Universitat Autònoma de Barcelona

Continuous wastewater treatment by Trametes versicolor immobilized on lignocellulosic support

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Continuous wastewater treatment by *Trametes versicolor* immobilized on lignocellulosic support

PhD Thesis

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Departament d'Enginyeria Química, Biològica i Ambiental

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GLÒRIA CAMINAL SAPERAS, Investigadora Científica de l'Institut de Química Avançada de Catalunya (IQAC) CSIC i PAQUI BLÁNQUEZ CANO, Professora Agregada del Departament d'Enginyeria Química, Biològica i Ambiental de la Universitat Autònoma de Barcelona.

CERTIFIQUEM,

Que la Llicenciada en Ciències Ambientals, Maria Josefina Torán ha realitzat sota la nostra direcció, als laboratoris del Departament d'Enginyeria Química, Biològica i Ambiental de la Universitat Autónoma de Barcelona el treball que amb el títol **"Continuous wastewater treatment by *Trametes versicolor* immobilized on lignocellulosic suppor**t**"**, es presenta en aquesta memòria, la qual constitueix la seva tesi per optar al Grau de Doctor en Ciència i Tecnologia Ambientals per la Universitat Autònoma de Barcelona.

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

a Bellaterra, Maig de 2018,

Dra. Glòria Caminal Saperas Dra. Paqui Blánquez Cano

“Poder decir adiós, es crecer.”

Gustavo Cerati

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Abstract

Over the last century, the water pollution has become a major problem which affects a large part of population and the environment. It is originated principally due to the discharges of untreated or inadequately treated wastewater in water bodies. Conventional wastewater treatment plants (WTTPs) typically remove organic compounds, but they are not designed to remove other pollutants such as micropollutants, so they can be discharged into the environment together with the effluents from the WTTPs.

Among the possible treatments, white-rot fungi (WRF) have become a promising alternative for the wastewater treatment because it can remove a wide range of micropollutants due to their nonspecific ligninolytic enzymatic system. WRF have been studied for the removal of a wide range of micropollutants in real wastewater, but the bacteria overgrowth usually produced a decline in removal efficiencies and consequently short-term operations are obtained.

The present thesis proposes the application of a bioreactor system using the WRF *Tramentes versicolor* immobilized on a lignocellulosic support, to solve this problem and to allow the continuous long-term wastewater treatment.

First of all, a substrate screening was performed in order to select the best lignocellulosic material for fungal growth. The pallet wood was selected for the following experiments. Immobilization studies were performed in a fluidized bed bioreactor. Good results were obtained with complex wood pellets, but the process results not scalable, hence new systems were proposed.

A trickle-bed bioreactor and a packed-bed bioreactor were developed and operated in a continuous long-term treatment with *T. versicolor* immobilized on pallet wood. Both reactors were employed for the treatment of wastewater from different sources: hospital wastewater with pharmaceutical active compounds, food-processing industrial wastewater with humic acids and rural area wastewater with pesticides.

The optimization of operational conditions is a key issue to improve the reactor performance. In the trickle-bed bioreactor, the recycling ration and the total volume were optimized. Meanwhile, in the packed-bed bioreactor preliminary studies of pH, fungal biomass, wood sorption and aeration were carried out.

In conclusion, both bioreactors systems with *T. versicolor* immobilized on pallet wood are a good alternative for the continuous long-term treatment of different wastewaters. The trickle-bed bioreactor achieved 61% of PhACs removal from hospital wastewater during 85 days; 50% humic acid removal from industrial wastewater for 26 days; and 84% diuron removal from synthetic tap water during 18 days. In the packed-bed bioreactor treating real wastewater, more than 90% removal of diuron was obtained during 50 days

Resumen

En el último siglo, la contaminación del agua se ha convertido en un problema importante que afecta a una gran parte de la población y al medio ambiente. La contaminación se debe principalmente a las descargas de aguas residuales no tratadas o tratadas inadecuadamente en cuerpos de agua. Las plantas convencionales de tratamiento de aguas residuales generalmente eliminan los compuestos orgánicos, pero no están diseñadas para la eliminación de otros contaminantes como los microcontaminantes, por lo que estos pueden ser descargados junto con los efluentes directamente al medio ambiente.

Entre las posibles tecnologías para el tratamiento de aguas residuales, los hongos de podredumbre blanca se han convertido en una alternativa prometedora porque pueden eliminar una amplia variedad de microcontaminantes debido a que presentan un sistema enzimático ligninolítico inespecífico. Los hongos de podredumbre blanca se han estudiado para la eliminación de una amplia gama de microcontaminantes en aguas residuales, pero el crecimiento excesivo de bacterias nativas del agua residual por lo general produce una disminución en las eficiencias de eliminación acortando la operación en continuo de los biorreactores.

Como alternativa a este problema, la presente tesis propone la aplicación de un biorreactor utilizando el hongo de podredumbre blanca *T. versicolor* inmovilizado sobre un soporte lignocelulósico. Esta estrategia permitiría el tratamiento en continuo de aguas residuales durante largos periodos de operación.

En primer lugar, se realizó un estudio con el objetivo de seleccionar el material lignocelulósico óptimo para el crecimiento de *T. versicolor* eligiéndose la madera de palé para los siguientes experimentos. Los posteriores estudios de inmovilización se realizaron en un biorreactor de lecho fluidizado. Se obtuvieron buenos resultados con el hongo auto-immobilizado sobre madera formado un pellet, pero el proceso no resultó escalable por lo cual se propusieron nuevos sistemas alternativos.

Se desarrolló y operó un biorreactor de filtro percolador y un biorreactor de lecho fijo utilizando *T. versicolor* inmovilizado sobre madera de palé para el tratamiento en continuo de aguas residuales durante largos periodos de operación. Ambos reactores se emplearon para el tratamiento de aguas residuales de diferentes orígenes: aguas residuales

hospitalarias con compuestos farmacéuticos activos, aguas residuales industriales de procesadoras de alimentos con ácidos húmicos y aguas residuales de áreas rurales con pesticidas.

La optimización de las condiciones operacionales resulta una cuestión clave para mejorar el rendimiento de los reactores. Por un lado, en el biorreactor de lecho percolador, se optimizaron la relación de recirculación y el volumen total de trabajo. Por otro lado, en el biorreactor de lecho fijo se realizaron estudios preliminares de pH, cantidad de biomasa, sorción en la madera y aireación.

En conclusión, ambos sistemas con *T. versicolor* inmovilizados sobre madera de palé resultaron ser una buena alternativa para el tratamiento en continuo de diferentes aguas residuales durante largos periodos de tiempo. El biorreactor de filtro percolador logró eliminar el 61% de los compuestos activos farmacéuticos presentes en aguas residuales hospitalarias durante 85 días; el 50% de eliminación de ácido húmico presentes en aguas residuales industriales durante 26 días; y la eliminación del 84% de diuron durante 18 días utilizando agua sintética. En el biorreactor de lecho fijo se obtuvo más del 90% de eliminación de diuron durante 50 días operando con agua real de origen rural.

Resum

En el darrer segle, la contaminació de l'aigua s'ha convertit en un problema important que afecta a una gran part de la població i al medi ambient. La contaminació es deu principalment a les descàrregues d'aigües residuals no tractats o tractats inadequadament. Les plantes de tractament d'aigües residuals convencionals solen eliminar els compostos orgànics, però no estan dissenyades per eliminar altres contaminants com els microcontaminants, de manera que aquests es podem descarregar directament al medi ambient juntament amb el efluents.

Entre els possibles tractaments, els fongs de podridura blanca s'han convertit en una tecnologia prometedora per al tractament d'aigües residuals, ja que poden eliminar una àmplia gamma de contaminants complexos a causa del seu sistema enzimàtic ligninolític inespecífic. S'han estudiat els fongs de podridura blanca per a l'eliminació de diferents microcontaminants en aigües residuals, però el creixement de microorganismes natius de les aigües residuals ha fet que l'operació en continu en reactor duri poc temps degut a la disminució en les eficiències d'eliminació.

Com alternativa a aquest problema, la present tesi proposa l'aplicació d'un bioreactor utilitzant el fong de podridura blanca *T. versicolor* immobilitzat sobre un suport lignocel·lulòsic per resoldre aquest problema i permetre així el tractament en continu de les aigües residuals durant llargs períodes de temps.

En primer lloc, es va realitzar una selecció del substrat amb l'objectiu de seleccionar el millor material lignocel·lulòsic per al creixement fúngic i la fusta de palets es va seleccionar per als següents experiments. Es van realitzar estudis d'immobilització en un bioreactor de llit fluïditzat. Es van obtenir bons resultats amb pellets formats amb un cor de fusta, però el procés resultant no es escalable, per això es van proposar nous sistemes.

Es va desenvolupar i operar un bioreactor de filtre percolador i un bioreactor de llit fix. Ambdós van operar en continu durant llargs períodes de temps amb *T. versicolor* immobilitzats sobre fusta de pallets i van ser emprats per al tractament d'aigües residuals procedents de diferents fonts: aigües residuals hospitalàries amb compostos farmacèutics actius, aigües residuals industrials de processament d'aliments amb àcids húmics i aigües residuals de zones rurals amb plaguicides. L'optimització de les condicions operatives és un

tema clau per millorar el rendiment del reactor. Al bioreactor de llit percolador, es van optimitzar la relació de recirculació i el volum total. D'altra banda, en el bioreactor del llit fix es van realitzar estudis preliminars de pH, biomassa fúngica, sorció sobre la fusta i aeració.

En conclusió, ambdós sistemes amb *T. versicolor* immobilitzat sobre fusta de palet van resultar ser una bona alternativa per al tractament en continuo durant llargs períodes de temps de diferents aigües residuals sense problemes operacionals.

El bioreactor de filtre percolador va aconseguir el 61% d'eliminació de fàrmacs en l'aigua de l'hospital durant 85 dies; el 50% d'eliminació d'àcid humic de les aigües residuals industrials durant 26 dies; i el 84% d'eliminació de diuron durant 18 dies en l'aigua sintètica. En el bioreactor de llit fix es va obtenir més del 90% d'eliminació de diuron durant 50 dies tractant aigües residuals reals.

List of abbreviation

ABT	1-aminobenzotriazole
ABTS	2,2-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) diammoniumsalt
AU	Activity units
BLD	Below limit of detection
BLQ	Below limit of quantification
BOD	Biological oxygen demand
CBZ	Carbamazepine
CFU	Colony forming unit
COD	Chemical oxygen demand
CPFR	Continous plug flow reactor
CRT	Cellular residence time
CSTR	Continuous stirred-tank reactor
DW	Dry weight
FA	Fulvic acids
FBR	Fluidized-bed reactor
HA	Humic acids
HPC	Heterotropic plate count
HOBT	1-hydroxy-benzotriazole
HPLC	High performance liquid chromatograpy
HRT	Hydraulic residence time
HS	Humic substances
HWW	Hospital wastewater
LiP	Lignin peroxidase
LME	Lignin modifying enzyme
LMPs	Lignin-modifying peroxidases
LOD	Limit of detection
LOQ	Limit of quantification
MnP	Manganese peroxidase
ND	Non-detected
NDV	N-desmethylvenlafaxine
ODV	O-desmethylvenlafaxine
PhAC	Pharmaceutically active compound
PUF	Polyurethane foam
SD	Standard deviation
TBR	Trickle-bed bioreactor
TSS	Total suspendid solids
TP	Transformation product
TU	Toxicity units
VA	Violuric acid

VLX	Venlafaxine
WRF	White-rot fungi
WW	Wastewater
WWTP	Wastewater treatment plant

Content

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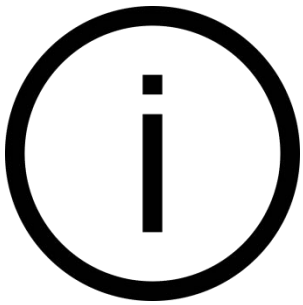
CHAPTER 1

General introduction



1.1 Water pollution and micropollutants

According to the FAO report, 4000 km³ per year are calculated to be the global freshwater withdrawals (United Nations World Water, Report 2017). Approximately 44% of this water is consumed (primarily in agriculture and secondly for industrial purpose) and 56% is discharged into the environment mostly as agricultural drainage and as wastewater (municipal and industrial wastewater) (Figure 1.1).

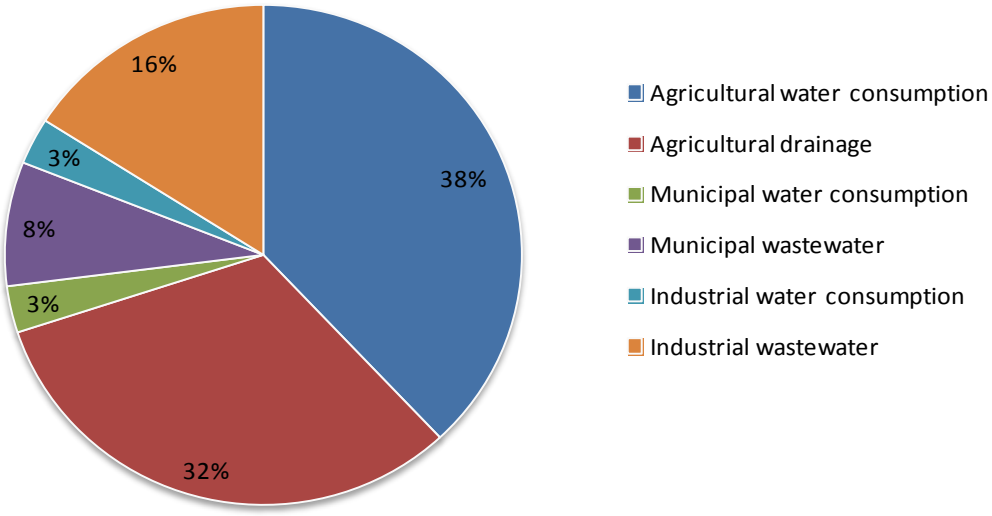


Figure 1.1. Fate of global freshwater withdrawals: consumption and wastewater production (modified from United Nations World Water, Report 2017).

The degradation of water quality is caused mostly by the discharge of untreated wastewater or inadequately treated wastewater. Conventional wastewater treatment plants (WWTP) remove organic compounds, but they are not designed to remove other contaminants such as micropollutants (Margot et al., 2015). In consequence, micropollutants are discharged with treated wastewater effluents and have been detected in surface and groundwater (Daughton and Ternes, 1999; Petrovic et al., 2003). This is a big problem, especially when the WWTP effluent is reused for irrigation in agriculture (Hong et al., 2018).

Micropollutants can be defined as "*substances that, even at trace concentrations, may have a negative effect on the environment and/or human health due to their characteristics: bioaccumulative, persistent and toxic*" (Sauvé and Desrosiers 2014; Mir-Tutusaus et al, 2018). This group includes some common categories such as personal care products,

plasticizers, pesticides, pharmaceuticals, endocrine disruptors, flame retardants, etc (Diamond et al., 2011).

The effects of the micropollutants over human health and the environment are mostly unknown (Gavrilescu et al., 2014). Moreover, micropollutants usually appear as a complex mixture with probably an unwanted synergic effect (Bolong et al., 2009; Petrie et al., 2015). There is also a lack of information about the pathway of exposures and the transformation or degradation process that occurs to the micropollutants in the environment (Luo et al., 2014).

There are many feasible sources and routes for the appearance of micropollutants in the aquatic environment, being the untreated urban wastewater and WWTP effluents the main ones (Jurado et al., 2012; Luo et al, 2014; Petrovic et al., 2003; Tambosi et al., 2010). As mentioned before, WWTP are not designed for the micropollutants removal, hence additional water treatment methods should be added to conventional WWTPs or on-site treatment should be included before the wastewater arrives to the WWTPs.

Activated carbon, sand filtration, membrane reactor, advance oxidation, ozonation and bioremediation are some of the techniques studied for micropollutants removal (Li et al., 2018; Rossi et al., 2013). Regarding bioremediation, the use of White Rot Fungi (WRF) is the alternative studied in this thesis.

- **Pharmaceutical Active Compounds**

Pharmaceutical Active Compounds (PhACs) have been detected all over the world in surface water, in groundwater and even in drinking water (aus der Beek et al., 2016; Jayasiri, 2013; Valcárcel et al., 2011) (Figure 1.2). PhACs include different compounds with therapeutic effects in humans and animals health (Castellet, 2017). Even at a very low concentration, they can promote drug resistance or tolerance in the original target organisms and unwanted effects in non-target organisms such as changes in activity, aggression and reproductive behaviors of fish (Brodin et al., 2014).

As other micropollutants, the effluents from WWTPs are the principal sources of PhACs in the environment (Daughton and Ruhoy, 2009; Huerta et al., 2016; Kümmerer, 2009). After the drug ingestion by humans, the PhACs are metabolized and excreted in urine and/or faeces in the parental form and/or in a transformation product (hydroxylated,

hydrolyzed and conjugated compounds) (Badia-Fabregat, 2014b; Margot et al., 2015; Shafy and Mansour, 2013).

High PhACs concentrations are detected in Hospital wastewater (HWW), but since the HWW is collected together with the municipal wastewater, the PhACs concentrations are 100 times diluted (Souza and Féris, 2016; Verlicchi et al., 2012). PhACs are also detected in effluents from pharmaceutical industry and landfills leachate (Heberer, 2002; Li and Randak 2009; Sim et al., 2011). Finally all these effluents reach the WWTPs, which become the main source of PhACs (Badia-Fabregat, 2014b).

Most of the WWTPs operate with the biological process of conventional activated sludge, but as mentioned before conventional WWTPs cannot completely remove many of the PhACs (Radjenovic et al., 2007; Verlicchi et al., 2012). Hence, it is necessary to develop and to apply effective treatments to completely remove PhACs from wastewaters.

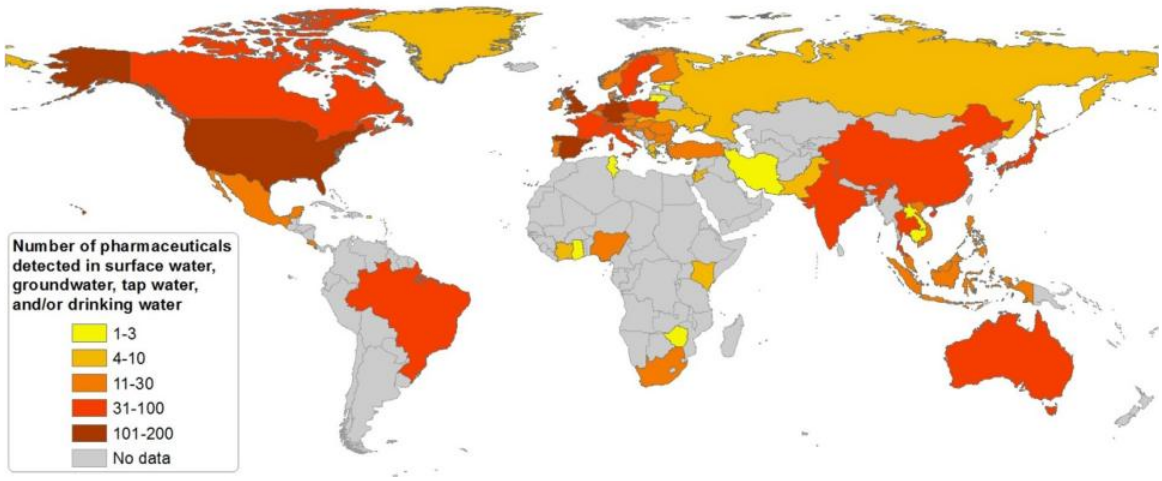


Figure 1.2. Pharmaceutical substances detected in surface waters, groundwater or tap/drinking water (modified from Aus der Beek et al., 2016 - literature review 2000-2013).

- **Industrial wastewater**

Industrial wastewater is one of the most important sources of water pollution. During the last century industrial wastewater was continuously discharged into aquatic environment (Shi, 2002). The uncontrolled industrial wastewater (in most of the cases untreated) caused negative effect to the ecosystem and humans life (Brusseau et al., 2004).

Each type of industry generates an industrial wastewater with specific characteristics and contaminants: oils, acids, phenols, sulfates, solids, dyes, phosphates, nitrates, chromium,

heavy metals, COD, BOD among others (Shi, 2002). Hence, the wastewater treatment technology must be designed for the treatment of each type of effluent produced.

In particular, food-processing companies are increasing over the world due to the exponential population growth. In consequence, increases the discharge of food processing effluents on aquatic environment (Meybeck et al., 1998; Noukeu et al., 2016).

Effluents from food processing industries contain high levels of suspended solids, COD, BOD, nitrates and phosphates. The color of the wastewater is caused by the content of organic matter such as humic substances (Noukeu et al., 2016). Due to their characteristics, these effluents are not suitable to be discharged into natural ecosystems without treatment. Hence, in this thesis the treatment of a humic-rich wastewater from a food-processing industry was studied.

- **Pesticides**

According to the FAO, the term pesticides "*refer to insecticides, fungicides, herbicides, disinfectants and any substance or mixture of substances intended for preventing, destroying or controlling any pest, including vectors of human or animal disease, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport or marketing of food, agricultural commodities, wood and wood products or animal feedstuffs, or substances which may be administered to animals for the control of insects, arachnids or other pests in or on their bodies"* (FAO Statistical Pocketbook 2015).

Since the middle of the last century there has been a continuous growth of pesticide usage (numbers and quantities) (Rahman, 2009). Figure 1.3 shows the pesticide per hectare used around the world from 2007 to 2012.

Pesticides enter the aquatic environment directly (spills during transportation, percolation from disposal sites) and/or mostly indirectly through runoff and infiltration processes of the pesticide present in the soil (Hamza et al., 2016; Hou and Wu, 2010; Tortella et al., 2010).

In general, pesticides or their transformation products travel in the environment and can arrive to other ecosystems producing toxic effects on non target species causing damage to biodiversity and ecosystems health (Aktar et al., 2009; Taylor et al., 2003).

