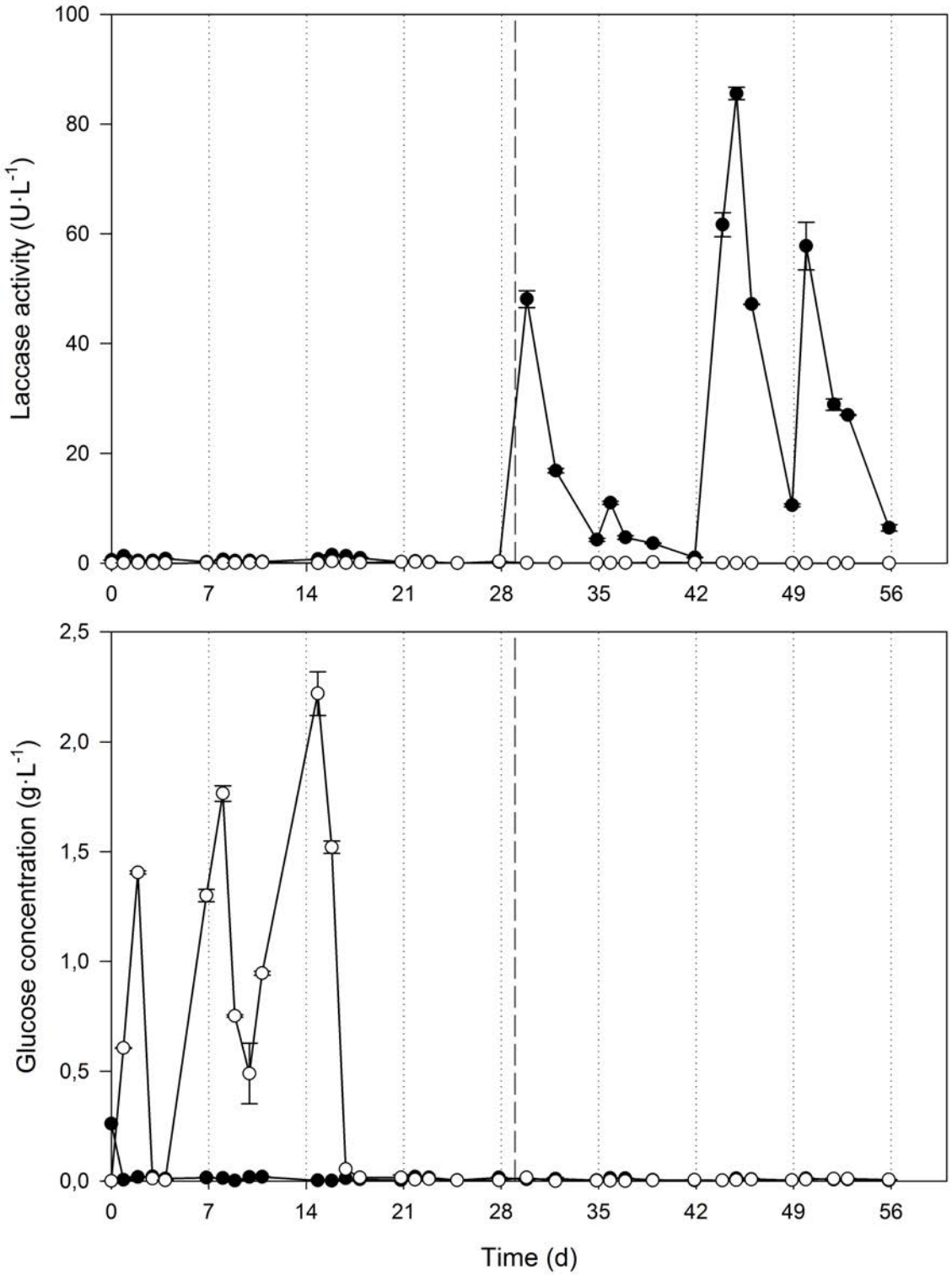

Figure 5.2: Evolution of laccase activity and glucose concentration during the continuous treatments with an HRT of 3 d. Black circles represent the reactor with T. versicolor and white circles, the uninoculated reactor. Vertical dotted lines represent weekly partial biomass renovation; the vertical dashed line, the change of wastewater.

Table 5.2: Pharmaceuticals initial concentration and removal percentage during the treatments (means in steady state).

Therapeutical group	Compound	HWW4						HWW5					
		RA			RB			RA			RB		
		C ($ng{\cdot}L^{-1}$)	Removal (%)		Removal (%)			C ($ng{\cdot}L^{-1}$)	Removal (%)		Removal (%)		
Analgesics and anti-inflammatories	Acetaminophen	> 20000	> 99.3	± 0	> 85.7	± 28		> 20000	> 99.5	± 1	100.0	± 0	
	Diclofenac	951.3	99.8	± 0	95.9	± 8		bld		-		-	
	Ibuprofen	>20000	> 85.5	± 19	> 88.7	± 6		3960.14	84.6	± 2b	93.0	± 2b	
	Ketoprofen	5109.3	-3.6	± 84	71.5	± 20		2432.33	-54.4	± 72b	79.8	± 25b	
	Phenazone	bld		-		-		9.23	-314.0	± 139	-147.3	± 192	
	Total	46061.9	81.9	± 15	85.6	± 14		26404.07	82.9	± 6b	97.0	± 2b	
Anthelmintics	Thiabendazole	blq	70.0	± 0	70.0	± 0		9.10	97.7	± 0b	92.3	± 0b	
	Albendazole	blq		-	-1338.7	± 2724		blq		-		-	
	Total	0.9	53.3	± 0	-266.8	± 651		9.32	95.4	± 0b	90.1	± 0b	
Antibiotics	Azithromycin	bld		-		-		45.36	-95.5	± 107b	88.5	± 21b	
	Ciprofloxacin	366.4	47.1	± 25	-32.8	± 67		266.90	-7.2	± 16	-2.5	± 29	
	Ronidazole	bld	-7745.2	± 15490	-16726.3	± 3627		bld	-18135.5	± 1690	-28321.6	± 9351	
	Sulfamethoxazole	1130.4	78.2	± 9a	29.0	± 31a		55.85	34.8	± 76	-42.6	± 87	
	Trimethoprim	748.3	52.3	± 35a	-27.8	± 54a		81.80	-26.9	± 96	-42.1	± 67	
	Ofloxacin	2537.1	71.1	± 14a	3.8	± 21a		459.39	-57.8	± 168	-65.4	± 54	
	Total	4783.0	67.5	± 17a	1.0	± 17a		909.60	-42.3	± 89	-45.0	± 30	
Anticoagulants	Warfarin	10.0	94.8	± 0	94.8	± 0		bld		-		-	

Table 5.2: Pharmaceuticals initial concentration and removal percentage during the treatments (means in steady state).

Therapeutical group	Compound	HWW4						HWW5					
			RA			RB			RA			RB	
		C ($ng{\cdot}L^{-1}$)	Removal (%)			Removal (%)		C ($ng{\cdot}L^{-1}$)	Removal (%)			Removal (%)	
	Total	10.0	94.8	±	0	94.8	± 0	bld			-		-
Antihypertensives	Valsartan	112.7	34.2	±	22	43.4	± 46	55.46	-44.6	±	63	15.6	± 18
	Total	112.7	34.2	±	22	43.4	± 46	55.46	-44.6	±	63	15.6	± 18
β-blockers	Atenolol	59.4	14.3	±	37a	78.7	± 23a	154.45	67.5	±	24	60.9	± 19
	Propanolol	bld			-		-	bld			blq		blq
	Sotalol	251.6	-151.9	±	29	-205.2	± 65	bld	-10442.4	±	20885	-17997.6	± 35995
	Total	327.5	-114.1	±	27	-143.3	± 54	171.16	32.9	±	23	3.5	± 41
Diuretics	Furosemide	bld	-664.4	±	1431	-1018.9	± 2124	161.88	97.6	±	0	96.2	± 3
	Hydrochlorothiazide	408.4	58.4	±	34	23.1	± 14	650.78	10.9	±	12	21.8	± 2
	Total	412.3	51.6	±	45	13.2	± 10	812.66	28.2	±	10	36.6	± 1
Drug against	Tamsulosin	bld			-	-58.3	± 117	7.30	41.6	±	5b	98.7	± 0b
prostatic hyperplasia	Total	bld			-	-58.3	± 117	7.30	41.6	±	5b	98.7	± 0b
Histamine H1 and H2	Ranitidine	1830.3	96.7	±	3	89.9	± 6	20.43	-2.4	±	107	-77.3	± 61
receptor antagonists	Loratadine	1.2	46.9	±	33	75.5	± 0	bld	-89.6	±	113	-116.7	± 135
	Total	1831.5	96.7	±	3	89.8	± 6	20.71	-3.6	±	105	-77.9	± 62
Lipid regulators	Atorvastatin	14.9	94.9	±	7	90.1	± 15	13.77	95.6	±	2	71.1	± 28

Table 5.2: Pharmaceuticals initial concentration and removal percentage during the treatments (means in steady state).

Therapeutical group	Compound	HWW4						HWW5					
			RA			RB			RA			RB	
		C ($ng{\cdot}L^{-1}$)	Removal (%)			Removal (%)		C ($ng{\cdot}L^{-1}$)	Removal (%)			Removal (%)	
	Fluvastatin	bld			-	-306.2	± 612	bld			-		-
	Gemfibrozil	6364.1	100.0	±	0a	83.6	± 3a	1921.34	85.1	±	19	90.3	± 19
	Total	6381.8	99.9	±	0a	83.5	± 3a	1937.80	85.1	±	19	90.1	± 19
Psychiatric drugs	10.11-epoxyCBZ	673.2	43.6	±	9	81.3	± 30	1816.91	25.4	±	6b	82.6	± 35b
	2-hydroxyCBZ	1661.1	99.9	±	0	76.7	± 46	> 2000	> 74.9	±	50b	> 18.3	± 24b
	Acridone	126.0	-183.5	±	43a	74.5	± 0a	225.85	-295.8	±	221b	6.5	± 62b
	Alprazolam	bld			-		-	10.04	-17.7	±	41	4.4	± 41
	Carbamazepine	251.4	61.0	±	19a	16.0	± 8a	270.09	22.7	±	7	11.6	± 7
	Citalopram	297.4	39.0	±	18a	-18.2	± 5a	351.00	-4.4	±	38	-22.0	± 14
	Diazepam	bld	-58.3	±	117		-	bld	-58.3	±	117	1.9	± 4
	Fluoxetine	bld			-		-	bld			-		-
	Norfluoxetine	12.5	90.1	±	0	-26.5	± 233	bld			-		-
	Olanzapine	131.5	98.7	±	2	-44.6	± 153	66.98	99.2	±	0b	-73.9	± 104b
	Setraline	98.8	98.3	±	0	98.3	± 0	127.85	98.7	±	0	55.1	± 50
	Razodone	36.3	98.0	±	0a	-112.5	± 94a	20.86	69.7	±	54	29.7	± 54
	Venlafaxine	495.3	41.0	±	41a	-18.2	± 7a	146.18	-19.2	±	79	-78.8	± 46
	Total	3791.9	54.8	±	24	47.6	± 22	5044.96	30.0	±	24	34.7	± 18

Table 5.2: Pharmaceuticals initial concentration and removal percentage during the treatments (means in steady state).

Therapeutical group	Compound	HWW4						HWW5					
			RA			RB			RA			RB	
		C ($ng{\cdot}L^{-1}$)	Removal (%)			Removal (%)		C ($ng{\cdot}L^{-1}$)	Removal (%)			Removal (%)	
Synthetic glucocorticoid	Dexamethasone	121.8	77.2	±	44	33.5	± 76	bld	-34501.3	±	23481	-32983.9	± 23315
	Total	121.8	77.2	±	44	33.5	± 76	bld	-34501.3	±	23481	-32983.9	± 23315

Bql: below limit of quantification; bld: below limit of detection. [a,b]Statistically different ($p < 0.05$).

Table 5.3: Chemical oxygen demand of effluent of reactors during the treatment.

Time (d)	COD (mg·L-1)	
	RA	RB
0	215	215
7	358	1900
21	3141	1115
42	379	1260
56	292	1335

the reactors during the first period of operation (HWW4). However, *T. versicolor* produced the enzyme because when biomass was removed from the reactors, rinsed and placed in stirred Erlenmeyers with defined medium (ex situ assay), laccase activity could be measured. Ex situ assays detected laccase activity in RA but not in RB (data not shown). During the second period of operation (HWW5) laccase profile in RA was irregular with peaks of over 45 $U{\cdot}L^{-1}$ at Days 30, 45 and 50. Laccase activity in the uninoculated reactor remained insignificant throughout the treatment. *T. versicolor* remained active during the whole treatment. However, it could not be asserted whether HWW4 interfered with the assay, the production of laccase, or some compounds in HWW4 inactivated the laccase. In complex matrices, the purification of the laccase is required prior to the measurement of the activity and it should be taken into accout in future studies (Annibale et al., 2006). As previously discussed (Mir-Tutusaus et al., 2016), laccase production was sign of *T. versicolor* activity, but its absence was not an indication of the fungus inactivity. In fact, RA showed high removal capacity when laccase activity was very low.

Glucose was added at *T. versicolor* consumption rate thus glucose concentration remained at values close to zero throughout the treatment in the fungal reactor. In RB glucose accumulated during the first two weeks of operation until it was colonized by HWW-native microorganisms and remained insignificant from that point onwards. Total COD is presented in Table 5.3. RA did not significantly increase initial COD except around the day of the pH incident. This agreed with the observed loss of pelleted morphology, as free hyphae increased the COD load. Contrarily, RB consistently exhibited COD around 1200 $mg{\cdot}L^{-1}$. The profiles of TSS concentration are presented in Figure 5.3. RA profile was mostly constant at around 120 $mg{\cdot}L^{-1}$, but the control RB did not achieve a steady state, exhibiting a much more irregular profile with peaks of over

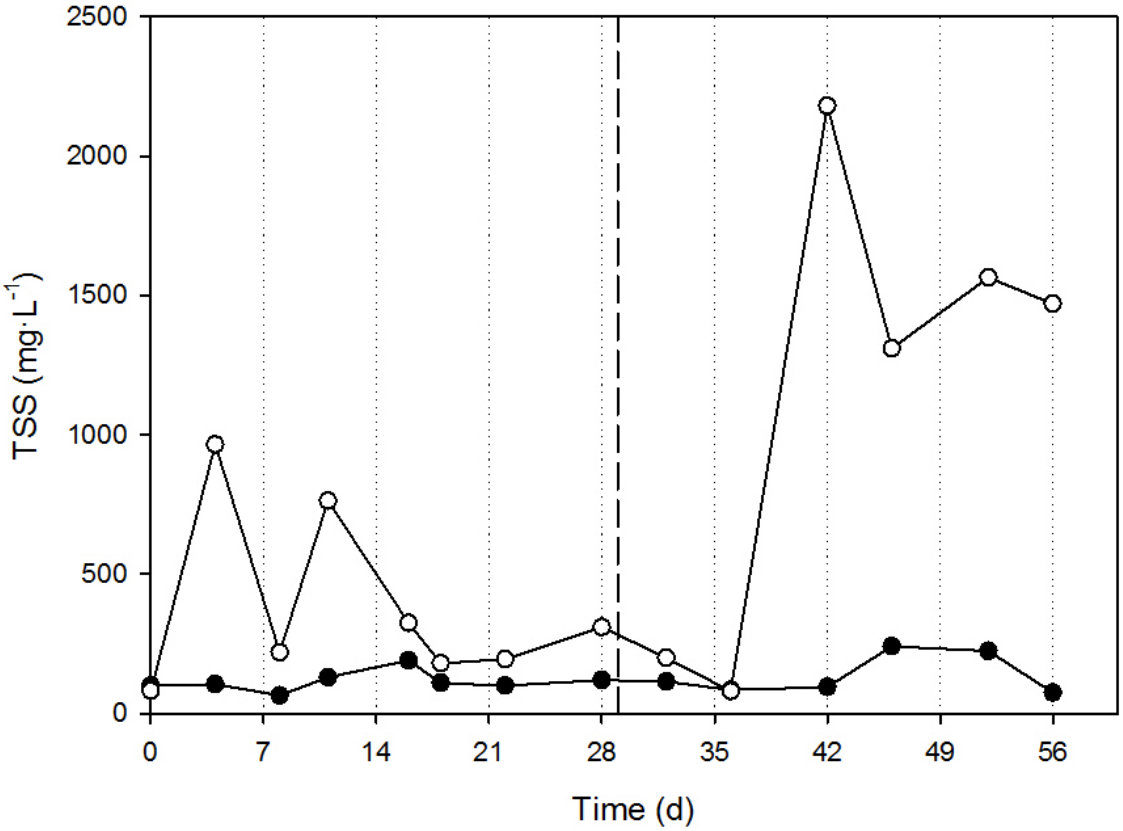

Figure 5.3: Evolution of total suspended solids concentration during the treatments. Black circles represent the reactor with T. versicolor; white circles, the uninoculated reactor. Dotted lines represent weekly partial biomass renovation; the dashed line, the change of wastewater.

2000 mg·L^{-1} at the end of the treatment. Neither COD nor TSS levels reached the standard of 125 mg·L^{-1} and 35 mg·L^{-1}, respectively, in either reactor according to the EEC (1991); but the objective was to remove PhACs. The system is, as stated before, an on-site treatment prior to discharge to the sewer network.

5.2.2 PhACs removal and toxicity assessment

46 out of the 81 PhACs analyzed were detected during the treatments (Table 5.2). 35 and 34 compounds were detected in raw wastewater HWW4 and HWW5, respectively, whereas after the pretreatment only 34 and 32. The two pretreated wastewaters used as influent had different PhAC composition. Overall, HWW4 had higher concentrations of detected PhACs than HWW5. The most common families of PhACs detected were analgesics and anti-inflammatories, antibiotics and psychiatric drugs, as the hospital has

an important psychiatric pavilion. Initial concentrations of individual pharmaceuticals can be found in Table 5.2. The analgesics and anti-inflammatories family contribute the most to the final concentration, especially due to the high concentrations of ibuprofen and acetaminophen. Psychiatric drugs, led by 2-hydroxycarbamazepine and 10,11-epoxycarbamazepine (both known metabolites of carbamazepine), are the second group in concentration. Lipid regulators family ranked 3rd followed by antibiotics family.

Excluding analgesics and anti-inflammatories, which is a family known to be easily degraded, the amount of compounds was 17.8 $\mu g \cdot L^{-1}$ and decreased to 4 ± 1 $\mu g \cdot L^{-1}$ and 9 ± 1 $\mu g \cdot L^{-1}$ in reactors RA and RB, respectively, corresponding to a removal of 78 ± 7% and 48 ± 4%. In HWW5, when RA was recovering from the pH incident, the removal percentages excluding analgesics and anti-inflammatories were around 30-35% for both reactors.

Time-course profile of concentrations of the different families during the treatments is presented in Figure 5.4. Analgesics and anti-inflammatories were present in a high concentration, but were rapidly removed by both the inoculated and the uninoculated reactor with removal values of above 80%, as both bacteria and fungi are reported to remove these compounds (Langenhoff et al., 2013). Both reactors exhibited an increase in removal capacity from day 9 to day 27, around the time of the change in wastewater, which contained approximately half of the concentration of PhACs than HWW4. From that point onwards, both RA and RB behaved steadily with removal values of around 90%.

Antibiotics initial concentration was around 5000 $ng \cdot L^{-1}$. *T. versicolor* was able to remove 90% of its initial load but gradually lost removal capacity to values around 50%. RB did not significantly remove antibiotics; its concentrations remained equal or increased. Since the pH incident, RA did not recover removal capacity. RB continued to exhibit higher antibiotics concentration than the inlet when treating HWW5.

The presented psychiatric drugs family excludes carbamazepine (CBZ) and its transformation products (TP); carbamazepine case is discussed below because it is an especially recalcitrant compound not removed in conventional WWTPs and information on the TPs was available (Clara et al., 2004). *T. versicolor* was able to remove around 50% of the initial load of psychiatric drugs, although its initial removal was 86%. The uninoculated reactor did not remove this family and presented higher concentrations than the inlet during all the treatment.

The concentration profile of other pharmaceutical compounds is particularly different in the two wastewaters. The first 27 days showed nearly constant concentrations of around 900 $ng \cdot L^{-1}$ in the fungal reactor and around 2500 $ng \cdot L^{-1}$ in RB. This translates to removal values of circa 90% for RA and 70% for RB. During the second

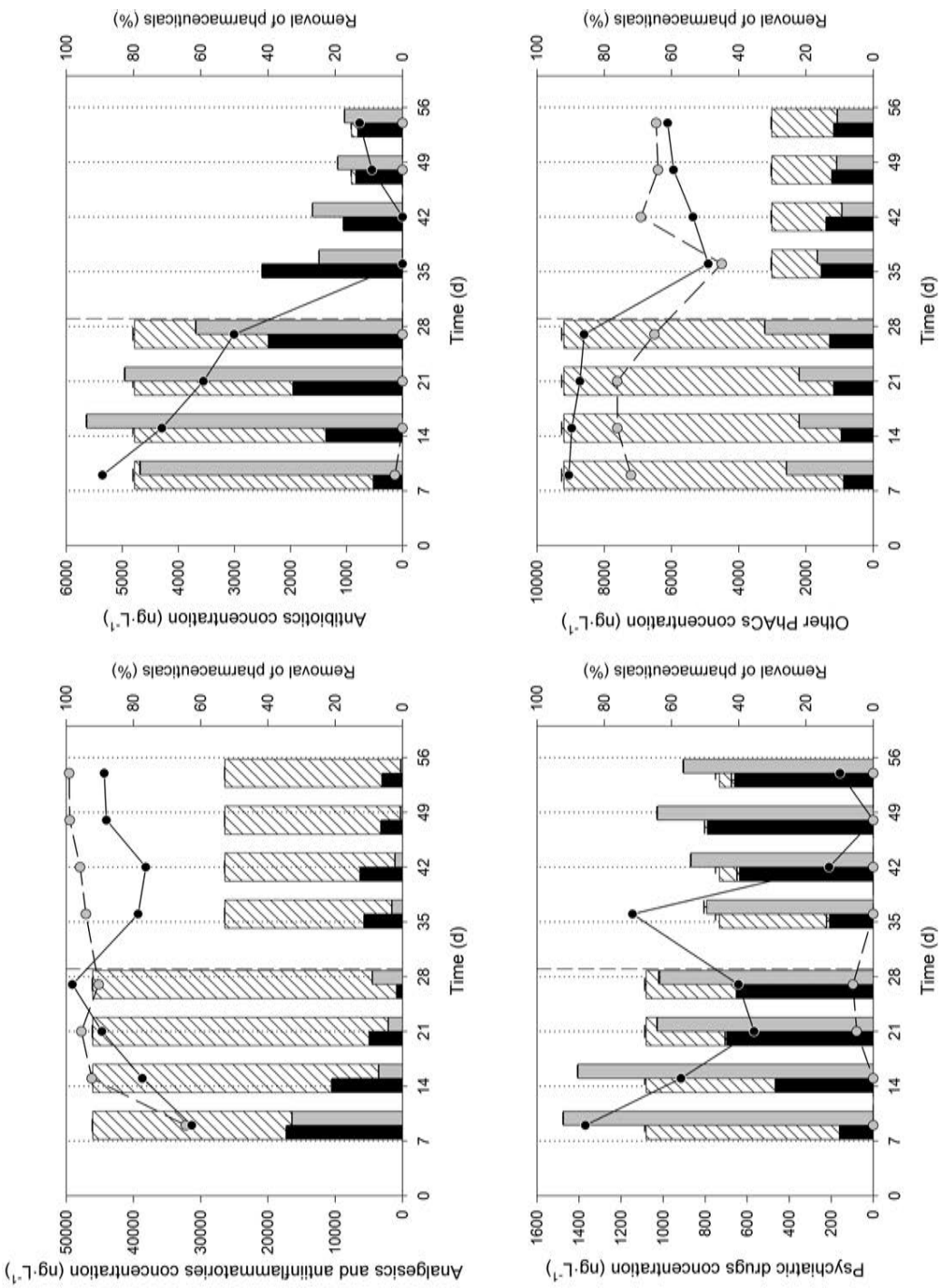

Figure 5.4: Pharmaceuticals concentration (in bars) and degradation (in circles) by families: analgesics and anti-inflammatories (top left), antibiotics (top right), psychiatric drugs not including carbamazepine and carbamazepine transformation products (bottom left) and the rest of PhACs (bottom right). Black bars/circles represent the reactor with T. versicolor and gray bars/circles, the uninoculated reactor; white patterned bars, the initial concentrations of each family. Dotted lines represent weekly partial biomass renovation; the dashed line, the change of wastewater.

part of the treatment, both reactors behaved similarly with removals around 60%. None of the compounds detected had anti-fungal activity.

The case of carbamazepine and its transformation products 10,11-carabamazepine, 2-hydroxycarbamazepine and acridone is presented in Fig. 4. Carbamazepine is mainly metabolized in the liver, generating the analyzed transformation products, among other metabolites as well as several glucuronide conjugates (Kaiser et al., 2014). Both HWW4 and HWW5 contained similar concentrations of CBZ and its TPs, excluding 10,11-expoxyCBZ, with a concentration much more higher in HWW5 than in HWW4. The fungal bioreactor was able to remove from 50 – 80% of CBZ, around 50% of 10,11-epoxyCBZ and nearly 100% of 2-hydroxyCBZ, but the concentration of acridone increased. While recovering from the pH incident, RA behaved differently: it retained the 2-hydroxyCBZ removal capacity and the concentration of acridone increased, but it exhibited low removals of CBZ and 10,11-epoxyCBZ. RB was not able to remove CBZ from the wastewaters. It showed, nonetheless, good removal values of carbamazepine TPs but lost removal capacity from day 27 onwards. After day 42 RB regained its ability to remove 10,11-epoxyCBZ and small amounts of 2-hydroxyCBZ; concentration of acridone also increased.

Regarding the toxicity assessment, the fungal reactor maintained the toxicity at 0 TU during the whole treatment. Contrarily, the control bioreactor raised toxicity to 27 TU.

5.2.3 Evolution of bacterial and fungal populations

The evolution of fungal and bacterial populations was studied by DGGE analysis and DNA sequencing. Two DGGE gels (one for bacteria and one for fungi) were run with samples collected from the liquid matrix of RA (RA), from the pelleted biomass in RA (PRA) and from the liquid matrix of RB (RB). The results are presented in Figure 5.7 and Figure 5.8, and the DGGE profiles in Figure 5.6. Microbial communities in both reactors changed during the treatment and longer operations might be needed to achieve a steady state.

64 prominent bands from the fungal DGGE were excised and sequenced, obtaining 97% coverage of the phylotypes associated with the quantitative DGGE band matrix. Representative sequences were submitted to the GeneBank database under the accession numbers KX530041 to KX530058. While only two phyla (Ascomycota and Basidiomycota) were represented in the fungal sequences, these were composed by 7 different genera: *Candida*, *Isaria*, Phialemoniopsis and *Trichoderma* from phyla Ascomycota and *Asterotremella*, *Trametes* and *Tremella* from phyla Basidiomycota (Table 5.4).

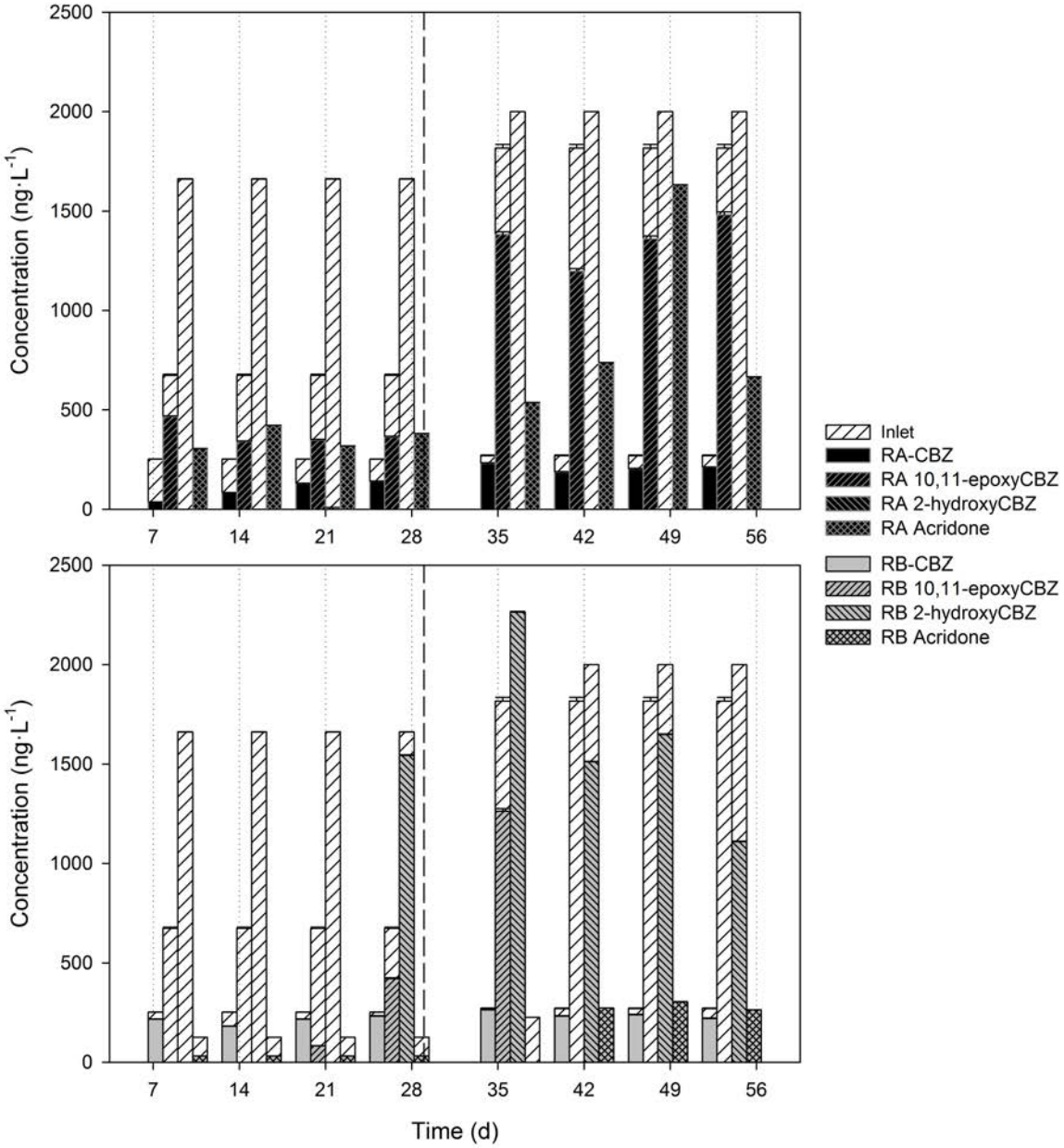

Figure 5.5: Carbamazepine and carbamazepine transformation products concentration. Black bars represent the reactor with T. versicolor and gray bars, the uninoculated reactor; white patterned bars, the initial concentration of each product. Dotted lines represent weekly partial biomass renovation; the dashed line, the change of wastewater.

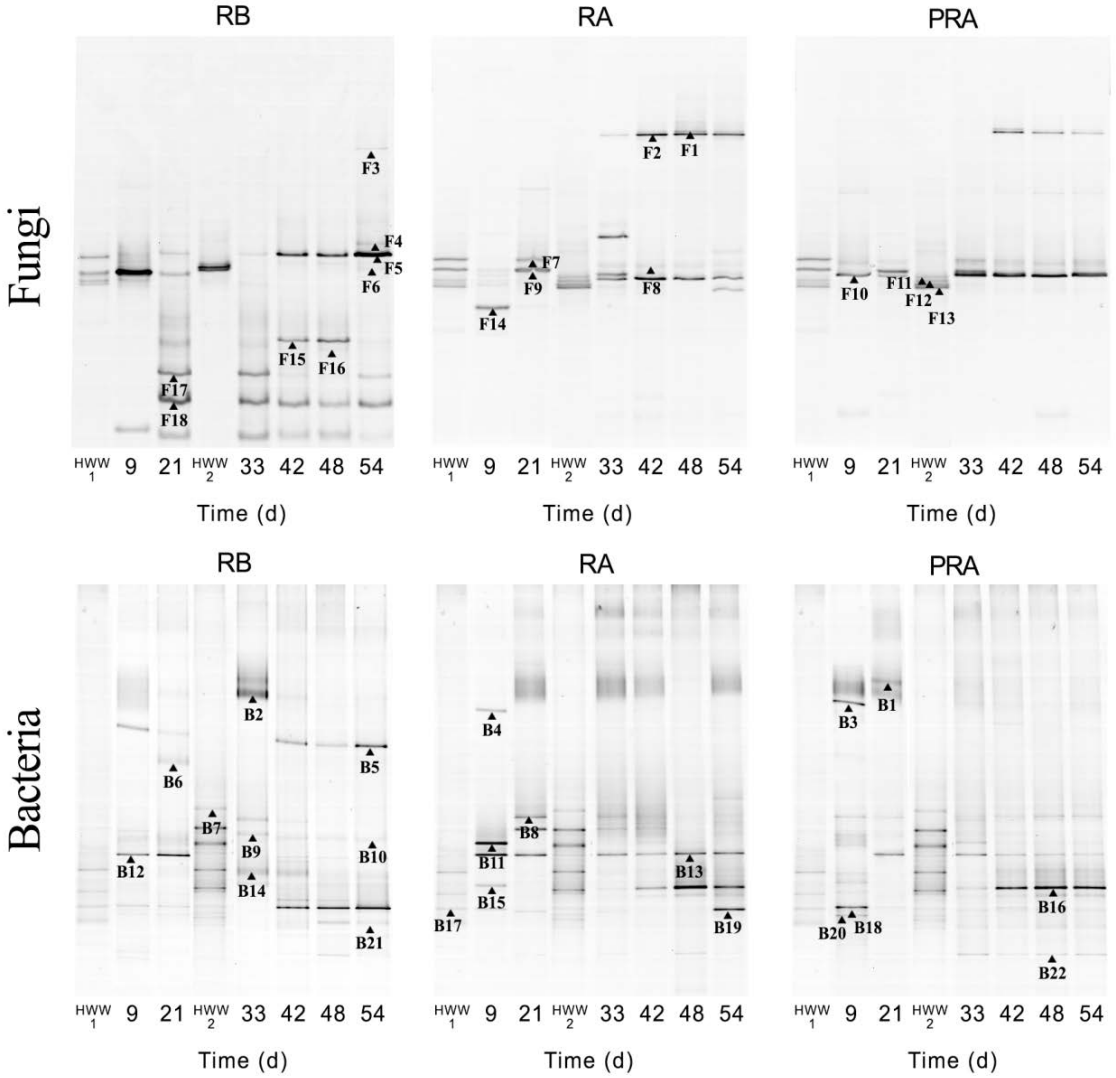

Figure 5.6: DGGE profiles of fungal and bacterial communities detected in the inoculated reactor (RA), pellet fraction of the inoculated reactor (PRA) and non-inoculated reactor (RB) using the primer sets EF4-ITS4/ITS1-ITS2 and 341f-907r respectively. Representative bands (▲) are labeled according to their source. (F) Fungi, (B) Bacteria.

At the genus level, *Trametes* was consolidated all along the operation in pellet samples (PRA). When pellets started losing shape, *T. versicolor* was also found in the supernatant samples (RA). *Tremella* and *Asterotremella* were dominant in RA reactor initially, until *Candida* was established in mid-late period after the pH incident. Moreover in RB reactor *Asterotremella* and *Trichoderma* predominated before the change of WW, then a substitution of the former for Phialemoniopsis was observed from day 21 onwards.

In parallel, 74 prominent bands from the bacterial DGGE were excised and sequenced. In this case the community coverage also stood in 97%. Sequences from each phylotype

Table 5.4: Phylogenetic affiliations of bacterial 16S rRNA gene and fungal ITS sequences obtained from the reactors after DGGE.

DGGE band	Closest cultured BLAST match	Accession number	Similarity (%)	Phylogenetic affiliation (Phylum)
F01-02	*Candida sojae*	KJ722419	100	Ascomycota
F03-06	*Phialemoniopsis curvata*	AB278180	98	Ascomycota
F07, F9	*Asterotremella humicola*	KC118118	100	Basidiomycota
F08, F10	*Trametes versicolor*	KR261581	100	Basidiomycota
F11-13	*Isaria cf. farinosa*	FN548150	99	Ascomycota
F14	*Tremella exigua*	KP986514	100	Basidiomycota
F15	*Trichoderma asperellum*	KR856224	100	Ascomycota
F16-18	*Trichoderma asperellum*	KR856224	100	Ascomycota
B01	*Flavobacterium oncorhynchi*	KT354259	100	Bacteroidetes
B02	*Flavobacterium sp.*	JF915323	99	Bacteroidetes
B03-04	*Elizabethkingia miricola*	LN995715	100	Bacteroidetes
B05	*Chryseobacterium meningosepticum*	AF207076	100	Bacteroidetes
B06	*Bacteroides oleiciplenus*	NR_113070	95	Bacteroidetes
B07	*Faecalibacterium prausnitzii*	HQ457025	100	Firmicutes
B08	*Dyadobacter fermentans*	LN890052	100	Bacteroidetes
B09	*Dyadobacter sp.*	DQ207362	100	Bacteroidetes
B10	*Microvirgula aerodenitrificans*	LN997979	100	Proteobacteria (β)
B11	*Lactococcus lactis*	KU942499	100	Firmicutes
B12	*Burkholderia gladioli*	KT862889	100	Proteobacteria (β)
B13	*Pandoraea sputorum*	LN995687	100	Proteobacteria (β)
B14	*Cyanobacterium TDX16*	KJ599678	95	Cyanobacteria
B15	*Acetobacter aceti*	KR261398	99	Proteobacteria (α)
B16	*Rhizobium sp.*	KT387839	100	Proteobacteria (α)
B17	*Comamonas aquatica*	LN558648	99	Proteobacteria (β)
B18	*Comamonas aquatica*	KT716080	99	Proteobacteria (β)
B19	*Paenibacillus sp.*	JX469414	99	Firmicutes
B20	*Stenotrophomonas sp.*	KR922087	100	Proteobacteria (γ)
B21	*Magnetospirillum sp.*	KM289194	99	Proteobacteria (α)
B22	*Microbacteriaceae bacterium*	KR082269	100	Actinobacteria

Abbreviation: BLAST, Basic Local Alignment Search Tool.

were submitted to GeneBank under accession numbers KX523866 to KX523887. These sequences represented 5 phyla (Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes and Proteobacteria) consisting of 17 different genera. Proteobacteria was the most widely represented phylum with Acetobacter, *Burkholderia*, *Comamonas*, Magnetospirillum, Pandoraea, Rhizobium and Sternotrophomonas genera. For this reason, classes within Proteobacteria were taken into account to evaluate the data. Bacteroidetes followed with four affiliated genera, namely, Bacteroides, Dyadobacter, *Elisabethkingia* and Flavobacterium. Faecalibacterium, Lactococcus and Paenibacillus genera made up for the Firmicutes phylum (Table 5.4). Results revealed a co-dominance of Betaprotoebacteria and Bacteroidetes in RB during all operation with some fluctuations. In RA and PRA the class Betaproteobacteria was generally persistent all along the operation. Additionally, Bacteroidetes were abundant in early-mid stages of the operation while Alphaproteobacteria class took over at mid-late operation.

5.3 Discussion

The consortia established in the reactors were well adapted to lower pH, as both reactors were controlled at pH 4.5, and to aerobic conditions, as the reactors were aerated. Differences in bacterial populations between RA and RB were due to the presence of pelleted fungal biomass (i.e. in RA, contrarily to RB, Firmicutes were present until Day 9 and Proteobacteria α were predominant from Day 42 onwards). Differences in removal percentages cannot be directly linked to laccase production, as RA showed high removal values even when laccase activity was very low or not detected at all. This decoupling between laccase activity and PhACs removal can be explained by the diverse removal pathways of PhACs, some of which can be removed by mechanisms other than the laccase system –notably the cytochrome P450 in the case of fungal systems (Blánquez et al., 2004; Jelic et al., 2012; Marco-Urrea et al., 2010b). Therefore, removal capacity will be discussed in terms of microbial community shifts.

Despite the differences, good removals are observed in the analgesics and anti-inflammatories family in both reactors. Several microorganisms are known capable of degrading some compounds of this group. In particular, acetaminophen and ibuprofen have been largely studied and can be degraded by bacteria as well as fungi (Bragança et al., 2016; Langenhoff et al., 2013; Nguyen et al., 2013). The two compounds account for the high removal of this family in both reactors. RB removal did not reach 80-90% until microorganisms fully colonized the reactor, around Day 18, when glucose reached near zero values. RA removal increase during the first 14 days could only be partially explained by the growth of microorganisms other than *T. versicolor*. A second contribution could be the increase in concentration of ketoprofen in RA, hence reducing the overall removal. *T. versicolor* can degrade well over 80% of ketoprofen in the same matrix and at the same HRT when the compound is in spiked concentration (Mir-Tutusaus et al., 2016). This fact demonstrated that the concentration increase in this non-spiked matrix is probably due to deconjugation of glucuronide conjugates of ketoprofen, and that ketoprofen conjugated compounds were in fact at higher concentrations. This was in agreement with Jelic et al. (2015), which stated that an 80% of the ketoprofen is excreted of the human body as a glucuronide-conjugated. In addition, the ability of *T. versicolor* to cleave conjugates of pharmaceutical compounds has been reported before in similar fungal systems(Badia-Fabregat et al., 2015a; Cruz-Morató et al., 2013a). The described ketoprofen concentration augment was not mirrored in RB, indicating that the microbial consortium did not reverse-transform conjugated compounds or that the community also removed such reverse-transformed products.

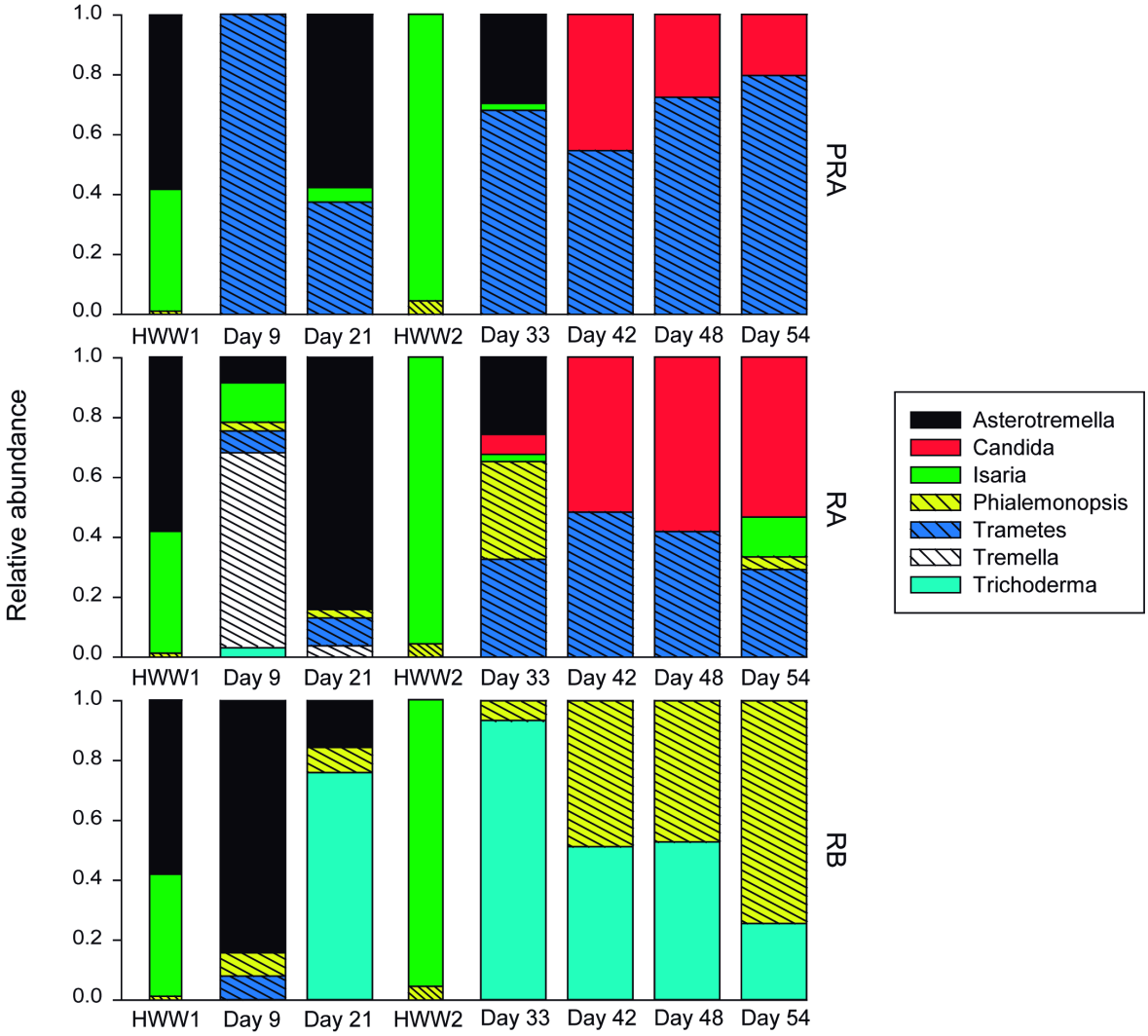

Figure 5.7: Phylogenetic assignment of fungal sequences from the HWWs, the liquid matrix (RA) and pelleted biomass (PRA) of the inoculated reactor, and from the liquid matrix of the non-inoculated reactor (RB). Data is presented in form of relative abundance, previously calculated with a semi-quantitative DGGE matrix and sequenced bands from the DGGE gels. Narrow bands represent the initial fungal composition of the two wastewaters; broad bands, the fungal composition during the treatments.

The case of diclofenac is of interest because it is widely used and not efficiently removed in conventional WWTP (Verlicchi et al., 2012). It was present only in HWW4 and completely removed during the fungal treatment, in accordance with Cruz-Morató et al. (2014). The uninoculated reactor also achieved a >90% removal; diclofenac degraders have been found in activated sludge with associated removal rates as low as 40% (Bouju et al., 2016). To our knowledge, this is the first time diclofenac has been reported to be completely removed by biostimulated wastewater-native microorganisms, although with an HRT of 3 days. This was achieved under non-steady conditions in terms of TSS, although diclofenac removal was indeed constant. The responsible *Candida*tes could be

bacteria within the Proteobacteria (*Burkholderia*, *Comamonas* or *Microvirgula*) and Bacteroidetes (*Elisabethkingia*) phyla or fungi as *Asterotremella* or Phialemoniopsis. The genus *Elisabethkingia* contains species associated with meningitis and to this date none of them have been described (nor studied) as degraders. Some species of *Burkholderia* have been found to degrade several pollutants such as chlorinated compounds (Zhang et al., 2013b). *Comamonas* representatives have been found to degrade steroids, dyes and 4-chlorophenol (Jadhav et al., 2008; Linares et al., 2008; Tobajas et al., 2012). *Microvirgula* is a well-known genus of aerobic denitrifiers, which has also been reported to degrade several dyes (Han et al., 2012). Regarding the fungal *Candida*tes, Phialemoniopsis genus is usually related to eye infections and not reported to degrade PhACs. *Asterotremella* proliferation in the fungal pellets, as can be seen in Figure 5.7, correlated with the decrease in PhACs removal. Microscopic observations were not carried out so this fact could not be confirmed. Although the liquid fraction in the pellet samples is very low, the *Asterotremella* percentage is higher than the corresponding to the liquid fraction, so it evidences an interaction between fungi. In addition, scarcely any references can be found about the yeast and none of them regarding its ability to biotransform any compounds. Thus, we propose that *Asterotremella* was not involved in the biotransformation of diclofenac.

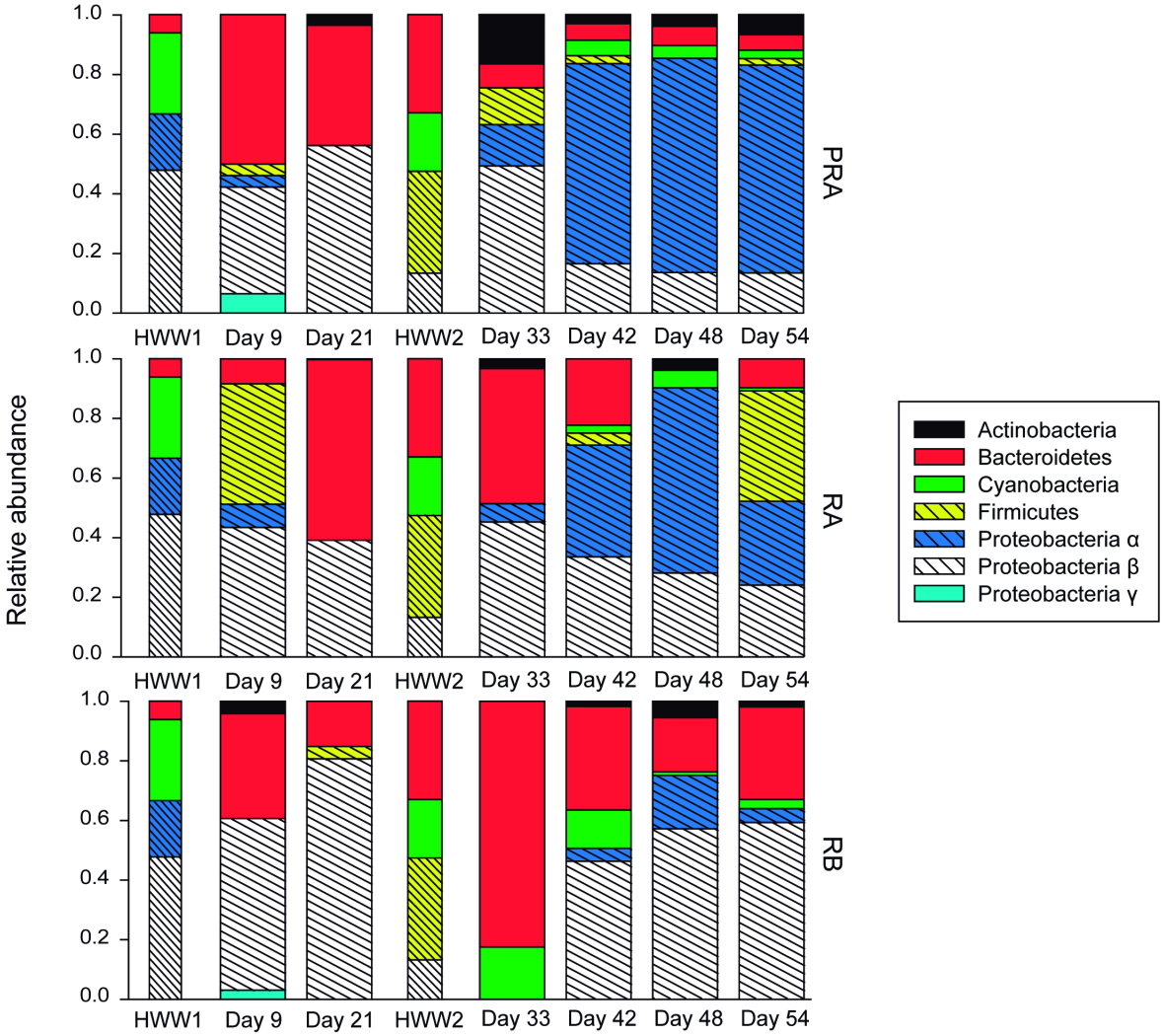

Figure 5.8: Phylogenetic assignment of bacterial sequences from the HWWs, the liquid matrix (RA) and pelleted biomass (PRA) of the inoculated reactor, and from the liquid matrix of the non-inoculated reactor (RB). Data is presented in form of relative abundance, previously calculated with a semi-quantitative DGGE matrix and sequenced bands from the DGGE gels. Narrow bands represent the initial bacterial composition of the two wastewaters; broad bands, the bacterial composition during the treatments.

Antibiotics are resistant to bacterial biodegradation but not to fungal degradation. This trend can be observed in Figure 5.4 and Table 5.2, where no removal is appreciated in the uninoculated reactor. Contrarily, the fungal reactor, whose main biomass was pelleted fungi, showed very high removals of all the antibiotics detected during HWW4. The decrease in removal efficiency of RA could be clearly attributed to the loss of predominance of *T. versicolor* to *Asterotremella* in the fungal pellets, as seen in Figure 5.7. The fungal reactor behaved equally as RB during HWW5 treatment, with no antibiotics removal. As presence of *T. versicolor* is demonstrated by DGGE results (Figure 5.6) and activity of the fungus, by laccase activity results (Figure 5.2), the absence

of removal could be attributed to inhibition of *T. versicolor* or its degrading enzymes. Pandoraea and Rhizobium were the main bacterial genera present in the liquid matrix and pellets during the stated period. *Trichoderma* was present in the HWW and established without apparent difficulties in the non-inoculated bioreactor. However, antagonism with *Trametes* was expected to take place in reactor A to the detriment of *Trichoderma*, as *Trametes* was the one that prevailed. Similarly, *Isaria* was also abundant in the HWW but was not able to establish, not even in the control reactor. *Candida* was the main fungal genus in the liquid matrix and was present in the fungal pellets while no antibiotics removal was observed; additionally, a decrease in *Candida* between days 42-56 resulted in a slight increase in antibiotics removal. Therefore, *T. versicolor* inhibition could be caused by the presence of *Candida*.