Over the past decades, several methods have been developed for pesticides removal in different matrices. There are basically three methods to remedy the presence of pesticides: biological, chemical, and physical remediations. In this thesis, the pesticides bioremediation is studied.

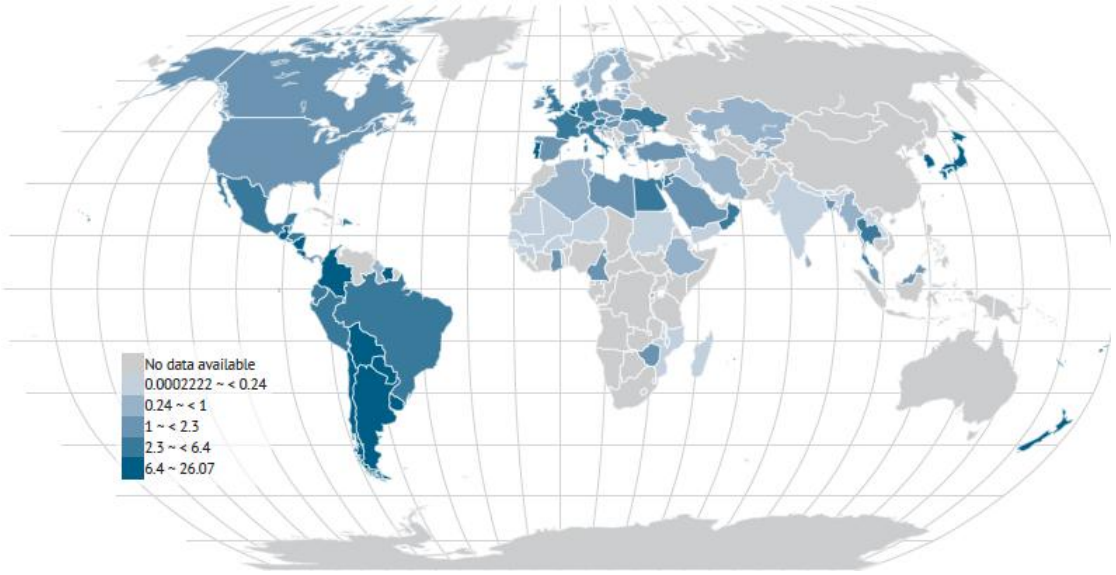


Figure 1.3. Pesticides per hectare of arable land (kg/ha 2007 to 2012) (FAO Statistical Pocketbook 2015).

1.2 White Rot Fungi (WRF)

WRF is a heterogeneous group of saprotrophic fungi used in fungal bioremediation. WRF include mostly basidiomycetes and some ascomycetes (Barrasa et al., 2014). The principal characteristic of this group is the capacity to degrade lignin from decaying wood (Lee et al., 2004; Kirk et al., 1976). Some relevant species include *Trametes versicolor, Pleurotus ostreatus, Ganoderma lucidum, Phanerochaete chrysosporium* and *Irpex lacteus* among others.

WRF present a powerful and unspecific enzymatic system. On one hand, WRF produce extracellular enzymes, peroxidases and laccases, which confer a high tolerance to toxic compounds (Hatakka, 1994; Hofrichter, 2002; Hofrichter et al., 2010; Morozova et al., 2007; Rodríguez-Couto and Toca-Herrera, 2006; Thurston, 1994). On the other hand, WRF have the intracellular enzymatic complex cytochrome P450, which is present in many fungi and other microorganisms and is also similar with the cytochrome P450 system present in

some mammalian and human (Bernhardt, 2006; Cerniglia, 1997; Cruz-Morató, 2013b; Subramanian and Yadav, 2008; van den Brink et al., 1998).

1.2.1 Bioremediation by WRF

As mentioned before, WRF present a nonspecific ligninolytic enzymatic system which allows the removal of a wide variety of xenobiotics with complex structures (Christian et al., 2005). Therefore, the use of fungi for the wastewater treatment has become a promising alternative technology (Harms et al., 2011).

WRF can degrade xenobiotic in a co-metabolic way, hence the addition of supplementary nutrient sources (C and N) is necessary (Cruz-Morató, 2013b; Pointing, 2001). Moreover, WRF could also mineralize and incorporate some compounds (Badia-Fabregat et al., 2014a; Mir-Tutusaus et al., 2018). But, since micropollutants are presented at very low concentration, an additional carbon source is needed to maintain fungal growth (Mir-Tutusaus et al., 2018). In conclusion, despite the degradation is co-metabolic or not, the nutrient addition is usually necessary.

However, the co-metabolic degradation is an advantage when the contaminant is toxic because prevents the need to internalize it in fungal cell (Barceló et al., 2013).

Fungal biodegradation has been applied to remove a diverse kind of contaminants such as: pesticides (Bumpus et al., 1993; Mir-Tutusaus et al., 2014; Quintero et al.,2007; Rubilar et al., 2007); polychlorinated biphenyls (Novotný et al., 1997); polycyclic aromatic hydrocarbons (Valentín et al., 2007); humic substances (Zahmatkesh et al., 2016); components of munitions wastes (Bumpus and Tatarko, 1994); olive mill wastewater (Cerrone et al., 2011; Olivieri et al., 2006); dyes and pigments (Blánquez et al., 2002, 2004); UV filters (Badia-Fabregat et al., 2012); organochlorine compound (Marco-Urrea et al., 2009a); pharmaceuticals (Marco-Urrea et al., 2009b, 2010a, 2010b; Mir-Tutusaus et al., 2017a, 2017b; Rodríguez-Rodríguez et al., 2010a, 2010b) and endocrine disruptor (Nguyen et al., 2013).

1.2.2 *Trametes versicolor*

T. versicolor is a filamentous WRF from the polyporaceae family (Cruz-Morató, 2013b) (Figure 1.4). It is an aerobic saprotrophic fungus and plays an important role as wood degrader (Rabinovich et al., 2004). The enzymes laccase, manganese peroxidase and lignin peroxidase are expressed by *T. versicolor* (Hofrichter et al., 2010; Vrsanska et al., 2016). Many different names have been used for this fungus, for instance *Polyporus versicolor* and *Coriolus versicolor*.

Figure 1.4. *Trametes versicolor.*

1.2.3 Limitations in wastewater treatment by WRF and possible solutions

Different types of fungal reactors to treat liquid effluents can be found, but a significant decline in the removal performance of bioreactors with real wastewater under non-sterile conditions commonly occurs. This performance deterioration is generally caused by the overgrowth of bacteria (Mir-Tutusaus et al., 2018; Yang et al., 2013).

Bacteria and fungi generally compete for the substrate. In addition, the presence of bacteria decreases the growth of the fungus, damage its mycelium and reduce the expression of certain fungal enzymes (España-Gamboa et al., 2016; Espinosa-Ortiz et al, 2015). Therefore, it is necessary to develop technologies or strategies in fungal bioreactor which favor the fungal competitiveness over bacteria.

Different strategies have been suggested in the literature to suppress bacterial growth under non-sterile conditions such as reduction of medium pH, limitation of nitrogen in the medium, partial biomass renovation, selective disinfection, wastewater pretreatment, immobilization of fungal cultures and optimization the HRT (Espinosa-Ortiz et al., 2015; Mir-Tutusaus et al., 2018).

- **Operation at optimal fungal pH**

Acid pH is the optimal one for the fungal growth, meanwhile bacteria preferentially growth in a pH near neutrality. Hence, the control and use of acid medium or wastewater in bioreactor treatments favor fungal competitiveness. However, this is a temporary strategy because bacteria can adapt to acid conditions in long-term operation (Libra et al., 2003).

- **Operation at optimal carbon to nitrogen ratio**

The ratio between carbon and nitrogen may play a role in favoring fungal over bacterial populations, especially in the systems with nutrient addition (Leite et al., 2017; Mir-Tutusaus et al., 2018). The use of media with limited nitrogen concentrations restricts the bacterial increase and benefit the fungal growth (Espinosa-Ortiz et al., 2015; Libra et al., 2003). Badia-Fabregat et al. (2017) pointed out that lower C/N ratio in the extra nutrients supplied increases the fungal/bacterial ratio in a fluidized bed bioreactor. In contrast, high C/N ratios increase white-rot fungal production of lignin modifying enzymes (Eggert et al., 1996; Levin et al., 2002). Therefore, C/N ratio might be important for the maintenance of the fungal predominance (Pedroza-Rodríguez and Rodríguez-Vázquez, 2013).

- **Suppressing bacteria**

Another strategy to favor fungi in the competition is the direct suppression of bacteria. Since the wastewater sterilization is not possible at industry scale, the use of ozone as bactericide and wastewater pretreatment have been suggested as alternatives (Mir-Tutusaus et al., 2018).

The use of bactericides such as ozone has been used for the limitation of bacterial growth in fungal reactor systems (Amin et al., 2013; Cheng et al., 2013; Espinosa-Ortiz et al., 2015). The addition of wastewater pretreatments can potentially reduce the inlet concentration of bacteria. Mir-Tutusaus (2016) used a coagulation-flocculation pretreatment, which successfully reduced the bacterial concentration in a hospital wastewater in four order of magnitude from 10^7-10^8 colony forming unit (CFU)·mL^{-1}, allowing then the operation of a *T. versicolor* fluidized bed reactor with high PhACs removals in continuous mode during 91 days.

- **Partial biomass renovation**

The fungal biomass renovation facilitates the maintenance of a young fungal culture, which promotes the fungal activity and avoids operational problems, and therefore allows for continuous long-term operation of fungal reactors (Blánquez et al., 2006). The partial renovation has been reported to be effective in maintaining the fungal activity also under non-sterile conditions employing a fluidized bed bioreactor with *T. versicolor* pellets and a cellular retention time of 21 days (Badia-Fabregat et al., 2015b; Mir-Tutusaus et al., 2017a).

- **Decoupling HRT and CRT**

Another strategy is the decoupling of the hydraulic retention time (HRT) and the cellular retention time (CRT), with the objective of increasing the CRT of WRF and using an HRT able to wash out other microorganisms present in the wastewater (Mir-Tutusaus et al., 2018). In systems working with immobilized fungi, the use of lower HRTs allows washing out the microorganism present in the wastewater while the fungus remains in the reactor for example using pelletized biomass (Espinosa-Ortiz et al., 2015). But, it must be considered that employing lowers HRTs usually lowers degradation values are obtained because extracellular enzymes and mediators produced by the fungus are also washed out (Badia-Fabregat et al., 2017; Cruz-Morató et al., 2013a; Nguyen et al., 2013; Mir-Tutusaus et al., 2018). In summary, both HRT and CRT must be optimized.

- **Microbial community structure in bioreactors**

The microbial community is not usually monitored in most of the recent studies working with fungal bioreactors under non-sterile conditions. Badia-Fabregat et al. (2017) indicated the need to make this monitoring; they reported the competitions of many indigenous fungi and bacteria with the inoculated *T. versicolor* in a fluidized-bed bioreactor for the removal of PhACs from a veterinary effluent.

Mir-Tutusaus et al. (2017a) also monitored the microbial diversity during a hospital wastewater treatment in a fluidized-bed bioreactor with *T. versicolor* pellets and the results confirmed the presence of *T. versicolor* during all the treatment.

Most of the microbiological studies were performed in bioreactors working with liquid effluents, where the samples could be taken from the liquid and in some cases from the fungal pellets when partial biomass renovation was performed.

Working with the fungus immobilized on a substrate, the sample extraction is more complicated and usually is done at the end of the experiment, in order to ensure a representative sample. Hence, in most cases, the monitoring of the microbial communities cannot be done during the treatment. In this thesis, microbiology samples were taken from the different experiments treating real wastewaters, but they were not analyzed because this is the first work applying *T. versicolor* immobilized on a substrate in a new type of bioreactor, hence more studies are required before carrying out a microbiological monitoring of the system. However, the samples are stored if necessary to analyze them.

- **Immobilization**

The immobilization of the fungus can be done by the auto-immobilization in pellet form, over a carrier and over a membrane (Hai et al., 2013). On one hand, the use of the pelletized biomass increases the fungal concentration used in a bioreactor and avoids some operational problems. On the other hand, the use of a lignocellulosic material as a carrier and substrate allows white rot fungi to outcompete bacteria because the lignocellulosic substrate acts as a selective carbon source. Immobilization strategy is deeply discussed in the next section.

1.2.4 Immobilization of fungal biomass

Fungal dispersed mycelium produces some operational problems in the bioreactor such as mycelia growth on the reactor walls and agitators and foam formation (Espinosa-Ortiz et al., 2015). The immobilization of fungal biomass reduces theses difficulties. Two types of immobilization have been described and used: auto-immobilization in pellet form and immobilization over a carrier.

- **Auto-immobilization**

The auto-immobilization can be accomplished by the growth of the fungus in form of pellets. Espinosa-Ortiz et al. (2015) reviewed several reactor configurations employing

fungal pellets for the wastewater treating. The application of pelletized biomass in bioreactors has been successfully applied at lab-scale mostly under sterile conditions, but the application at pilot-scale is still lacking and more studies are required (Espina-Ortiz et al., 2015).

- **Immobilization onto carriers**

The immobilization can also be carried out by growing the fungus onto a carrier. Several studies have reported the use of different types of supports to immobilize white-rot fungi to be employed in different type of bioreactors. Some authors reported the immobilization of WRF onto inert carriers. In this case, the nutrient addition is necessary to maintain the fungal activity. They usually employed nutrient media (for example Kirk media) or the nutrients were continuously added to the reactor (Medina-Moreno et al., 2012; Rodarte-Morales et al., 2012a).

Rodarte-Morales et al. (2012a) studied the removal of selected pharmaceuticals in a spiked Kirk-media under sterile conditions. They compared the removal performance of *P. chrysosporium* cultured as free pellets or immobilized in polyurethane foam (PUF) using two types of bioreactor (stirred tank reactor and fixed bed reactor). They obtained better removal values with the immobilized fungus on PUF using a fixed bed reactor.

Medina-Moreno et al. (2012) developed a mathematical model for the decolorization of a synthetic dye reactive blue 4 in a fixed bed bioreactor with the white-rot fungus *Trametes subectypus* immobilized in stainless steel sponges. They employed modified Kirk-medium and worked under sterile conditions.

Pakshirajan and Kheria (2012) reported the continuous biological treatment of coloured wastewater from a textile dying industry employing *Phanerochaete chrysosporium* immobilized onto PUF in a rotating biological contact reactor. They worked with diluted wastewater in media with glucose.

Alginate beads, PUF, nylon sponge and stainless steel sponges have been also used as support materials to immobilize the white-rot fungus *T. hirsuta* for laccase production and textile dyes effluent decolorization (Rodríguez-Couto et al., 2004).

Other authors reported the fungal immobilization on lignocellulosic carriers, which usually improve the removal efficiencies in fungal bioreactorS compared with

immobilization on inert carriers (Palli et al., 2016). Immobilization on lignocellulosic materials is deeply discussed in the next section.

1.2.5 Lignocellulosic materials: an alternative for fungal immobilization

The most abundant organic compounds present in the environment are the lignocellulosic materials (Fornasiero and Graziani, 2012). They consist of three polymeric constituents: cellulose, hemicelluloses and lignin (Anderson et al., 2009). Lignin presents a very complex structure and it is hardly degraded by microorganisms (Härtig and Lorbeer, 1993). A schematic representation of the lignocellulose complex is shown in Figure1.5.

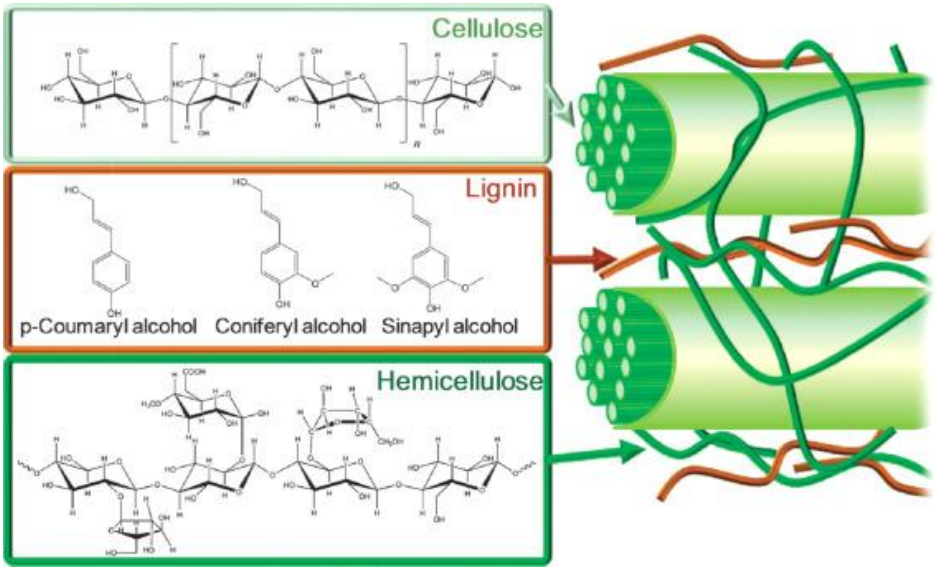


Figure 1.5. Structural representation of lignocellulosic biomass with cellulose, hemicellulose, and building blocks of lignin (Salvachúa Rodríguez, 2003).

As mentioned before, lignocellulosic materials have already been used as substrates for fungal growth in biodegradation technology. Table 1.1 shows different lignocellulosic materials used as a carrier in fungal treatment for the degradation of pollutants. The use of lignocellulosic materials as support for the immobilization allows using the material as a selective nutrient source for WRF. Hence, lignocellulosic materials can be used as support and substrate at the same time. Based on the natural environment where WRF commonly grow, wood is the material most frequently selected for fungal immobilization.

Table 1.1. Studies employing lignocellulosic substrates for bioremediation.

Degraded Pollutant	Fungi	Substrates	System	References
Polichlorphenols	*T. versicolor; P. chrysosporium*	Sawdusts, starch and maize flour	Solid-phase cultures	Lestan et al., 1996.
PAHs	*P. ostreatus; P. chrysosporium; T. versicolor*	Sawdusts and wheat straw	Solid-phase cultures	Novotný et al., 1999.
2,4,6-trichlorophenol	*P. chrysosporium; T. versicolor; Lentinula edodes*	Pine wood	Fixed-bed bioreactor	Ehlers and Rose, 2005.
Polichlorphenols	*T. versicolor*	Sawdust of Pinus radiata	Solid-phase cultures	Ford et al., 2007.
Molasses distillery wastewater	*P. ostreatus*	Wheat straw and maize stalks	Solid-phase cultures	Pant and Adholeya, 2007.
Naproxen	*T. versicolor*	Wheat-straw	Solid-phase cultures	Rodríguez-Rodríguez et.al, 2010b.
Naproxen	*T. versicolor*	Wheat strarw, rabbit feedstock, maize stalks, wheat straw, pine stardust and rice	Solid-phase cultures	Borràs et al., 2011.
Remazol Red	*Bjerkandera sp.*	Wood chip	Packed bioreactor	Jonstrup et al., 2013.
Carbamazepine, naproxen	*P. chrysosporium*	Wood chips	Fixed-bed bioreactor	Li et al., 2015a.
Carbamazepine, naproxen	*P. chrysosporium*	Wood chips	Countercurrent seepage bioreactor	Li et al., 2015b.
2-naphthalensulfonic acid polymers	*P. ostreatus; Bjerkandera adusta*	Straw	Trickle-bed bioreactor	Palli et al., 2016
PhACs	*T. versicolor*	Pine bark	Solid-phase cultures	Llorens-Blanch et al., 2017.

Ehlers and Rose (2005) immobilized cultures of white-rot fungi (*T. versicolor, P. chrysosporium, Lentinula edodes*) on pinewood chip to use in fixed-bed trickling reactors to biodegrade phenol and 2,4,6-trichlorophenol. Palli et al. (2016) operated a trickle-bed bioreactor inoculated with *P. ostreatus* attached on straw for the treatment of real petrochemical wastewater during three months.

Li et al. (2015a) operated a fixed-bed bioreactor packed with *P. chrysosporium* immobilized over wood chips for naproxen and carbamazepine removal during 28 days. The same authors (Li et al., 2015b) reported a countercurrent seepage bioreactor with *P. chrysosporium* immobilized onto wood chips for the naproxen and carbamazepine removal using Kirk medium. They obtained higher removal efficiencies during 165 days.

Yum and Pierce (1998) investigated the ability of wood-chip reactors with *P. chrysosporium* to degrade 4-chlorophenol and 2,4-dichlorophenol. Meanwhile, Jonstrup et al. (2013) run a bioreactor packed with wood as carrier material and inoculated with *Bjerkandera sp.* for Remazol Red decolorization from synthetic water. They obtained 65–90% decolorization efficiency during 12 days and then bacterial growth caused a drop in the efficiency.

Moreover, the use of lignocellulosic materials could bring other advantages: they are readily available, economical (Saba et al., 2016) and represent a physical support for immobilized cultures, which gives more protection against environmental perturbations like changes in the pH and toxic chemical exposure (Rodríguez-Couto et al., 2004).

1.3 Bioreactors

Different configurations such as stirred-tank reactor (Rodarte-Morales et al., 2012b), airlift (Zhou et al., 2006), fluidized-bed bioreactor (Badia-Fabregat et al., 2016; Cruz-Morató et al., 2014; Mir-Tutusaus et al., 2016), trickle-bed bioreactor (Ehlers and Rose, 2005; Li et al., 2015b) and membrane bioreactor (Nguyen et al., 2013) have been used for wastewater treatment.

In this section the description and principal characteristics of the bioreactors employed in this thesis are presented.

The **Continuous Stirred-Tank Reactor (CSTR)** is a conventional bioreactor widely used. CSTR consists of a tank fed with the medium or wastewater (which contained the pollutant) and the inoculum. To ensure the adequate mixing in the reactor, air is supplied usually at the bottom of the reactor. Figure 1.6 shows a schematic diagram of a typical stirred-tank bioreactor.