The profile of psychiatric drugs concentration (excluding the carbamazepine family) in RA is very similar to the antibiotics profile in RA: a decrease in removal capacity is observed, well correlated with the invasion of the fungal pellets by *Asterotremella*. After the change in HWW, RA exhibited very low removal values, probably due to the hypotheses discussed above. Special attention can be paid to the antidepressant venlafaxine, a very recalcitrant compound typically detected in HWW and urban wastewaters (Evgenidou et al., 2015). Venlafaxine is usually poorly removed, even in similar fungal reactors in sterile conditions (Badia-Fabregat et al., 2015b). RA removed up to 95% of venlafaxine at Day 7. Interestingly, similar results can be found in the bibliography using the same fungal system in non-sterile conditions. Cruz-Morató et al. (2014) reported 90% removal and attributed it to the synergistic action of fungal and bacterial enzymes.

Other pharmaceutical compounds included antihypertensives, anthelmintics, anticoagulants, β-blockers, diuretics, tamsulosin, H1 and H2 antagonists, lipid regulators and dexamethasone. The main contributors of this miscellanea family are gemfibrozil and ranitidine, with extremely different concentrations in HWW4 and HWW5. Gemfibrozil concentration was 6364 and 1921 $ng{\cdot}L^{-1}$ and ranitidine was 1830 and 20 $ng{\cdot}L^{-1}$ for HWW4 and HWW5 respectively. Both compounds could be removed well above 80% by RA and RB, except ranitidine in HWW5, where the initial concentration was very low. This combination of good degradability and lower initial concentrations between HWWs resulted in the decrease in overall removal of this miscellanea family observed in HWW5.

Carbamazepine removal in the fungal reactor was well correlated with *T. versicolor* presence in the fungal pellets in HWW4 and averaged 60%, which was in accord with the bibliography (Zhang and Geißen, 2012). The decreased removal of CBZ and

10,11-epoxyCBZ in HWW5 could be due to the already discussed *Candida* presence. The increase in acridone concentration was attributed to the biotransformation of CBZ, 10,11-epoxyCBZ and acridine, this last one not analyzed in this study (Golan-Rozen et al., 2015). The ability to completely remove 2-hydroxyCBZ remained unaltered during the whole treatment. Cruz-Morató et al. (2013a) also found an increase in acridone concentration when non-sterile wastewater was treated, but complete removal when treating a sterile matrix. Contrarily, 46% removal of 2-hydroxyCBZ was achieved in sterile conditions while in our study the complete removal was obtained in non-sterile matrices. Therefore, other microorganisms may play a role in acridone accumulation and 2-hydroxyCBZ removal in non-sterile fungal operations. Jelic et al. (2011) deduced that 10,11-epoxyCBZ could appear by deconjugation of glucuronides. Glucoronidases are a type of transferase enzyme present in white-rot fungi and used in the catabolism of organic pollutants. Transferases catalyze the formation of glucoside, glucuronide, xyloside, sulphate or methyl conjugates from several compounds, increasing its solubility and reducing its toxicity (Harms et al., 2011). In fact, conjugation of xenobiotics has been widely reported (Hundt et al., 2000; Ichinose et al., 1999; Kondo et al., 1990). The deconjugation of ketoprofen in the fungal reactor –but not in the uninoculated reactor– has been suggested above. Therefore, it is proposed that *Trametes versicolor* could enhance the deconjugation of such compounds.

In general, the global removals of both treatments RA and RB are similar due to ibuprofen and acetaminophen being the main contributors to the overall PhACs concentration and both being easily removed. Activated sludge in WWTP is also reported to remove several of these compounds. However, when highly recalcitrant xenobiotics are taken in account, like the compounds in antibiotic and psychiatric drugs families, the fungal treatment overpowered the uninoculated reactor. These families are not only very recalcitrant but also the main contributors to effluent overall toxicity and therefore environmental risk (Lucas et al., 2016). Fungal effluent exhibited lower concentrations of such products and lower toxicity values, as discussed below.

In an attempt to evaluate the capacity of both reactors to degrade or transform other compounds not included in the chemical analysis an acute toxicity bioassay with the bacterium *Vibrio fischeri* was performed. In addition, the approach could be used to evaluate the risk involved with the disposal of a potentially fungal-treated and non-treated hospital effluent into the sewage system. The Environmental Protection Agency (2004) recommends 0.3 TU as a threshold for acute toxicity and 1.0 TU for chronic toxicity. The fungal treatment succeeded in maintaining the acute toxicity at 0.0 TU during the whole treatment. This indicated that *T. versicolor* removed non-analyzed

toxic compounds and that no toxic metabolites were generated, or that potential toxic intermediates were also degraded, as pointed out by Cruz-Morató et al. (2013a). The absence of toxicity suggested the possibility of disposal of the effluent to the sewage system. Contrarily, the uninoculated reactor raised the acute toxicity of the initial HWW to 27 TU, implying that the effluent should not be disposed of, that non-analyzed toxic compounds were not removed or that toxic metabolites were formed –and not degraded.

5.4 Conclusions

T. versicolor in pelleted morphology was maintained in a fungal reactor treating flocculated non-sterile real hospital wastewater for two months with an HRT of 3 d. A partial biomass renovation strategy was used to maintain *T. versicolor* activity throughout the treatment, with a CRT of 21 d. A DGGE and sequencing approach confirmed that *T. versicolor* survived during the whole treatment. Regardless, longer operations might be needed to achieve a steady community structure.

81 pharmaceutical compounds were analyzed and 46 were detected. Fungal treatment consistently removed most of the detected PhACs, including the most recalcitrant ones. Treated wastewater effluent did not exhibit any toxicity and therefore the operation might have removed potential toxic metabolites. Some interspecies interactions favored and some obstructed removal of some PhACs. To the best of the authors' knowledge this is the first time a fungal treatment was implemented for 2 months treating non-sterile HWW.

6

Optimization of some process variables

Influence of some process variables in a continuous treatment of non-sterile hospital wastewater by *Trametes versicolor* and novel method for inoculum production

Based on the homonymous article Mir-Tutusaus, J.A., Caminal, G., Sarrà, M., 2017. Journal of Environmental Management. *In press*.

Abstract

Micropollutants such as pharmaceutical active compounds, present at high concentration in hospital wastewater (HWW), pose both environmental and human health challenges. Fungal reactors can effectively remove such contaminants and produce non-toxic effluents, but their ability to operate for a long period of time is yet to be demonstrated in real hospital wastewater. Several process variables need to be studied beforehand. Here, variables pellet size, aeration and carbon-to-nitrogen ratio are studied in continuous operations with real HWW. Moreover, a novel strategy for inoculum production that could reduce economical and operational costs is proposed and tested. Optimum pellet size was found to be 2 mm and an aeration of 0.8 $L \cdot min^{-1}$ was needed to maintain fungal viability. A carbon-to-nitrogen ratio of 7.5 was selected and the pellet production time was reduced from 6 to 3 days. The novel low-cost inoculum preparation produced pellets with the same characteristics as the traditionally prepared ones.

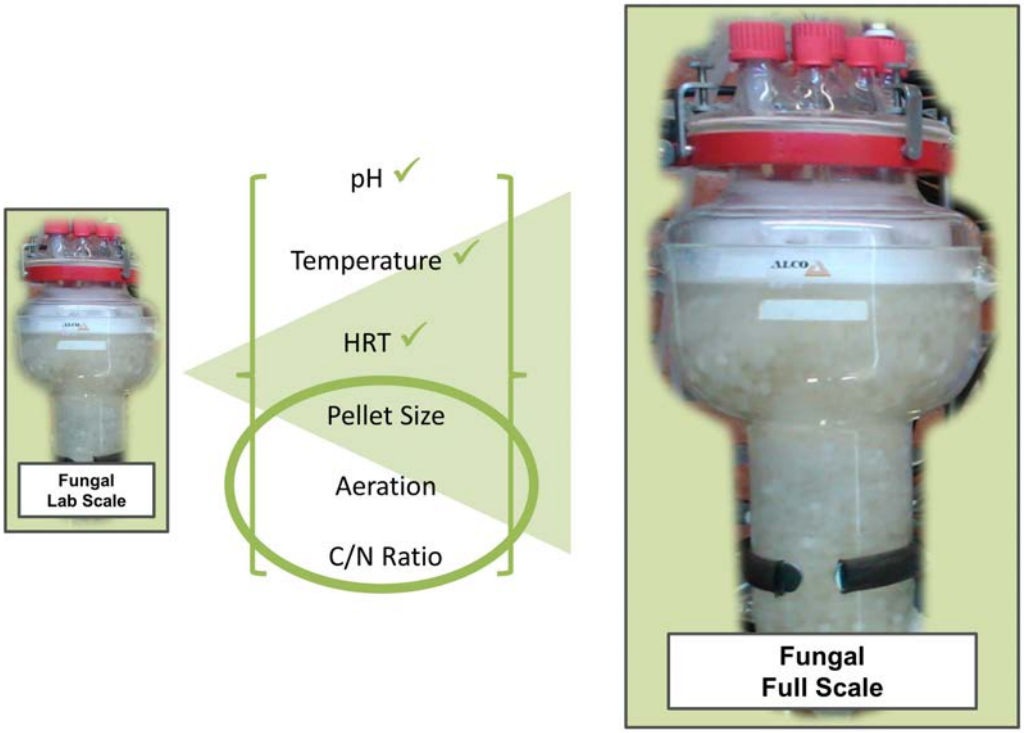

Figure 6.1: Graphical abstract.

6.1 Introduction

Hospital wastewater (HWW) is a main source of pharmaceutical active compounds (PhACs) and is discharged untreated to public sewer. Wastewater treatment plants (WWTPs) receive these effluents but are not designed to remove such contaminants and thus the pollutants reach water bodies. PhACs tend to be recalcitrant and can accumulate, posing a threat to the environment and human health (Verlicchi et al., 2015). Therefore, a specific treatment to remove these contaminants on-site (e.g., a hospital) is of high interest. Treatments based on white-rot fungi (WRF) have been tested, because, among other capabilities, WRF's non-specific enzymatic machinery makes the fungal treatment a potential mechanism for the removal of these pollutants. Laccase is a multicopper oxidase with low substrate specificity, and the main secreted enzyme of the WRF Trametes versicolor. Laccases have been widely researched for degradation of PhACs (Hata et al., 2010; Tran et al., 2010). A deeper review on the topic can be found elsewhere (Viswanath et al., 2014). Laccase can play a role in micropollutant degradation, but also the cytochrome P450 can transform

micropollutants(Marco-Urrea et al., 2010a; Mir-Tutusaus et al., 2014).

Cultures of WRF have demonstrated in a sterile condition their ability to degrade a wide range of contaminants, including PhACs(Marco-Urrea et al., 2010a; Mir-Tutusaus et al., 2014). Therefore, WRF could reduce the pharmaceutical load of HWW onsite prior to discharge to the WWTP. When non-sterile wastewater was used *T. versicolor* removed contaminants but the longevity of the treatment reduced significantly (Badia-Fabregat et al., 2015b). This could be a drawback for the application in full-scale plants. Recently, promising results have been published extending the fungal viability inside the reactor –therefore the length of operation, by the addition of a coagulation-flocculation pretreatment (Mir-Tutusaus et al., 2016). Weekly partial biomass renovations also extended operation length (Blánquez et al., 2006).

Several fungal pelleted reactor configurations exist and some of them are summarized in Espinosa-Ortiz et al. (2015). The present research focuses on air-pulsed fluidized bed reactors using pelleted *T. versicolor*. Such reactors have been operated with good removal values and temperature, cellular residence time (CRT), hydraulic residence time (HRT) and pH have been studied (Blánquez et al., 2006, 2007; Mehna et al., 1995). Nevertheless, few studies focused on other important variables. In particular, fungi morphology is critical and has been discussed elsewhere (Žnidaršič and Pavko, 2001). In fungal pelleted reactors, pellet size gains significance as it affects oxygen and mass transfer, but few studies were found regarding effect of pellet size in removal efficiency. Aeration could also be of importance in wastewater treatment, as pointed out by Badia-Fabregat et al. (2015a). In addition, aeration was identified as one of the main costs of operation (Gabarrell et al., 2012); therefore, decreasing the aeration without losing removal capacity could lower the operation costs. Little information can be found regarding the effect of carbon-to-nitrogen ratio (C/N). It has been studied in solid systems and in immobilized biomass in a bubble reactor (Pedroza-Rodríguez and Rodríguez-Vázquez, 2013; Rousk and Bååth, 2007), but, to the best of the author's knowledge, no bibliography was found in fluidized bed systems. Moreover, most studies focused on growth and/or enzyme production while the main parameter in HWW treatment is PhACs removal.

In addition, as a wastewater treatment needs to be economically viable, a novel approach to produce inoculum for the pellet growth has been studied. Pellet production and inoculum preparation traditionally requires careful handling and use malt extract, which is very expensive. Borràs et al. (2008) previously tackled the problem of low-cost pellet production. In the present article, we propose a low-manipulation, low-cost inoculum preparation.

The general objective is to gain information about the system in order to optimize several variables prior to scaling up the operation. For this reason the variables pellet size, aeration and carbon-to-nitrogen ratio have been studied. A novel approach for inoculum production has also been investigated. The HWW was spiked with ketoprofen (KTP), ibuprofen (IBP), and naproxen (NP) for analytical purposes.

6.2 Results and discussion

Several operational variables, such as pellet size, aeration and C/N ratio, were studied during five 21-days continuous operations, labeled A-E. The experimental setup is summarized in Table 6.2. Pellet size experiments (A, B) used HWW6 and aeration and C/N ratio experiments (C, D, and E) used HWW7. Both HWWs were physically and chemically characterized and results are presented in Table 6.1. Physicochemical characteristics of the wastewaters were mostly equivalent, and in the same range as other wastewaters described (Cruz-Morató et al., 2014; Verlicchi et al., 2015). A reduction in TSS, COD, DIC and DOC was observed due to the coagulation-flocculation pretreatment, a known behavior already reported (Mir-Tutusaus et al., 2016). KTP, IBP and NP content of the two wastewaters was not relevant due to the spiking of 10 $mg{\cdot}L^{-1}$ of each compound. This value was 100-fold higher than those typically found in wastewaters from this hospital, in the range of 2-20 $\mu g{\cdot}L^{-1}$ (Mir-Tutusaus et al., 2017a).

Table 6.1: Physicochemical characterization of the hospital wastewaters.

	HWW6		HWW7	
	Non flocculated	Flocculated	Non flocculated	Flocculated
pH	7.9	7.8	7.6	7.5
Conductivity ($mS{\cdot}cm^{-1}$)	1.7	1.7	4.0	3.9
Absorvance at 650 nm	0.191	0.000	0.136	0.009
Chloride ($mg\ Cl{\cdot}L^{-1}$)	288.6	290.7	1112.2	1078.2
Sulfate ($mg\ S{\cdot}L^{-1}$)	70.0	67.3	52.9	50.9
Nitrate ($mg\ N{\cdot}L^{-1}$)	4.7	4.4	2.1	1.3
Nitrite ($mg\ N{\cdot}L^{-1}$)	2.4	2.1	2.9	2.4
Phosphate ($mg\ P{\cdot}L^{-1}$)	0.60	0.61	0.01	0.01
Ammonia ($mg\ N \cdot L^{-1}$)	8.0	7.1	6.3	5.9
TSS ($mg{\cdot}L^{-1}$)	152	38	122	22
COD ($mg\ O_2{\cdot}L^{-1}$)	315	164	178	109
DIC ($mg{\cdot}L^{-1}$)	61 ± 2	53 ± 3	54 ± 2	50 ± 4
DOC ($mg{\cdot}L^{-1}$)	58 ± 5	46 ± 2	50 ± 3	35 ± 5

Table 6.2: Experiment ID and variables studied in the experimental setup.

Experiment	Pellet size (mm)	C:N ratio (mol:mol)	Aeration ($L \cdot min^{-1}$)
A	2.0	7.5	0.8
B	7.0	7.5	0.8
C	2.0	20	0.8
D	2.0	30	0.8
E	2.0	7.5	0.4

6.2.1 Pellet size and aeration operations

Operations A and B, presented in Figure 6.2, were used to study the effect of pellet size in a continuous HWW treatment; operations A and E (Figure 6.2) were used to analyze the effect of aeration. In order to enable a better comprehension for the reader, experiment A is presented in Figure 6.2 as well as in Figure 6.4. As an inversely proportional relationship was reported between the amount of inoculum and the pellet size (Sharma and Padwal-Desai, 1985), 20 $mL \cdot L^{-1}$ and 4 $mL \cdot L^{-1}$ of mycelial suspension were used to control the pellet diameter during the pellet production phase. The first amount produced a small pellet size (around 2±1 mm) and the latter a large pellet size (around 7 mm). In addition, not only pellet production time was reduced from 5–6 days to 72h when small pellets were prepared, but the risk of contamination decreased as well.

Treatment A showed a rapid peak of laccase on the first 24h of about 35 $U \cdot L^{-1}$ and an also steep decrease of laccase activity until Day 7-8, when production of laccase was recovered. The maximum peak of laccase activity was 50 U·L-1 and could be observed on Day 16. Although some fungal strains are able to produce other lignin modifying enzymes, *T. versicolor* ATCC#42530 under this culture conditions only produced laccase (Font et al., 2003). But, as presented in Section 6.1, this does not mean that laccase is the main enzyme involved in the degradation of the 3 pharmaceuticals studied.

In fact, prior to discussing ketoprofen, ibuprofen and naproxen removal, one must understand its degradation mechanisms in *T. versicolor*: ketoprofen is metabolized inside the cell by the cytochrome P450, with no involvement of laccase and non-relevant adsorption phenomena (Marco-Urrea et al., 2010b); distinctly, ibuprofen pathway is less clear but does not involve laccases, manganese peroxidases nor the P450 system, and a maximum adsorption of 20% was found in batch cultures (Marco-Urrea et al., 2009); lastly, naproxen can be vastly degraded by laccase, with P450 playing also a key role and adsorption being non-relevant (Marco-Urrea et al., 2010a). More than 80% removal of KTP, IBP and NP was obtained from the first day onwards during treatment A. Since that

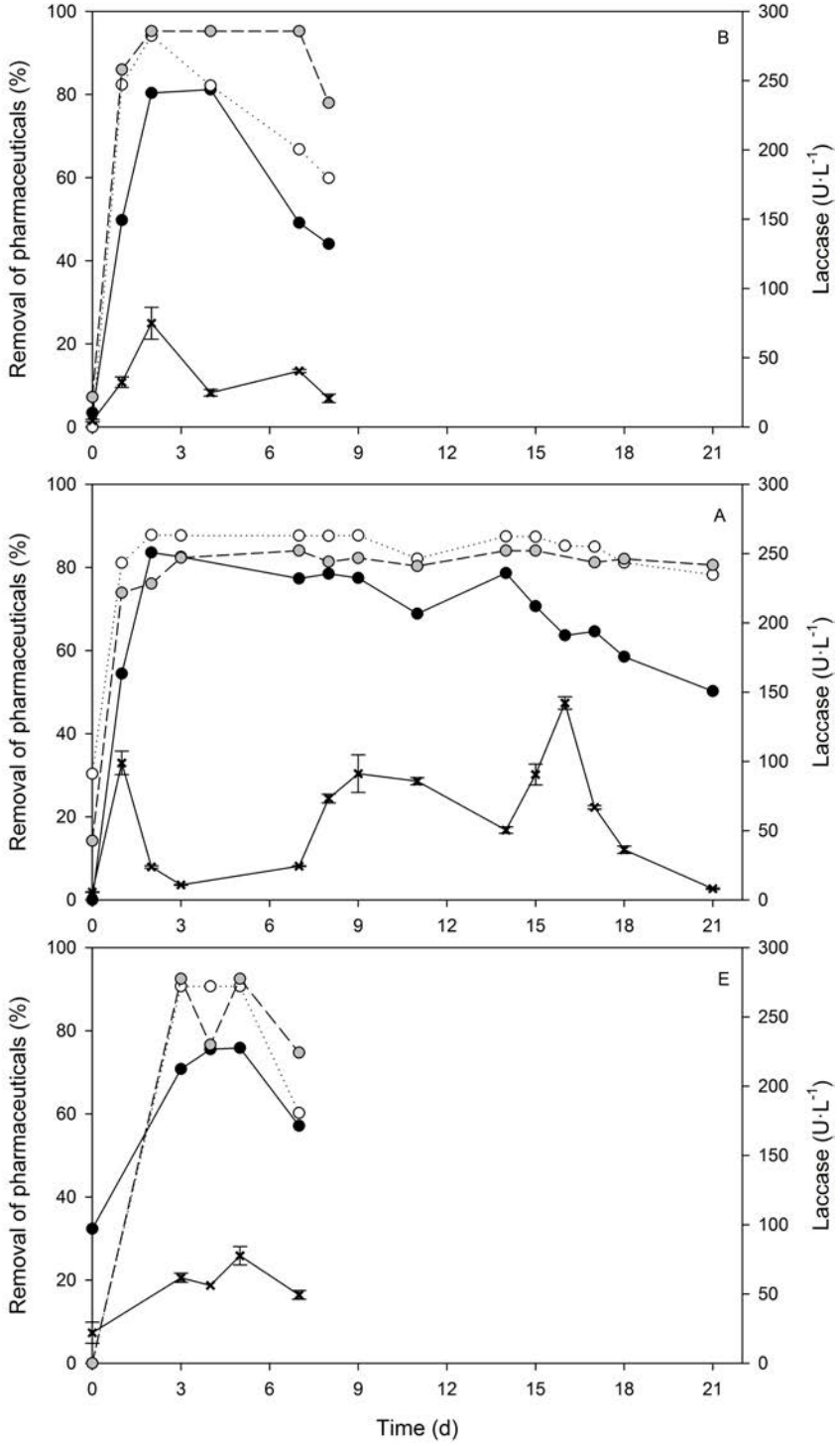

Figure 6.2: Removal profiles for ketoprofen, ibuprofen and naproxen and laccase activity profiles in experiments B (large pellet size, high aeration), A (small pellet size, high aeration) and E (small pellet size, low aeration). Legend: black, gray and white circles represent the ketoprofen, ibuprofen and naproxen removal percentages, respectively. Black crosses represent laccase activity.

Table 6.3: Chemical oxygen demand, ammonium nitrogen and pellet size of the experiments A-E. COD and N-NH4+ were measured at start, mid, and end of the treatment; pellet size, at the initial and end of the treatment.

	COD ($mg \cdot L^{-1}$)			$N\text{-}NH_4^+$ ($mg \cdot L^{-1}$)			Pellet size (mm)		
Experiment	0d	14d	21d	0d	14d	21d	0d		21d
A	61	160	1640	11	151	109	2.0 ± 0.4		2.5 ± 0.4
B	73	1400[a]	-	11	166[a]	-	6.7 ± 1.7		-
C	107	1390	715	13	120	125	1.7 ± 0.3		1.4 ± 0.4
D	112	1750	785	12	76	68	2.3 ± 0.4		2.6 ± 0.7
E	107	1462[a]	-	13	174[a]	-	1.3 ± 0.5		-

[a]Sample taken on the last day of treatment: 8th day for experiment B, 7th day for experiment E.

day, the operation behaved constantly until Day 14, when a slow decrease in KTP removal could be observed. IBP and NP were steadily removed throughout the treatment. Pellet diameter slightly increased during the treatment, as can be observed in Table 6.3. This fact confirmed that biomass was active and justified the visually good aspect of the reactor at the end of the treatment. In order to capture loss of pelleted biomass other than visually, total COD was measured throughout the treatment and is presented in Table 6.3. The overall aim of this type of fungal treatment was pharmaceutical removal, not COD removal and thus COD is discussed only as a confirmation of biomass loss. In fact, some fungal processes maintain or even increase COD of the effluent (Badia-Fabregat et al., 2015b; Mir-Tutusaus et al., 2017a). In spite of the visually good aspect of pellets, COD on the last day amounted 1640 $mg \cdot L^{-1}$, a 10-fold increase of the COD at mid-treatment. This increase could be due to a proliferation of free mycelia in the reactor. This could suggest the breakdown of pellets, which would be in accord with previous studies that already identified 21 days as an appropriate CRT for this process (Blánquez et al., 2006).

Operation B, with a mean pellet diameter of 6.7 mm, behaved similarly as A for only 3 days, although with higher removals for IBP and NP. After that, KTP removal was reduced rapidly to <50%, followed by NP. Experiment B retained IBP removal capacity until Day 7. The operation finally lasted 8 days, when pelleted biomass was lost. Pellet size could not be determined because *Trametes* was found only in form of free mycelium, visually observed and confirmed by the COD of 1400 $mg \cdot L^{-1}$. Pellet morphology is a key factor in self-immobilized systems, as free mycelia exits the reactor, decreasing the fungal concentration. Mass transfer and oxygen limitation inside the pellets may be the cause of the loss of biomass observed, leading to autolysis (El-Enshasy, 2007): it was hypothesized that the lack of oxygen and nutrients in the center of the pellet created a hollow center with low oxygen concentration and low quantity of viable hyphae. Wittier

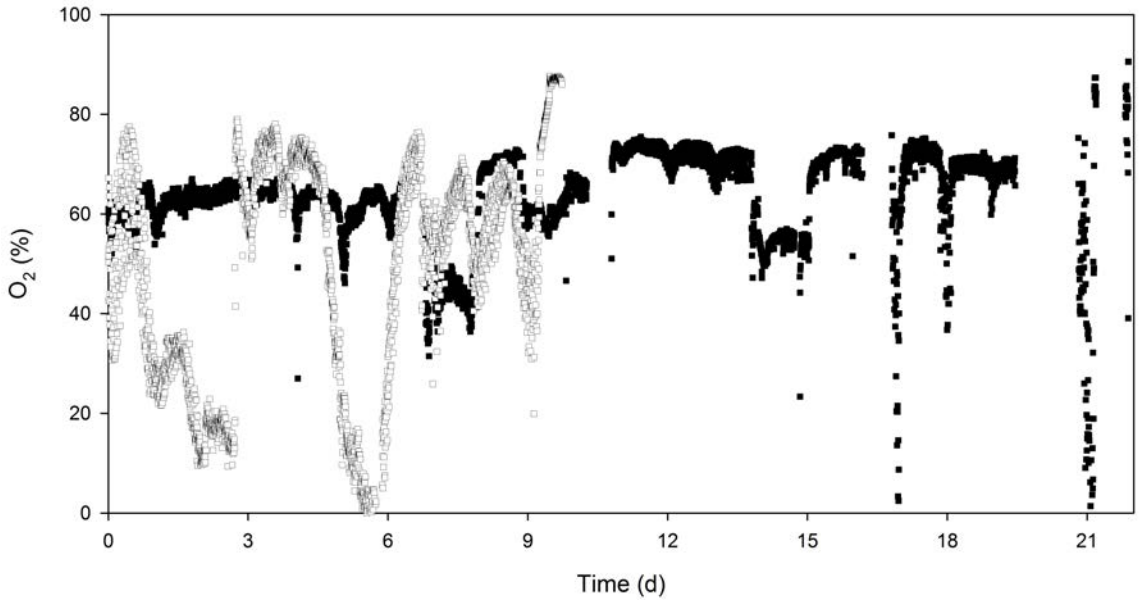

Figure 6.3: Oxygen concentration during the experiments A (in black squares) and E (in white squares).

et al. (1986) proposed a critical diameter, strain-and-cultivation-condition dependent, above which lack of nutrients and of oxygen would create this hollow center. Therefore, the critical diameter of *Trametes versicolor* in this system was lower than 6.7 mm. As a 2.0 mm diameter pellet produced a stable treatment, favored mass and oxygen transfer, and did not affect negatively on the operation of the reactor, this pellet size was selected for the consecutive experiments.

Studies have reported a relationship between diameter and protein production (Feng et al., 2004), but few focused on micropollutants removal. In this study, as a result of 6.7 mm pellets yielding very short operations due to the death of the fungus, it remained unclear the impact of pellet diameter in removal of PhACs. Additionally, as the matrix was not sterile, native wastewater microorganisms could tamper the draw of conclusions. Therefore, to understand the pellet size repercussions in the removal of PhACs, in vivo batch experiments were conducted in Erlenmeyer flasks in sterile conditions. Results are presented in Table 6.4, and as it can be observed, small pellets (2±1 mm) produced higher removal values than larger pellets (3±1 mm) for all three compounds at 8 and 24h –except for ketoprofen removal at 8 h, which is statistically equal in both pellet sizes. Pellets as large as 6.7 mm were not studied because its longevity was low, as discussed above. The three PhACs were removed at rates following IBP>NP>KTP in both pellet diameters, in accordance with the results in bioreactor.

In regards to aeration optimization, operation E is presented in Figure 6.2 and used half of the aeration used in the other experiments. In this operation laccase production remained at 20 $U{\cdot}L^{-1}$. Although laccase activity was rather low in operation E and in general in all three treatments but in the same range as similar operations (Badia-Fabregat et al., 2015b; Rodríguez-Rodríguez et al., 2012), removals escalated to 90% in the case of IBP and NP but only to 75% for KTP. This fact reinforced the idea of a decoupling between detection of laccase activity and pharmaceutical load removal. A decrease in KTP and NP could be observed as in the above reported operation B. The reactor was stopped by the end of the week, when all pellets were lost. This observation was confirmed by total COD concentration, which increased to 1462 $mg{\cdot}L^{-1}$. Therefore, no diameter measurement could be performed. An oxygen profile is presented in Figure 6.3 for operations A and E. As it could be observed, oxygen in experiment E dropped to below 20% on Day 3, when pelleted biomass was already damaged. Oxygen concentration subsequently increased due to the reduction of viable fungal biomass, and dropped again below 20% on Day 6 due to growth of microorganisms. Afterwards O_2 increased due to death of microorganisms and the operation was stopped. Sudden drops of oxygen in operation A were produced by a malfunction in the oxygen measurement. The pellets were approximately 1.3 mm in diameter, well below the proposed critical diameter. Therefore, nutrient limitation inside the pellets was rejected as cause of the loss of pelleted biomass. In conclusion, an aeration of 0.4 $L{\cdot}min_{-1}$ was insufficient to maintain the fungus in this reactor. Higher aeration values would increase the cost of a full-scale operation and in consequence such experiments were ruled out. In addition, high concentrations of O_2 are attributed to oxidative stress (Higashiyama et al., 1999). As an aeration of 0.8 $L{\cdot}min_{-1}$ proved to be the best-performing aeration tested, it was selected to conduct the subsequent experiments.

6.2.2 Carbon-to-nitrogen ratio operations

Experiments A (C/N 7.5), C (C/N 20) and D (C/N 30), presented in Figure 6.4, were studied in to evaluate the effect of C/N ratio in the removal of pharmaceuticals, as all other variables were fixed. Laccase profiles of all 3 operations were equivalent the first week. Afterwards, experiments A and C remained equivalent during the second week of treatment, with laccase activity at 20-30 $U{\cdot}L^{-1}$. Overall, laccase activity in A was maintained until the last week, until the second week in C and it was lost on the first week in D. In general it is accepted that a high carbon-to-nitrogen ratio, mimicking ligninolytic conditions, is required for laccase production (Eggert et al., 1996). Limiting

conditions of carbon or nitrogen have also been reported to trigger ligninolytic enzymes production (Reddy, 1995). It may seem that the opposite behavior has been observed in our experiments, as higher C/N ratios produced less laccase. However, fungal viability should be taken into account: low C/N ratios favor fungal over bacterial growth (Demoling et al., 2007; Rousk and Bååth, 2007). An increasing loss of fungal viability was observed in experiments A, C and D, in this order and could be quantified by COD determination at Day 14, presented in Table 6.3: D operation had the higher COD value, followed by C and finally A, with a low COD value. The three operations presented an increasing profile of COD and N-NH_4^+ (Table 6.3) until pelleted biomass was lost: at the end of the 3rd week in operation A and at the end of 2nd week in treatments C and D. N-NH_4^+ was found to be the lowest in experiment D (C/N 30), followed by C (C/N 20) and A (C/N 7.5).

In regards to KTP, IBP and NP removal all experiments reached values above 80% during the first week. Removals dropped to 40% on the 8th and on the 9th day in operations C and D, respectively, but quickly recovered. Removal capacity was lost on reactor C from Day 14 onwards and reactor D did not recover 80% removal values, except for ibuprofen. In general, ketoprofen was the first compound to be affected when the system lost removal capacity. This is in accord with other studies, as KTP is slower removed than IBP and NP (Badia-Fabregat et al., 2015b). As discussed, KTP degradation is intracellular, IBP does not involve laccase nor P450 and naproxen can be degraded by P450 and laccase (Marco-Urrea et al., 2009, 2010b,a). Naproxen removal values consistently fell below the IBP removal values in all experiments, especially at the end of the operations. The fact that experiments did not accumulate high concentrations of laccase enzyme, and that laccase activity was close to zero during the lasts weeks of treatment could be an explanation to NP being slower removed than IBP. In terms of PhACs removal, as well as of preservation of pelleted biomass and laccase production, the experiment with a C/N ratio of 7.5 performed better than with C/N ratios of 20 or 30. Therefore, a C/N ratio of 7.5 was selected.

6.2.3 Inoculum production

Preparation of inoculum for pellet production is largely described and it usually requires a mycelial suspension, which involves several steps of agar plate subculturing and careful manipulation (Casas López et al., 2005; Nair et al., 2016). Scaling-up the production of fungal inoculum for environmental applications would require a cheaper and easier methodology (Leštan and Lamar, 1999). Therefore, a process flow diagram

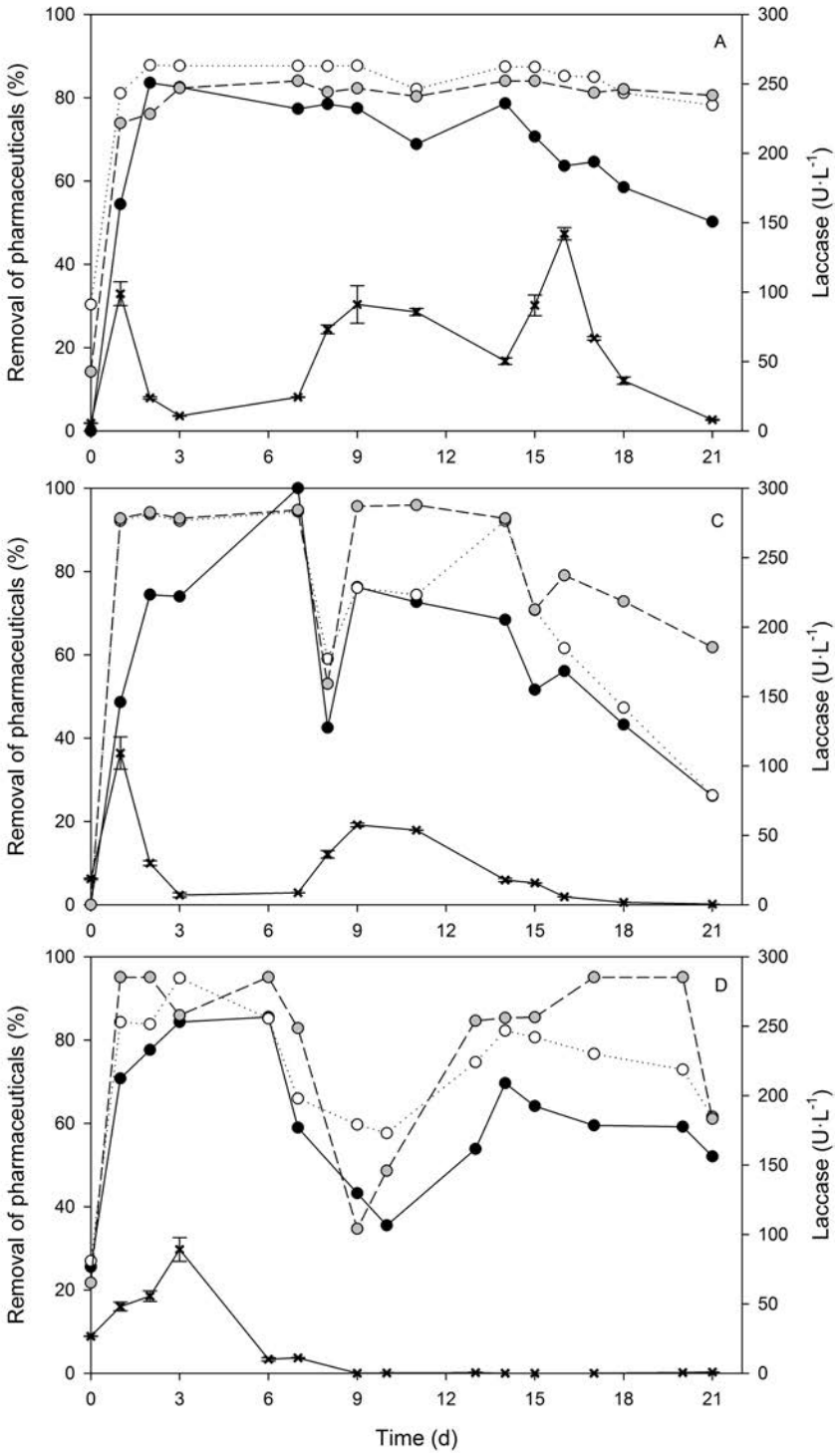

Figure 6.4: Removal profiles for ketoprofen, ibuprofen and naproxen and laccase activity profiles in experiments A (C/N 7.5), C (C/N 20) and D (C/N 30). Legend: black, gray and white circles represent the ketoprofen, ibuprofen and naproxen removal, respectively. Black crosses represent laccase activity.

(PFD) of the fungal operation, including the novel mycelial suspension preparation, is proposed in Figure 6.5. Following the PFD, a sufficient amount of pellets along with depleted media from the first batch of the production reactor (batch fluidized bed reactor, FBR) was homogenized and used as inoculum for the subsequent pellet production batch. Pellet production was performed as described in Section 3.2. The pellets and exhausted media would be separated in a settler and pumped along with fresh flocculated HWW to be treated in the continuous FBR as explained in Section 3.3.4.

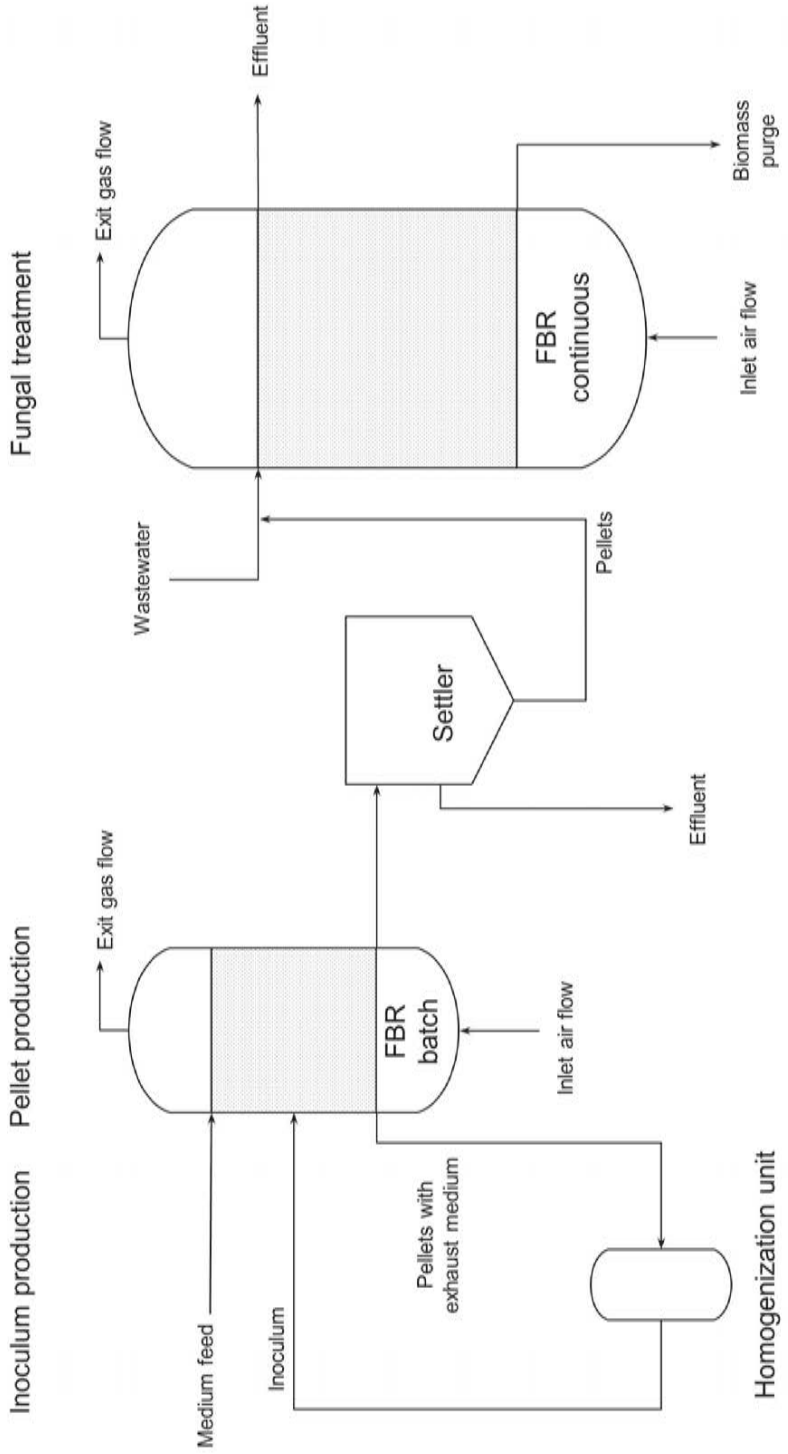

Figure 6.5: Process flow diagram of the treatment, including first, the novel inoculum preparation step; second, the pellet production phase; lastly, the fungal treatment.

Table 6.4: Removal percentages of ketoprofen, ibuprofen and naproxen with small (2 mm) and larger (3 mm) pellets.

Pharmaceutical active compounds	Removal at 8h (%)		Removal at 24h (%)	
	Small pellets	Large pellets	Small pellets	Large pellets
Ketoprofen	28 ± 3*	25 ± 6*	55 ± 3	44 ± 7
Ibuprofen	54 ± 0	43 ± 5	100 ± 0	71 ± 4
Naproxen	39 ± 2	31 ± 6	81 ± 2	68 ± 5

*Statistically equal

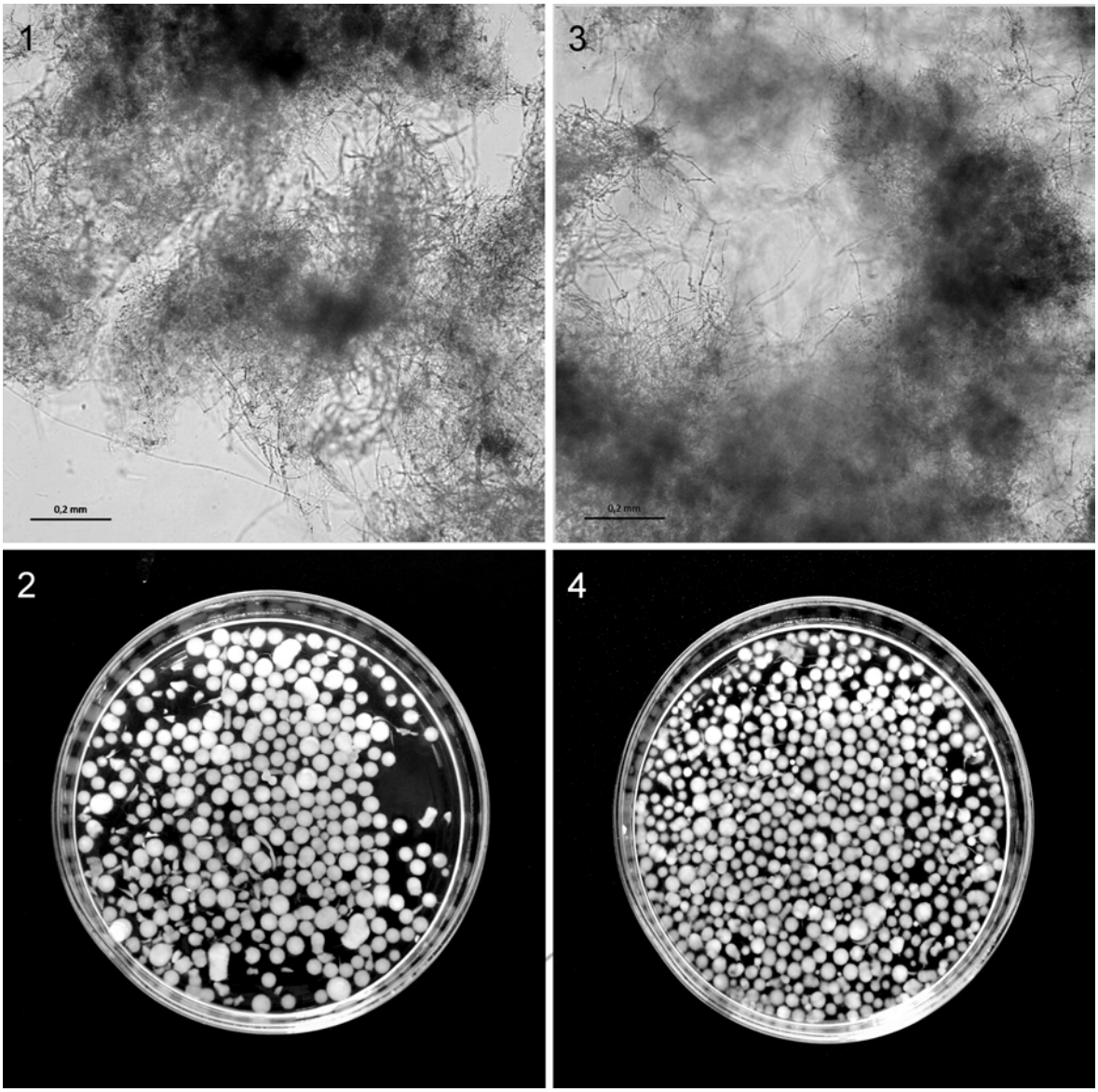

Figure 6.6: Mycelium traditionally prepared (1) and pellets produced with it (2) observed under a microscope. Mycelium prepared by the novel production process (3) and pellets produced with it (4) observed with the naked eye.

In order to validate the novel mycelial suspension preparation process a mycelial suspension was prepared by the traditional method described in Borràs et al. (2008) and by the novel method, namely, homogenizing pelleted biomass. Images 1 and 3 in Figure 6.6 illustrate both mycelial suspensions. Substantial differences could not be observed at 5.0x. For comparison purposes, the same amount of dry cell weight of mycelial suspension was used as inoculum for the pellet production batches. In this case, slight differences could be observed on the pellets produced with traditional inoculum (batch 1) and novel inoculum (batch 2), presented in images 2 and 4, respectively, in Figure 6.6. Mycelial suspension prepared by the traditional and novel method produced 2±1 mm and 2±0.5 mm pellets, respectively. Both batches consumed 7 $g{\cdot}L^{-1}$ of glucose in 72h and produced similar laccase activity profiles (data not shown). In order to further validate the inoculum production process, the ability of *T. versicolor* pellets produced by the traditional and novel method to remove KTP, IBP and NP was evaluated in a batch degradation experiment. The results presented in Table 6.4 showed that pellets derived from both inoculum preparation methods produced statistically equivalent removal values of 15, 50 and 26% for KTP, IBP and NP, respectively, in 8 h. The novel low-manipulation, low-cost method for mycelial suspension (inoculum) preparation reliably reproduced pelleted morphology so it should be applied in future works to approximate full-scale operation.

6.3 Conclusions

This chapter studied three variables for which there was little information: pellet size, aeration and C/N ratio. Pellet size was important in fungal survival and a critical diameter of 6.7mm is proposed; moreover, 2 mm pellets demonstrated better removal capacity than 3 mm pellets. A $0.8L{\cdot}min^{-1}$ aeration and a 7.5 C/N ratio were chosen for extending the treatment length. Pellet production time was reduced from 5–6d to 72h by increasing the mycelial inoculum amount, which also reduced the risk of contamination during the pellet production phase. Finally, the low-cost inoculum production method formed similar pellets to those traditionally prepared.

7

Long-term fungal treatment

Long-term continuous treatment of non-sterile real hospital wastewater by *Trametes versicolor*

Abstract

Hospital wastewater is commonly heavily polluted with pharmaceutically active compounds that pass through WWTPs and end up in water bodies, posing ecological and health risks. White-rot fungal treatments can cope with a wide variety of micropollutants while remaining ecologically and economically attractive. Unfortunately, bacterial contamination impeded so far a successful fungal treatment. This work embodied a 91-day long-term robust continuous fungal operation treating real non-sterile wastewater. Evolution of microbial community was followed combining 16S rDNA PCR-DGGE and sequencing. The operation was able to maintain a pharmaceutical load removal of over 80% while keeping the white-rot fungus active and predominant through the operation.

7.1 Introduction

Pharmaceutically active compounds (PhACs) occurrence in water bodies remains an issue despite persistent efforts of the scientific and global community. They can be found in relevant concentrations and pose a wide range of risks to the ecosystem, to the receiving wastewater treatment plant (WWTP) and to human health (Collado et al., 2014; Evgenidou et al., 2015; Ferrando-Climent et al., 2014; Verlicchi et al., 2012). Wastewater treatment plants (WWTPs) are in fact the primary source of PhACs into the environment, since they are not designed nor operated to remove these micropollutants (Deblonde and Hartemann, 2013). Hospital wastewater (HWW) contains higher concentrations of PhACs, which make hospitals a good target for on-site treatment (Gros et al., 2012;

Verlicchi et al., 2010). Otherwise, HWW is usually discharged untreated to the sewer network, thus contributing to the release of PhACs into WWTP influent (Grandclément et al., 2017; Verlicchi et al., 2010, 2012).

Removing such micropollutants from water streams is not trivial, although milestones have been achieved in that direction. While some studies dealt with the degradation of single pollutants, real streams have mixtures of several contaminants, thus a robust process should be capable of coping with the removal of most micropollutants. Amongst the biological oxidation processes, white-rot fungi (WRF) have proved especially well-suited for removing and degrading a wide range of pharmaceuticals (Jelic et al., 2012; Marco-Urrea et al., 2010a; Nguyen et al., 2013; Yang et al., 2013a). A versatile enzymatic system comprising both intracellular (e.g., cytochrome P450 system) and extracellular enzymes (e.g., laccase-mediators system) allows these fungi to transform most of the PhACs, usually very recalcitrant, to more biodegradable compounds or even achieve complete mineralization Pointing (2001); Badia-Fabregat et al. (2014). Therefore, a fungal system for treating hospital effluents has been regarded as a feasible approach, as some pharmaceuticals would be removed and other transformed into more biodegradable compounds, suitable for the posterior conventional activated sludge treatment (CAS) in the WWTP. This approach suggests a single fungal process that can remove most pharmaceutical load, rather than using a specific treatment for every compound.

However, full scale applications of this technology do not exist at the moment. Developments in this direction depend on overcoming several shortcomings, namely: (1) maintaining a stable activity of the fungal pellets over prolonged periods of time and (2) preserving a good performance in non-sterile conditions, as sterility would be unviable from the economic and ecological perspective (Espinosa-Ortiz et al., 2015; Gabarrell et al., 2012). On one hand, non-sterility reduces the length of bioreactor operation due to native microorganisms exerting competitive pressure in WRF survival. This aspect has been partly resolved by introducing a pretreatment step that reduces the initial concentration of microorganisms in the influent (Mir-Tutusaus et al., 2016). A partial biomass regeneration strategy could also help to increase the length of the bioreactor operation (Blánquez et al., 2006). On the other hand, removal efficiency can be higher in non-sterile matrices than in sterile conditions due to the consortium established (Ferrando-Climent et al., 2015; Gros et al., 2014). Additionally, in non-sterile matrices bacteria could degrade the more biodegradable transformation products of the xenobiotic parent compounds transformed by the WRF (Badia-Fabregat et al., 2015a; Cruz-Morató et al., 2014; Nguyen et al., 2014). The interactions between the inoculated

fungus and the microbial community existing in the wastewater and developed during the treatment seem to be case-dependent and are highly complex (Badia-Fabregat et al., 2015a). This fact calls for a thorough study of the microbial communities developed during a more stable, long-term operation. Furthermore, identification of native HWW PhACs degraders could lead to a better understanding of the process and to an improvement in the reactor operation.

Some studies optimized values for pH, temperature, growth conditions, aeration, pellet size, biomass renovation and nutrients addition (Badia-Fabregat et al., 2015a; Blánquez et al., 2006; Borràs et al., 2008; Mir-Tutusaus et al., 2017b). Those previous studies enabled this unprecedentedly long-term operation of a fungal fluidized bed bioreactor treating real non-sterile wastewater. In summary, the objectives of the study are to prove the concept of a long-term fungal treatment of real HWW, to evaluate the bacterial and fungal communities arisen during the treatment and to assess the removal efficiency for PhACs.