The complete mixture is produced when the particles enter the tank and are immediately dispersed all over its volume. The particles leave the tank according to their statistical population (Metcalf and Eddy, 1991). In a CSTR, the same conditions exist everywhere inside the reactor. In this thesis, a trickle-bed bioreactor combined with a reservoir bottle that works like a CSTR was used with extracellular enzymes and without fungal biomass.

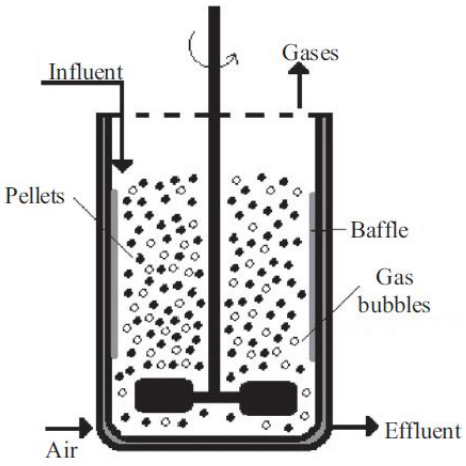


Figure 1.6. Example of a CSTR scheme working inoculated with fungal pellets (modified from Espinosa-Ortiz et al., 2015).

The **Plug Flow Reactor (CPFR)** consists of a straight pipe/tank in which the medium or wastewater transits (Figure 1.7). The particles enter and leave the tank with the same sequence, and present the same conditions at cross-section areas with a minimum longitudinal dispersion (Metcalf and Eddy, 1991). In this thesis, a CPFR adaptation was employed for the wastewater treatment experiments. A packed-bed channel filled with a support for fungal immobilization was employed working as a CPFR.

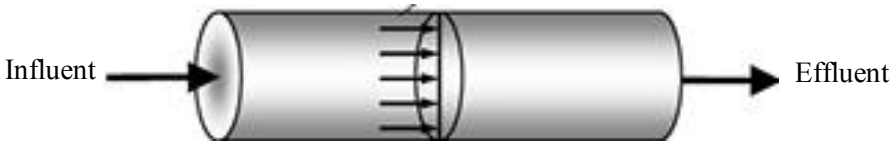
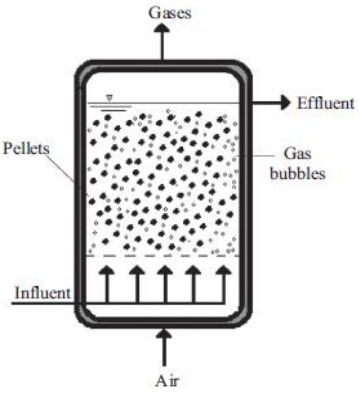


Figure 1.7. Scheme of a CPFR.

The **Fluidized-Bed Reactor (FBR)** is characterized by the fluidization of the solid material in the medium or the wastewater (Nelson et al. 2017). The fluid velocity must be sufficient to maintain the particles in suspension. For example, fungal pellets fluidized in

bioreactor are largely employed for wastewater treatment (Figure 1.8). A good mixing is achieved in this type of reactor due to the fluidization processes, resulting in good mass transfer.



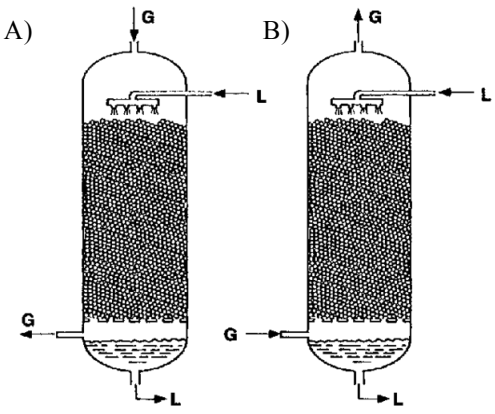
In this thesis, a fluidized-bed bioreactor was employed. The fluidization and the homogenization in the bioreactor were achieved by entering air in pulses. The air pulses allow the control of pellet growth and maintain the pellets form (Lema et al., 2001).

Figure 1.8. Fluidized-bed bioreactor (modified from Espinosa-Ortiz et al., 2015).

Packed-bed bioreactors use a support material for the microbial attachment. The reactors can work completely filled with the medium or wastewater (such as anaerobic filter) or dosed intermittently (trickle-bed bioreactor) (Metcalf and Eddy, 1991). The major disadvantage of this type of bioreactors is their poor mass transfer (Wang et al., 2007).

Trickle-bed reactors (TBR) are cylindrical reactors in which a liquid phase and a gas phase flow together through a fixed bed of permeable particles in which the microorganisms are adhered (Jørgensen, 1986; Satterfield, 1975).

The gas and liquid can operate in downwards concurrent flow or in countercurrent flow. The modes of operation are illustrated in Figure 1.9 (Duduković et al., 2002). The recirculation of the wastewater trough the TBR is a key issue that must be studied in depth because it is not clear how the recirculation improves or not the reactor performance (Metcalf and Eddy, 1991).



In this thesis, a trickle-bed bioreactor in downward concurrent flow was packed with *T. versicolor* immobilized on a lignocellulosic support and employed for the wastewater treatment of different kinds of pollutants.

Figure 1.9. Trickle-bed reactor. (A) Concurrent donwflow. (B) Countercurrent flow. (modified from Duduković et al., 2002)

1.4 Background in our research group

This thesis has been done in the BioREM Research Group. This group has a long experience in the treatment of pollutants by microorganisms (fungi, algae and bacteria). Regarding bioremediation by fungus, several thesis have been done during the last 20 years. A brief summary is presented below.

1995

Dr. Xavier Gabarrell i Durany began with the research line of the use of ligninolityc fungi for wastewater treatment. He studied the continuous treatment of black bleach from paper industry in a stirred tank reactor, an air lift and a trickle-bed bioreactor with *Phanerochaete chrysosporiu* immobilized on polyurethane foam and nylon.

1997

Dr. Xavier Font i Segura studied the treatment of black bleach from paper industry employing *T. versicolor* pellets and fungus immobilization on sodium alginate, polyurethane foam and nylon. He employed different types of bioreactor: air-lift, Biostat, fluidized-bed bioreactor (FBR) and fixed-bed bioreactor. The best result was obtained using a FBR with *T. versicolor* pellets in batch mode after 6 days.

2001

Dra. Silvia Romero Solé studied the ability of *T. versicolor, Phanerochaete Chrysosporium, Bjerkandera sp*. and *Rhizoctonia solani* to discolor industrial wastewater. She studied also the treatment application in different types of bioreactors; the best result was obtained with a fluidized-bed bioreactor.

2005

Dra. Paqui Blánquez Cano studied the decolorization of wastewater from textile industry. She reported the partial biomass renovation strategy working in FBR with *T. versicolor* pellets. The fungal biomass renovation facilitates the maintenance of a young fungal culture, which promotes the fungal activity and avoids operational problems, and therefore allows continuous long-term operation of a fungal reactor.

2007

Dr. Ernest Marco Urrea studied the biodegradation of chlorinated aliphatic hydrocarbons (trichlorethylene and perchlorethylene) by different WRF (*Trametes versicolor, Irpex lacteus* and *Ganoderma lucidum*) at Erlenmeyer scale.

2010

Dr. Eduard Borràs Camps studied the ability of *Trametes versicolor* to bioremediate polycyclic aromatic hydrocarbons in different matrices. First, he studied the growth of the fungus on different lignocellulosic materials to be employed in degradation systems. The bioslurry was the most effective treatment for contaminated soil after 12 days

2012

Dra. Nuria Casas Collet studied the dyes degradation by the fungus *T. versicolor*. She reported the involvement of the laccase in most of the cases. However, better results were obtained comparing the use of the whole fungus with the use of only laccase. Finally, she studied the influence of different parameters in a fluidized-bed bioreactor for dye decolorization, concluding that "sequential batch" is the best option.

2012

Dr. Carlos Rodríguez Rodríguez described the application of *Trametes versicolor* for the elimination of pharmaceuticals from sewage sludge.

2013

Dr. Carles Cruz Morató studied the biodegradation of pharmaceuticals by *Trametes versicolor*. He first focused on individual PhACs (ketoprofen, diclofenac and carbamazepine) degradation at Erlenmeyer scale and sterile conditions. Then, a FBR under sterile conditions was employed for the degradation of carbamazepine and clofibric acid, operated in both continuous and batch mode. And finally, he reported a FBR treating real hospital wastewater in batch mode during 8 days.

2014

Dra. Marina Badia Fabregat presented her thesis called "Study of relevant factors in the treatment of effluents by fungi for the degradation of emerging contaminants". She operated a fluidized bed bioreactor with *T. versicolor* and biomass renovation in continuous mode during 26 days for the removal of PhACs from reverse osmosis concentrate and veterinary hospital wastewater.

2016

Dr. Guillem Llorens Blanch studied the removal of pharmaceuticals from WWTP streams by biological and physical processes. He employed the utilization of fungal biopiles with forestry by-products as substrates during 42 days under non-sterile conditions.

2017

Dr. Francesc Castellet Rovira studied the PhACs removal capabilities for different WRF (including *T. versicolor).* A fluidized-bed bioreactor was operated with *Pleurotus ostreatus* and *S. rugosoannulata* pellets in spiked hospital wastewater during 32 days under sterile conditions and 10 days under non-sterile conditions. He also studied the possibility of the implementation of a Biosand Filter as a HWW pretreatment to prevent microbial competition in the fungal bioreactors.

2017

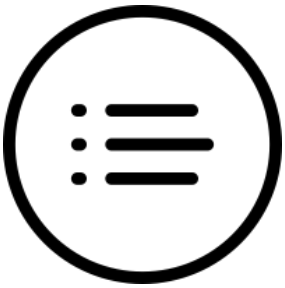
Dr. Josep Anton Mir- Tutusaus implemented a combination of a coagulation-flocculation pretreatment, which decreased the initial concentration of microorganisms. Employing this pretreatment, he reported a long-term treatment of real non-sterile hospital wastewater with an average removal of 80% during 91days in a fluidized bioreactor with *T. versicolor* pellets and weekly biomass renovation.

The ability of *T. versicolor* to degrade different pollutants has been widely described in our research group. The degradation pathways, enzymes implication and transformation products were also previously studied. Most of the works have been done with *T. versicolor* pellets in a fluidized-bed bioreactor. The first experiments were conducted under sterile conditions in batch and then in continuous mode reactor with non-sterile wastewater. Some operational parameters have been optimized, such as nutrient addition, pellets size, and aeration among others. Fungal biomass renovation was established as a suitable strategy to maintain the fungus activity. Most of the thesis studied the degradation of PhACs and focused on the hospital wastewater treatment. A significant decline in the removal performance commonly occurs with real wastewater due to bacterial overgrowth, hence two strategies were proposed: the wastewater pretreatment in order to diminish the initial bacterial concentration and the fungus immobilization on lignocellulosic substrates.

On one hand, the wastewater pretreatment was successfully developed and applied in the hospital wastewater treatment in the thesis by Mir-Tutusaus (2017b). Consequently, this strategy was applied in the present thesis. On the other hand, the strategy of immobilization on lignocelullosic substrates is studied in this thesis.

CHAPTER 2

Objectives



The main goal of the present thesis is to develop a continuous long-term wastewater treatment based on the white-rot fungus *Trametes versicolor* immobilized on a lignocellulosic material. In order to achieve that general goal, the following specific objectives were formulated:

- To select an optimal lignocellulosic substrate and methodology for *T. versicolor* immobilization.
- To determine the ability of *T. versicolor* to degrade selected contaminants under sterile conditions and to study the enzymes involved.
- To develop a bioreactor system and select operational conditions for the wastewater treatment with *T. versicolor* immobilized on a lignocellulosic material.
- To study, monitor and operate a long-term continuous bioreactor for the treatment of different real wastewater (hospital wastewater, industrial wastewater, rural wastewater).

CHAPTER 3

Materials and methods

3.1 Microorganisms

3.1.1 Fungal strain

The fungus *Trametes versicolor* ATCC#42530 was acquired from the American Type Culture Collection. It was maintained by subculturing every 30 days on malt extract (2% w/v) agar plates (pH 4.5) at 25°C.

3.1.2 Mycelial suspension

For the production of the mycelial suspension, four agar plugs were cutted from fungal growing area on Petri dishes. These cubes were used to inoculate 500 mL Erlenmeyer flasks containing malt extract medium (2% w/v) previously sterilized at 121°C during 30 minutes. Erlenmeyer flasks were maintained with orbital agitation (135 rpm) at 25°C. After 5-6 days, the resulting fungal mass was separated from the media using a strainer, homogenized (Ystral GmgH X/10/20) and stored in sterilized saline solution (0.8% w/v NaCl) in a ratio of 1:1 (v/v) under sterile conditions. The resultant suspension can be immediately used for the formation of pellets or kept at 4°C (Blánquez, 2005).

3.1.3 Pellets production

Pellets were obtained as previously described (Font et al., 2003); 1 mL of the homogenized mycelial suspension was inoculated to 1 L Erlenmeyer with 250 mL of malt extract (2% w/v, pH 4.5). The culture was maintained during 5-6 days with orbital agitation (135 rpm) and 25ºC. Final pellets were separated from the media with a strainer, washed with MilliQ water and stored in a 0.8% (w/v) NaCl sterile solution.

3.2 Chemicals and culture media

3.2.1 Chemical and reagents

Ketoprofen, ibuprofen and naproxen were purchased from Sigma-Aldrich (Barcelona, Spain). All the pharmaceuticals and the corresponding isotopically labelled standards used 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).

High-performance liquid chromatography (HPLC) grade ethanol, methanol and acetonitrile were supplied by Merck (Darmstadt, Germany). All the solvents were of high purity grade.

The laccase mediators Violuric acid (VA), hydrated 1-hydroxy-benzotriazole (HOBT), 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) were obtained from Sigma-Aldrich. Purified laccase of *T. versicolor* was purchased from Fluka (reference 53739). The enzyme was lyophilized and kept at -20°C. The cytochrome P450 inhibitor 1-aminobenzotriazole (ABT) was purchased from Sigma-Aldrich.

Regarding the pesticides: diuron, simazine, cypermethrin, chlorpyrifos, malathion, dicofol and irgarol were purchased from Sigma-Aldrich (Barcelona, Spain). Triallate was supplied by LGC-Standards. The corresponding isotopically labelled standards were of high purity grade (>90%) and they were purchased from Toronto Research Chemicals TRC (Ontario, Canada), CDN isotopes (Quebec,Canada) and Sigma–Aldrich (Steinheim, Germany).

Coagulants Hyfloc AC50 and flocculants Himoloc DR3000 were provided by Derypol, S.A. (Barcelona, Spain). Thiamine hydrochloride, malt extract, glucose, ammonium chloride, ammonium tartrate, humic acid and other chemicals were purchased from Sigma-Aldrich (Spain). Polystyrene sulfonate standards were purchased from Polymer standard service (Germany). All other chemicals used were of analytical grade.

3.2.2 Culture media

The treatment medium was used for degradation experiments. This medium was modified from Kirk et al. (1978), containing glucose as carbon source, and ammonium tartrate as nitrogen source. Before sterilization, the medium was adjusted at pH = 4.5.

The growth medium was employed for the production of complex wood pellets inside the bioreactor, as previously described Borràs et al. (2008).The thiamine was added directly to the medium in the bioreactor after sterilization. Table 3.1 and Table 3.2 describe the detailed composition of the media.

Table 3.1. Composition of the treatment and growth media.

Component	Concentration	
	Treatment medium	Growth medium
Glucose ($g \cdot L^{-1}$)	8	8
Macronutrients ($mL \cdot L^{-1}$)	100	100
Micronutrients ($mL \cdot L^{-1}$)	10	10
2.2-dimethyl succinic acid ($g \cdot L^{-1}$)	1.16	--
Ammonium tartrate ($g \cdot L^{-1}$)	3.3	--
NH_4Cl ($g \cdot L^{-1}$)	--	2.1
Thiamine (10 $mg \cdot L^{-1}$)	--	10

Table 3.2. Composition of macronutrients and micronutrients.

Micronutrients	Concentration ($g \cdot L^{-1}$)
Nitrile triacetic acidic	1.5
$MgSO_4 \cdot 7H_2O$	3.0
$MnSO_4 \cdot H_2O$	0.5
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

Macronutirents	Concentration ($g \cdot L^{-1}$)
KH_2PO_4	20
$MgSO_4 \cdot 7H_2O$	5
$CaCl_2$	1

3.2.3 Humic acid stock solution

Humic acid (HA) powder (4 g) was dissolved in 200 mL of NaOH solution (0.1 M) and mixed for 30 min. The solution was centrifuged (7000 rpm, 20 min) to remove the particulates. Then 100 mL of phthalate buffer (0.5 M, pH 4) was added to the particulate-free HA solution and pH was adjusted at 4.7-5 with HCl. The buffered solution was centrifuged again and the supernatant was used as HA stock solution (Zahmatkesh et al., 2016).

3.3 Lignocellulosic substrates and *T. versicolor* immobilization

Several lignocellulosic substrates were employed for the culture of *T. versicolor*: pine bark, nutshell, hazelnut shell and pallet wood. All of them are waste products. The substrates were kept at room temperature until its use. The pallet wood was kindly provided by Timgad S.A. (Polinyà, Barcelona, Spain).

3.3.1 Fungus pre-grown on lignocellulosic substrates

For the screening of substrates by *T. versicolor* colonization, each lignocellulosic substrate was autoclaved at 121 °C during 30 min immersed in tap water and then strained to remove excess water. Cultures were performed in Schott bottles (250 mL, 95 x 105 mm; Duran, Inc) equipped with 4 port screw caps, 3 of them were hermetically closed and 1 was kept open, using a 0.45 μm filter as passive air intake (Figure 3.1). Under sterile conditions, 10 g of each sterile substrate (with 100% of the water holding capacity) were placed in each bottle and inoculated with 3 mL mycelial suspension. Cultures were incubated in static conditions at 25°C during 9 or 30 days depending on the experiment.

In the experiments with the trickle-bed bioreactor with a total volume of 0.25 L (TBR 1), the inoculation of the pallet wood was performed in the same way as described before during 9 days. The wood size was 2 x 1 x 0.5 cm.

In the experiments with the trickle-bed bioreactors with a total volume of 0.6 L and 2.5 L (respectively TBR 2 and TBR 3) and the packed-bed channel bioreactor, cultures were performed in a polyvinyl chloride box (80 x 60 x 50 cm) covered with aluminum foil (Figure 3.1). Under sterile conditions, the wood was placed in the box and inoculated with mycelial suspension (1mL·2.5g wet wood^{-1}). Cultures were incubated in static conditions at 25°C during 9 days. The wood size was 3 x 2 x 1 cm.

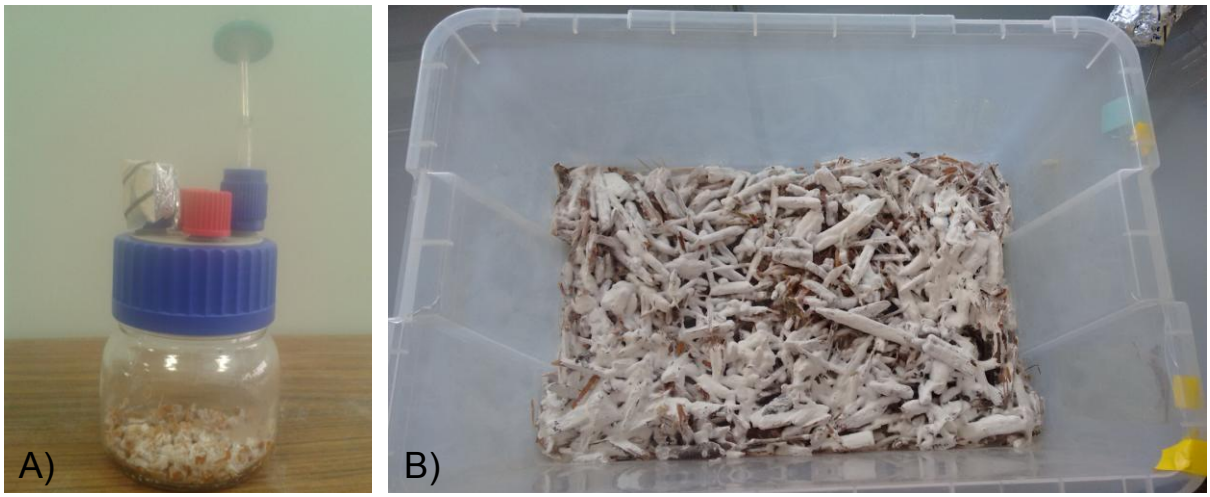


Figure 3.1. Culture of *T. versicolor* growth on pallet wood in (A) Schott bottles and (B) polyvinyl chloride box.

3.3.2 Complex wood pellets

Cultures of *T. versicolor* grown on wood (3 x 3 x 2 mm) during 9 days in Schott bottles were used as inoculum. The complex wood pellets production was done in Erlenmeyer flasks or in a fluidized-bed bioreactor.

A 1 L Erlenmeyer flask with 250 mL of malt extract (2% w/v, pH 4.5) was inoculated with 5 g of inoculum. The culture was maintained during 5-6 days with orbital agitation (135 rpm) and 25ºC.

For the formation of the complex wood pellets inside the bioreactor, 30 $g \cdot L^{-1}$ of inoculum were added to a 1,5 L glass air-pulsed fluidized-bed bioreactor under sterile conditions, which is described in section 3.5.1. After 6 days, the medium was withdrawn from the reactor and the pellets removed from the bioreactor.

In both cases (Erlenmeyer flasks and fluidized-bed bioreactor), when the pellets were grown, the complex wood pellets were manually separated from the simple pellets that were also produced.

3.4 Wastewater effluents

3.4.1 Hospital wastewater effluent and coagulation-flocculation pretreatment

Hospital wastewater (HWW) was collected directly from the sewer manifold of Sant Joan de Déu Hospital (Cornellá de Llobregat, Catalonia). Hospital wastewater was freshly

collected prior to every experiment. The HWW was coagulated and flocculated, and then freezed until use.