7.2 Results and discussion

Physicochemical characteristics of the wastewater used are summarized in Table 7.1. All values fell within the range of similar wastewaters, either from the same hospital or from other hospitals of the same region (Mir-Tutusaus et al., 2016, 2017a). The approximately 75% decrease of both TSS and COD after flocculation was also reported previously. The wastewater was taken from a hospital with an important psychiatric ward, therefore a high concentration of psychiatric drugs could be observed. Out of 74 compounds analyzed, 45 were detected and 11 detected compounds belonged to the psychiatric drugs group. Nearly 7 $\mu g \cdot L^{-1}$ of psychiatric drugs were measured, with carbamazepine, venlafaxine and their transformation products (TPs) being the most abundant. The presented psychiatric pharmaceuticals concentration was substantially lower than larger psychiatric hospitals, but more compounds were found (Yuan et al., 2013b). As it is common in HWW, the analgesics and anti-inflammatories family contributed the most to the overall pharmaceutical load (Badia-Fabregat et al., 2015b; Cruz-Morató et al., 2014), with ibuprofen, salicylic acid (a transformation product of aspirin) and acetaminophen (also known as paracetamol), in that order, being the most important of the group.

Table 7.1: Physicochemical characterization of the hospital wastewater.

	Non flocculated	Flocculated
pH	8.3	8.9
Conductivity ($mS \cdot cm^{-1}$)	2.1	1.9
Absorvance at 650 nm	0.239	0.082
Chloride ($mg\ Cl \cdot L^{-1}$)	343.0	359.9
Sulfate ($mg\ S \cdot L^{-1}$)	287.7	288.4
Nitrate ($mg\ N \cdot L^{-1}$)	n.d.	n.d.
Phosphate ($mg\ P \cdot L^{-1}$)	3.33	1.20
Ammonia ($mg\ N \cdot L^{-1}$)	9.8	10.0
TSS ($mg \cdot L^{-1}$)	276	73
COD ($mg\ O_2 \cdot L^{-1}$)	507	128

7.2.1 Reactor performance

Real-time determination of micropollutant concentration in non-spiked matrices is usually not feasible. In this case, as the ability of active *T. versicolor* to remove the microcontaminants present in HWW was already established, this treatment relied on the measurements of laccase activity and glucose concentration as a measure of fungal activity. Laccase activity profile during the operation is presented in Figure 7.1. Two periods could be observed: the first period, from the start until the 63rd day, with peaks of around 50 $U \cdot L^{-1}$ which corresponded to the weekly partial biomass regenerations and a mean value around 30 $U \cdot L^{-1}$; the second period, after Day 63, where laccase activity levels remained close to zero. Through this partial biomass restoration strategy a young culture was maintained in the bioreactor (Blánquez et al., 2006); this fact and the rather long hydraulic residence time of 3 d allowed a relatively stable and high laccase activity. In fact, several studies reported no laccase activity in fungal MBRs (Yang et al., 2013a; Nguyen et al., 2013) without biomass renovation, and in chapter 8 a much lower laccase activity was observed when the HRT was set at 12 h. Extracelular enzymes can be produced but degraded or inactivated by products from other microorganisms (Yang et al., 2013b), and low laccase activity was also reported even when fungal pelleted biomass was maintained in an FBR (Badia-Fabregat et al., 2015b). Therefore, as *T. versicolor* was confirmed by DGGE (Table 7.3), a shift in the microbial communities might have inactivated laccase by the end of the operation. In fact, a high concentration of *T. versicolor* was maintained throughout the operation (discussed in Section 7.2.4) even when no laccase could be detected, confirming the non-correlation between absence of laccase activity and fungal inactivation.

Although laccase activity was being detected and glucose concentration was close to

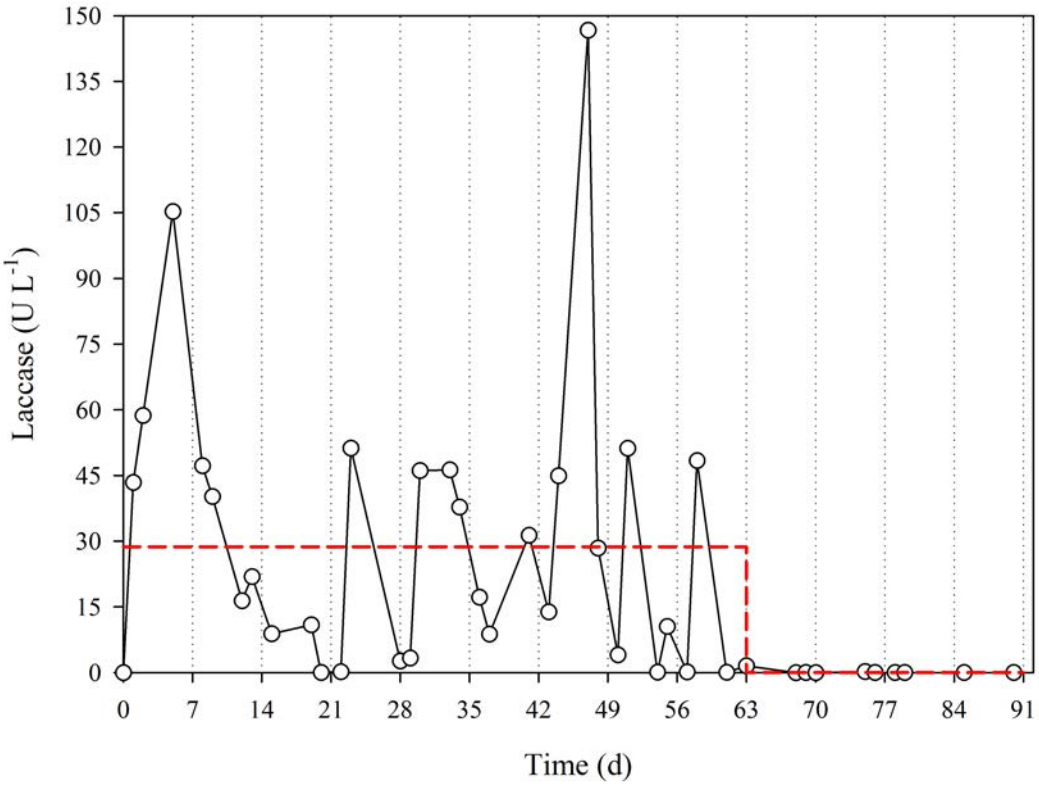

Figure 7.1: Evolution of laccase activity during the continuous treatment. The dotted red line represents mean laccase activity values; vertical dotted lines represent the weekly partial biomass regeneration.

zero, the reactor gained a pinkish color between days 33 and 37, when reactor biomass was replaced by fresh pellets. Even though laccase activity was low at the end, it could not be linked to the inactivation of the fungus, as demonstrated previously –although the presence of laccase could be indeed linked to fungal activity (Mir-Tutusaus et al., 2016). As glucose was added at consumption rate, glucose concentration remained insignificant during the treatment (data not shown).

7.2.2 Pharmaceutical load removal

Time-course profile of concentrations of pharmaceutically active compounds is presented in Figure 7.2, organized by families. The initial concentration of PhACs was around 45 $\mu g \cdot L^{-1}$, mainly contributed by analgesics and anti-inflammatories, followed by psychiatric drugs, antihypertensives and other pharmaceuticals. The increase of analgesics and anti-inflammatories concentration at Day 8 is worth mentioning, mainly

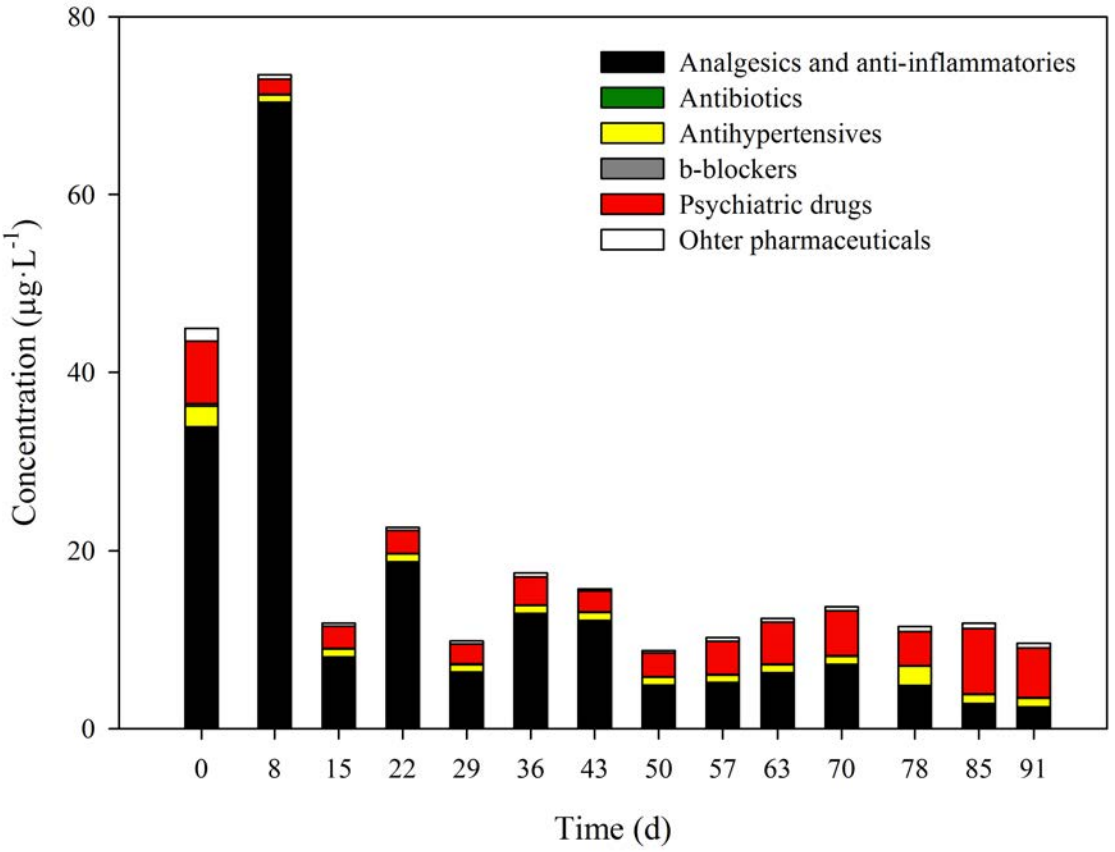

Figure 7.2: Concentration profile of pharmaceutically active compounds during the treatment.

contributed by ketoprofen and naproxen. Although *T. versicolor* has proved the ability of degrading ketoprofen and naproxen both in sterile, defined media and in non-sterile HWW (Marco-Urrea et al., 2010b,a; Mir-Tutusaus et al., 2016), it was also demonstrated that it deconjugated some compounds such as ketoprofen and naproxen (Badia-Fabregat et al., 2015a; Cruz-Morató et al., 2013a; Mir-Tutusaus et al., 2017a). Briefly, conjugation is a chemical transformation used to detoxify xenobiotics, performed in the liver in humans, which enables xenobiotics to be more water-soluble and excreted through urine. Cleavage of these conjugated compounds by *T. versicolor* was the most likely explanation for the increase in the concentration at Day 8. This fact established that the PhAC concentration in the influent was equal or higher than at Day 8.

From the second week of operation onwards it was considered as a pseudo-steady state, given that 5 times the HRT had passed: as can be observed in Figure 7.2, the global

pharmaceutical concentration remained constant since Day 15, representing an overall 82% removal. From the point of view of *T. versicolor*, on the one hand, it was indeed considered a steady state because the biomass renovation ensured a constant concentration of the fungus. On the other hand, from the point of view of other microorganisms (bacteria and fungi), it could not be considered steady state as a constant evolution of these populations was revealed through a DGGE + sequencing approach (Section 7.2.4). During this period and for the first time a fungal treatment successfully removed pharmaceutical active compounds from a real non-sterile wastewater in a long-term operation. PhAC concentration was maintained around 15 $\mu g \cdot L^{-1}$, although the removal profile diverged between groups. For example: concentration of analgesics and anti-inflammatories decreased with time, probably due to adaptation of native wastewater analgesics and anti-inflammatories degraders, as was concluded in a previous study (Mir-Tutusaus et al., 2017a); however, removal capacity of the psychiatric drugs group declined. This latter event is further discussed in Section 7.2.3.

Taking into account the profile of laccase activity and information of PhAC removal, it could be established that laccase was not essential to PhAC degradation. It was not unexpected, as degradation pathways in WRF are diverse. Notably, the cytochrome P450 mechanism is known to play a role in several micropollutants transformation (Blánquez et al., 2004; Jelic et al., 2012; Marco-Urrea et al., 2010b). Therefore, although laccase could play a role in PhAC degradation –and is in fact heavily studied in that direction (Morozova et al., 2007; Viswanath et al., 2014)–, the non-detection of laccase activity should not be considered an indicator of decrease in removal efficiency in similar systems. However, higher concentrations of psychiatric drugs were found by the end of the treatment, when laccase activity was not detected. This behavior is further discussed in Section 7.2.3.

As the pharmaceutical load was successfully maintained constant around 10-15 $\mu g \cdot L^{-1}$ and the novel method of mycelial suspension production by homogenization of pelleted biomass proposed previously (Mir-Tutusaus et al., 2017b) was used throughout the long-term operation, the novel method of mycelial suspension preparation was therefore validated. This improvement could substantially reduce the ecological and economic burden of mycelial preparation at industrial scale, and together with the also validated use of low-cost medium, the technology become more attractive economically.

The average removal of compounds during the steady state period (from Day 15 onwards) is presented in Figure 7.3. Most analgesics and anti-inflammatories were

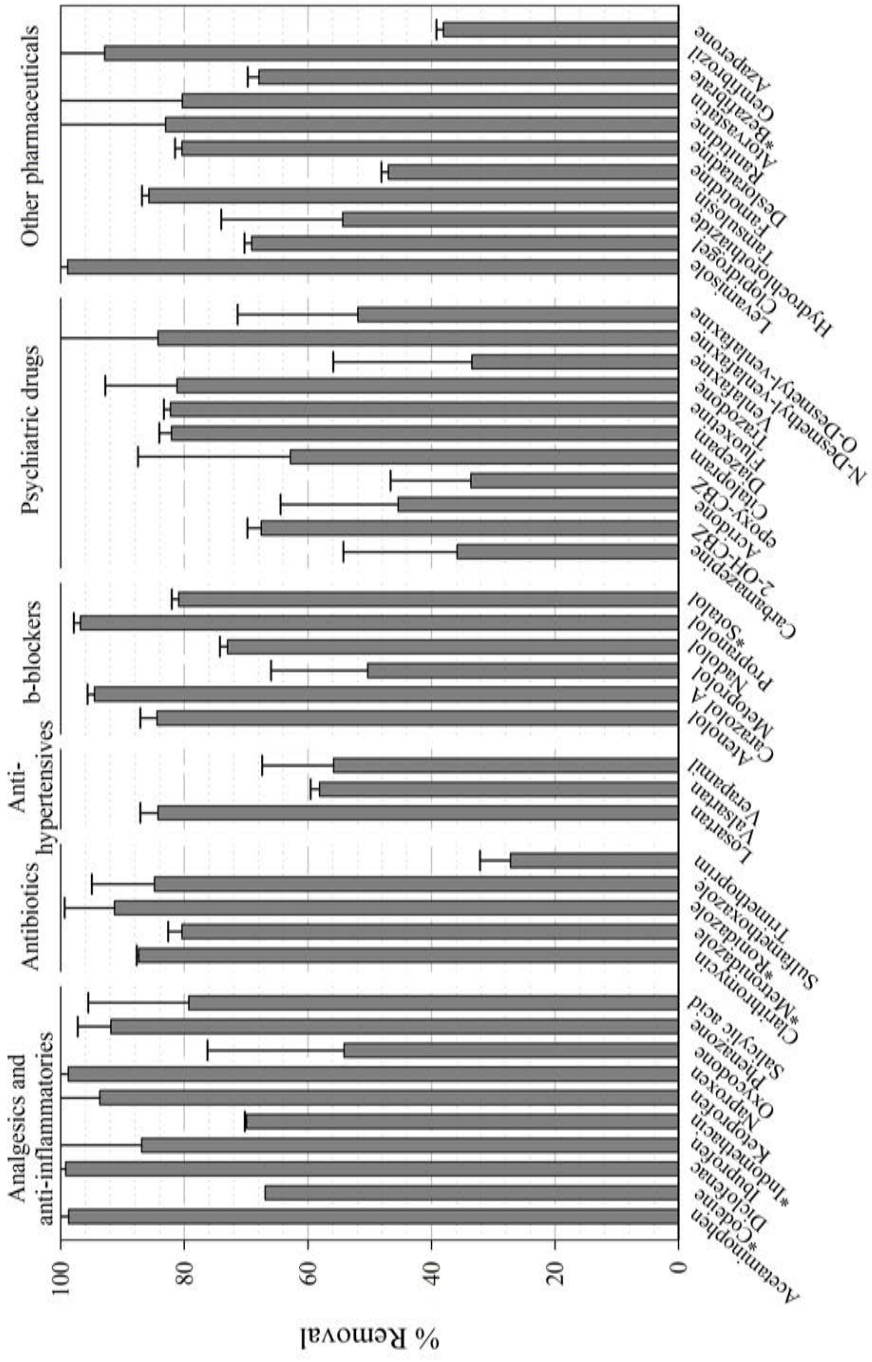

Figure 7.3: Removal values of pharmaceutically active compounds. Error bars represent the standard deviation of 12 samples during the steady state. Asterisks mark compounds whose initial concentration contained >80% of left-censored data.

removed over 80%, or more if we took into account deconjugation phenomena, except for oxycodone. Oxycodone was similarly removed by around 60% in a batch fungal treatment (Badia-Fabregat et al., 2015b), while removal data in activated sludge greatly varies between 0 and 50% (Baalbaki et al., 2016; Yang et al., 2017). The fact that diclofenac was completely removed throughout the treatment is of interest because it is widely used and not removed in WWTPs (Verlicchi et al., 2012). In addition, diclofenac, ibuprofen and naproxen are all classified by the Global Water Research Coalition (GWRC, 2008) as high priority pharmaceuticals and were all completely removed by the treatment.

Antibiotics were well removed over 80% with the exception of trimethoprim. This was in accord with the bibliography, as low removal values for trimethoprim are produced in fungal processes (36% - 50% (Badia-Fabregat et al., 2015b; Mir-Tutusaus et al., 2017a)) and lower in activated sludge, even in more complex A2/O-UV WWTPs (Wang et al., 2014; Yang et al., 2017). The other detected antibiotics are usually poorly or not removed in CAS and MBR systems (Joss et al., 2006), so the fungal treatment remained an interesting alternative. In fact, this long-term treatment slightly improved clarithromycin and sulfamethoxazole removal, but greatly improved ronidazole degradation in comparison with shorter fungal continuous and batch operations (from 0-30% to >90%) (Badia-Fabregat et al., 2015b; Mir-Tutusaus et al., 2017a).

Antihypertensives and β-blockers were removed between 50 and >95%. These families included atenolol > metoprolol = propranolol > sotalol, listed in order of priority in GWRC report (Global Water Research Coalition, 2008). Out of the 11 psychiatric drugs detected, 4 were carbamazepine and its TPs and 3 were venlafaxine and its TPs; these are discussed in Section 7.2.3. The other psychiatric drugs were removed around 80% and citalopram around 60%. Diazepam removal value during this long-term operation was especially high compared to the 0-50% achieved previously in shorter-term operations and batches (Badia-Fabregat et al., 2015b; Mir-Tutusaus et al., 2017a).

In regards to the miscellanea group, anthelmintic levamisole, antiplatelet agent clopidrogel, drug against prostatic hyperplasia tamsulosin, H1 and H2 receptor antagonists desloratadine and ranitidine, and lipid regulators atorvastatin, bezafibrate and gemfibrozil were removed around 70% or above, whilst diuretic hydrochlorothiazide, H1 and H2 receptor antagonist famotidine and veterinary tranquilizer azaperone were removed around 50% or below. In general, removal values for this family and all other groups were higher than in previous studies (Badia-Fabregat et al., 2015b; Mir-Tutusaus et al., 2017a) probably due to the partial biomass restoration strategy (Blánquez et al., 2006) and the prior optimization of carbon-to-nitrogen ratio in

the nutrients addition, aeration and pellet size (Mir-Tutusaus et al., 2017b).

7.2.3 Carbamazepine and venlafaxine removal and transformation products

Both carbamazepine and venlafaxine are psychiatric drugs widely used and commonly detected in hospital wastewater, WWTP influent and also WWTP effluent, because both compounds are very recalcitrant to bacterial degradation. In fact, carbamazepine and venlafaxine were, in this order, amongst the most detected psychiatric drugs in Europe (Herrmann et al., 2015; Mackuak et al., 2016; Mir-Tutusaus et al., 2017a; Santos et al., 2013; Yuan et al., 2013a). CBZ is metabolized in the human liver by a 95% and produces mainly CBZE, which then is transformed to acridone; CBZ transformation also produces 2-OH-CBZ, other TPs and conjugated compounds (Pearce et al., 2009). Further discussion on CBZ degradation pathway on fungi and humans can be found elsewhere (Section 9.2.3). Carbamazepine (CBZ) and its TPs 2-hydroxycarbamazepine (2-OH-CBZ), acridone and epoxy-carbamazepine (CBZE) concentration profiles are presented in Figure 7.4 (A). The long-term operation removed around 50% of the parent compound CBZ up until Day 57, when CBZ concentration started to increase until reaching influent concentration levels. While CBZ degradation was respectable, 2-OH-CBZ, acridone and CBZE were produced by transformation of the parent compound, but the treatment still succeeded in achieving good removal values of CBZ TPs. By the end of the treatment, though, CBZ TPs were not efficiently removed, even when no transformation of CBZ was occuring.

Venlafaxine (VEN) is highly metabolized in humans by cytochrome P450 in liver cells producing primarily O-desmethylvenlafaxine (ODV) –with antidepressant activity– and being N-desmethylvenlafaxine (NDV) a minor metabolic pathway (Celada et al., 2004). Concentration profiles of venlafaxine and its TPs are presented in Figure 7.4 (B). NDE was generally completely removed throughout the treatment. VEN and ODV exhibited low concentrations during the first 6 weeks of steady state, which slowly rose up to concentrations similar than those in the influent. It is worth noting that VEN could only originate from the influent, but ODV could derive from the influent or from transformation of VEN. Taking into account both groups of CBZ + TPs and VEN + TPs, they all followed a similar pattern, with low concentration at the start, followed by an increase from Day 57. This behavior was similarly described in a shorter term fungal operation, where removal of psychiatric drugs decreased over time(Mir-Tutusaus et al., 2017a). In this case, as CBZ and CBZE can be removed by both laccase and the intracellular cytochrome P450 (Golan-rozen et al., 2011; Hata et al., 2010), the decrease

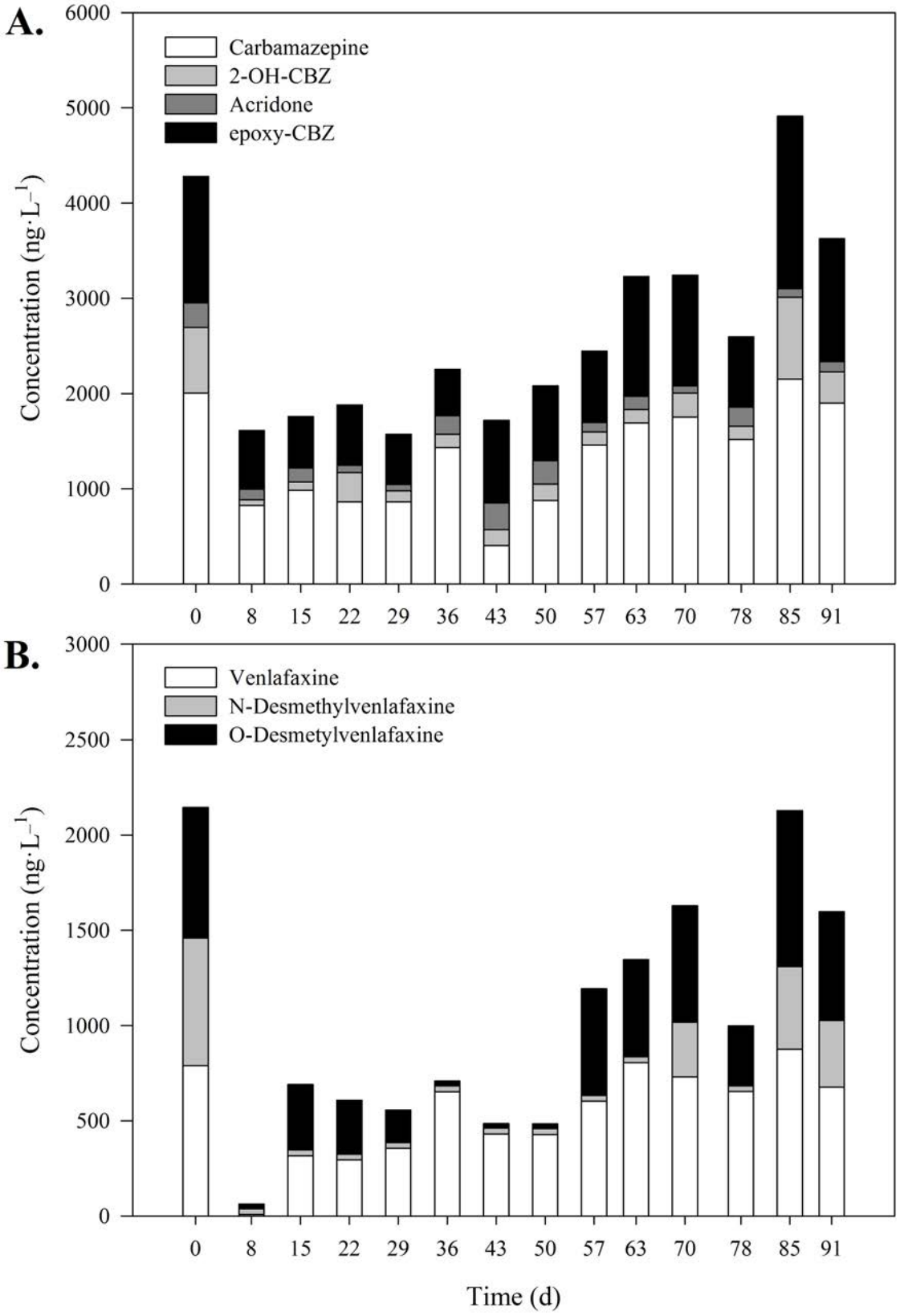

Figure 7.4: Concentration profile of carbamazepine and its transformation products (A) and venlafaxine and its transformation products (B) during the treatment.

in laccase activity might have affected CBZ removal. No information was found regarding venlafaxine degradation by laccase. Additionally, a synergistic effect was hypothesized between fungal and bacterial enzymes that led to a high removal of VEN in a batch treatment (Cruz-Morató et al., 2014); an increase in CBZ and its TPs degradation was also observed when non-sterile wastewater was treated, in contrast to sterile WW. Therefore, the microbial community might have played a role in the psychiatric drugs removal diminution (further discussed in Section 7.2.4).

7.2.4 Evolution of bacterial and fungal populations in the bioreactor

The evolution of microbial populations in the liquid matrix was assessed using DGGE and sequencing of prominent bands. This work was carried out by Eloi Parladé, from the Environmental Microbiology group in the Genetics and Microbiology Department (UAB). A total of 45 bands were recovered from bacteria and 33 bands from fungi, representing 90 and 98% coverage in the quantitative DGGE band matrix, respectively (data not shown). Unidentified bands were not considered in further analyses.

Bacterial sequences belonged to the Bacteroidetes, Firmicutes and Proteobacteria phyla. Results displaying the relative abundances of phyla (or classes, in the case of proteobacteria) are presented in Table 7.2. On one hand, Gammaproteobacteria were abundant in the early operation of the bioreactor but oscillated along time displaying three peaks at days 15, 43 and 78. Bacteroidetes behaved similarly, their abundance varied throughout the experiment, although with lower relative abundances. On the other hand, the Betaproteobacteria was the only group present during all the operation, with particularly high relative abundances (from 0.18 to 1) throughout the mid-late period. This bacterial taxon showed to be well adapted to fungal treatment in a previous work (Mir-Tutusaus et al., 2017a). Firmicutes were only found at the influent and did not colonize the reactor.

Half of the taxonomical groups detected in this study have been usually found in activated sludge treatments (Guo et al., 2017; Jo et al., 2016; Ye et al., 2016; Zhang et al., 2016). The acidic pH of the reactor, the HRT of 3 days and the presence of *Trametes* and its enzymes and microbial products might have affected the bacterial composition developing in the bioreactor. The loss of psychiatric drugs removal from Day 57 onwards could be attributed to the bacterial shift that led to a major abundance of Betaproteobacteria over the other groups. Even if there was no degradation of psychiatric drugs by bacteria in an earlier period, they may have played a role in the

Table 7.2: Phylogenetic assignment of bacterial sequences from the liquid matrix of the bioreactor. Data is presented in form of relative abundance, previously calculated using the semi-quantitative DGGE matrix and the sequenced bands from DGGE gels. Symbols: "-", absent; "+", ≥ 0.01%; "++", ≥ 10%; "+++", ≥ 40%. Unidentified bands (10% for bacteria and 2% for fungi) were not represented.

Bacteria	Time (d)												
	0	8	15	22	29	36	43	50	57	63	70	78	91
Bacteroidetes	+	++	-	+	-	+	++	+++	-	+	+	++	-
Firmicutes	++	-	-	-	-	-	-	-	-	-	-	-	-
Alphaproteobacteria	+	+	-	+++	+++	+	-	++	-	++	++	+	++
Betaproteobacteria	++	++	+	+	+	+++	++	+++	+++	+++	+++	++	+++
Gammaproteobacteria	+++	+++	+++	++	+++	++	+++	-	-	++	++	+++	++

Table 7.3: Phylogenetic assignment of fungal sequences from the liquid matrix of the bioreactor. Data is presented in form of relative abundance, previously calculated using the semi-quantitative DGGE matrix and the sequenced bands from DGGE gels. Symbols: "-", absent; "+", ≥ 0.01%; "++", ≥ 10%; "+++", ≥ 40%. Unidentified bands (10% for bacteria and 2% for fungi) were not represented.

Fungi	Time (d)												
	0	8	15	22	29	36	43	50	57	63	70	78	91
Fusarium	+++	+++	+++	+++	+++	+++	-	-	++	+	++	++	++
Trametes	-	+	+++	+	+	+	+	+++	+++	+++	+++	+++	+++
Clitopilus	+++	-	-	-	-	-	-	-	-	-	-	-	-

further degradation of fungal metabolites, thereby allowing an increased reactor removal capacity (Mikesková et al., 2012).

In regards to fungal populations (Table 7.3), although many fungi are typically present in wastewater (Viegas et al., 2014), the DGGE + sequencing approach identified three fungal genera in the liquid fraction of the bioreactor: *Clitopilus* (Basidiomycota) *Trametes* (Basidiomycota) and *Fusarium* (Ascomycota).

Clitopilus was only found at the beginning and did not colonize the liquid fraction of the reactor at the conditions of operation. Contrarily, *Fusarium* rapidly colonized the liquid fraction up to a relative abundance of ≥0.92 until Day 43, but concentration of *Trametes* in the liquid steadily rose due to the breakdown of pellets into free, dissolved mycelium (Table 7.3). A sporadic increase in turbidity and appearance of pink pigmentation was observed between days 33 and 37, coinciding with the appearance and increase in abundance of a Gammaproteobacterium. Recent studies reported the appearance of pink-hued contaminant strains belonging to this class (Jain et al., 2016). Interestingly, this event occurred during the same period when *Trametes* overtook the

dominance of the bioreactor. The biomass renovation of Day 37 helped in outcompeting *Fusarium* for the rest of the operation, observed in the DGGE and confirmed with the qPCR (data not shown). Weekly partial biomass renovation was most likely a major contributor to the predominance of *T. versicolor* throughout the operation. *Fusarium* is a genus of filamentous fungi which includes plant pathogens, but has been studied for dye, PAH and steroid degradation (Porri et al., 2011; Zhang et al., 2013a; Zhao et al., 2017). Therefore, its ability and role in the removal of PhACs during the operation cannot be disregarded. *Trametes* concentration in the pellets was maintained and it seemed that the partial biomass restoration strategy produced a balance between *Trametes* in the form of free mycelium and in the shape of pellet (data not shown).

7.3 Conclusions

A long-term fungal operation removing PhACs at environmental concentration from non-sterile and non-spiked hospital wastewater has been demonstrated for the first time. The fungus confirmed the ability of bioremediating most compounds from this matrix heavily contaminated with native wastewater microorganisms. Rather than using a specific treatment to remove every single micropollutant, the fungal approach allowed a single bioreactor step, in the same operating conditions, to remove the vast majority of compounds from the wastewater.

The mycelial suspension production described previously (Mir-Tutusaus et al., 2017b) and the biomass renovation strategy were validated, the latter as means to ensure a high concentration of *Trametes*. A balance between *Trametes* in pellet shape and as free mycelium was observed by qPCR for the first time (Eloi Parladé, personal communication, September 1, 2017). A negative correlation between a Gammaproteobacterium and WRF *T. versicolor* was described for the first time, although no effect on laccase production or removal efficiency was observed.

The treatment significantly reduced the pharmaceutical load of every compound detected. It removed 4 PhACs listed in the initial watch list of the EU Commission, 3 proposed to be introduced in it, and 18 compounds listed in the priority list of the Global Water Research Coalition (2008). All these PhACs were removed well over 80% with the exception of carbamazepine, bezafibrate, hydrochlorothiazide, metoprolol and trimethoprim, with a removal value of around 50%. Increasing the HRT or the fungal concentration could help in improving the removal of pharmaceuticals.

In summary, this manuscript demonstrated a long-term fungal operation and signified a robust step towards industrial-scale fungal PhAC removal from non-sterile

wastewater, with obvious applications in hospitals and pharmaceutically polluted wastewater streams.

8

Fungal MBR treatment

Removal of micropollutants in a non-sterile membrane bioreactor inoculated with *Trametes versicolor*

Abstract

Micropollutants pose both environmental and human health challenges. Among the possible treatments, membrane bioreactors have been tested for micropollutant removal and fungal reactors can effectively remove such contaminants. However, few fungal MBR operations can be found, although with promising results. Here, several fungal MBRs are operated with the objective of improving micropollutant removal. In order to achieve that, the effect of ozonation and biomass renovation in the removal of five selected micropollutants was studied. The partial biomass renovation allowed for a continuous production of laccase enzyme and the ozonation improved diclofenac and sulfamethoxazole removal.

8.1 Introduction

Micropollutants have been detected in almost every compartment of the environment (Frédéric and Yves, 2014). They are a diverse group of compounds including pharmaceutically active compounds (PhACs), pharmaceuticals and personal care products (PPCPs), hormones, pesticides and industrial chemicals. Present in the environment at $ng{\cdot}L^{-1}$ to $\mu g{\cdot}L^{-1}$, these micropollutants can be very resistant to degradation (Jelic et al., 2015). Consequently, several approaches have been suggested to remove such compounds from wastewater.

Membrane bioreactors (MBRs) combine biological degradation and membrane filtration in a single step, allowing for longer solids retention times (SRT) and more flexibility in operation (Hai et al., 2009). Typically, MBRs for removal of micropollutants

use a pore size of 0.04 – 0.4 μm to physically retain bacterial flocs and virtually all suspended solids within the reactor (Le-Clech et al., 2006; Tadkaew et al., 2011). SRT, together with HRT affect the removal efficiency of many microcontaminants in wastewater treatment plants (WWTP) (Grandclément et al., 2017). Therefore, the longer SRT allowed in an MBR may represent an improvement over conventional active sludge (CAS) in terms of micropollutant removal efficiency for some compounds (Grandclément et al., 2017; Kaya et al., 2013; Verlicchi et al., 2015).

White-rot fungi (WRF) embody a whole other approach in removing organic microcontaminants from wastewater. WRF have proven especially well-suited for removing and degrading a wide range of compounds (Cruz-Morató et al., 2013a; Mir-Tutusaus et al., 2014). A versatile enzymatic system comprising both intracellular (e.g., cytochrome P450 system) and extracellular enzymes (e.g., laccase-mediators system) allows these fungi to transform most of the micropollutants, usually very recalcitrant, and even achieve mineralization in the case of diclofenac and benzophenone-3 (Badia-Fabregat et al., 2014; Marco-Urrea et al., 2010c; Pointing, 2001).

The combination of these two technologies, namely, membrane bioreactors and fungal treatment, has been tested previously and showed promising results in continuous dye removal (Hai et al., 2008). Conversely, moderate results were found when operating a continuous reactor for longer periods of time removing several micropollutants, which resulted in a deteriorated removal capacity and unstable operation (Nguyen et al., 2013; Yang et al., 2013a). In summary, some common drawbacks of the fungal technology could be identified, mostly resulting from low fungal viability inside the reactor: rather slow fungal degradation requiring long hydraulic retention times, bacterial contamination destabilizing fungal removal capacity (Gao et al., 2008; Libra et al., 2003), and loss of the extracellular enzymes and mediators with discharged water (Hai et al., 2012; Zhang and Yu, 2000). The inherent capacity of MBRs to retain the fungal biomass can reduce the HRTs because they permit longer SRTs. A biomass renovation strategy could help in maintaining the fungal activity by keeping fresher fungal biomass inside the reactor and thus delaying bacterial contamination (Blánquez et al., 2006). An additional approach involving disinfection with ozone, which can be selective for bacteria and not fungi, could further diminish the bacterial contamination (Cheng et al., 2013; Sankaran et al., 2008). Finally, the use of larger pore-sized membranes could wash out bacteria while retaining fungal biomass –thus improving fungal viability, but extracellular enzymes such as laccase would also be washed out; therefore, a compromise should be found between a low enough HRT to enable bacteria to be washed out but high enough to allow for a significant free laccase

and mediators concentration.

The general objective of this study is to increase pharmaceutical removal of a membrane bioreactor inoculated with *T. versicolor* during a continuous treatment of non-sterile synthetic wastewater. In order to accomplish this, different approaches have been studied: a large pore size membrane (~100 μm) to wash out the contaminating bacteria, a partial biomass renovation and an ozone-based disinfection method. Results were evaluated in terms of micropollutant removal.

8.2 Results and discussion

8.2.1 Performance stability and laccase activity during the MBR operation

Two identical MBR systems were operated for 70 days: one inoculated with the fungus and with partial biomass renovation (Fungal, F); and one inoculated with the fungus, with the same partial biomass renovation strategy and partial ozonation (Fungal-ozone, FO_3). The FO_3 was tested under two different ozonation intervals (as explained in Section 3.3.5). A third MBR was included as control (C), operated for 42 days and inoculated with the fungus but without partial biomass renovation nor ozonation. During the last week of operation of the control MBR all fungus was washed out from the reactor but the operation continued; this last week has been referred to as non-inoculated (NI) period. All reactors were operated under non-sterile conditions and contamination was due to air-borne microorganisms and microorganisms present in non-sterile surfaces.

Operating parameters such as pH, temperature and DO in both experimental MBRs were stable throughout the treatment. pH fluctuated around 6-7 in both the supernatant and the permeate, temperature was maintained at 25ºC and DO remained around 6 mg $O_2 \cdot L^{-1}$. In the case of the control MBR, the pH decreased to 5.2, similar to the influent pH (4.5). The large pore size used in the membrane and a low HRT of 12 h allowed the washout of bacteria. In fact, around 0.6 $g \cdot L^{-1}$ of solids were lost through the membrane every week. However, the partial biomass restoration allowed to maintain the MLSS concentration around 2.0 $g \cdot L^{-1}$in the experimental MBRs; the control MBR lost all fungal biomass by Day 28. Membrane fouling, causing TMP build-up did not occur (data not shown) probably due to the large pore size of the membrane.

The laccase activity profile is presented in Figure 8.1. Laccase activity in the MBR permeate was similar in both experimental reactors: the profile was irregular during the

first weeks of operation until stabilization around Day 31. A peak of 110 μM·min^{-1} laccase activity was found on the first week. From that point onwards, laccase activity fluctuated around 5 μM·min^{-1} until the end of the operation. The low laccase activity could be attributed to the low HRT (12 h): when HRTs of 3 days were used in fluidized bed reactors, much higher laccase activity values were measured. Laccase in the supernatant was also consistently detected, with similar low values than those observed in the permeate (data not shown). The fact that laccase activity was found in the permeate confirmed the passage of enzyme through the coarse-pore membrane. In the case of the control MBR, laccase was only detected for two weeks in permeate, supernatant and fungal biomass. This was in accord with previous studies, which detected laccase only during the first days of operation, even when longer HRTs of 24 and 48 h were applied (Nguyen et al., 2013; Yang et al., 2013a). This fact, and a comparison with the control reactor determined that the weekly biomass restoration strategy ensured this relatively stable (although low) laccase activity. Therefore, biomass restoration strategies should be used in similar systems for maintaining stable laccase activities. Although laccase enzyme is not the only responsible enzyme for micropollutant degradation (cytochrome P450 is notably involved in the transformation of several organic pollutants (Badia-Fabregat et al., 2012; Golan-rozen et al., 2011; Marco-Urrea et al., 2010b)), laccase activity detection has been largely linked to micropollutant removal (Kuhar et al., 2015; Mir-Tutusaus et al., 2014; Viswanath et al., 2014). Additionally, as recommended elsewhere, laccase activity was also used as a *T. versicolor*-activity indicator (Mir-Tutusaus et al., 2016).

Mycelium-bound laccase activity was always detected in biomass from both experimental MBRs, giving an additional proof of *T. versicolor* activity throughout the treatment. Ozone has been studied for the denaturation of an alpha-amylase, so a laccase activity decrease could be expected (Martínez-Gallegos et al., 2014). However, as similar laccase activity profiles were measured in the ozonated and the non-ozonated MBRs, one can conclude that the ozonation did not negatively affect laccase activity in the reactors: the fact that laccase was continuously produced by the fungus and that only between 0.66 and 1.5 L of supernatant were ozonated weekly (this accounts only for 4.7-10.7% of the wastewater treated weekly) might be responsible for this behavior.

8.2.2 Biomass properties

Biopolymers secreted by or attached onto microorganisms have been largely described (Chang et al., 1999). Those biopolymers can be divided between extracellular polymeric

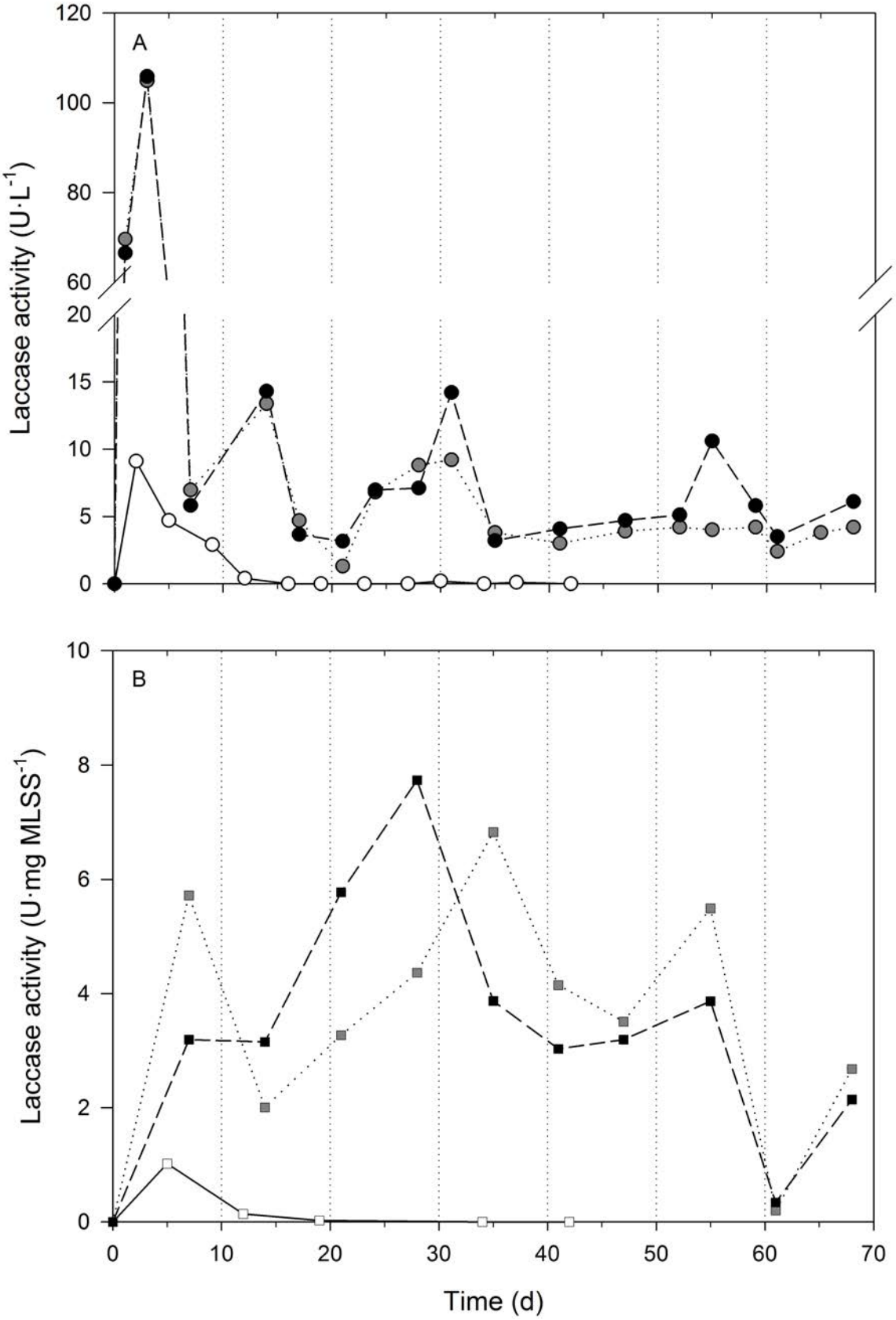

Figure 8.1: Laccase activity profile in the MBR permeate (top, circles) and mycelium-bound laccase activity (bottom, squares) in the F MBR (black symbols), FO_3 MBR (grey symbols) and contro MBR (white symbols).

substances (EPS) and soluble microbial products (SMP), both of which can be again divided to polysaccharides and proteins. Figure 8.2 presents EPS and SMP concentration profile during the operation, as a sum of both polysaccharides and proteins. It is worth mentioning that EPS and SMP are not molecules with a specific structure; they are rather operationally defined: they can be molecules, colloids, particles and they should not be compared between studies unless the methods used to measure them are identical (Chang et al., 1999). The increase of EPS and SMP can be attributed to the release of organic cellular constituents due to autolysis of cells, accumulation of unmetabolized and/or intermediate products of organic molecules and to cell stress (Aquino and Stuckey, 2004; Laspidou, 2002).

A notable increase in EPS (from 20 to 140 mg·g $MLVSS^{-1}$) was observed in the non-ozonated (F) reactor. However, such increase was not measured in the control or in the FO_3 MBR, which maintained EPS concentrations around 30-60 mg·g $MLVSS^{-1}$ during the whole operation. A similar profile can be observed when taking into account SMP concentration: an increase from around 100 to 232 mg·L^{-1} in the case of the F MBR and a more constant behavior of the ozonated reactor, maintaining the SMP between 100 and 130 mg·L^{-1}. The SMP concentration in the C MBR remained between 150 and 170 mg·L^{-1}, probably contributed by unmetaboilized substrate, as no fungus was observed since Day 28 onwards. EPS and SMP are linked to membrane fouling, which is a major issue in MBR technology when it comes to full-scale plant maintenance and operating costs (Chang et al., 1999). However, membrane fouling did not occur in any operation.

As fungal pellets were the main biomass of the operation, EPS and SMP could be mainly contributed by loss of pelleted morphology, leading to the formation of free hyphae, residual MEB and byproducts secreted by the fungus. However, contribution of bacteria to the biopolymers should not be underestimated. A previous study with a more conventional sludge MBR used the same EPS and SMP quantification methodology (Luo et al., 2015). When comparing this results with that study, the non-ozonated fungal MBR exhibited a similar profile than the sludge MBR when salinity was built-up (although SMP concentration was lower and EPS higher in the fungal case). That same study also reported a more constant, although slightly increasing EPS and SMP concentration over time when no salinity build-up was performed, very much like the behavior of the FO_3 MBR. In summary, the partial ozonation strategy led to a reduced concentration of both EPS and SMP concentrations.

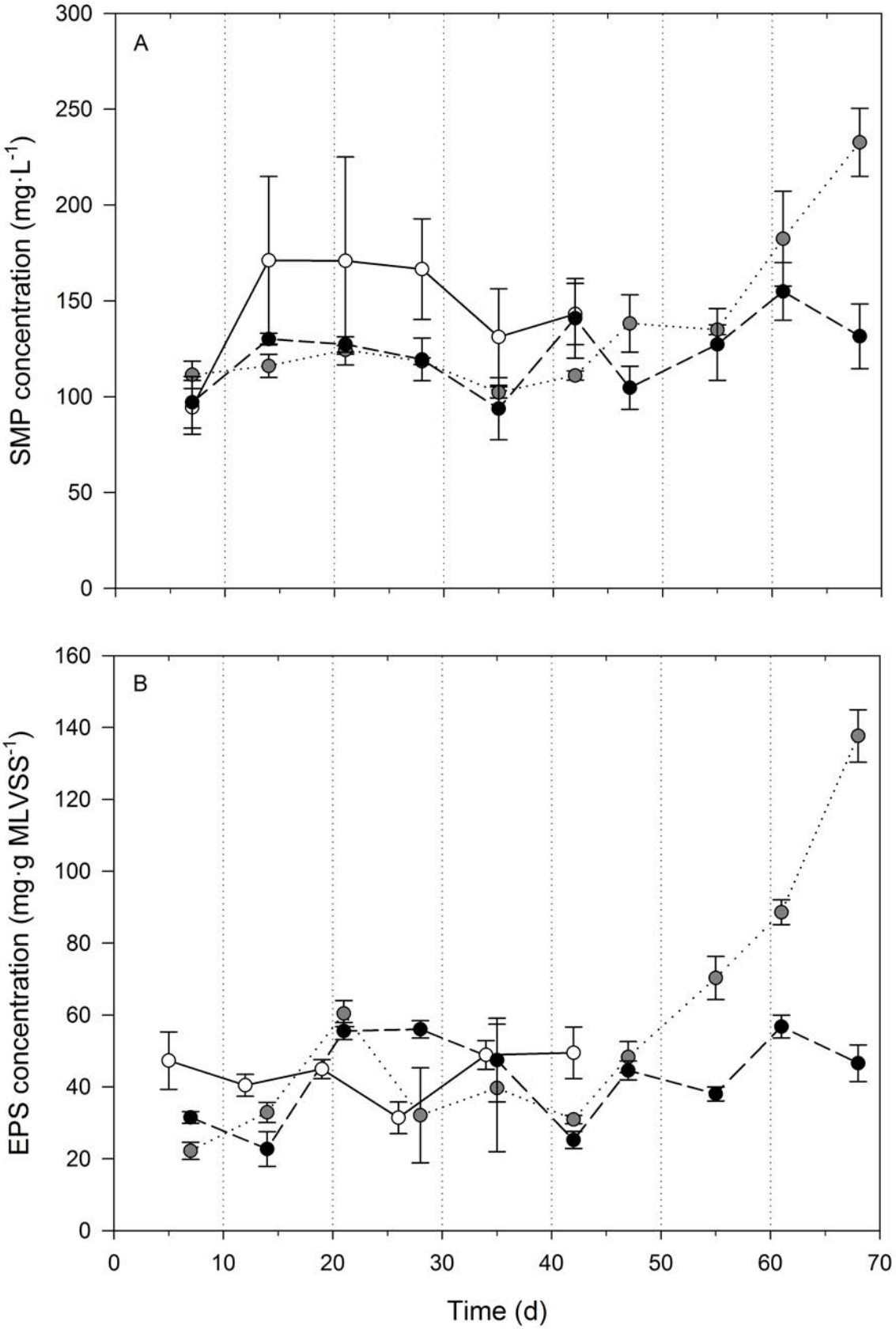

Figure 8.2: Variation of SMP (A) and EPS (B) concentration during the MBRs operation. Black circles represent the F MBR, grey circles, the FO_3 MBR and white circles, the control MBR.

Table 8.1: Micropollutant removal values from the batch ozonation tests and from the FO_3 reactor supernatant ozonation cycles. Values are means standard deviations of 2 samples; for the MBR supernatant, values are means standard deviations of 8 samples.

Matrix	TrOC removal (%)														
	CBZ			SMX			OXB			DCF			BPA		
MilliQ water	72	±	0	33	±	9	87	±	1	100	±	0	44	±	3
Synthetic wastewater	33	±	8	68	±	2	0			70	±	7	18	±	4
MBR supernatant	55	±	13	7	±	66	0			46	±	28	0		

8.2.3 Effect of ozonation on micropollutant degradation

Partial ozonation was tested in order to lengthen the fungal MBR operation by selectively disinfecting bacteria. However, in order to understand the contribution of ozonation in the overall removal of micropollutants in the FO_3 MBR, samples for HPLC analysis were taken before and after the ozonation; therefore, the degradation due to ozonation of the compounds in the FO_3 MBR is presented in Table 8.1. For comparison purposes, the degradation of micropollutants by ozonation was also studied in spiked MilliQ water and sterile synthetic wastewater.