The coagulation-flocculation pretreatment was carried out in a jar-test apparatus (Flocculator from Stuart Scientific, Staffordshire, UK). This pretreatment involved the addition of the coagulant Hyfloc AC50 and the flocculant Himoloc DR3000. The coagulant and the flocculant concentrations were modified depending on the characteristics of HWW batch in order to achieve an absorbance at λ_{600} of 0 ± 0.1, although in general, coagulant was added at a range of 37-150 $mg \cdot L^{-1}$, whereas the flocculant concentration was 4.5-15 $mg \cdot L^{-1}$. The jar test involved 2 min of coagulation at 200 rpm, 15 min of flocculation at 20 rpm and 30 min of settling.

3.4.2 Industrial humic rich-wastewater

Industrial wastewater was collected from the effluent of a wastewater treatment plant of a food-processing company (Eindhoven, The Netherlands) and stored at 4°C until use.

3.4.3 Wastewater with pesticides

The wastewater was collected from the Llobregat River Basin located in the northeast of Catalonia (Spain). The first sampling campaign was performed during February 2017 and water was collected from 14 selected sites. Based on the results obtained in this first sampling campaign, one point (irrigation channel in Gavà – Figure 3.2) was chosen for the water collection to use in the bioreactor experiments. The wastewater was collected on September 2017, fractionated in 500 mL amber PET bottles and stored at -20°C. During the experiment, every day one bottle was thawed to fill the influent tank.

Figure 3.2. Irrigation channel in Gavà, Barcelona.
Sample point for the fungal bioreactor experiments.

3.5 Fungal reactors

3.5.1 Fluidized-bed bioreactor

A glass fluidized-bed bioreactor with a useful volume of 1.5 L was employed (Figure 3.3). The central body is a cylindrical vertical column in which fluidization occurs. The air is introduced at the bottom by crossing a porous plate, generating small bubbles rising though the liquid phase (Blánquez, 2005).

The head or top of the reactor has a wither diametric than the central body, reducing the liquid up-flow velocity and achieving good separation of solid/liquid/gas phase. The head has several ports that are used for the pH probe, air exit system, scum collection system, nutrient feeding, acid and base entry and sampling (Blánquez, 2005)..

The homogenization in the bioreactor is achieved by entering air in pulses. The pulsation flow is generated by a pneumatic transmission disturbance in the air pulse form to the culture broth contained in the bioreactor (1 second air pulse every 4 seconds). The aeration rate was 0.8 $L \cdot min^{-1}$. The pH was controlled to be constant at 4.5 adding HCl 1 M or NaOH 1 M. The temperature was maintained at 25°C (Blánquez, 2005)..

For continuous treatment of the different wastewaters, the reactor was filled with 1.5 L of the wastewater and the fungal biomass. Immediately, the inlet and outlet pumps were turned on. The hydraulic retention time (HRT) was fixed at 3 days.

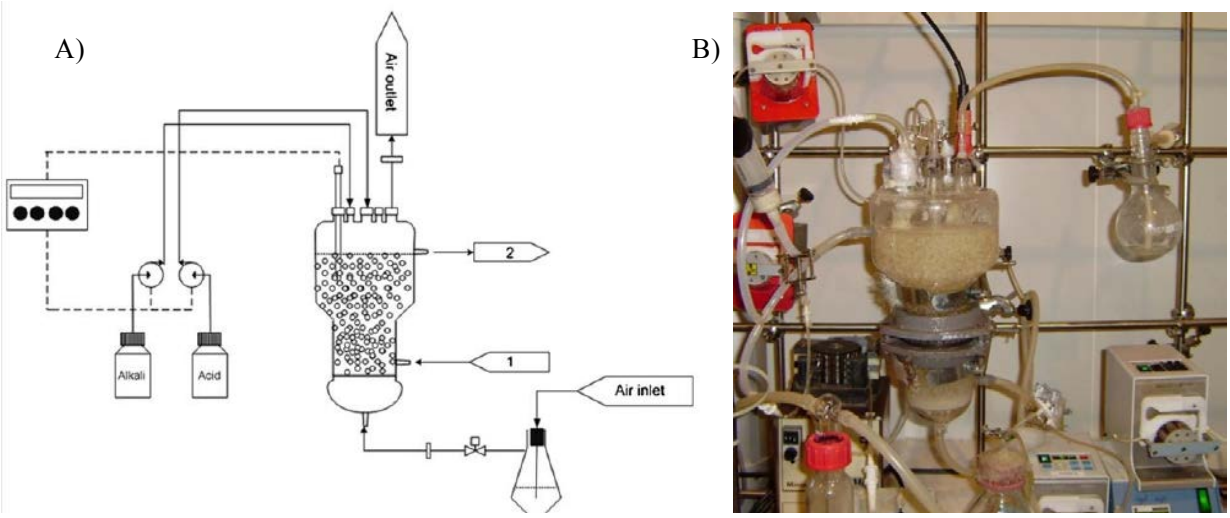


Figure 3.3. Air-pulsed fluidized-bed bioreactor. (A) Schematic diagram. 1: inlet. 2: outlet (Blánquez et al., 2006). (B) Reactor in operation.

3.5.2 Trickle-bed bioreactor

The trickle-bed bioreactor system used in this thesis is formed by a reservoir bottle, an inlet pump, an outlet pump, a recirculation pump, a pH probe connected to a pH controller, and a fixed-bed (Figure 3.4). Humidified air was introduced at the top of the reactor. The pH was controlled in the reservoir bottle at 4.5 by adding 1 M HCl or 1 M NaOH. The temperature was maintained at 25°C. No nutrients were added to the reactor. A magnetic stirrer was used to mix the reservoir bottle. The reactor was provided with an external bottom-to-top recirculation loop.

The fixed-bed contains the wood inoculated with the fungus; hence the water is in direct contact with the fungus when it is recirculated throw the fixed-bed. The fixed-bed is not completely filled with water; it is maintained always under aerobic conditions. Finally, it must be taken into account that the compounds degradation could be carried out in the fixed-bed when intracellular enzymes are involved and also in the reservoir bottle when extracellular enzymes act. In summary, the trickle-bed bioreactor includes the fixed-bed and the reservoir bottle which acts as a stirred-tank bioreactor.

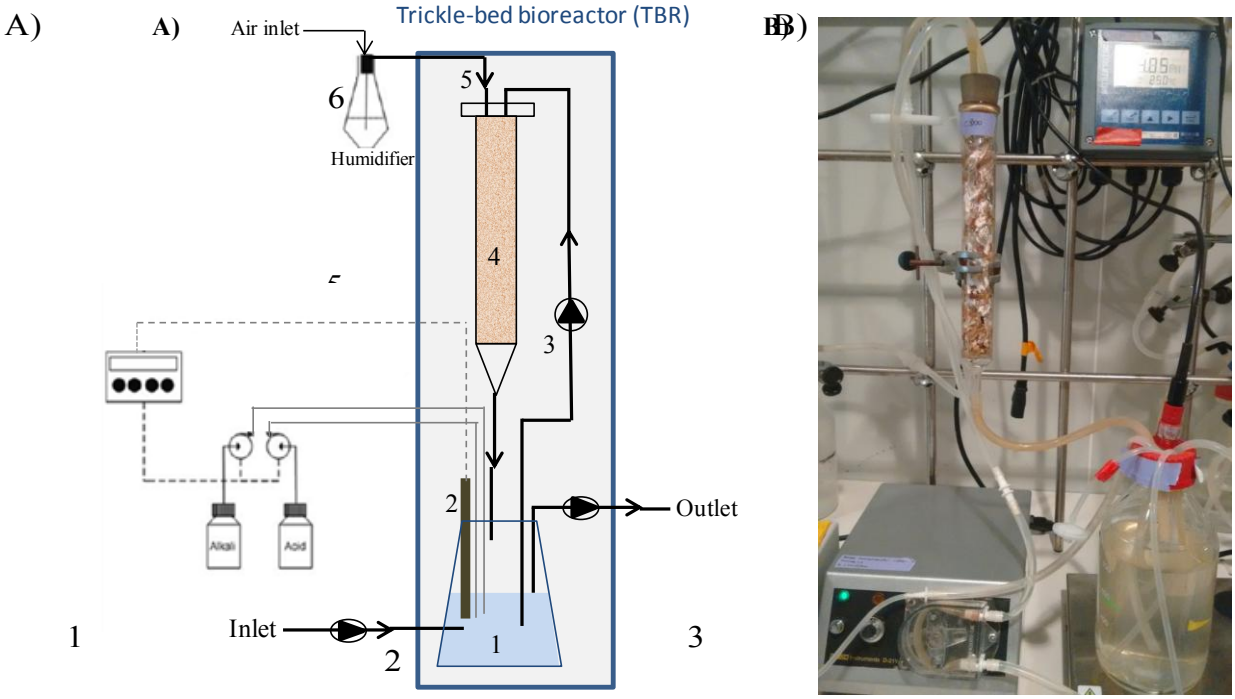


Figure 3.4. Trickle-bed bioreactor. (A) Schematic diagram. 1: reservoir bottle. 2: pH controller. 3: recirculation pump. 4: fixed-bed. 5: air supply. (B) Reactor in operation.

The fixed-bed was filled with *T. versicolor* culture growth on wood during 9 days. In order to adapt the biomass to the new environment, tap water (pH 4.5) was continuously recirculated to ensure the humidity conditions. After 5 days of adaptation, the continuous process was started (Figure 3.5).

For the continuous process, first of all the reservoir bottle was filled with the wastewater up to the total volume; which is used for HRT calculation. Then, the recirculation pump, influent pump and effluent pump were turned on at the same time for the continuous process.

Figure 3.5. Process diagram of the wastewater treatment in trickle-bed bioreactor

Different trickle-bed bioreactors (TBR) were used along this thesis depending on the experiments and the total volume. Table 3.3 shows the TBR and fixed-bed details. The fixed-bed used in the TBR 1 was acquired from ALCO S.A. (Barcelona, Spain). At its bottom part a welded glass disc with 3 mm holes was placed to retain the wood and to avoid clogging problems in the reactor. The fixed beds used in the TBR 2 and the TBR 3 were built by the author and a mesh was used to retain the wood. The wood sizes employed for the culture of *T. versicolor* were different depending on the fixed-bed size.

Table 3.3. Trickle-bed bioreactor (TBR) details.

Code	Fixed-bed details			Trickle-bed bioreactor details		
	Material	Diameter (cm)	Height (cm)	Wood amount (g wet weight)	Wood size (cm)	Total volume (L)
TBR 1	Glass	3	23	60	2 x 1 x 0.5	0.25
TBR 2	Methacrylate	5	40	180	3 x 2 x 1	0.6
TBR 3	Methacrylate	8	50	600	3 x 2 x 1	2.5

3.5.3 Packed-bed channel bioreactor

A horizontal channel was constructed using a polyvinylchloride gutter. The channel body contained the wood (3 x 2 x 1 cm) inoculated with *T. versicolor* or non inoculated and the wastewater (Figure 3.6). Its internal dimensions were 100 cm length and 5.5 cm radius. The nominal volume of the reactor was 4 L and the liquid volume of treatment 1 L, which was used to calculate the HRT fixed at 3 days in all the experiments. A scheme of the packed-bed channel reactor is shown in Figure 3.7.

The liquid feed entrance is located in one edge of the packed-bed channel and the outlet is in the opposite end. A pump was used for the feeding and the outlet. A magnetic stirrer was used to mix the influent. Samples were taken from the effluent tank. The pH was monitored manually at different points of the channel, influent and effluent. No nutrients were added to the reactor.

Figure 3.6. Process diagram of the wastewater treatment in packed-bed channel bioreactor.

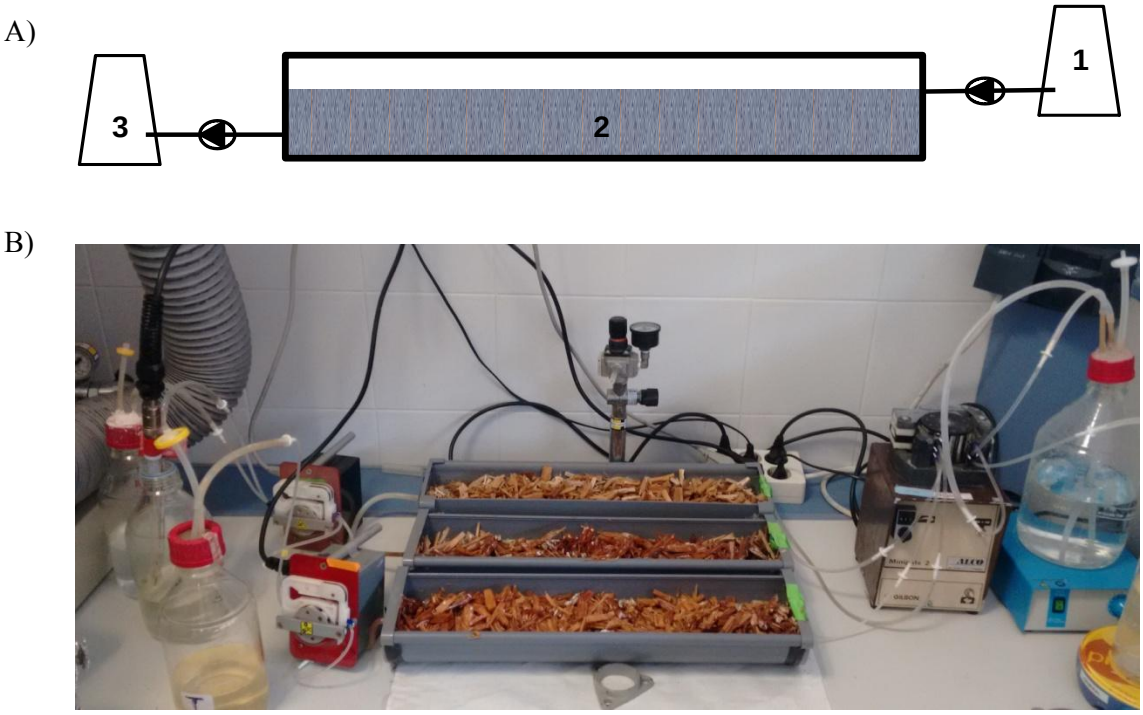


Figure 3.7. Packed-bed channelsbioreactor. (A) Schematic diagram. 1: influent tank. 2: bioreactor. 3: effluent tank. (B) Reactors in operation.

3.6 Degradation experiments

3.6.1 Hospital wastewater treatment

Some experiments were run with spiked PhACs, in order to allow daily analysis in our laboratory and to obtain immediate results, which could let to make decisions about the operation of the reactor. Table 3.4 summarizes the experiments performed for the hospital wastewater treatment.

The matrix was spiked with ibuprofen, ketoprofen and naproxen at a final concentration of 10 $mg \cdot L^{-1}$ each one. Taking into account that PhACs are found in concentrations in the order of $\mu g.L^{-1}$ in HWW (Verlicchi et al., 2012), a higher concentration is used in this work for analytical purposes as mentioned before. These compounds were selected due to the well known ability of *T. versicolor* to degrade them involving different metabolic pathways (Marco-Urrea et al., 2009b, 2010a, 2010b).

For the HWW treatment, first experiments were run in a **fluidized bed bioreactor** to study the best growing conditions. *T. versicolor* was inoculated in two different ways: fungal culture growth over wood and complex wood pellets. The effects of different wood sizes and times of incubation were tested. Two types of water were used spiked with PhACs: synthetic tap water and hospital wastewater (HWW). In the case of the HWW, a coagulation-flocculation pretreatment was applied in order to reduce the bacterial concentration.

The **trickle-bed bioreactor** was also tested for the removal of PhACs from HWW. The first experiments were performed to study the removal of spiked compounds (ibuprofen, ketoprofen and naproxen at 10 $mg \cdot L^{-1}$ each one) from coagulated-flocculated HWW. For that purpose, two reactors TBR 1 were run in parallel: one filled with fungal culture growth over wood and the other with non-inoculated sterile wood as control reactor.

Next, a validation experiment was performed keeping the same conditions as before, but studying the removal of the PhACs present in the coagulated-flocculated HWW at real concentrations. The TBR 1 was also employed in the validation experiment.

A third reactor (TBR 1) was set with raw HWW (without wastewater pretreatment) spiked with ibuprofen, ketoprofen and naproxen (final concentration of 10 $mg \cdot L^{-1}$ each one)

with the aim to study if it is necessary the coagulation-flocculation pretreatment in this system.

Finally, a scale-up of the system was made for the treatment of spiked PhACs from coagulated-flocculated HWW. In this case, the total volume was 2.5 L and TBR 3 was employed.

Table 3.4. Characteristics of HWW treatment experiments.

Reactor	Wastewater	Spiked?	Total volume (L)	Fungal form
Fludized-bed bioreactor	SW	Yes	1.5	Fungal culture growth over wood
	SW	Yes	1.5	Complex wood pellets formed in Erlenmeyer flasks
	SW	Yes	1.5	Complex wood pellets formed inside bioreactor
	SW	Yes	1.5	Simple pellets
	CF HWW	Yes	1.5	Complex wood pellets formed inside bioreactor
	CF HWW	Yes	1.5	Simple pellets
Trickle-bed bioreactor	CF HWW	Yes	0.25	Fungal culture growth over wood
	CF HWW	No	0.25	Fungal culture growth over wood
	Raw HWW	Yes	0.25	Fungal culture growth over wood
	CF HWW	Yes	2.5	Fungal culture growth over wood

SW=Synthetic tap water // CF HWW = coagulated-flocculated hospital wastewater

3.6.2 Humic acids degradation

3.6.2.1 Laccase *in vitro* experiments

The experiments were performed in 150 mL Erlenmeyer flasks containing 50 mL of laccase solution at 500 $AU \cdot L^{-1}$ in malonate buffer (pH 4.5). The effect of mediators was investigated by the addition of HOBt, ABTS and VA up to a final concentration of 1 mM. All flasks were spiked with 2 mL of HA stock solution. The Erlenmeyer flasks were kept in a shaker incubator for 24 hours (25^{o}C, 130 rpm) under sterile conditions. 2 mL samples at designated times were analyzed by means of size exclusion chromatography (SEC). All experiments were done in triplicate (Zahmatkesh et al., 2016; Marco-Urrea et al., 2009b).

3.6.2.2 Biodegradation of humic-rich wastewater in bioreactors

The continuous treatment of real wastewater with HA was done in the fluidized-bed bioreactor and in the trickle-bed bioreactor.

In the **fluidized-bed bioreactor** experiments, two reactors were set-up in parallel: one inoculated with *T. versicolor* pellets and the other non-inoculated as a control. Simple pellets of *T. versicolor* were produced in Erlenmeyer flasks with malt extract as previously described; 3.5 g $DW \cdot L^{-1}$ of pellets were added to the bioreactor and filled with 1.5 L of the wastewater. For the continuous treatment, the influent was fed with a HRT of 3 days. Every week, 1/3 of the biomass was replaced by fresh one, as determined by Blánquez et al. (2006). In both reactors, nutrients for maintenance (glucose as a carbon source and ammonia tartrate as a nitrogen source) were added to the reactors with a molar C/N ratio of 7.5 at a consumption rate of 1200 mg glucose· g fungal $DW^{-1} \cdot d^{-1}$.

In the **trickle-bed bioreactor** experiments, also two reactors were run in parallel: one filled with wood pre-inoculated with *T. versicolor* and the other with sterile wood as a control. No nutrients were added. The TBR 1 was used with a total volume was 0.25 L.

3.6.3 Pesticides biodegradation

3.6.3.1 Biodegradation experiments at Erlenmeyer scale

- ***Diuron and 3,4-dichloroaniline degradation at high concentration***

All experiments were carried out using *T. versicolor* pellets. Degradation experiments for diuron were performed in 500 mL Erlenmeyer flasks containing 4 or 9 g of wet pellets in 100 mL (equivalent to 1.4 g $DW \cdot L^{-1}$ and 3.2 g $DW \cdot L^{-1}$) of treatment medium depending on the experiment. Each experiment was conducted in triplicate and included uninoculated controls to evaluate abiotic losses and heat-killed controls to evaluate adsorption. Diuron or 3,4-dichloroaniline were added at a final concentration of 8 $mg \cdot L^{-1}$ and incubated under continuous orbital shaking (130 rpm) at 25 °C during 7 days.

- ***Laccase in vitro experiments***

Degradation of pesticides was performed in 150 mL Erlenmeyer flasks containing 50 mL of laccase solution at 500 $AU \cdot L^{-1}$ in malonate buffer (pH 4.5). The effect of mediators was investigated by the addition of HOBT, ABTS and VA at final concentration of 1 mM. All flasks were spiked with 10 $mg \cdot L^{-1}$ of diuron. The flasks were incubated for 3 days under

continuous orbital shaking (130 rpm) at 25°C. All experiments were done in triplicate (Marco-Urrea et al, 2009b).

- ***Cytochrome P450 inhibition experiments***

To study the involvement of cell bound enzymes, *T. versicolor* was incubated in presence of 1-aminobenzotriazole (ABT) as the cytochrome P450 inhibitor to hinder the production of cell bound enzymes. Fungal pellets (3.2 g $DW \cdot L^{-1}$) were incubated in treatment medium (100 mL Erlenmeyer flasks containing 25 mL media) for 7 days by triplicate. The medium was spiked with 8 $mg \cdot L^{-1}$ of diuron (Marco-Urrea et al, 2009b).

- ***Degradation experiment for selected pesticides***

This experiment was carried out in order to study the degradation of 8 selected pesticides from different families by *T. versicolor* pellets: diuron, simazine, triallate, cypermethrin, chlorpyrifos, malathion, dicofol and irgarol.