As can be observed, the standard deviations (SD) increased and the removal decreased with the complexity of the matrix. Accurate results were measured in MilliQ water; higher SDs were observed in a more complex matrix and higher in the MBR supernatant, which also included free hyphae and free bacteria. Some studies highlight that suspended solids have a minor influence in oxidation efficiency (Huber et al., 2005). However, a competition for ozone between soluble and particulate matter has also been reported elsewhere (Cesbron et al., 2003). In this case, the high complexity of the matrix seemed to play a role in the decreased removal efficiency of micropollutants by ozonation.

Margot et al. (2013)reported better degradation of hydrophilic or negatively charged compounds (such as sulfamethoxazole and diclofenac) in comparison with more hydrophobic compounds in a pilot WWTP. Ozonation also efficiently removed carbamazepine and bisphenol A by over 80% in that same study. A lower degradation was observed in the present study, probably because contact time was much lower. OXB and BPA poor degradation by ozonation under these conditions could be explained by their high hydrophobicity.

As ozonation of the MBR supernatant significantly removed only CBZ and DCF, but

only a 10.7% of the supernatant was ozonated weekly, only around a 5% of CBZ and DCF removal could be explained by ozonation.

8.2.4 Micropollutant removal during the MBR operation

The psychiatric drug carbamazepine (CBZ), the antibiotic sulfamethoxazole (SMX), the UV filter oxybenzone (OXB), the anti-inflammatory drug diclofenac (DCF) and the industrial chemical bisphenol A (BPA) were selected based on their diverse physicochemical properties and their widespread occurrence in wastewater. Removals of the five micropollutants are presented in Figure 8.3, which belong to the same 70d operation, where an increase in ozonation was performed at Day 35. Overall, the ozonated MBR performed slightly better than the non-ozonated reactor; these differences were made more evident when a higher ozonation (Figure 8.3B) was used. SMX and DCF removal values specially benefited from this fact, significantly increasing from below 20 to >40% and from 70 to 80%, respectively. However, ozonation seemed not to affect CBZ or OXB removal.

No significant removal was observed during the non-inoculated period, with the exception of a 50% removal of BPA; the C MBR removed an average of 30 and 20% of OXB and DCF, respectively, while BPA removal at around 55% was in the range of the NI period. An average removal value of 30 and <10% of CBZ and SMX, respectively, was observed in the non-ozonated reactor. OXB and BPA were degraded over 80% and DCF around 55-60%. Better removal values were detected in the ozonated reactor when ozonation dose was increased, averaging >80% removal of OXB, DCF and BPA, and around 40% of CBZ and SMX. Our study may be compared to two previous studies using a fungal MBR with a membrane pore size of 0.4 μm and no biomass restoration nor ozonation, but with an HRT of 48h instead of 12h (Nguyen et al., 2013; Yang et al., 2013a). In spite of the much lower HRT, degradation of CBZ, DCF and BPA in the Fungal non-ozonated MBR was higher than in Nguyen et al. (2013) and Yang et al. (2013a): CBZ removal increased from 20% in the previous studies to 30% in the present study, DCF from 20-50 to >60% and BPA from 40-70 to >90% (SMX was not studied and OXB was equally removed). The larger pore size and the biomass renovation strategy may have played a role in the improved performance. On the one hand, a larger pore size allowed for bacteria to be washed out from the reactor; on the other, the biomass restoration maintained a young and active fungal culture (Blánquez et al., 2006) and detectable levels of laccase activity throughout the treatment (Figure 8.1).

Degradation mechanisms of SMX, OXB, DCF and BPA in *T. versicolor* involved laccase

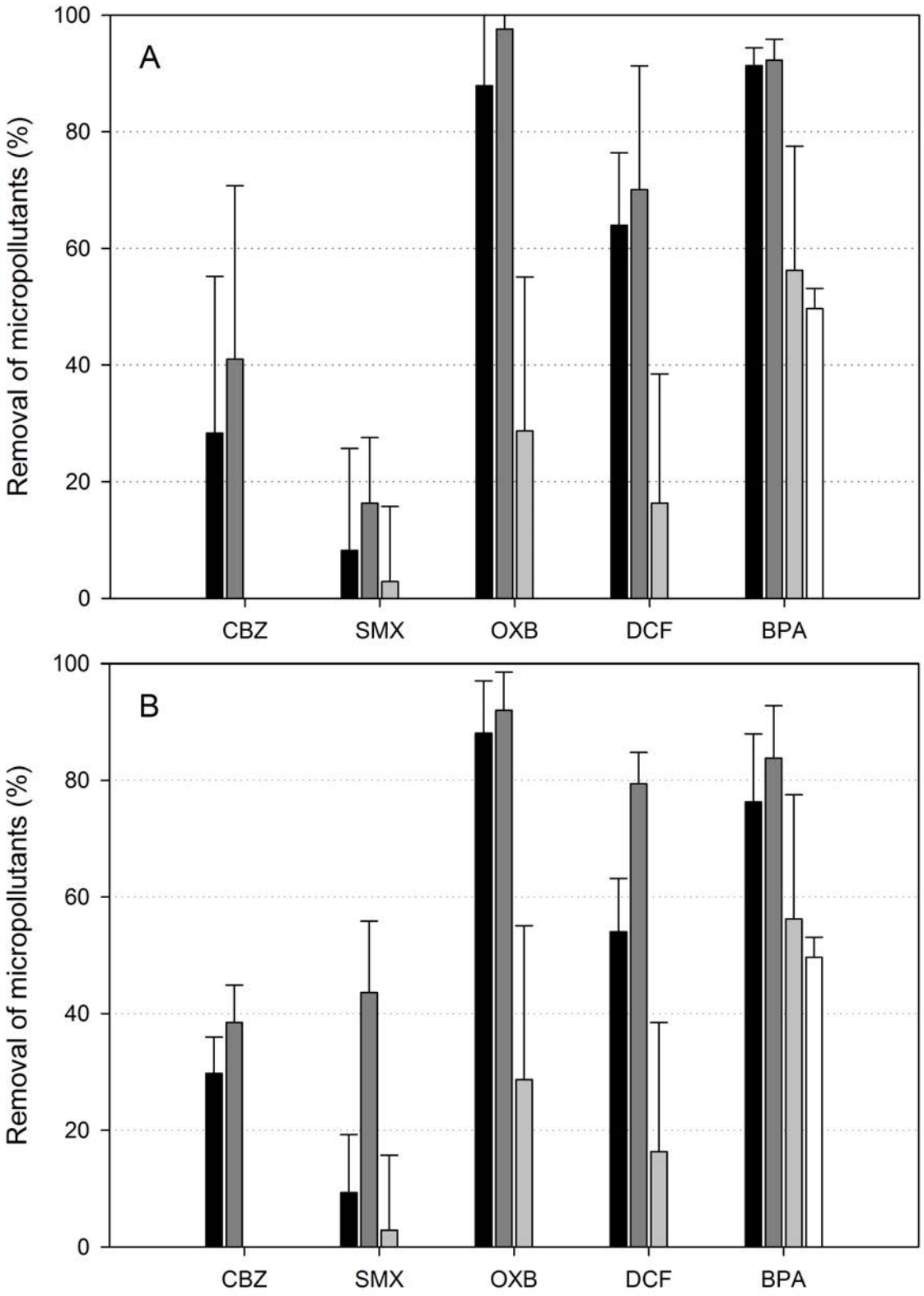

Figure 8.3: Removal of five trace organic contaminants by the MBRs. Black bars represent the F MBR (A and B); dark grey bars, the FO_3 MBR ozonated twice a week (A) and the FO_3 MBR ozonated three times a week (B); light grey bars, the control MBR (A and B); white bars, the NI period (A and B).

or laccase and mediators system, but not CBZ transformation (Nguyen et al., 2013; Rahmani et al., 2015); CBZ degradation pathway, in fact, involves the cytochrome P450 system (Golan-rozen et al., 2011); cyt P450, in addition to laccase, also played a role in diclofenac degradation (Marco-Urrea et al., 2010c). As 4 out of 5 compounds could be degraded by laccase, and a relatively constant laccase activity was maintained, this fact could have been a major explanation for the enhanced degradation attained. The ozonation strategy decreased fungal stress in the FO_3 MBR, as illustrated by the decreased EPS and SMP concentration (Aquino and Stuckey, 2004) without affecting laccase production, thus allowing for an even better removal of SMX and DCF.

Micropollutant transformation by WRF often leads to detoxification (Jelic et al., 2012), but an increase in toxicity of fungal-treated media has also been reported (Marco-Urrea et al., 2009). Therefore, a toxicity analysis was performed. The results showed no increase of toxicity and the bacterial luminescence toxicity (BLT) assay resulted in a $TU_{IC(REF)_{20}} < 1$ for all samples, which revealed no toxicity of the permeate, indicating that toxic compounds were not produced or that they were also removed.

8.3 Conclusions

A submerged membrane fungal reactor was operated for 70 days while a synthetic wastewater spiked with 5 micropollutants was continuously fed. Some conclusions drawn from this study are listed below:

- Partial biomass restoration ensured a constant laccase activity throughout the treatments. Maintaining a moderate enzyme activity could be important for the degradation of 4 out of the 5 compounds studied.

- Partial ozonation of the supernatant was examined in comparison with a non-ozonated fungal reactor. Ozonation significantly reduced EPS and SMP concentration (indicative of cell stress).

- A combination of a large pore-sized membrane and a low HRT of 12h allowed for the washout of bacteria.

- The partial ozonation allowed for an increased removal of SMX and DCF but the ozonation itself did not play an important role in micropollutant degradation.

- The combined strategies partial biomass restoration and large pore size increased the removal of all studied micropollutants in comparison with similar systems.

This work provided a proof of concept of several approaches that can be used in order to improve operational stability and micropollutant removal of similar fungal MBR systems. However, variables should be further studied: on the one hand, ozonation for

the decreased cell stress could be increased while keeping in mind the operation costs; on the other, HRT and pore size fine-tuning would give insight into the complex compromise between bacteria washout and enzyme retention.

9

Coupling with UV/H_2O_2

Prospects on coupling UV/H_2O_2 with activated sludge or a fungal treatment for the removal of pharmaceutically active compounds in real wastewater

Abstract

9.1 Introduction

Hospital wastewater contains a complex mixture of hazardous chemicals and harmful microbes, which can pose a threat to the environment and public health. Although the contribution of hospital facilities to the total volume uploaded in the municipal WWTP usually range between 0,2 and 2% (Carraro et al., 2016), there is not a specific directive or guideline for the management of hospital wastewater effluents in Europe, and national legal regulations quite rarely define how to manage and treat hospital wastewaters before its disposal (Rodriguez-Mozaz et al., 2017). Therefore, hospital effluents are usually discharged in the municipal sewer system without any previous pretreatment. The common practice of co-treating hospitals and urban wastewaters jointly at a municipal WWTP is considered as an inadequate solution for the removal of compounds such as PhACs by many authors, because dilution of the highly polluted effluents such as hospitals can be detrimental for their biological removal (Badia-Fabregat et al., 2015a; Joss et al., 2006; Pauwels and Verstraete, 2006; Verlicchi et al., 2015). Therefore, the use of alternative wastewater treatments at the source point for this kind of effluents has been highly recommended by many authors (Cruz-Morató et al., 2014; Joss et al., 2006; Pauwels and Verstraete, 2006; Verlicchi et al., 2010, 2015). Extensive research has been performed in the last year in the development of appropriate decentralized treatment for the hospital effluents as it has been reviewed lately (Verlicchi et al., 2015). However, the

application of full-scale dedicated treatment of the effluents in hospitals has only been implemented in a limited number of places (Rodriguez-Mozaz et al., 2017). In the case of psychiatric hospitals, on-site wastewater treatment can be particularly recommended since the effluents contain remarkable loads of psychiatric drugs (Herrmann et al., 2015; Yuan et al., 2013a). These type of pharmaceuticals are known to be more recalcitrant than most of PhACs in conventional WWTP and in the natural environment (Baena-Nogueras et al., 2017; Calisto and Esteves, 2009; Verlicchi et al., 2012). They have also been targeted as contaminants to be prioritized by several authors (Ashton et al., 2004) as well as by Global Water Research Coalition (2008). In addition, the use of antidepressants has significantly increased in most OECD countries in the last years, as a reflection of the prevalence of mental illness, increase in health coverage, new treatment opportunities and population ageing (Organization for Economic Cooperation and Development2015). An increase in the worldwide consumption of this type of PhACs class can thus be foreseen in the next years.

As the concern is perfectly justified, important efforts have been devoted by the scientific community to remove these pollutants from wastewater streams. Physical, chemical and biological processes have been tested with varying degrees of success. Conventional activated sludge (CAS) process is the standard practice in conventional WWTPs, which usually does not achieve high removal efficiencies of micropollutants (Verlicchi et al., 2015). As CAS is common practice, it has also been deeply studied regarding PhACs degradation (Collado et al., 2012; Ferrando-Climent et al., 2012; Rubirola et al., 2014). Some anti-analgesics and anti-inflammatories are well removed by CAS but other drug families such as psychiatric drugs and antibiotics are more resistant to bacterial degradation. In fact, WWTPs are among the main sources of pharmaceutical release into the environment and so the removal of such compounds is of high importance (Collado et al., 2013; Fatta-Kassinos et al., 2011; Verlicchi et al., 2015). Other biological processes such as fungal treatment have also emerged in order to degrade and/or remove PhACs from wastewater streams. Particularly white-rot fungi (WRF) have succeeded on degrading a wide range of pollutants thanks to its unspecific enzymatic systems (Cruz-Morató et al., 2013a; Nguyen et al., 2013; Rodarte-Morales et al., 2011). Fungal operations perform reasonably well in terms of PhACs removal but some compounds remain in its effluents (Cruz-Morató et al., 2014). Both CAS and fungal processes may enhance its PhAC removal efficiency thanks to an AOP pretreatment; however, a biological treatment prior to an AOP could not only improve AOP degradation efficiency but also its economic cost and ecological footprint (Oller et al., 2011).

Advanced Oxidation Processes (AOPs) are being largely studied in regards to PhACs

degradation, and an increasing number of journal articles have been published in recent years. Promising results are available and a rigorous overview of pharmaceuticals treated by AOPs can be found in M. Klavarioti's review (Klavarioti et al., 2009). Although these technologies are usually capable of destroying a specific pharmaceutical, total mineralization is highly unlikely (Cavalcante et al., 2015). In some cases transformation products (TPs) can be more toxic than the parent compounds, but typically they are more biodegradable than the original substrate. In those cases, a posterior biological oxidation process would be feasible and with a considerable lower cost (Oller et al., 2011).

Some studies discussed the coupling of selected AOPs with some biological processes, mainly CAS-based and using the AOP as a polishing step (Vidal et al., 2004; Sirtori et al., 2009; Hörsch et al., 2003; Giannakis et al., 2015). Synergies seem to have been identified between these treatments, but more thorough and wider-spectrum studies need to be performed to fully understand the interactions between the systems and to give a framework of feasible combinations from which to decide on. Oller et al. (2011) reviewed some studies which appled an AOP as a post-oxidation step after a primary biotreatment, in order to improve biodegradability of COD. However, to the best of the authors' knowledge, no studies were found comparing AOP and biological treatments with the same real wastewater. Therefore, the main objective of the study was to evaluate the effect of coupling a UV/H_2O_2 process with activated sludge or a fungal treatment in terms of the removal of a broad set of PhACs present in a real non-sterile hospital wastewater.

9.2 Results and discussion

General physicochemical parameters of the HWW studied were in the same range than previously sampled hospital wastewater from the same sewer manifold and from other hospitals (Mir-Tutusaus et al., 2016, 2017a). The coagulation-flocculation pretreatment reduced the absorbance at 650 nm to zero, the COD from 174 to 87 mg $O_2 \cdot L^{-1}$ and total suspended solids from 108 to 16 mg$\cdot L^{-1}$. The use of this pretreatment step can be highlighted as necessary, as several studies have reported low removal efficiencies while operating UV/H_2O_2 and fungal reactors with raw wastewater, due to high suspended solids and microorganisms concentration, respectively (Mir-Tutusaus et al., 2017a; Verlicchi et al., 2015). Therefore, raw wastewater was pretreated with a coagulation-flocculation process before launching any of the experiments.

Figure 9.1summarizes the experiments carried out on the coupling of AOP and the

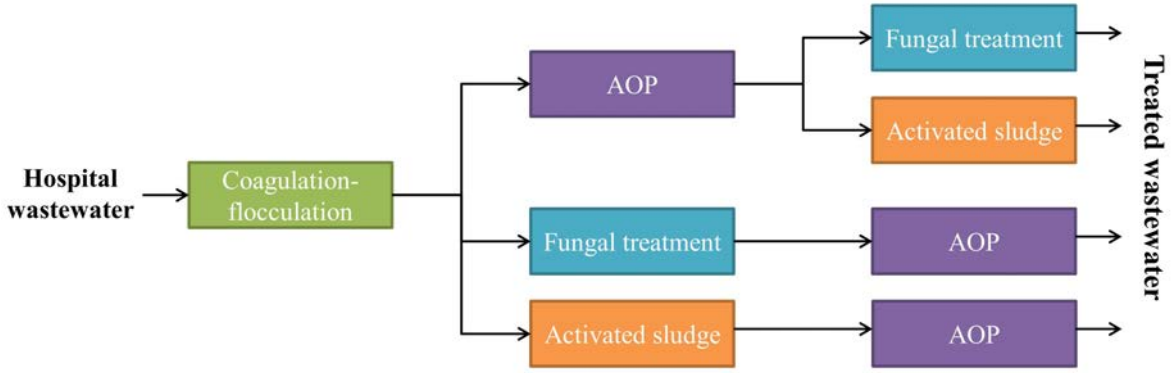

Figure 9.1: Diagram of the coupled treatments. FG represents the fungal treatment; AOP, the UV/H_2O_2 treatment; AS, the activated sludge process.

biological treatments. On one hand, as presented in Figure 9.1, the UV/H_2O_2 was studied as a step for both removing micropollutants and incrementing the biodegradability of the effluent before the subsequent fungal or activated sludge treatments (Oller et al., 2011). On the other hand, fungal and AS treatments were evaluated as a first step for reducing the pharmaceutical load prior to the UV/H_2O_2 stage, which would be considered as polishing step. In order to ensure robustness in the results, only 23 PhACs detected in raw wastewater at 10 times its limit of quantification have been discussed –out of the 77 selected. Figure 9.1 shows initial and final concentrations of those 23 compounds. The analgesics and anti-inflammatories family was the main contributor to the total concentration of PhACs in the raw wastewater, a common trend in ubran and hospital wastewaters (Frédéric and Yves, 2014; Verlicchi et al., 2010, 2015). However, a high concentration of the psychiatric drugs family can be highlighted, as a large psychiatric ward was located within the hospital. Levels of carbamazepine and lorazepam, for example (4118 and 538 $ng{\cdot}L^{-1}$, respectively), were considerably higher than the levels found in urban hospitals (1200 $ng{\cdot}L^{-1}$ and n.d., respectively) (Verlicchi et al., 2012).

9.2.1 Performance of biological treatments

Since both fungal and AS operations were carried out using the same initial wastewater, a comparison between the removal efficiencies of the two biological treatments was easily carried out. The single fungal step removed 78% of the PhACs studied, or a 66% without taking into account the analgesics and anti-inflammatories family, which are

easily degraded and accounted for roughly half of the pharmaceutical load. The fungus completely removed acetaminophen, diclofenac, ibuprofen, ciprofloxacin, furosemide, hydrochlorothiazide, ranitidine, atorvastatin, gemfibrozil, 2-hydroxyCBZ and trazodone (Table 9.1). Results are in accordance with previous studies, with good removal values achieved for analgesics and anti-inflammatories (94%) as well as for antibiotics (91%) and around 42% removal of psychiatric drugs (Cruz-Morató et al., 2013a; Mir-Tutusaus et al., 2017a). In the case of the activated sludge treatment, 63% of the initial pharmaceutical load was eliminated, or a 33% disregarding the analgesics and anti-inflammatories family. The AS step completely removed the analgesics and anti-inflammatories acetaminophen, ibuprofen and naproxen (Table 9.1). This was also in accordance with previous studies, as this family of PhACs is reportedly well removed in CAS (Verlicchi et al., 2012). This treatment also removed nearly 80% of the antibiotic ciprofloxacin, probably by adsorption to the biomass as it occurs in full-scale WWTPs (Verlicchi et al., 2012). Gemfibrozil is usually not transformed by CAS (Verlicchi et al., 2010), but our lab-scale experiment did partly remove the compound. In general, the AS treatment exhibited lower removal efficiencies than fungal treatment for most of the compounds analyzed, in agreement with those removal efficiencies usually reported in WWTP (Verlicchi et al., 2012; Wang et al., 2014). Fungal operation was thus more efficient than reference AS operation for the treatment of the hospital wastewater and could be regarded as a standalone treatment of such PhAC-polluted streams. In addition, fungal treatment was particularly efficient in eliminating the most hazardous compounds: it removed a 91% of antibiotics, a 42% of psychiatric drugs and a 94% of analgesics and anti-inflammatories, therefore reducing the environmental risk posed by these effluents, as it was previously assessed by Lucas et al. (2016).

On the other hand, the increase of concentration of PhACs during biological wastewater treatment is usually attributed to conjugation/deconjugation phenomena and desorption from solids. Conjugation is a mechanism used by several organisms to detoxify xenobiotics, and involves the covalent addition of a molecule to a compound. In the human liver, the conjugation leads to the formation of water-soluble compounds that can be excreted through urine, being glucuronidation the most common conjugation pathway in the biotransformation of xenobiotics (Sanchez and Kauffman, 2010). Therefore, PhACs, its transformation products and conjugated forms are often found in wastewaters, but the analytical methods are usually used to measure the non-conjugated molecules.

Table 9.1: Physicochemical characterization of the hospital wastewaters.

Family	Pharmaceutical	Initial concentration ($ng \cdot L^{-1}$)	Final concentration ($ng \cdot L^{-1}$)						
			AOP	FG	AS	AOP+FG	AOP+AS	FG+AOP	AS+AOP
Analgesics and anti-inflammatories	Acetaminophen	27569 ± 954	bld	3 ± 0	3 ± 0	bld	3 ± 0	6 ± 1	bld
	Diclofenac	1448 ± 398	bld	bld	1045 ± 35	bld	bld	bld	bld
	Ibuprofen	26939 ± 3506	7128 ± 595	113 ± 4	bld	bld	bld	60 ± 5	bld
	Ketoprofen	3169 ± 1205	581 ± 132	3450 ± 506	2342 ± 390	3778 ± 554	1052 ± 175	886 ± 201	892 ± 203
	Naproxen	6883 ± 799	bld	1578 ± 60	bld	bld	bld	995 ± 214	bld
	Salicylic acid	19379 ± 2549	3515 ± 872	>80000 ± 8076	884 ± 219	4362 ± 440	148 ± 37	>40000 ± 9926	1192 ± 296
Antibiotics	Ciprofloxacin	6738 ± 846	2921 ± 289	bld	1713 ± 499	bld	1247 ± 363	bld	bld
	Ofloxacin	2052 ± 111	2 ± 0	757 ± 1	3887 ± 95	bld	5319 ± 130	2 ± 0	1212 ± 38
Antihypertensives	Valsartan	414 ± 118	60 ± 4	bld	328 ± 40	bld	232 ± 28	79 ± 5	132 ± 9
β-blockers	Atenolol	1370 ± 574	93 ± 39	136 ± 2	11 ± 1	797 ± 14	694 ± 55	97 ± 40	46 ± 19
Diuretics	Furosemide	2188 ± 601	bld	bld	1053 ± 27	bld	bld	bld	bld
	Hydrochlorothiazide	1670 ± 411	bld	bld	1565 ± 100	bld	178 ± 11	bld	47 ± 1
H1 and H2 antagonists	Ranitidine	1970 ± 509	bld	bld	2439 ± 20	bld	bld	bld	bld
Lipid regulators	Atorvastatin	77 ± 12	bld	bld	41 ± 0	bld	bld	bld	bld
	Gemfibrozil	13955 ± 1051	7655	bld	6259	bld	4718	bld	2773
Psychiatric drugs	10,11-epoxyCBZ	28505 ± 238	622 ± 63	12675 ± 32	15655 ± 2823	1195 ± 3	1254 ± 226	2442 ± 246	bld
	2-hydroxyCBZ	335 ± 465	35 ± 2	bld	5465 ± 278	bld	1045 ± 53	bld	605 ± 41
	Acridone	493 ± 135	542 ± 11	5710 ± 1	257 ± 34	1402 ± 0	1544 ± 207	153 ± 3	251 ± 5
	Carbamazepine	4118 ± 314	2067 ± 52	1897 ± 48	2952 ± 75	790 ± 20	1897 ± 48	1545 ± 39	892 ± 23
	Citalopram	898 ± 107	53 ± 1	264 ± 0	663 ± 7	196 ± 0	253 ± 3	224 ± 5	123 ± 2
	Lorazepam	538 ± 178	154 ± 13	543 ± 27	901 ± 53	275 ± 14	1214 ± 71	bld	612 ± 50
	Trazodone	225 ± 31	bld	bld	96 ± 13	bld	bld	bld	bld
	Venlafaxine	5766 ± 295	1295 ± 16	2504 ± 66	4202 ± 30	435 ± 11	1056 ± 8	1800 ± 22	462 ± 6
Total†		137320 ± 12857	23207 ± 1217	29631 ± 747	50876 ± 4521	8867 ± 617	21705 ± 1379	8290 ± 781	8045 ± 397
Removal (%)†			83 ± 12	78 ± 5	63 ± 150	94 ± 4	84 ± 15	94 ± 5	94 ± 2

†Without taking into account salicylic acid concentration

Deconjugation in activated sludge and fungal treatments has been described elsewhere (Badia-Fabregat et al., 2015a; Jelic et al., 2015), and it probably occurred in the present study as can be inferred by the increase in concentration observed for some compounds (Table 9.1). Deconjugation has not been described in AOPs and it is not observed in this study either: first, no increase has been detected after the AOP; second, increase of concentration of ketoprofen, ofloxacin, 2-hydroxyCBZ, acridone and lorazepam after the AOP+FG and AOP+AS experiments suggests that their corresponding conjugated forms were still present after the UV/H_2O_2 treatment, although not measured by our analytical methodology. Some deconjugation therefore must have occurred in the biological treatments. Specifically, atenolol (Yuan et al., 2013b), salicylic acid (the main transformation product of acetylsalicylic acid)(Kuehl et al., 2006), citalopram (Dalgaard and Larsen, 1999), ketoprofen, naproxen, ibuprofen, diclofenac, gemfibrozil, ofloxacin, carbamazepine, 2-hydroxyCBZ, 10,11-epoxyCBZ, acridone and lorazepam (Jelic et al., 2015) are excreted to some extent as conjugates.