The experiments were performed in sets of 500 mL Erlenmeyer flasks, filled with 120 mL of treatment medium spiked with 1 mL of pesticides stock solution at a final concentration of 5 $\mu g \cdot L^{-1}$ of each pesticide. Stock solution of selected pesticides was prepared dissolved in methanol and stored at -20°C protected from light with aluminum foil. Pellets were added at a final concentration of approximately 3.3 g $DW \cdot L^{-1}$.

Apart from the experimental treatment, abiotic and killed controls were also included in the experiments. In abiotic control, only treatment medium containing the target compounds without fungus was used to assess potential physicochemical degradation. In the case of the killed control, the same amount of dead fungus, killed by autoclaving (30 min at 121ºC), was used in order to determine the removal due to sorption onto the biomass.

The treatments were done by triplicate during 14 days. The entire content of the flasks was sacrificed at each sample point. The experiments were carried out at 25ºC and 130 rpm of orbital agitation.

3.6.3.2 Pesticide biodegradation in bioreactors

For the degradation experiments in the bioreactor, the matrix was spiked with diuron at a final concentration of 10 $mg \cdot L^{-1}$. Diuron was chosen as target compound because it was the

compound most frequently detected in the sampling campaign at high concentrations. Moreover, diuron is classified as a Priority Hazardous Substance by the European Commission (Directive 2000/60/CE).

The first experiments were done in the **trickle-bed bioreactor** (TBR 1) with the aim to obtain the best operation conditions for the diuron removal from synthetic tap water; with a total volume of 0.25 L. For that purpose a Central Design Composite method was employed to select the best recycling ratio and total volume. More details can be found in section 5.3.3. Based on the results obtained, a trickle-bed bioreactor was tested for real wastewater treatment, and the pesticides removal at real concentration was studied. In this case, the experiments were done with the TBR 2 with a total volume of 0.6 L. Two reactors were set up in parallel: one with wood inoculated with *T. versicolor* and the other with wood non-inoculated as a control.

The first experiments with the **packed-bed channels** were carried out in order to study different operational conditions to maximize the diuron removal from synthetic tap water (spiked with diuron at a final concentration of 10 $mg \cdot L^{-1}$). For this purpose, four channels were operated varying the amount of inoculated wood with the fungus, adding aeration in the reactor and operating at submerged conditions in total or partial time. The best operational conditions were selected to perform a continuous long-term reactor with synthetic tap water and real wastewater, in both cases spiked with diuron (10 $mg \cdot L^{-1}$).

3.7 Analytical methods

3.7.1 Analysis of pharmaceutically active compounds

3.7.1.1 Spiked wastewater

The samples were filtered through a Millipore Millex-GV PVDF 0.22 μm membrane and placed in amber vials. The analyses were carried out using a Dionex Ultimate 3000 HPLC system equipped with a UV detector. The separation was performed with a GraceSmart RP 18 column (250mm x 4.6mm, particle size 5 um). The mobile phase consists of MilliQ water with a pH adjusted to 3.5 with methane sulfonic acid (Pump A) and acetonitrile (Pump B). The flow rate was 1.5 $mL \cdot min^{-1}$ 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. A sample volume of 20 µl was injected from Dionex autosampler and the detection was carried out at 210 nm. All determinations were performed at 30°C (Mir-Tutusaus, 2017b).

3.7.1.2 Non-spiked wastewater

Samples were filtered through 0.45 µm PVDF filters (Millipore, Barcelona, Spain) and kept in PET containers at -20ºC until PhAC analysis. These analyses were performed by the chemical analytical group of Institut Catala de Recerca de l'Aigua (ICRA), led by Dra. Sara Rodríguez and Dr. Damià Barcelò. The methodology of the analysis is included in Annexes.

3.7.2 Humic acid size exclusion chromatography (SEC)

Samples for SEC analysis were prepared by separating Humic acid (HA) and Fulvic acid (FA). Each experimental sample (2 mL) was acidified (pH <2) by adding 20 µl HCl (37%), and centrifuged (14000 rpm, 20 min). The supernatant acid was separated as FA and the precipitate was re-suspended by adding 2 mL NaOH (0.1 M) and used as HA portion of the sample. Both FA and HA portions of the samples were used in SEC analysis. The SEC was conducted using Phenomenex column (Yarra™ 3 µm SEC-2000, LC Column 300 x 7.8 mm, Ea) connected to a high-pressure liquid chromatography (HPLC) system (Dionex Ultimate 3000) to detect changes in the concentration and Molecular Weight of HA and FA molecules during the experiments. The mobile phase was 25% acetonitrile in ultra pure water supplemented with 10 mM sodium phosphate buffer (pH 7). The flow rate of the mobile phase was 1 $mL \cdot min^{-1}$ and the injection volume was 10 µl. Polystyrene sulfonate standards were used for the calibration of the column (~200 – 20000 Da). The separations were done at 30°C by means of a column oven for 16 min and the eluted substances were detected at 254 nm (Zahmatkesh et al., 2016).

3.7.3 Pesticides analysis

3.7.3.1 Diuron and 3,4-dichloroanline analysis in spiked media

The samples were filtered through a Millipore Millex-GV PVDF 0.22 µm membrane and placed in ambar vials. 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 5 um). The mobile phase consisted of a mixture of water with sulfuric acid (1 $mL \cdot L^{-1}$) and methanol (35:65, v/v) and was delivered in isocratic mode at a flow rate of 1 $mL \cdot min^{-1}$. A sample volume of 40µl was injected from the Dionex autosampler and the detection was carried out at 210 nm. All determinations were performed at 30°C.

3.7.3.2 Pesticides analysis from real wastewater

The samples from the degradation experiments were analyzed by the Water and Soil Quality Research Group from Institute of Environmental Assessment and Water Research (IDAEA-CSIC, Barcelona, Spain). The methodology of the analysis is included in Annexes.

- ***Sample preparation***

The samples were strained to separate the fungal pellets. The liquid medium obtained was centrifuged at 10000 rpm for 15 minutes. After centrifugation, 100 µL of deuterated standards pesticides solution was added to the supernatant up to a final volume of 100 mL. The deuterated standard solution was prepared at a final concentration of 0.5 $\mu g \cdot L^{-1}$ of each pesticide in ethanol. Finally, the sample was divided (50 mL) into two ambers vials and kept at -20°C until the analysis. The fungal pellets were frozen in aluminium foil and also kept at -20°C until the analysis.

3.7.4 Biomass measurements

3.7.4.1 Dry cell weight

The biomass was vacuum filtered through washed, dried, and pre-weighed Whatman GF/C glass fiber filters (Whatman, Maidstone, England), and subsequently dried at 105°C to a constant weight.

3.7.4.2 Ergosterol extraction and analysis

Ergosterol was measured in a homogeneously-mixed sample of lignocellulosic cultures employing a modified method used by Novotný et al. (1999). The lignocellulosic cultures were completely triturated; 0.5 g of sample were placed in a test tube and extracted with 1

mL cyclohexane and 3 mL KOH-methanol mixture (10% w/v) for 90 min at 70°C. Ultrasonication was applied for the first 15 min (Selecta, Spain); then 1 mL distilled water and 2 mL of cyclohexane were added and the tube was vortexes for 30 seconds and centrifuged at 3500 rpm during 5 minutes. The organic phase was recovered and the aqueous phase was washed twice with 2 mL cyclohexane. The organic phases were pooled and evaporated to dryness under N_2. The dry ergosterol was dissolved in 1 mL methanol at 40°C for 15 minutes, vortexes for 30 seconds and centrifuged in eppendorf vials at 6000 rpm for 3 minutes. Finally, the solution was transferred to amber vials and analyzed in a Dionex 3000 Ultimate HPLC equipped with a UV detector at 282 nm (reverse phase Grace Smart RP18 column, 250 mm x 4 mm, particles size 5 um). Methanol was isocratically supplied at 1 $mL \cdot min^{-1}$. The retention time was 7.9 minutes. The ergosterol content was expressed in milligrams per gram of solid dry weight ($mg \cdot g \cdot DW^{-1}$) (Borràs, 2010).

- **Determination of biomass content from ergosterol content**

In order to calculate the fungal biomass from the ergosterol determination, it was used a calibration curve reported by Rodríguez-Rodríguez et al. (2010b). They presented the correlation between the ergosterol and the pelletized biomass indicating that ergosterol content of *T. versicolor* ATCC 42530 corresponds to 6.61 $mg \cdot g\ DW^{-1}$ of fungal biomass.

3.7.5 Other analysis

- ***Glucose***

The glucose concentration was measured with an enzymatic glucose analyzer YSI model 2700 (Yellow Spring Instruments & Co., USA). The allowable range of glucose concentration is 0 to 20 $g \cdot L^{-1}$. The analysis is based on the glucose enzymatic oxidation to peroxide using glucose oxidize immobilized on the membrane and the subsequent reduction of peroxide on a platinum anode. Samples were filtered with filters of 0.45 μm nylon syringe filters (Millex Millipore, Barcelona) before the analysis.

- ***Laccase***

The laccase activity was analyzed by the activity test defined by Wariishi et al., (1992) and modified by Kaal et al., (1993). The samples were filtered before the analysis with filters of 0.45 μm nylon syringe filters (Millex Millipore, Barcelona, Catalonia). The

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). The changes in the absorbance at 468 nm were monitored for 2 min on a Varian Cary 3 UV/Vis spectrophotometer at 30ºC. Activity units per liter ($AU \cdot L^{-1}$) are defined as the micromoles per liter of DMP oxidized per minute. The molar extinction coefficient of DMP was 24.8 $mM^{-1} \cdot cm^{-1}$ (Wariishi et al., 1992).

- ***Color***

In experiments with humic acids, the color was monitored by measuring the light absorbance at 450 nm with UNICAM 8625 UV/VIS spectrometer.

- ***Water characterization***

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 (1995), The N-NH4 concentration and chemical oxygen demand (COD) were analyzed by using commercial kits LCH303 and LCK114 or LCK314m respectively (Hach Lange, Germany).

Total suspended solids (TSS) were measured according to Standard Methods (APHA 1995). Chloride, sulfate, nitrate and phosphate anions were quantified by a Dionex ICS-2000 Ion Chromatograph (Dionex Coporation, Synnyvale, USA) equipped with Dionex IonPac AS18-HC column (250 mm x 4 mm) eluted at 1 $mL \cdot min^{-1}$ with a 13 mM KOH aqueous solution.

- ***Fiber content of lignocelulosic substrates***

These analyses were performed by the Chemical Analysis Service of the Universitat Autónoma de Barcelona (Bellatera, Spain). The methodology of the analysis is included in Annexes.

3.7.6 Holding capacity determination

Field capacity determination of different lignocellulosic substrates was performed in a cylinder especially prepared for this purpose. The cylinder was deposited in a tray with water, which covered the height of the substrate without exceeding it for 2 hours. After that,

it was left on a filter paper for 30 minutes to remove gravimetric water (wet weight) and then was dried at 105°C for 24 hours (dry weight). Field capacity of substrates was calculated as the difference between wet and final dry weight. It was expressed as g H2O· g DW^{-1}. Each sample was performed by triplicate (Borràs et al., 2011).

3.7.7 Toxicity tests

A Microtox bioluminiscence assay was used to perform acute toxicity test. This method relies on the decrease in the percentage of emitted light by the biolumniscinet bacterium *Vibrio fischeri* upon contact with a filtered sample at pH 7. Bioluminescent bacteria *V. fisheri* and test reagents for Microtox analyses were supplied by Strategic Diagnostics Inc. (Newark, DE, USA). The 50% effective concentration (EC50) was measured after 15 min of exposure. Effluent toxicity was expressed in toxicity units (TU), calculated as TU=100/EC50. An effluent was considered toxic when its TU was over 25 as it is set by local sewage disposal regulation (Generalitat de Catalunya, 2003).

3.7.8 Statistical analysis

For pharmaceuticals and pesticides removal calculations, those compounds detected below the limit of detection (BLD) and below the limit of quantification (BLQ) were considered to have a concentration of half the limit detection and half the limit of quantification, respectively (EPA, 2000). One-factor analysis of variance (ANOVA) for experimental data was done with Sigmaplot 14.0.

CHAPTER 4

Pharmaceutically Active Compounds

Hospital wastewater is a major source of pharmaceutically active compounds (PhACs), which are not all removed in conventional wastewater treatment plants. White rot fungi can degrade PhACs, but their application has been limited to non-sterile conditions due to the competition with other microorganisms for growth. In this chapter, immobilization of *Trametes versicolor* on different lignocellulosic supports was studied as strategy to ensure fungal survival under continuous treatment conditions. A fluidized-bed bioreactor and a trickle-bed reactor with *T. versicolor* immobilized on pallet wood were employed for the removal PhACs. Promising results were obtained with the trickle-bed bioreactor during 85 days treating hospital wastewater.

4.1 Introduction

Pharmaceutically active compounds (PhACs) are increasingly detected in the surface of different environmental water compartments (Benotti et al., 2009; Watkinson et al., 2009), as not all are removed in conventional wastewater treatment plants (Joss et al., 2008).

Many studies have reported the ability of white-rot fungi (WRF) to degrade PhACs due to their unspecific oxidative enzyme system (Marco-Urrea et al., 2009b; Prieto et al., 2011). It has been proved the ability of WRF to degrade analgesics (Eibes et al., 2011; Marco-Urrea et al., 2009b, 2010a, 2010b), antibiotics (Accinelli et al., 2010; Rodarte-Morales et al., 2011), psychiatric drugs (Hata et al., 2010; Li et al, 2015a, 2015b) and lipid regulators (Tran and Urase Kusakabe 2010).

The key issue, in practice, for applying the white-rot technology is to design and establish a suitable reactor. Different configurations such as a stirred-tank reactor (Rodarte-Morales et al., 2012a), airlift (Zhou et al., 2006), fluidized-bed bioreactor (Badia-Fabregat et al., 2015b; Cruz-Morató et al., 2014; Mir-Tutusaus et al., 2016), trickle-bed bioreactor (Ehlers and Rose, 2005), packed-bed bioreactor (Li et al., 2015a, 2015b) and membrane bioreactor (Nguyen et al., 2013) have been used for wastewater treatment.

T. versicolor has also been proven to degrade PhACs in real wastewater (Badia-Fabregat et al., 2015a, 2016; Cruz-Morató et al., 2013a, 2014; Zhang and Geißen, 2012). A significant decline in the removal performance of the continuously operated bioreactor under non-sterile conditions, especially with a real matrix, commonly occurs. This performance deterioration is generally caused by the overgrowth of bacteria, which impose an inhibition on fungal growth and enzyme production (Yang et al., 2013).

Many authors have studied some strategies to foster fungal growth such as using nitrogen-limiting conditions, maintaining an acidic pH, using immobilized or encapsulated mycelium, pretreating wastewater and employing selective carbon sources (Gao et al., 2008; Libra et al., 2003; Mir-Tutusaus et al., 2016). Regarding the wastewater pretreatment, Mir-Tutusaus et al. (2016, 2017a) used a coagulation-flocculation method to reduce the bacterial concentration in a hospital wastewater from 10^7-10^8 to 10^3-10^5 CFU·mL^{-1}, allowing then a longer-term operation of a *T. versicolor* fluidized-bed bioreactor with high PhACs removals.

The use of a selective carbon source, such as a lignocellulosic material, allows the fungi to outcompete bacteria. Taking into account that in hospital wastewater treatment by fungal bioreactor usually is necessary to add other carbon sources in order to maintain the fungus viability due to the low concentration of PhACs, irrespective if the degradation is co-metabolic or not. Easily biodegradable carbon sources (glucose) commonly used fostered bacterial growth, while the use of lignocellulosic substrate is a strategy to limit bacterial growth in the reactor.

Lignocellulosic materials have already been proven to successfully sustain fungal biodegradation (Ehlers and Rose, 2005; Rodríguez-Rodríguez et al., 2010b). Therefore, this strategy to prevent bacterial contamination in real wastewater treatment is studied in this thesis.

Moreover, the use of lignocellulosic material could bring other advantages: they are readily available, economical (Saba et al., 2016) and represent a physical support for immobilized cultures, which are more resilient to environmental perturbations and exposure to toxic chemical concentrations than suspension cultures (Rodríguez-Couto et al., 2004; Shin et al., 2002).

The objective of this chapter was to developed a white-rot fungus reactor and determine the best operational conditions for the continuous treatment of real hospital wastewater. To solve bacterial overgrowth, different strategies are studied and combined in this chapter: fungal immobilization, wastewater pretreatment and different types of reactors. With the best reactor´s configuration it is expected to create the conditions to ensure the viability and survival of *T. versicolor* during wastewater treatment, maintaining good levels of PhAC removal.

In first experiments, a substrate screening was performed in order to select the best lignocellulosic material for fungal growth. The selected one was employed in the following experiments.

Immobilization studies were performed in a fluidized-bed bioreactor. Different growth strategies were studied to obtain the best conditions for the fungal treatment. With the best one, two reactors were run in parallel for the removal of ibuprofen, ketoprofen and naproxen from synthetic tap water and real HWW.

A trickle-bed bioreactor was proved for the removal of selected PhACs. First experiments were done with spiked HWW and later on, a validation experiment was run for the treatment of PhACs at real concentration. Also, the scale-up of the system was performed in this chapter.

As mentioned in chapter 3, some experiments were done with spiked compounds at high concentration for analytical purpose and daily monitoring in our laboratory. The immediate information allows us to take decision about the reactor´s operation. Only, in the validation experiment working with PhACs at real concentration, the analysis was made by the chemical analytical group of Institut Catala de Recerca de l'Aigua (ICRA) at the end of the experiment.

4.2 Results

4.2.1 Colonization of substrates by *T. versicolor*: screening

An initial colonization screening of *T. versicolor* was performed in order to identify the most suitable substrate for fungal growth using the visual observation and ergosterol content as an indicator of active fungus (Bååth, 2001; Barajas-Aceves et al., 2002).

Ergosterol is a sterol found in cell membranes of fungi and microalgae; an advantage of using ergosterol is that it indicates only viable biomass since it is quickly degraded after cell death (Gutarowska and Zakowska, 2009).

The materials tested were: hazelnut shell, pallet wood, nutshell and pine bark. Field capacity and ergosterol content are presented in Table 4.1. Growth on pallet wood and pine bark yielded high amounts of biomass, with maximum values of 0.028 and 0.031 $mg \cdot g^{-1}$ DW after 9 days. According to Planinić et al. (2016), *T. versicolor* grows until the ninth day, whereupon it reached a stationary phase. Otherwise, 0.011 and 0.016 mg of ergosterol per g^{-1} DW were obtained with growth on hazelnut shell and nutshell, respectively. Figure 4.1 shows the colonization of *T. veriscolor* over the different lignocellulosic material studied.

Ergosterol values ranging from 0.025 to 0.05 $mg \cdot g^{-1}$ DW have been reported for *Trametes versicolor* grown on wheat straw and maize stalks, while bigger values were

obtained with agricultural wastes that were processed for animal feeding (Borràs et al, 2011).

Table 4.1. Screening of lignocellulosic substrates by *T. versicolor*. Field capacity and colonization after 9 d weight.

	Ergosterol ($mg \cdot gDW^{-1}$)	**Field capacity** ($gH_2O \cdot gDW^{-1}$)
Hazelnut shell	$0.012 \pm 7.4 \cdot 10^{-4}$	0.576 ± 0.02
Pallet wood	$0.028 \pm 8.3 \cdot 10^{-4}$	1075 ± 0.05
Nutshell	$0.016 \pm 5.8 \cdot 10^{-4}$	0.331 ± 0.02
Pine bark	$0.032 \pm 1.6 \cdot 10^{-3}$	2.112 ± 0.04

Pallet wood and pine bark yielded the highest concentrations of ergosterol, so a fluidized-bed bioreactor was loaded with the substrates and tap water to determine the best conditions of fluidization (data not shown). In the reactor with the pine bark, the water was totally brown after two days, probably due to the extractives (Ramos et al., 2013); this phenomenon has been a problem for PhAC analysis. Consequently, the pine bark was discarded because its use increased the colour of the water; using this water would require the application of another type of treatment to remove the added colour. Based on these results, pallet wood was chosen for the next experiments along this thesis work.

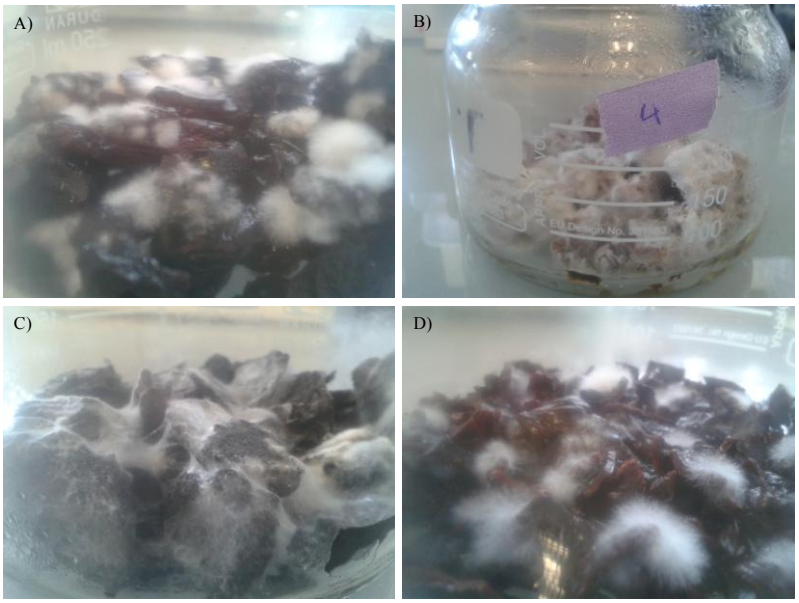


Figure 4.1. Colonization of lignocellulosic substrates by *T. versicolor* after 9 days: (A) pine bark, (B) pallet wood, (C) hazelnut shell and (D) and nutshell.

4.2.2 Immobilization studies of *T. versicolor* in Fluidized-bed bioreactor

A fluidized-bed bioreactor was used for immobilization studies of *T. versicolor* on pallet wood. Two ways of immobilization were proved: fungal culture growth over wood or the forming complex wood pellets. The immobilization was tested in a continuous treatment for the removal of selected PhACs from synthetic tap water and HWW.

4.2.2.1 *T. versicolor* culture growth over wood

The bioreactor was filled with synthetic tap water spiked with ibuprofen and ketoprofen (10 $mg \cdot L^{-1}$ each) and inoculated with *T. versicolor* culture growth over wood (30 g wet $weight \cdot L^{-1}$). To study the best growth conditions in immobilized cultures, growth with two sizes of wood (0.5 x 0.5 cm and 2 x 1 cm) and two incubation times (9 or 30 days) were tested. In all cases, after 6 days of treatment, the fungus detached from the wood and started to accumulate on the glass walls of the bioreactor before it achieved a stationary state (Figure 4.2). In all cases, only 0.06 $gDW \cdot L^{-1}$ of biomass was employed, it is a very small amount compared with other authors who usually employed 3 $gDW \cdot L^{-1}$ of biomass working in the same type of bioreactor (Badia-Fabregat et al., 2015a; Mir-Tutusaus et al., 2016).

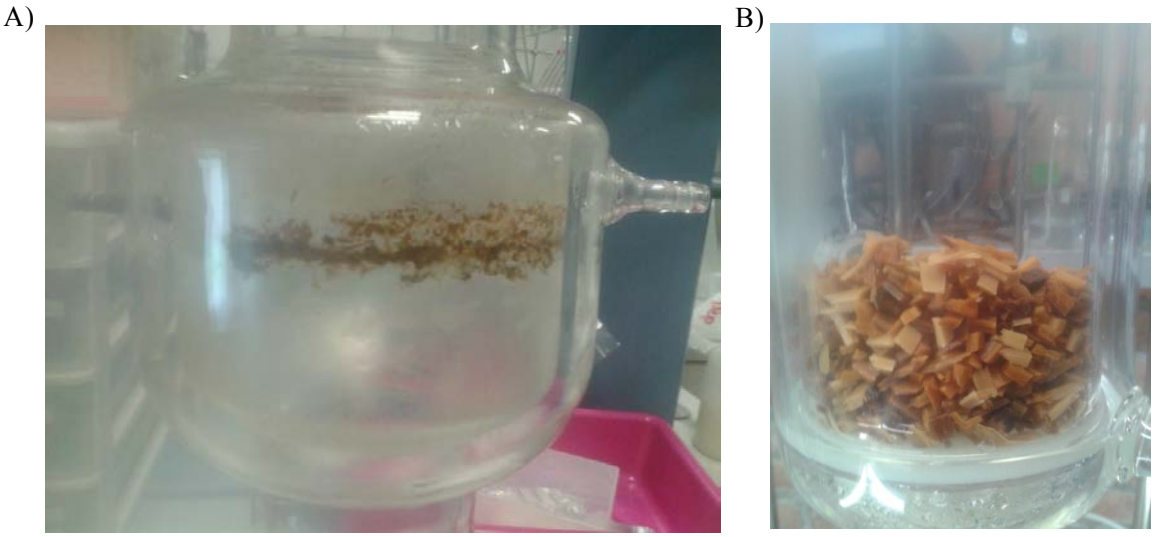


Figure 4.2. Fluidized bed-bioreactor with *T. versicolor* culture over wood after 6 days of treatment. (A) Fungus accumulate on the glass walls. (B) Wood without fungus because it was detached.

Figure 4.3 presents the results of ibuprofen and ketoprofen removal in each reactor. In the case of the reactor with 9 days culture of *T. versicolor* on wood (big size), 90% ibuprofen removal was detected and decreased to non-removal when the reactor was stopped. 50 % of ketoprofen was removed until day 5. Regarding the laccase activity, a peak was detected at day 2 and decreased to the end of the experiment.

Meanwhile, the reactor with 9 days culture of *T. versicolor* on wood (small size), stables values of ibuprofen removal (90%) were detected during all the treatment, but less than 60% of ketoprofen removal was observed. Low laccase activity was detected during the experiment (less than 2 $AU \cdot L^{-1}$).

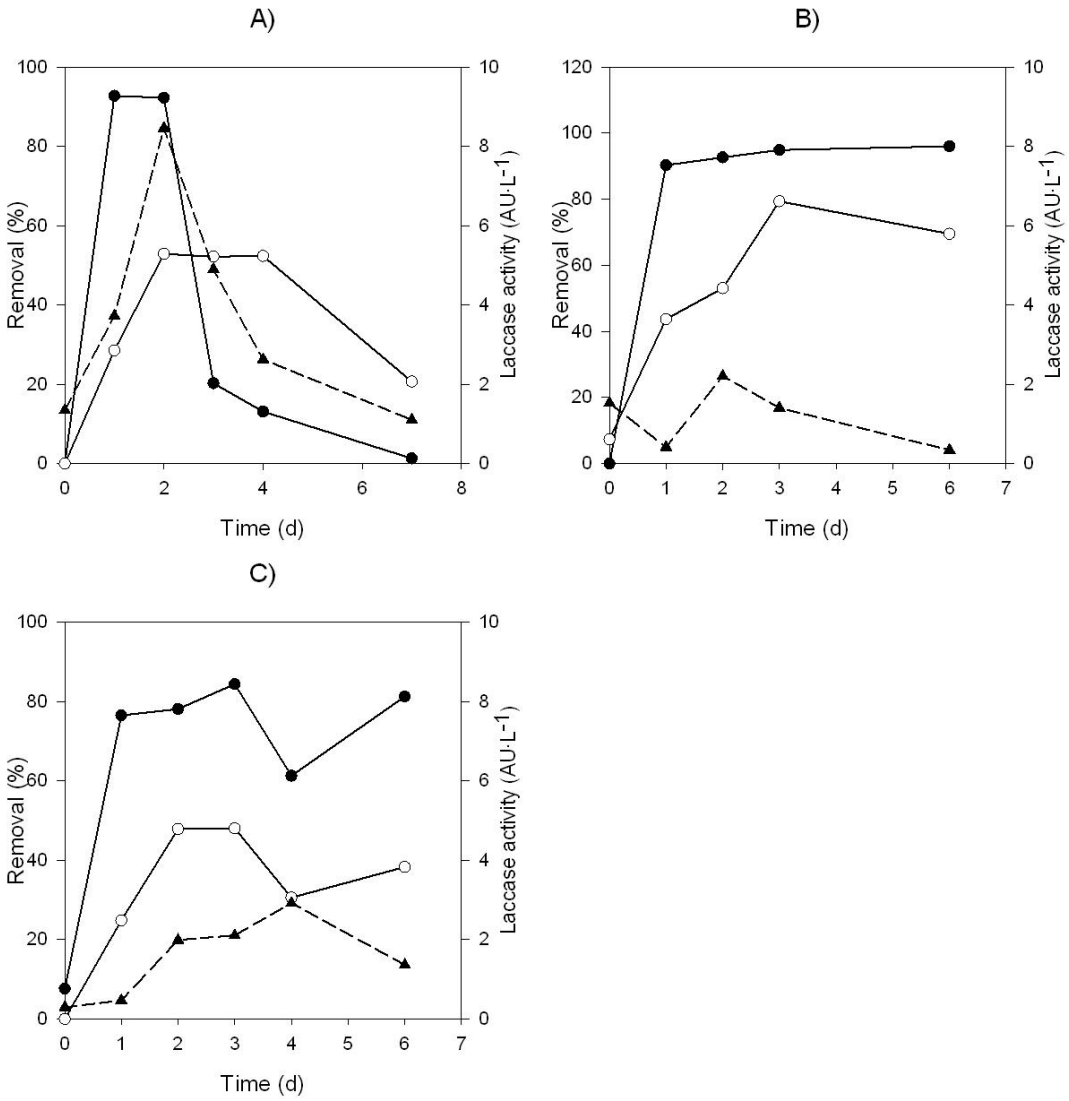


Figure 4.3. Fluidized-bed bioreactor with pre-grouth culture over wood. 9 days culture over wood with big size (A) and small size (B). 30 days culture over small size wood (C). Symbols: (●) ibuprofen, (○) ketoprofen and (▲) laccase activity.

Finally, the reactor with 30 days culture of *T. versicolor* on wood (small size), stables values of ibuprofen (80%) and ketoprofen (40%) removals were observed. In this case, also low laccase activity was detected (less than 2 $AU \cdot L^{-1}$).

No differences were observed between 9 and 30 days culturing of *T. versicolor* on wood. But, better results were obtained with the smaller size of wood, probably because it had more superficial area for sorption.

These results demonstrated that growing *T. versicolor* directly on the wood is not a good immobilization strategy for its subsequent use as inoculum in the bioreactor since it was observed that the fungus was removed from the wood, and consequently the removal of the PhACs decreased.

4.2.2.2 Complex wood pellet formation

- ***Complex wood pellet formation in Erlenmeyer flasks and reactor performance***

Taking into account that the previous results, when *T. versicolor* was directly pregrown on wood were not good, one step of complex wood pellet formation was added before continuous treatment was started in order enhance the adherence of the fungus to the wood.

Cultures of *T. versicolor* grown on wood (3 x 3 x 2 mm) during 9 days were used as inoculum. 5 g (wet weight) of inoculum were added to a 1 L Erlenmeyer flask containing 250 mL of malt extract. The pellets were obtained after 6 days of incubation. The complex wood pellets were manually separated from the simple pellets that were also produced.

The reactor was filled with 30 g (wet weight) of complex wood pellets and synthetic tap water that was spiked with ibuprofen and ketoprofen (10 $mg \cdot L^{-1}$ each). High removal of ibuprofen was obtained during all the treatments and over 80% ketoprofen removal was achieved from day 8 to the end (Figure 4.4). Laccase activity was measured in all the treatments, peaking at the beginning.

Good removal was obtained using complex wood pellets. Similar results were previously reported for complex wood pellets of sawdust and activated carbon with *Anthracophyllum discolor* for the biological treatment of wastewater contaminated with Reactive Orange 165 (Elgueta et al., 2012). In this case, approximately 1.7 $gDW \cdot L^{-1}$ of biomass was used, and if

compared with the previous studies when only 0.06 gDW·L^{-1} were employed, it should be expected to have better removal values.

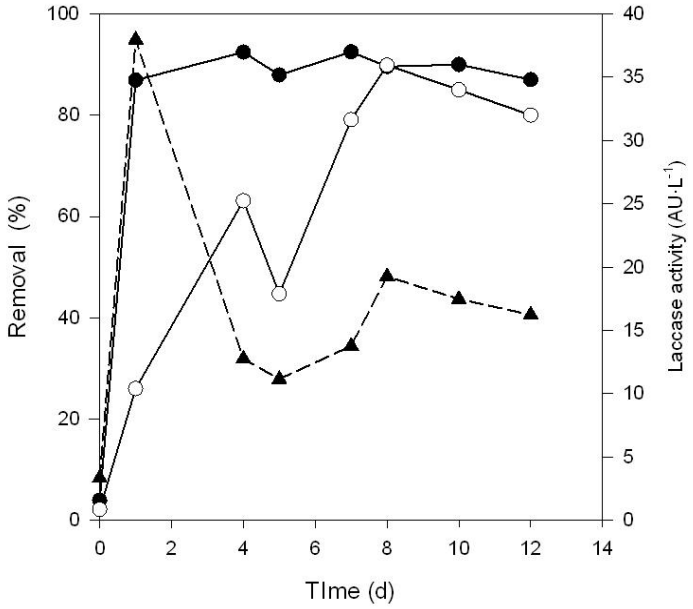


Figure 4.4. Treatment with complex wood pellets obtained in Erlenmeyer flask in fluidized-bed bioreactor with synthetic water. Symbols: (●) ibuprofen, (○) ketoprofen and (▲) laccase activity.

4.2.3 Comparison between simple pellets and complex wood pellet in a fluidized-bed bioreactor

Based on the good results obtained using complex wood pellets produced in Erlenmeyer flasks, pellets were directly produced in the bioreactor with growth medium under sterile conditions in order to make the process scalable (Borràs et al., 2008). For the pellet formation, two bioreactors were set up in parallel with growth medium: one inoculated with 9 days wood cultures (30 g wet weight·L^{-1}) (complex wood pellets), and the other was inoculated with a mycelial suspension (4 mL·L^{-1}) as a control (simple pellets). After 6 days, when the pellets were grown, the growth medium was withdrawn from both reactors. In the case of simple pellets, the pellets were maintained inside the reactor. Meanwhile, the complex wood pellets were manually separated from the simple pellets that were also produced (Figure 4.5). However, complete separation was not possible, and in these cases a mixture of complex wood pellets and simple pellets was returned to the reactor.

A)

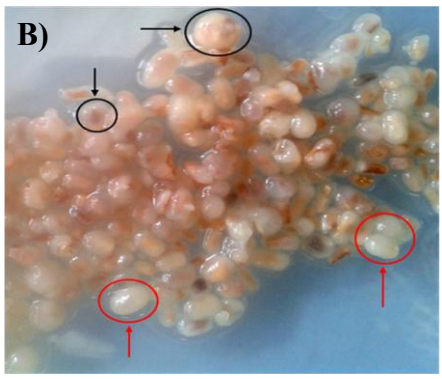


Figure 4.5. *T. versicolor* formed pellets structure. Complex wood pellets (black circles) and simple pellets (red circles).

Different experiments testing PhAC removal were carried out in parallel with simple and complex wood pellets in order to compare their reactor performance. The experiments were first done with synthetic tap water, and then coagulated-flocculated HWW was employed.

The results obtained for the experiments with synthetic tap water are presented in Figure 4.6. With complex wood pellets almost 100% of the ibuprofen was degraded, while ketoprofen degradation rates were greater than 80% for 31 days, after which they started to decrease until they reached 70% on day 41. Meanwhile, in the control reactor with simple pellets, the degradation of ibuprofen and ketoprofen were almost 100% and 90%, respectively, for only 8 days, after which they decreased until day 12. In both reactors, low laccase production was observed.

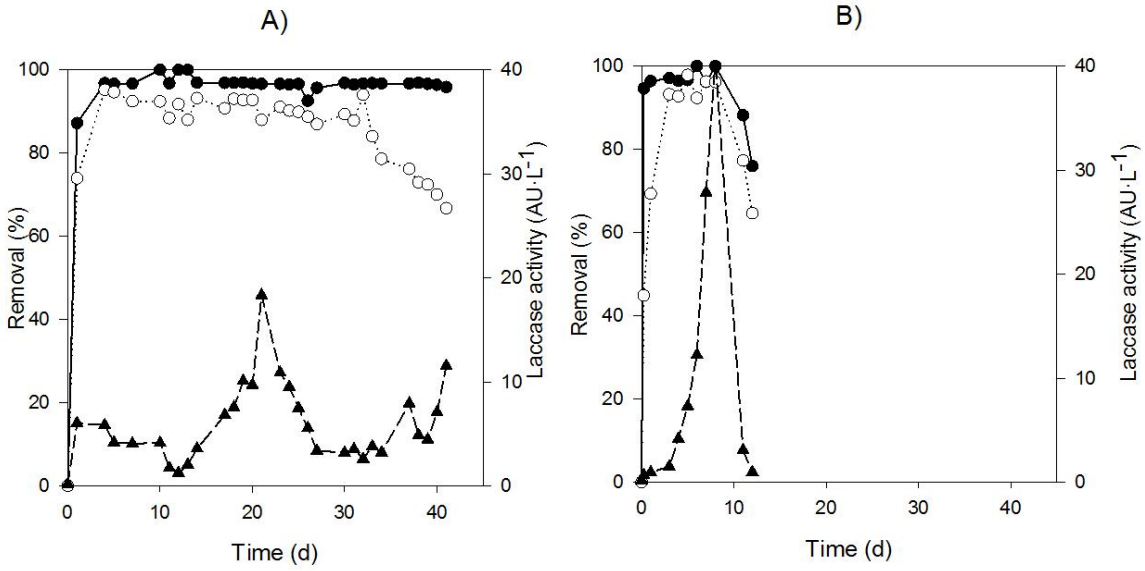


Figure 4.6. Synthetic tap water treatment in fluidized-bed bioreactor with (A) complex wood pellets and (B) simple pellets. Symbols: (●) ibuprofen, (○) ketoprofen and (▲) laccase activity.

The differences in the degradation profiles suggest that *T. versicolor* is able to use the pallet wood as the only source of nutrients (Valentín et al., 2009), and the immobilization could be a good strategy for the continuous treatment.

In the experiments with coagulated-flocculated HWW (Figure 4.7), the reactor that was inoculated with complex wood pellets operated in continuous mode for 28 days. The degradation profiles were ibuprofen, 90% for 18 days; ketoprofen, 75% for 14 days; and naproxen, 80% until day 21, at which point the degradation started to decrease. In the control reactor with simple pellets, the degradation was below 60% after 7 days, when it was stopped.

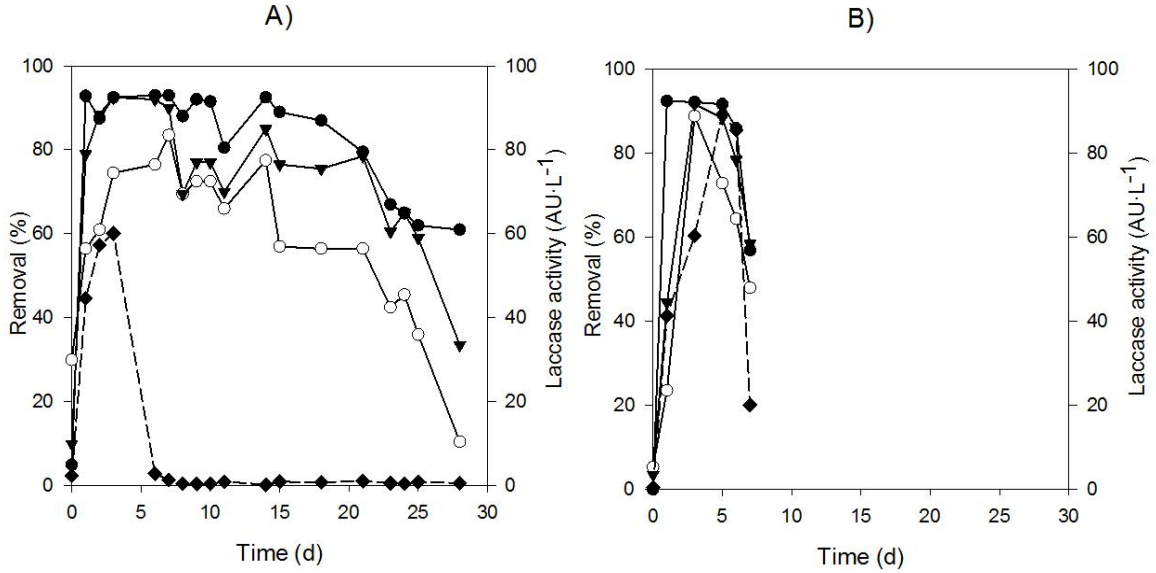


Figure 4.7. HWW treatment with complex wood pellets (A) and simple pellets (B) in fluidized-bed bioreactor. Symbols: (●) ibuprofen, (▲) naproxen, (○) ketoprofen and (♦)laccase activity.

The laccase profile, in both cases, showed a maximum peak and then started to diminish. On one hand, in the reactor with complex wood pellets, the laccase activity decreased from 60 $AU \cdot L^{-1}$ (day 3) to very low levels (less than 1 $AU \cdot L^{-1}$). On the other hand, in the reactor with simple pellets, the peak was at day 5 (89 $AU \cdot L^{-1}$) and decreased to 20 $AU \cdot L^{-1}$ at the end of the treatment (day 7). There is not a direct relationship between the laccase values and the PhAC´s removal, since the fungus can act as a multi-enzymatic reactor. In addition, when working with real wastewater, laccase detection confirms the fungus activity, on the contrary the low level of the enzyme is not an indication of fungus inactivity (Mir-Tutusaus et al., 2017a).

In both cases (with synthetic tap water and HWW), the complex wood pellets presented better removal than the simple pellets in long-term treatments. Since no external nutrients were added to the treatment, the complex wood pellets can use the wood as a carbon source, while the simple pellets lost their structure, probably due to a lack of nutrients. The lignocellulosic materials have been demonstrated as suitable substrates for the growth and survival of *T. versicolor* (Maciel et al., 2013; Walter et al., 2004).

Many studies demonstrated the advantages of using fungi immobilized on lignocellulosic substrates in different treatments (Bending et al., 2002). Elgueta et al. (2016) evaluated the atrazine dissipation in a biobed system that was inoculated with white-rot fungi that were immobilized on a pelletized support.

The results in this work indicated that pallet wood could be used as a single specific source of nutrients and support material for the formation of complex wood pellets. This kind of pellets could be used in the continuous treatment of PhACs in a fluidized–bed bioreactor for long time periods, achieving high removal for more than 40 days in synthetic tap water and 25 days in HWW. A decrease in the duration of the HWW treatment resulted, probably due to the accumulation of free mycelia in the reactor, which promotes bacterial growth. The free mycelia are derived from the complex wood pellets that lose their structure, taking into account that superficial hyphae could not easily access the carbon source (wood) that is in the centre of the pellet. Additionally, free mycelia come from simple pellets that were not separated at the beginning of the treatment. It was observed that these pellets broke and accumulated in the reactor.

The lower level of removal that was obtained in HWW compared with synthetic tap water is in according with previous studies. Zhang and Geißen (2012) operated a plate bioreactor with WRF immobilized on polyether foam for the degradation of carbamazipine and demonstrated a significant decrease in the performance when they employed a real effluent from a municipal wastewater treatment plant. Hai et al. (2009) demonstrated that the worse performance under non-sterile conditions in a decolourization reactor is caused by bacterial disruption, fungal morphology and enzyme washout in a continuous fungal reactor.

Complex wood pellets could be a good strategy for working with HWW, but the scaling up of the process is very complicated because after the formation of the pellets inside the

reactor, a manual step is needed to separate the complex wood pellets from the simple pellets that were formed at the same time. Additionally, the separation is not complete, and some simple pellets are introduced into the reactor.