Not taking into account carbamazepine and its TPs (discussed in detail in Section 9.2.3), the compounds detected at higher-than-initial concentration were treatment-specific: concentration of ofloxacin and lorazepam increased only after the activated sludge treatment, whilst ketoprofen and salicylic acid only after the fungal treatment. This fact could mean that the two treatments had distinct deconjugation capacities. In an attempt to shed light into the subject one can take into account the ketoprofen example: ketoprofen is well removed by fungal treatments in spiked matrices (Mir-Tutusaus et al., 2016), but increase in its concentration has been observed when treating real matrices (Badia-Fabregat et al., 2015a; Mir-Tutusaus et al., 2017a). Similarly, ketoprofen concentrations rose in the fungal treatments both before and after the AOP, which proved that conjugated forms of ketoprofen were present in the wastewater and that the UV/H_2O_2 treatment did not deconjugate –nor remove– them.

As exemplified with ketoprofen, deconjugation of PhACs with higher-than-initial concentrations could not be certainly quantified. Thus, concentration of the whole set of these pharmaceuticals and their transformation products and metabolites was undervalued to an unknown degree.

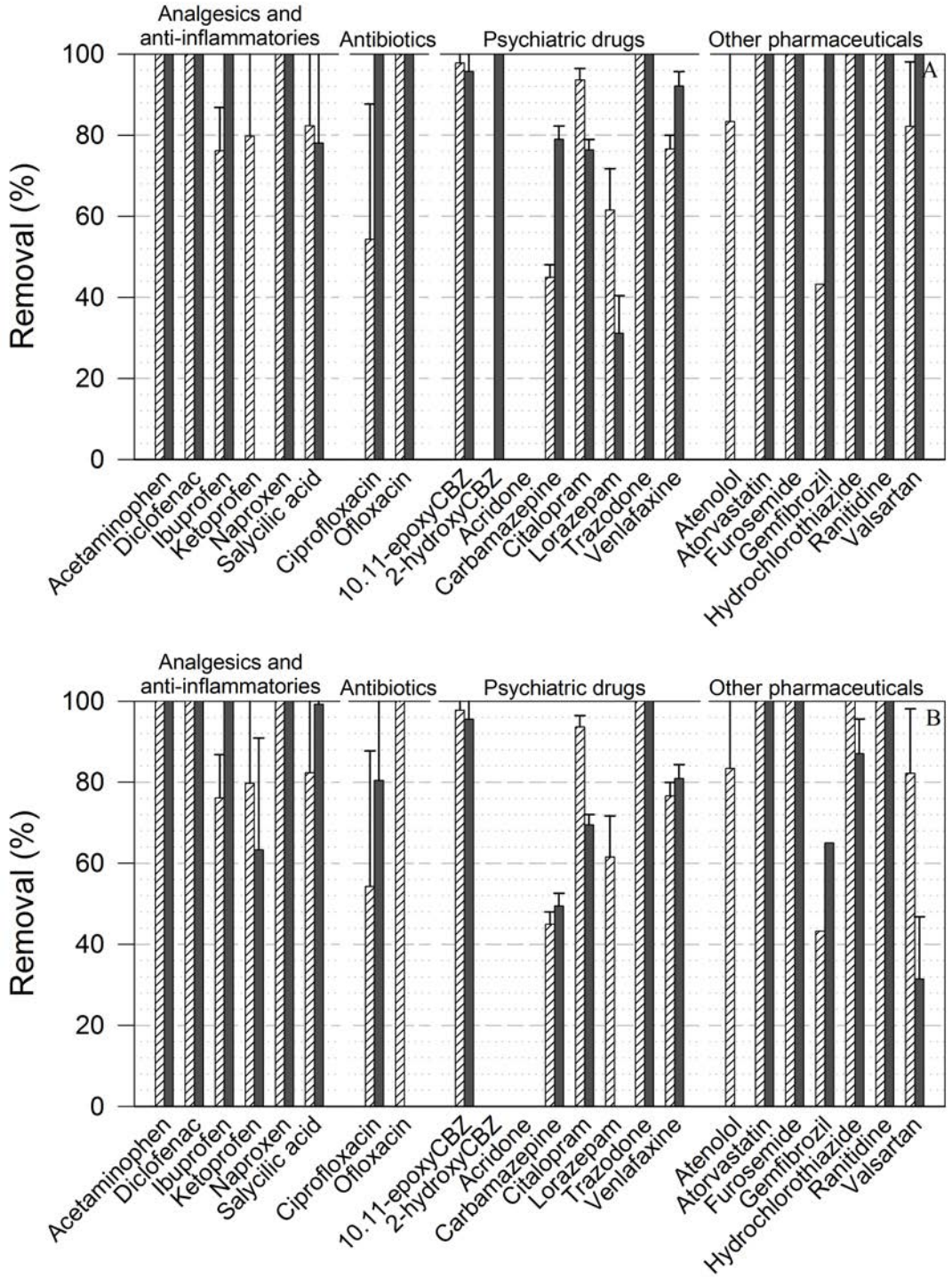

Figure 9.2: Cumulative removal percentages of pharmaceutically active compounds during the FG + UV/H_2O_2 (A) and AS + UV/H_2O_2 (B) treatments. White bars represent the removal of the fungal (A) and activated sludge (B) treatments; grey bars the overall removal of the corresponding biological step followed by UV/H_2O_2 process.

9.2.2 Performance of the coupled treatments

9.2.2.1 Biological treatments coupled with UV/H_2O_2

The combinations FG+AOP and AS+AOP are presented in Figure 9.2A and B, respectively. Psychiatric drugs were the family the most recalcitrant to this combination of treatments, although carbamazepine, citalopram and venlafaxine were >80% removed in the best-case scenario. Further discussion on CBZ and its TPs can be found in Section 9.2.3.

Placing the UV/H_2O_2 after the biotreatment aimed at degrading the remaining PhACs and the transformation products produced by the previous biological processes. In regards to the FG+AOP combination (Figure 9.2A), the UV/H_2O_2 furthered only slightly the removal of the analyzed compounds. Exceptions were CBZ TPs, produced by biotransformation of carbamazepine and deconjugation, and ofloxacin, whose removal was improved by the AOP.

Placing the AOP after the AS (Figure 9.2B) significantly improved the overall removal since AS removal efficiency was lower than FG. AS can indeed decrease the COD, improving the effectiveness of the subsequent AOP: since OH radicals produced in UV/H_2O_2 treatment have non-selective reactivity to organic materials, its effectiveness in PhAC degradation is lower when treating matrices with high COD or TSS content (Kim et al., 2009). Overall, placing the AOP after the biological treatment seemed to effectively increase the removal of the single treatment, allowing for a 94% removal of pharmaceutical load; more so if we take into account that both biotreatments deconjugated several compounds, which were in turn available for the posterior UV/H_2O_2 treatment.

9.2.2.2 UV/H_2O_2 coupled with biological treatments

Overall removal values of the studied AOP and both biological treatments on the same wastewater can be found in Table 9.1. The UV/H_2O_2 approach removed an overall 83% of pharmaceutical load, followed by the fungal treatment with a 78% and the activated sludge with a 63%. The AOP removed an 88% of psychiatric drugs, in comparison to a 42 and 26% of FG and AS, respectively. Contrarily, AOP antibiotic removal was lower, a 67% in comparison with a 91 and 36% of FG and AS, respectively. The AOP removal of the analgesics and anti-inflammatories family was slightly lower than the biological treatments. Figure 9.3 presents the cumulative removal efficiencies of UV/H_2O_2 process followed by biological treatments fungal (FG) and AS. The AOP step completely removed the analgesics and anti-inflammatories acetaminophen, diclofenac and naproxen, the antibiotic ofloxacin, furosemide, hydrochlorothiazide, ranitidine, atorvastatin and the

psychiatric drug trazodone. It also removed by more than 80% valsartan, atenolol, 10,11-epoxyCBZ (by far the major contributor to the psychiatric drugs family), 2-hydroxyCBZ and citalopram. These results are in accordance with previous studies where carbamazepine and gemfibrozil were poorly degraded by UV/H_2O_2 (Wols et al., 2013). It is noteworthy to remember that the wastewater was pretreated with coagulation-flocculation to lower the COD and TSS, which could have enabled the good removals observed with the AOPs: AOPs are in general used as a polishing step after biological treatment and therefore, treating wastewater with low loads of COD and suspended solids (Gomes et al., 2017; Lekkerkerker-Teunissen et al., 2012).

Placing the AOP step before a biological treatment generally aims at increasing the biodegradability of the biorecalcitrant compounds (Oller et al., 2011). This was the case when placing a FG treatment after the AOP (Figure 9.3A): although each single treatment was already able to remove around 80% of the initial pharmaceutical load, this value raised to a 94% when the AOP process was coupled with the fungal treatment. In the case of AS applied to AOP-treated effluent, (Figure 9.3B) AOP eliminated up to 74% of ibuprofen and the following biological treatment increased the final removal to 100%; and from 45% to 60% in the case of gemfibrozil, but did not significantly increase the removal of any other compound. In fact, the coupling only increased the overall removal from 83% (for AOPs) to an 84%. However, the AS might benefit from a previous AOP, as UV/H_2O_2 degraded, for example, part of the antibiotics family, known to be toxic to the activated sludge (Collado et al., 2013). In general, though, placing the advanced oxidation process before the biological treatment led to overall higher removal values than any single step.

9.2.3 Fate of carbamazepine and transformation products

The case of carbamazepine (CBZ) and its transformation products is of special importance as it is frequently found worldwide (Zhang and Geißen, 2010) and it is usually refractory to biodegradation during conventional wastewater treatments. Since carbamazepine is poorly removed in WWTPs and remains stable through the aquatic compartments (Zhang and Geißen, 2012), several approaches to remove it have been tested. Not only removal of carbamazepine but the presence of their metabolites and transformation products have been studied. A summary of carbamazepine transformation pathways in humans, UV/H_2O_2, WRF and activated sludge can be found in Figure 9.4 and it embodies a compendium of different studies (Golan-Rozen et al., 2015; Jarrott, 1999; Jelic et al., 2012; Kaiser et al., 2014; Kosjek et al., 2009;

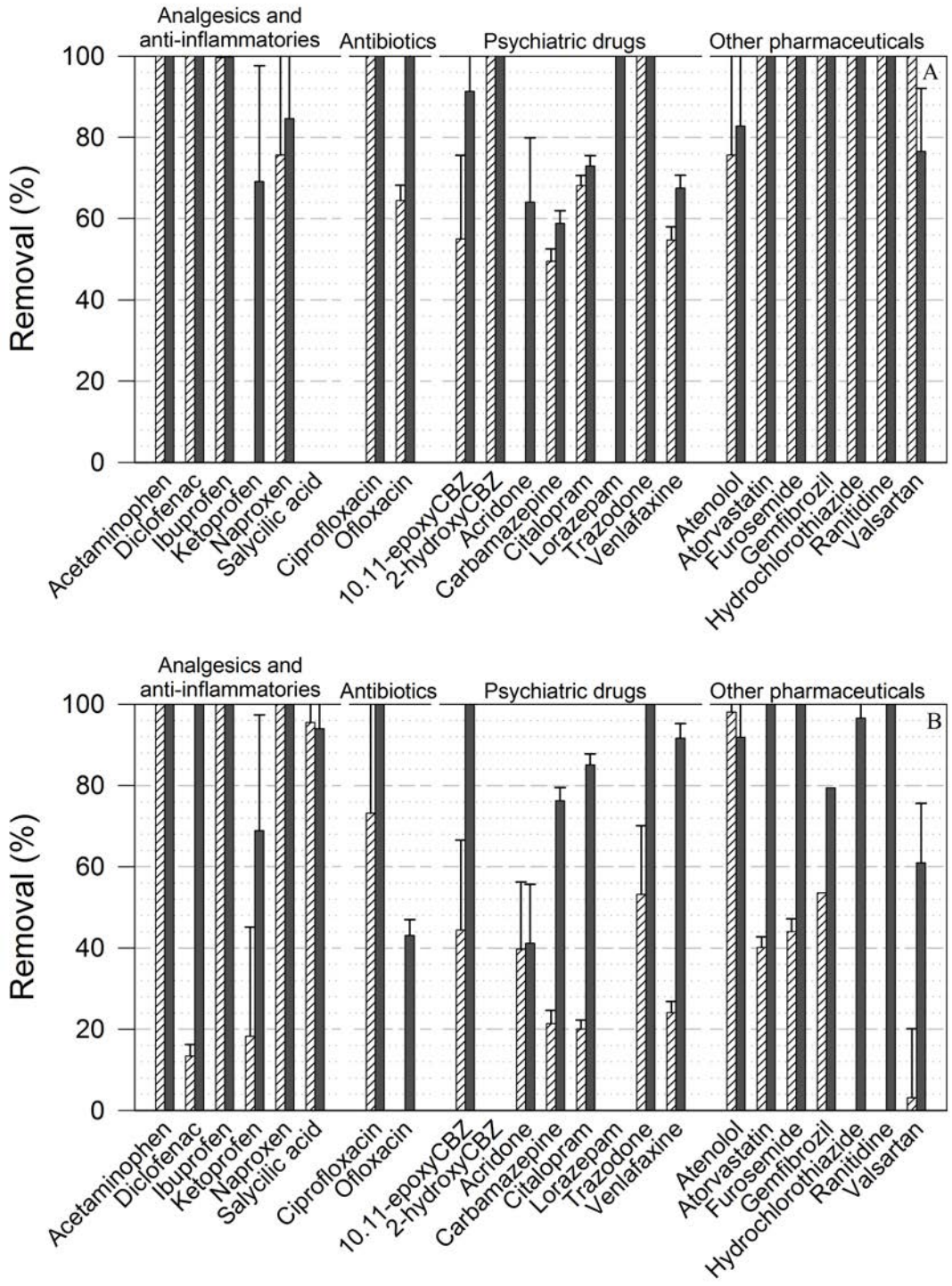

Figure 9.3: Cumulative removal percentages of pharmaceutically active compounds during the UV/H_2O_2 + FG (A) and UV/H_2O_2 + AS (B) treatments. White bars represent the removal of the UV/H_2O_2 treatment; grey bars, the removal of the coupled fungal (A) and activated sludge (B) treatments.

Table 9.2: Levels of carbamazepine and carbamazepine transformation products (mmol L^{-1}) in the initial wastewater and after each treatment.

Pharmaceutical	Initial concentration	Final concentration (nmol·L^{-1})						
	(nmol·L^{-1})	AOP	FG	AS	AOP+FG	AOP+AS	FG+AOP	AS+AOP
Carbamazepine	17	9	8	12	3	8	7	4
10,11-epoxyCBZ	113	2	50	62	5	5	10	bld
2-hydroxyCBZ	1	0	bld	22	bld	4	bld	2
Acridone	3	3	29	1	7	8	1	1
Total	134	14	88	98	15	25	17	7
Removal (%)		89	35	27	89	81	87	94

bld: below limit of detection

Lekkerkerker-Teunissen et al., 2012; Lynn et al., 1978; Mathieu et al., 2011; Pearce et al., 2009; Thorn et al., 2011). Several TPs are depicted but only 2-hydroxycarbamazepine, 10,11-epoxycarbamazepine and acridone were included in the present analysis.

The analyzed wastewater contained human metabolites of the CBZ: the parent compound, CBZ, is primarily metabolized in the human liver generating 10,11-epoxyCBZ (CBZE) as a main metabolite (Thorn et al., 2011). It is then further transformed to form acridine, acridone and the non-pharmaceutically active 10,11-dihydroxyCBZ (CBZD). A minor pathway is the transformation of CBZ to 2,3-epoxyCBZ to produce 2-hydroxyCBZ and 3-hydroxyCBZ. The initial load of CBZE in this study was 100-fold higher than 2-hydroxyCBZ (Table 9.2), because CBZE is the main route for CBZ transformation in humans and is produced and excreted at much higher concentration than CBZ itself (Thorn et al., 2011). In fact, CBZE was the compound with the highest concentration in the raw wastewater. Human metabolites include also several glucuronides of CBZ, CBZE, CBZD, 2-hydroxyCBZ and 3-hydroxyCBZ, which were not analyzed in this study (Lynn et al., 1978). UV/H_2O_2 and AS treatment seem to follow the same degradation pathway, namely, CBZE, acridine-9-carbaldehyde, acridine and acridone. White-rot fungal pathway resembles the human CBZE pathway (Jelic et al., 2012); and therefore, 2-hydroxyCBZ and 3-hydroxyCBZ could also be generated by WRF, as both humans and fungus have similar cytochrome P450 systems. In fact, fungi *C. elegans* and *U. ramanniana* were reported to produce such compounds when metabolizing CBZ (Kang et al., 2008).

Concentration of these compounds as well as carbamazepine in the wastewater before and after each treatment can be found in Table 9.2. The AOP treatment removed 90% of the overall initial concentration of CBZ and TPs. It removed around 50% of carbamazepine, although UV/H_2O_2 has been reported to degrade up to a 70% of CBZ in

some wastewaters (Giannakis et al., 2015). In contrast, 98% removal of the main metabolite, CBZE, was achieved whereas acridone concentration increased. Acridone is a byproduct of CBZE degradation although its increase does not account for the complete degradation of CBZE; AOPs are able to degrade the acridine-9-carbaldehyde, acridine and acridone (Kosjek et al., 2009), so acridone must have been degraded too.

The fungal treatment removed 54% of carbamazepine, the highest value for CBZ in the single treatments studied. This is in accordance with previous works, where removal values on the range of 50% were achieved (Mir-Tutusaus et al., 2017a). CBZE concentration decreased by 56% and acridone increased substantially, as expected when taking into account the fungal transformation pathway (Figure 9.4) and in agreement with previous studies (Mir-Tutusaus et al., 2017a). CBZE and 2-hydroxyCBZ might have been present as glucuronides and biological processes have been reported to deconjugate such glucuronides, which would have in turn undervalued the removal values (Badia-Fabregat et al., 2015a).

The AS process removed CBZ and CBZE lower than the fungal treatment did, but it didn't accumulate acridone. Acridone could have been also removed or intermediates could have accumulated. Carbamazepine is known to be very recalcitrant and poorly degraded in CAS, and acridone has been reported to be removed up to a 40% (Kosjek et al., 2009). Similarly to the fungal process, some compounds could have been present as glucuronides that the AS could deconjugate. This was confirmed by the increase in 2-hydroxyCBZ, which is not a byproduct of AS carbamazepine transformation, leaving the deconjugation of already present 2-hydroxyCBZ glucuronides as the only explanation.

When combining an activated sludge process with the AOP, the increase of 2-hydroxyCBZ produced by the AS is retained even when the AOP is placed after the bioprocess. Contrarily, the increase in acridone produced by the AOP can be avoided when placed after the AS. UV/H_2O_2 placed after the AS is the most convincing strategy to remove CBZ and its TPs. This combination removes around 76% of the initial CBZ, as if the removal percentages of AOP and AS separately were additive. Removal of CBZ and acridone were lower than other combinations, and 2-hydroxyCBZ increased, but as 10,11-epoxyCBZ was the main compound of the mixture, achieving complete removal of this compound led to this 94% overall removal of the carbamazepine and its transformation products.

The AOP already removed an impressive 89% of the compounds in real, flocculated HWW and the best coupling approach improved that value only by an additional 5% (up to 94% overall removal with AS+AOP treatment). Nevertheless, if we take into account

the conjugates, then placing the AOP after the biological treatments, as a polishing step, would be the top choice: the biological treatment would deconjugate and remove part of the compounds and the AOP would degrade the remaining pollutants and byproducts.

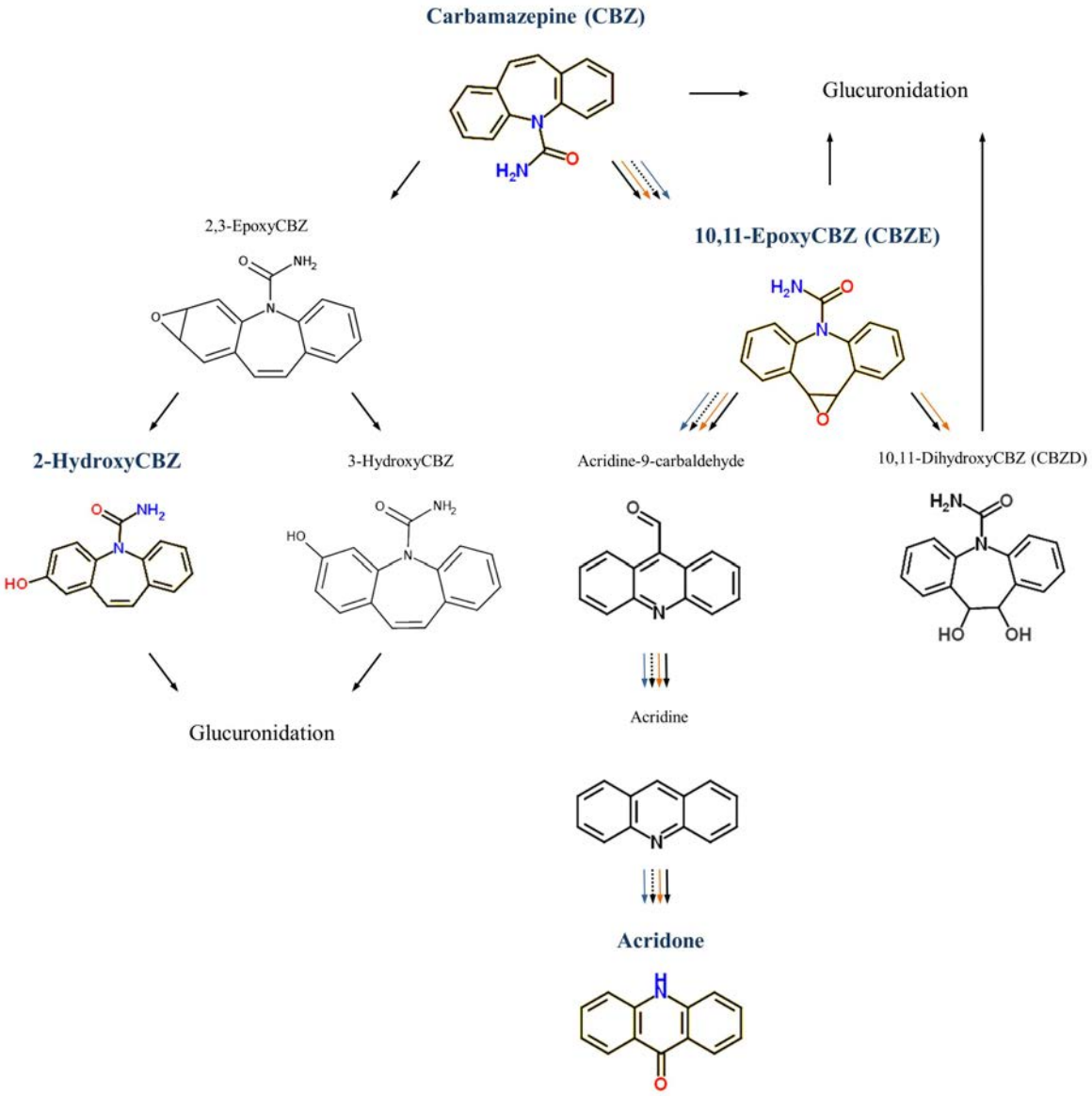

Figure 9.4: Transformation pathways of carbamazepine: in humans (solid lines), white-rot fungi (dotted lines), activated sludge (short-dashed lines) and UV/H2O2 (long-dashed lines). Analyzed compounds are presented in bold. Only relevant transformation products to this study have been represented(Golan-Rozen et al., 2015; Jarrott, 1999; Kaiser et al., 2014; Kosjek et al., 2009; Lekkerkerker-Teunissen et al., 2012; Lynn et al., 1978; Mathieu et al., 2011; Pearce et al., 2009).

9.3 Conclusions

Taking into account overall removal values of the studied compounds for each of the four treatment trains considered (namely AOP+AS, AOP+FG, FG+AOP and AS+AOP), three of them shared the highest removal value (94%), whereas AOP+AS was the treatment train with the lowest removal (84%).

Although placing an AOP prior to the CAS treatment is regarded in the bibliography as beneficial due to the increase of wastewater biodegradability (Bilińska et al., 2016), this study showed better results when the AOP step was placed after the AS. The AOP performance probably benefited from the decrease in COD and suspended solids promoted by the activated sludge, leading to higher removal values. This was in accordance with the bibliography, as AOPs are mainly used as a polishing step.

In regards to the coupling of UV/H_2O_2 with a fungal treatment, there are no significant differences between placing the AOP before or after the FG treatment. Being this the case, the fungal process should be placed as a pretreatment, so the following AOP could further decrease the concentration of parent compounds, transformation products and previously non-accessible, conjugated compounds.

10
Concluding remarks

The feasibility of a long-term white-rot fungal treatment for the removal of pharmaceutically active compounds from real non-sterile hospital wastewater has been demonstrated in the present thesis.

- Detection of laccase could be linked to *T. versicolor* activity, but the absence of laccase did not correlate with the fungus' inactivity.
- A continuous fungal treatment for hospital effluent was proposed, as easier operation and better results were obtained than with sequencing batch reactors.
- Sufficient aeration, a low pellet size and a low carbon-to-nitrogen ratio of the nutrients added proved to be important for favoring fungal survival.
- A combination of a coagulation-flocculation pretreatment, which decreased the initial concentration of microorganisms and a partial biomass renovation, which maintained an active fungal culture, allowed for a long-term fungal treatment of real, non-sterile hospital wastewater with an average removal of 80%.
- Fungal and bacterial competition were both observed during the treatments. However, a positive effect was observed for the removal of, at least, compounds belonging to the analgesics and antiinflammatories family.
- A partial ozonation and a partial biomass renovation strategy increased the removal efficiency of 5 micropollutants in a fungal MBR operated under a low HRT.
- The coupling of a fungal treatment and a UV/H_2O_2 process allowed for a 90% of the pharmaceutical active compounds present in hospital wastewater.
- Conjugation and deconjugation processes were a relevant factor during the fungal and activated sludge treatments. This fact undervalues to an unknown extent the removal capacity of both alternatives.

- DGGE and qPCR results confirmed the maintenance of *T. versicolor* activity throughout the treatments.

In general, this thesis has tackled several difficulties that hampered the feasibility of a fungal process for the treatment of hospital wastewater. The interaction with microorganisms, the influence of process variables, the alternative use of a membrane bioreactor and the coupling with physicochemical treatments have been studied and several problems detected in previous studies have been solved.

Future studies should include the operation of a pilot-scale treatment plant in a hospital and an effort to monitor the pharmaceutically active compounds as well as their conjugated forms.

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