4.2.4 Design of a new system: Trickle-bed bioreactor

Previous results obtained with a fluidized-bed bioreactor shows that the immobilization on the wood and pellets that were formulated with a wood core represented a good strategy to ensure the fungus survival in wastewater treatment, but the scaling up is complicated and the performance decreased in HWW. A trickle-bed bioreactor was studied as an alternative reactor configuration in examining the HWW treatment employing *T. versicolor* immobilized on wood.

The trickle-bed bioreactor system is formed by a reservoir bottle, an inlet pump, an outlet pump, a recirculation pump, a pH probe connected to a pH controller, and a fixed-bed (Figure 4.8). Humidified air was introduced at the top of the reactor. The pH was controlled in the reservoir bottle at 4.5 by adding 1 M HCl or 1 M NaOH. The temperature was maintained at 25°C. No nutrients were added to the reactor. The reactor was provided with an external bottom-to-top recirculation loop. For the continuous process, first the reservoir bottle was filled up to the total volume; which is used for HRT calculation.

The fixed-bed contains the wood inoculated with the fungus; hence the water is in direct contact with the fungus when it is recirculated throw the fixed-bed. The fixed-bed is not completely filled with water; it is maintained always under aerobic conditions. Finally, it must be taking into account that the PhACs removal could be carried out in the fixed-bed when intracellular enzymes are involved and also in the reservoir bottle when extracellular enzymes act. In summary, the trickle-bed bioreactor included the fixed-bed and the reservoir bottle.

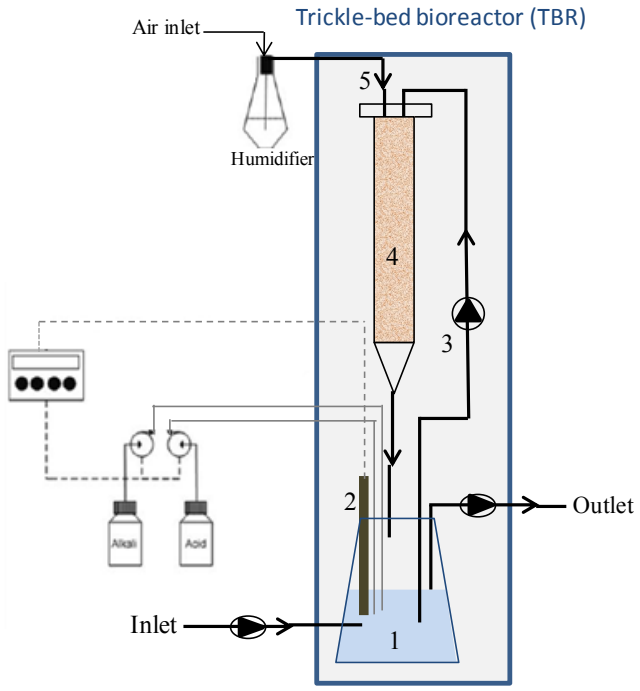


Figure 4.8. Schematic diagram of the trickle-bed bioreactor. 1: reservoir bottle. 2: pH controller. 3: recirculation pump. 4: fixed-bed. 5: air supply.

4.2.4.1 Trickle-bed bioreactor treating spiked HWW.

The reactor was packed with 60 g (wet weight) of 9-day wood culture of *T. versicolor* (size: 2 x 1 x 0.5 cm). Coagulated-flocculated HWW at pH 4.5 was spiked with naproxen, ketoprofen and ibuprofen (10 $mg \cdot L^{-1}$ each) was feed from the influent tank.

A first reactor was set up with the objective to determine an adequate recycling ratio (RR) for the continuous treatment. The RR can be defined as the ratio of the recirculated flow rate to the main influent flow rate. Four different RR (86, 173, 432 and 877) were tested for 45 days, and an increase in degradation was obtained with the highest RR (data no show). Based on these results, the recycling ratio of 877 was chosen for the next experiments. Higher RR means higher contact time between the contaminants and the fungus.

Two bioreactors were set up in parallel, one packed with 9-day wood culture and the other with sterile wood as a control. The bioreactors were continuously operated for 49 days to treat coagulated-flocculated HWW.

The results are presented in Figure 4.9. In the inoculated reactor, the following stable removal values were obtained throughout the treatment: ibuprofen 90%, ketoprofen 80% and naproxen 60%. The laccase profile was at its maximum until day 10 and then decreased to the end of the experiment, in concordance with previous studies (Ehlers and Rose, 2005). As mentioned before, the low laccase level obtained is not an indicator of the fungal inactivity.

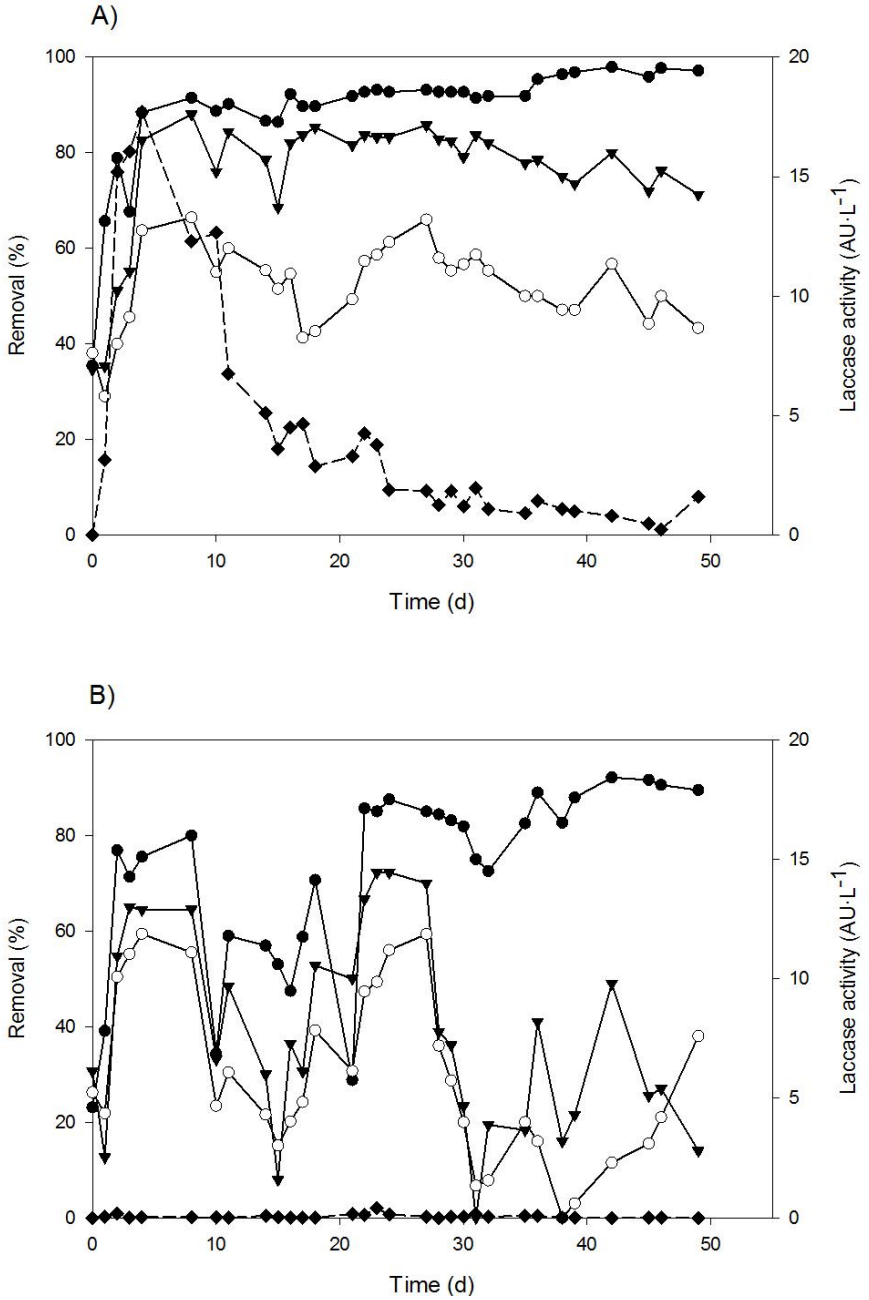


Figure 4.9. HWW treatment in trickle-bed bioreactor inoculated with *T. versicolor* (A) and non-inoculated as a control (B). Symbols: (●) ibuprofen, (▲) naproxen, (○) ketoprofen and (♦) laccase activity.

In the control reactor, until day 9, the removal value was over 50% for the three PhACs, probably due to the adsorption onto the wood. After day 9, the removal value started to decrease. In the case of ketoprofen and naproxen, the removal was very variable, and from day 30 till the end of the treatment the removal values were less than 30%. Regarding ibuprofen, high removal rates (>80%) were achieved from day 22 until the end of the treatment. As it has already been reported, ibuprofen can be easily degraded (Langenhoff et al., 2013; Marco-Urrea et al., 2009a).

The results obtained with the trickle-bed bioreactor indicate that *T. versicolor* grew well when it was attached to the wood, and the support size allowed the circulation of wastewater and air along the reactor to give suitable aeration for *T. versicolor*. The high porosity of the reactor (37.5%) avoided clogging problems and ensured good surface contact. Moreover, the wood was not visibly degraded during the whole process. Altogether, it makes this support a very suitable material for this type of processes.

In addition, the use of wood as a support and a substrate presents several advantages such as the reduction in production cost, due the wood's double function as a place of attachment and a source of nutrients. Rodríguez-Couto et al. (2003) already indicated that the use of a natural support provides the fungus an environment that is similar to its natural habitat and offers the possibility of reusing waste.

Excessive growth of bacteria was avoided by limiting the nutrient supply, carrying out a pretreatment of the HWW and controlling the pH, making possible a long-term operation. Folch et al. (2013) already reported the need to maintain pH values for fungal survival in wastewater treatment.

Böhmer et al. (2006) obtained good bioreactor performances decolourizing textile dyes with *T. versicolor* that was immobilized on pine wood chips under temporary immersion conditions. Jonstrup et al. (2012) achieved high decolorization by treating industrial textile wastewater in a trickle-bed bioreactor with a *Bjerkandera* sp. that was immobilized on wood and reported a drop in decolourization efficiency after 17–19 days of continuous operation, which was caused by reactor contamination. Additionally, Li et al. (2015a) found improved efficiencies of removing naproxen and carbamazepine when WRF was immobilized on wood chips, employing a fixed bed bioreactor for the treatment of synthetic water. Meanwhile, with real hospital wastewater, Mir-Tutusaus et al. (2017a) reported

wastewater treatment using a fluidized-bed bioreactor with coagulated-flocculated non-spiked HWW for 56 days. Therefore, with this system, we can combine the following strategies in order to promote fungus survival: coagulation-flocculation pretreatment of HWW, use of lignocellulosic material and controlling the pH value.

It is remarkable that the system in this work was able to operate in a continuous mode with high PhAC removal and without operational problems for 49 days while treating real wastewater. This result indicates the suitability of this system to treat coagulated-flocculated hospital effluents under non-sterile conditions.

- ***Physicochemical and biological parameters***

Table 4.2 shows the physicochemical parameters and biological characterization of the hospital wastewater before and after the coagulation-flocculation pretreatment and after the biological treatment. Most of the parameters of the raw HWW are in the same range as those of other hospital effluents (de Oliveira Schwaickhardt et al., 2017).

After the coagulation-flocculation pretreatment, an important reduction of the absorbance, heterotrophic plate count, TSS and COD was achieved, as previously reported Mir-Tutusaus et al. (2016). A reduction of three orders of magnitude was obtained for the bacterial concentration and almost 80% reduction of TSS.

Table 4.2. Physicochemical characterization of raw HWW, coagulated-flocculated HWW, effluent from fungal reactor and control reactor.

	Raw HWW	Flocculated HWW	Fungal reactor	Control reactor
Absorbance at 650 nm	0.3	0.009	0.019	0.015
Heterotrophic plate count ($CFU \cdot mL^{-1}$)	$5 \cdot 10^7 \pm 3 \cdot 10^7$	$3.5 \cdot 10^4 \pm 1.2 \cdot 10^4$	$6 \cdot 10^6 \pm 1 \cdot 10^6$	$5 \cdot 10^6 \pm 1 \cdot 10^6$
TSS ($mg \cdot L^{-1}$)	122	22	29.57	36.9
COD ($mg\ O_2 \cdot L^{-1}$)	178	109	351	273

Regarding the characterization of the effluent after fungal treatment and the control treatment (Table 4.2), an increase in the COD concentration was observed in both effluents, probably due to by-products or metabolites that were released during wood degradation by the fungus (Palli et al., 2016). As previously reported by Badia-Fabregat et al. (2015a), *T. versicolor* cannot remove wastewater COD, and it should be included as a pretreatment process only for contaminants removal.

On the other hand, the number of bacterial colonies in the effluent increase by two orders of magnitude compared with that of the initial coagulated-flocculated water; however, this value was lower than that of the initial raw water. In addition, the number of bacterial colonies in the fungal and control reactor remained stable at a value of lower than $1 \cdot 10^7$ during all treatment.

Additionally, the absorbance and TSS values that were obtained in fungal reactor effluent did not change after the treatment. These parameters, together with the heterotrophic plate count, allow the conclusion that bacterial growth was successfully limited by the operation strategies carried out.

The results of the Microtox analysis showed no toxicity in hospital wastewater before and after the treatment, demonstrating that the system does not increase the toxicity of the effluents. Taking into account that hospital wastewater may contain a wide variety of contaminants, with this test it was confirmed that the contaminants were not transformed in more toxics molecules.

4.2.4.2 Validation of the trickle-bed bioreactor

In previous section, a trickle-bed bioreactor with *T. versicolor* immobilized on wood was successfully operated to remove ibuprofen, ketoprofen and naproxen from coagulated-flocculated HWW during 49 days. Based on these good results, the validation of the same system was performed treating PhACs at real concentrations present in the HWW.

Two identical trickle-bed bioreactors were set-up in parallel: fungal and control reactor. The fungal reactor was filled with wood culture (*T. versicolor* pregrowth over the wood) and the control reactor with sterile wood. The reactors were continuously operated for 85 days treating coagulated-flocculated HWW. Micropollutant removal at real concentrations was studied in this system.

In Figure 4.10 are presented the images of the fungal reactor and the control reactor at the initial time (time: 0) and at the end of the treatment (day: 85). In the fungal reactor, it can be seen that the fungus remained attached over the wood. Meanwhile, in the control reactor there were some microorganisms growing over the wood.

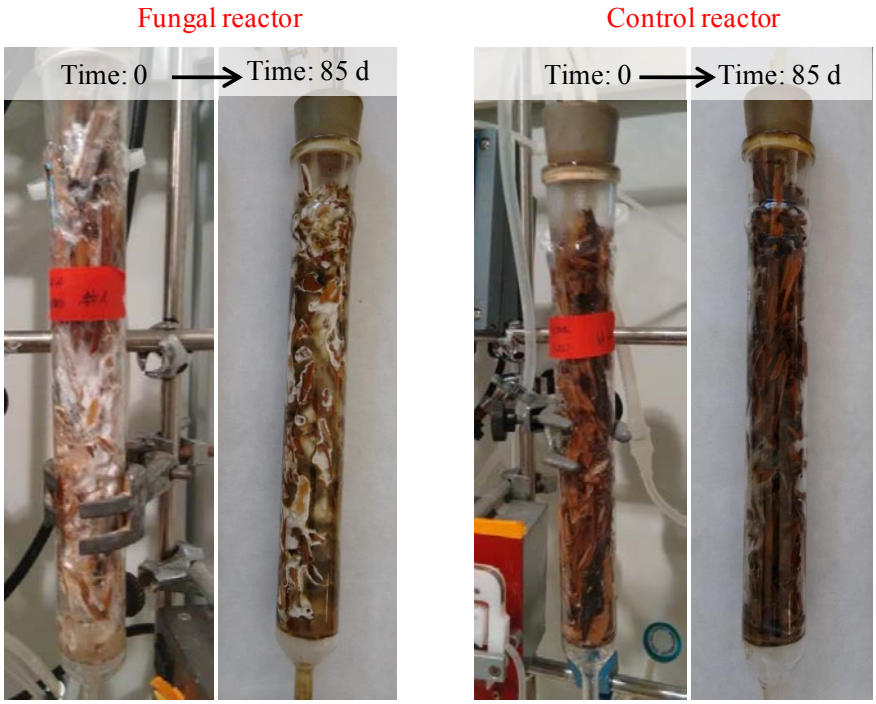


Figure 4.10. Trickle-bed bioreactors in validation experiments at the initial time and at the end of the treatment (day 85).

- ***Monitoring of the bioreactors***

In these experiments, naproxen, laccase activity, heterotrophic plate count, COD and TSS were measured during the treatment. Naproxen was spiked in the influent to a final concentration of 10 $mg \cdot L^{-1}$ in order to daily monitor the system. It was the only PhAC spiked.

In Figure 4.11 are presented the naproxen removal profiles of both reactors. Fungal reactor shows high stable removal value (78%), also higher than in the previous experiments. Since different HWW was employed in each experiment, probably the increase in naproxen removal is due to the presence of microorganisms able to remove naproxen acting in synergic way together with *T. versicolor*.

On the contrary, the control reactor presented instable and lower removal values along the treatment compared with the fungal reactor, meaning that the inoculation of *T. versicolor* is necessary to achieve a good removal.

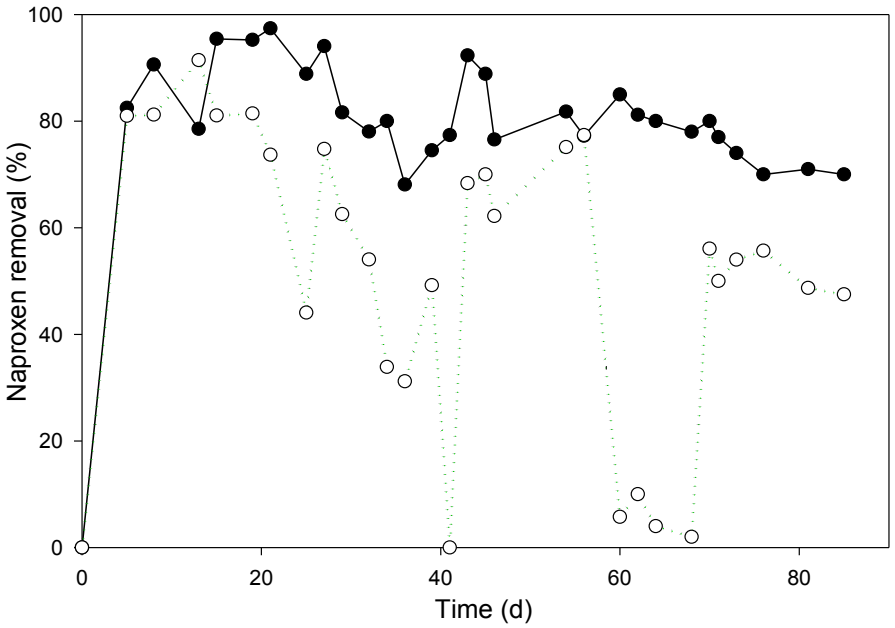


Figure 4.11. Naproxen removal during HWW treatment.
Symbols: (●) fungal and (○) control reactor.

The laccase activity profiles for the fungal and control reactors are presented in Figure 4.12A. Laccase activity was not detected in the control reactor; meanwhile in the fungal reactor, a peak of laccase activity was achieved at day 13 (19.8 $AU \cdot L^{-1}$), and started decreasing from day 29 to the end. As was reported before by other authors (Mir-Tutusaus et al., 2017a) and also in this work, the laccase production was sign of *T. versicolor* activity, but its absence was not an indication of the fungus inactivity.

Total COD is also presented in Figure 4.12B. Both reactors increased the initial COD during the treatment, but at the end of the treatment the COD values were almost the same than the initial one. On one hand, in the fungal reactor, COD increased significantly (+1000%) until day 25, which is in agreement with other authors working with *T. versicolor* (Badia-Fabregat et al., 2015a, 2015b). From day 46 to the end of the treatment, the total COD detected was very stable with a mean value of 130 $mg \cdot L^{-1}$. On the other hand, in the control reactor, COD presented two peaks at days 32 and 60, probably due to bacterial growth, as will be shown later in the TSS profile and heterotrophic plate count monitored in the reactor.

In the fungal reactor, the combination of the laccase activity and COD profiles can be used as an indicator of the fungus activity.

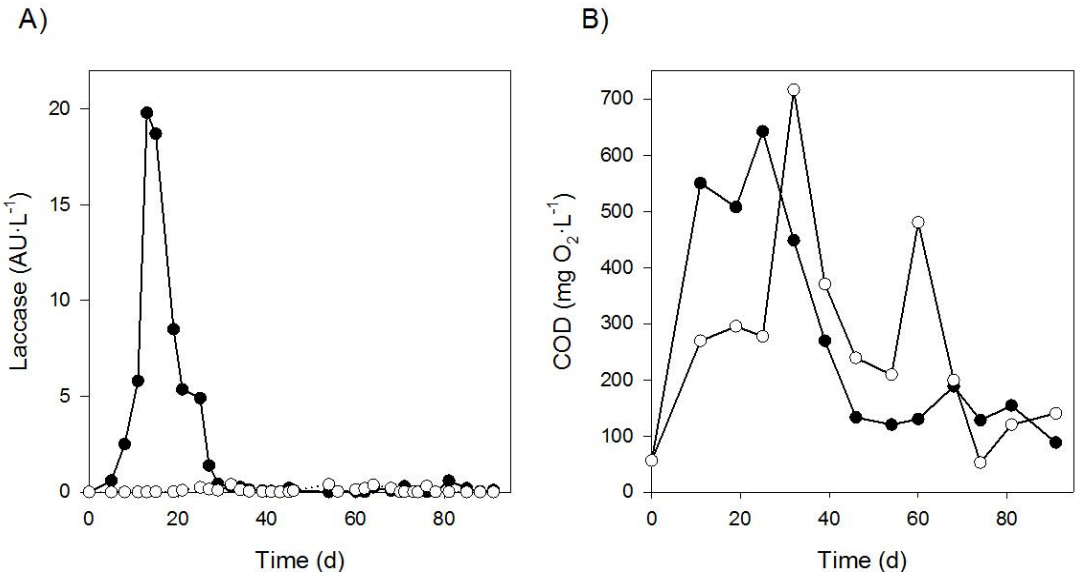


Figure 4.12. Evolution of laccase activity (A) and COD (B) in HWW treatment. Symbols: (●) fungal and (○) control reactor.

Bacterial growth usually limits the fungal activity, so it represents a key parameter for the reactor performance. In these reactors, different strategies were combined to limit the bacterial growth, being the most important the coagulation-flocculation pretreatment and the no-addition of nutrients. To study the bacterial content, COD, TSS and heterotrophic plate count were monitored in both reactors.

The profiles of TSS concentration are presented in Figure 4.13A. The fungal reactor profile was mostly constant at around 20 $mg \cdot L^{-1}$, but the control reactor did not achieve a stationary state, with two peaks of over 70 $mg \cdot L^{-1}$.

Heterotrophic plate count (HPC) for both reactors are presented in Figure 4.13B. After the coagulation-flocculation pretreatment applied to the raw wastewater, the influent water used in these reactors contained $4.5 \cdot 10^5 CFU \cdot mL^{-1}$. In fungal reactor, HPC increased by only one order of magnitude with a mean value of $3.4 \cdot 10^6$ $CFU \cdot mL^{-1}$. On the contrary, in the control reactor, bacterial content increased in three orders of magnitude by days 39 to 46, and higher bacterial concentration was observed during all the treatment. Probably due to the growth of endogenous microorganisms that had less nutrients competition. In the control reactor, the same peaks were detected for COD, TSS and heterotrophic plate count profiles.

Based on the TSS, COD and heterotrophic plate count results, it can be concluded that the bacterial growth is limited in the system inoculated with *T. versicolor*.

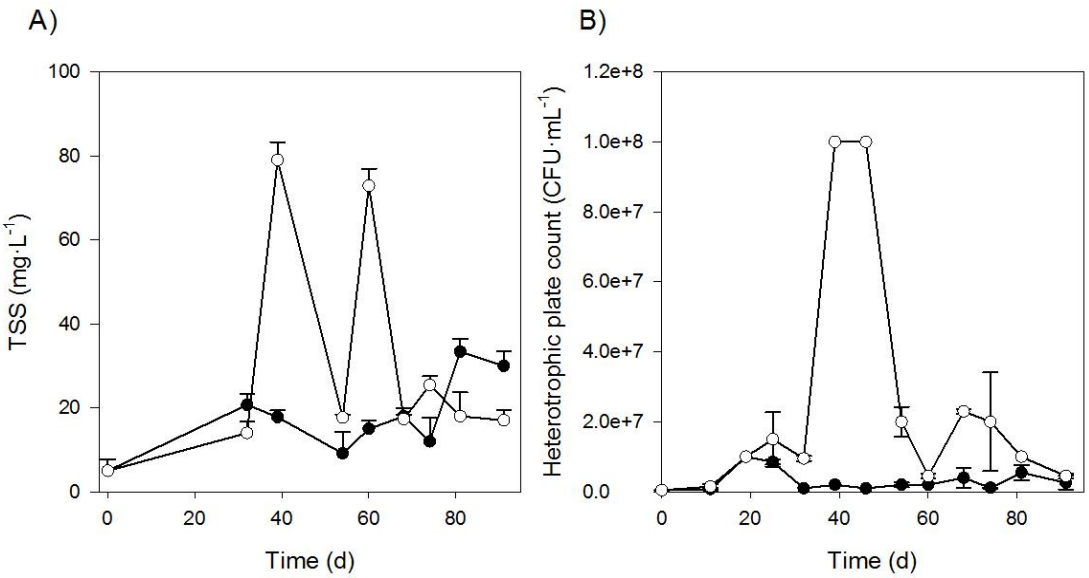


Figure 4.13. Evolution of (A) TSS and (B) heterotrophic plate count in HWW treatment. Symbols: (●) fungal and (○) control reactor.

In the same way as in previous section, the results of the Microtox analysis showed no toxicity in hospital wastewater before and after the treatment, demonstrating that the proven treatment does not increase the toxicity of the effluents.

- ***PhACs removal***

The analysis of the PhACs in raw hospital wastewater showed that 25 out of 68 analyzed compounds were detected in this effluent. The most common families detected were analgesic and anti-inflammatories, antibiotics, lipid regulators and psychiatric drugs, as the hospital has an important psychiatric pavilion. Initial concentration of individual pharmaceuticals is presented in Table 4.3.

The analgesic and anti-inflammatories families contribute the most to the final concentration, especially due to the high concentration of ibuprofen, ketoprofen and salicylic acid. Psychiatric drugs are the second group in concentration, led by carbamazepine and its metabolites (2-hydroxycarbamazepine and epoxy carbamazepine). Antibiotics family was the third with more concentration in the HWW, with a high amount of azithromycin.

Therapeutical group	Compound	Initial concentration	Removal (%)	
		($ng \cdot L^{-1}$)	Fungal reactor	Control reactor
Analgesic and anti-inflammatories	Acetaminophen	bld	-313.89 ± 586[a]	-2858.44 ± 2694[a]
	Ibuprofen	2065.73	--	--
	Ketoprofen	1231.60	--	--
	Phenazone	29.50	73.4± 23	48.79 ± 28
	Naproxen	>4000	--	--
	Salicylic Acid	>4000	90.09 ± 6	88.89 ± 7
	Total	>11337.30	88.91 ± 7[a*]	80.94 ± 1[a*]
Antibiotics	Azithromycin	2187.64	43.18 ± 20	42.57 ± 12
	Ofloxacin	1031.44	29.90 ± 4[a]	-14.26 ± 18[a]
	Trimetoprim	bld	-764.86 ± 1380	-3599.45 ± 10245
	Total	3220.11	38.66 ± 14[a]	23.20 ± 12[a]
b-blockers	Atenolol	48.95	12.34 ± 39	28.49 ± 39
	Propanolol	182.10	83.53 ± 20	74.94 ± 17
	Total	231.05	68.40 ± 13	64.94 ± 11
Diuretics	Hydrochlorothiazide	309.99	14.39 ± 38[a]	-18.48 ± 46[a]
	Total	309.99	14.39 ± 38[a]	-18.48 ± 46[a]
Lipid regulators	Gemfibrozil	406.34	80.54 ± 36	77.72 ± 40
	Pravastatin	219.74	95.72	95.70
	Total	626.08	85.86 ± 24	84.03 ± 26
Psychiatric drug	2-OH CBZ	1305.92	78.04 ± 4[a]	68.08 ± 10[a]
	Acridone	251.14	-20.72 ± 61[a]	51.71 ± 11[a]
	Carbamazepine	2425.91	42.04 ± 12[a]	23.91 ± 23[a]
	Citalopram	107.25	22.70 ± 110[a]	-44.92 ± 169[a]
	epoxy CBZ	2289.77	73.57 ± 12[a]	55.32 ± 19.6[a]
	Lorazepam	315.11	97.00 ± 5[a]	90.08 ± 14[a]
	N-Desmethyl-venlafaxine	222.75	68.01 ± 37	29.17 ± 63
	O-Desmetyl-venlafaxine	162.93	-28.60 ± 159	-54.20 ± 131
	Trazodone	bld	-314.27 ± 836	-159.01 ± 266
	Venlafaxine	501.73	22.84 ± 19[a]	15.88 ± 25[a]
	Total	7583.78	55.61 ± 6[a]	41.62 ± 13[a]

bld: below limit of detection. [a] Statically different ($p < 0.05$).

*Ibuprofen, ketoprofen and naproxen values were not included in total removal calculation.

Results from the treatment are referred to removal percentage since it cannot be distinguished between degradation and adsorption in the reactor experiments. However, the differences obtained between the fungal reactor and the control reactors (uninoculated) indicate the involvement of *T. versicolor* in the PhACs removal.

In this work only 0.49 g $DW \cdot L^{-1}$ of biomass were employed in the fungal reactor. Other authors also studied the PhACs removal from HWW with *T.versicolor* in bioreactor treatment, but they employed a higher biomass amount (in the range of 2-3 g $DW \cdot L^{-1}$) and included weekly partial biomass renovation (Badia-Fabregat et al., 2016; Mir-Tutusaus et al., 2017a). Hence, the biomass amount is the main difference compared with other systems for the PhACs removal using *T. versicolor.*

Analgesic and anti-inflammatories were present at high concentrations. The initial concentrations of ibuprofen and ketoprofen were 2065.7 and 1231.6 $ng \cdot L^{-1}$ respectively. A remarkable increase of these compounds were detected during all the treatment in both reactors (>4000 $ng \cdot L^{-1}$). These results were unexpected since ibuprofen and ketoprofen are well known easily degradable compounds by *T. versicolor*. Many authors reported the easily degradation of both compounds, specially ibuprofen, in bioreactor treatment of HWW employing *T. versicolor* (Badia-Fabregat et al., 2015b; Cruz-Morató et al., 2014; Mir-Tutusaus et al., 2017a). In addition, Marco-Urrea (2009b; 2010b) reported the biodegredatation pathway of ibuprofen and ketoprofen by *T. versicolor*. Moreover, in this thesis work, ibuprofen and ketoprofen were employed as target compounds to spike the HWW in different experiments, with high removal values.

Jelic et al., (2014) reported that 80% of ketoprofen is excreted in the urine, mainly as glucuronide metabolite. Ibuprofen is rapidly metabolized and eliminated in the urine, and it is also conjugated to glucuronide metabolites prior the human excretion. Since in this work glucoronide forms were not analyzed, probably the initial concentration of these two compounds was higher. For example, in the case of ketoprofen, taking into account that 80% is excreted in conjugated form, the initial concentration could be around 6000 $ng \cdot L^{-1}$, because the analytical method only quantifies the deconjugated form. Taking this into account, ibuprofen and ketoprofen were excluded from the removal calculation.

Therefore, excluding ibuprofen and ketoprofen, the initial amount of analgesic and anti-inflammatories was more than 4029.5 $ng \cdot L^{-1}$ (25% from the total PhACs). The removals

were 88% and 81% for the fungal and control reactors respectively, as both bacteria and fungi are reported to be able to remove these compounds (Langenhoff et al., 2013) (Figure 4.14).

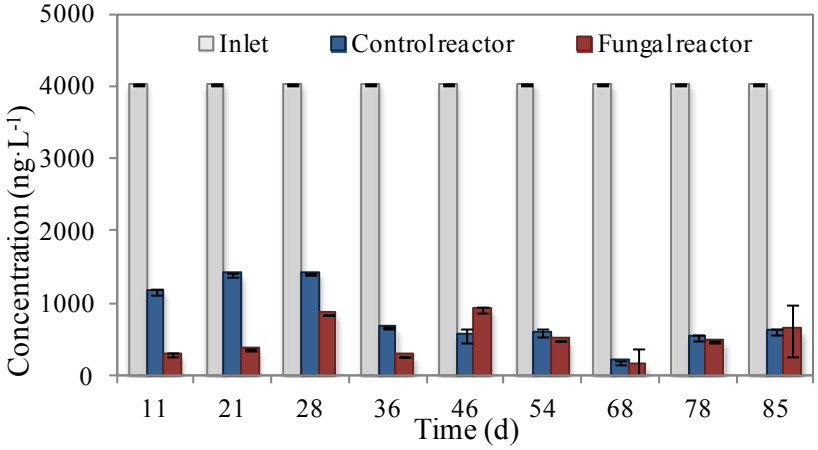


Figure 4.14. Analgesic and anti-inflammatories concentrations no including ibuprofen and ketoprofen.

Antibiotic initial concentration was 3219 ng·L^{-1}; *T. versicolor* was able to remove 39% of its initial load and the control reactor only 23%, as the Figure 4.15 shows. Mir-Tutusaus et al. (2017a) reported values ranging from 90% removal to no-removal in a fluidized-bed bioreactor with *T. versicolor* pellets (including weekly partial biomass renovation), and also detected an increase in the antibiotic concentration. Meanwhile, Cruz-Morató et al. (2014) indicated a total removal with a batch fluidized-bed bioreactor after 8 d of treatment with *T. versicolor* pellets. Both studies used a higher amount of biomass compared to this work (2.5-3.2 gDW·L^{-1} and 0.49 gDW·L^{-1}for the previous studies and for this work respectively).

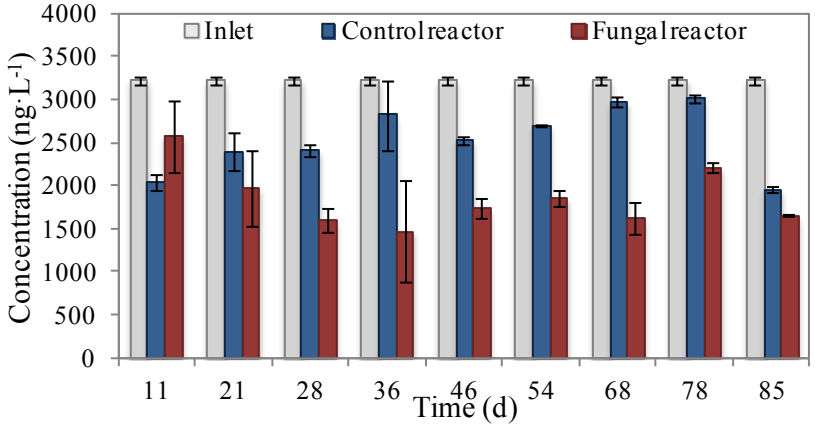


Figure 4.15. Antibiotics concentrations.

Diuretics concentration is presented in Figure 4.16. The fungal reactor removes 38% of its initial load until day 36, and then gradually loses its removal capacity. At the end, the concentration increased in 35%. In the control reactor, same trend was observed with 15% removal until day 54 and then an increase in the concentration by the end of the treatment. The increase in both reactors at the same time suggest that maybe desorption of the compound from the wood was occurring.

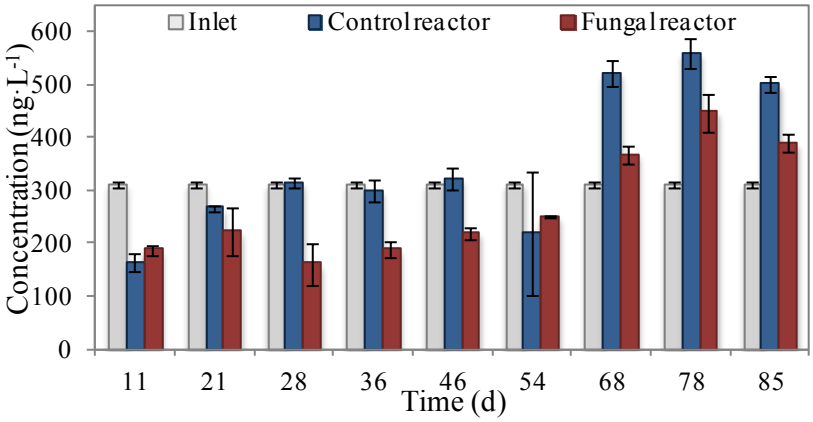


Figure 4.16. Diuretics concentrations.

Lipid regulators were detected at initial concentration of 626 ng·L^{-1}, high removal value were achieved for both reactors (85%). No differences between the fungal and control reactor were identified for the removal of lipid regulators (Figure 4.17). In both reactors, an increase of the concentration was detected at day 68, probably due an experimental error.

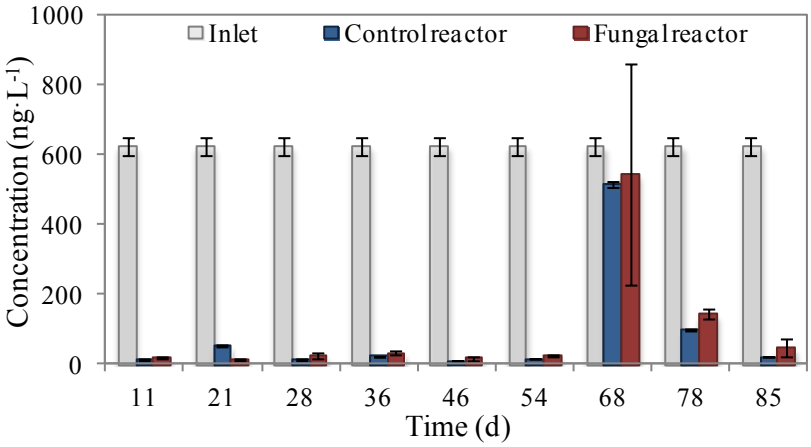


Figure 4.17. Lipid regulators concentrations.

B-blockers family was detected with an initial amount of 231 $ng \cdot L^{-1}$, both reactors presented the same removal profiles with an average value of 68% and 64% for fungal and control reactors respectively. The profile concentration is presented in Figure 4.18.

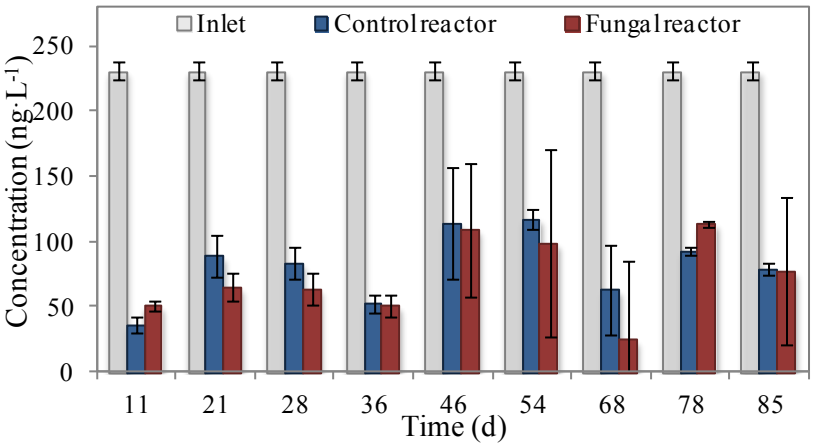


Figure 4.18. B-blockers concentrations.

Finally, psychiatric drugs represent 48% of the total PhACs detected in the HWW, with an initial amount of 7602 $ng \cdot L^{-1}$. The fungal reactor removes 56% of the psychiatric drugs and 42% are removed in the control reactor (Figure 4.19). Carbamazepine was the compound with higher amount detected at initial time. Alike, the case of carbamazepine, venlafaxine and its transformation products are discussed below, because an increase of some of these compounds was identified during the treatment.

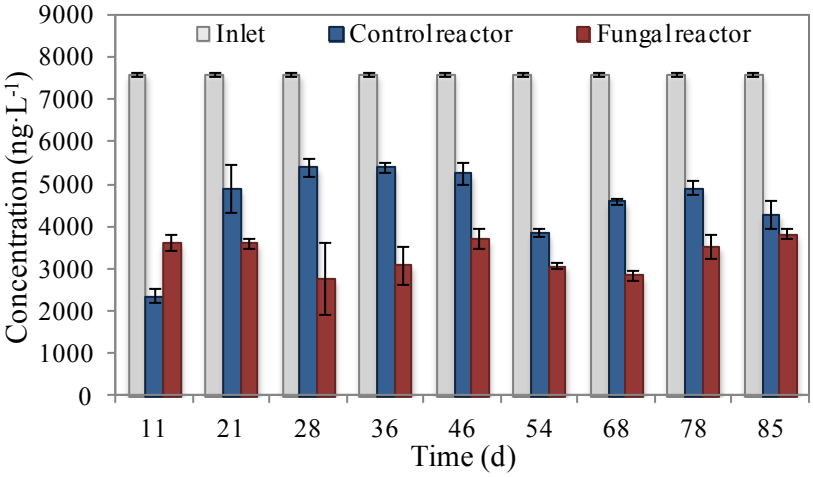


Figure 4.19. Psychiatric drugs concentrations.

Excluding ibuprofen and ketoprofen, the global removal for fungal and control reactors were 61% and 41% respectively, despite the small amount of biomass employed (0.49

$gDW \cdot L^{-1}$). The 20% difference in the PhACs removal between both reactors indicates the importance of the inoculation of *T. versicolor*.

- ***Carbamazepine removal and transformation products***

Carbamazepine (CBZ) is one of the most used drug for the treatment of epilepsy, trigeminal neuralgia and some psychiatric diseases (Fertig and Mattson, 2008). It is administered chronically and usually in high doses; in consequence, the CBZ production is high over the world (Jones et al., 2002).

CBZ is persistent to biodegradation and shows almost no removal in WWTP (Clara et al., 2004; Gebhardt and Schroder, 2007), which makes CBZ a compound with a high environmental relevance (Thanekar et al., 2018). Some studies have reported its presence in wastewaters effluents ranging from $ng \cdot L^{-1}$ up to $\mu g \cdot L^{-1}$ (Gros et al., 2010; Kasprzyk-Hordern et al., 2009) and also in drinking water at low $ng \cdot L^{-1}$ concentrations (Benotti et al., 2009; Ternes et al., 2002).

In human body, 70% of the CBZ is metabolized leading to the formation of more than 30 metabolites excreted mainly via urine (Kaiser et al., 2014). The major route of CBZ metabolism is the conversion to 10,11-epoxyCBZ (Epoxy CBZ) (Pearce et al., 2009). Then it is transformed in acridine, acridone and 10,11-dihydroxyCBZ. Minor metabolic pathways form 2-hydroxy-CBZ (2-OH-CBZ) and 3-hydroxy CBZ (3-OH CBZ). Human metabolites include also several glucuronides of CBZ, Epoxy CBZ, CBZD, 2-OH-CBZ and 3-OH CBZ. A summary of CBZ transformation pathway is presented in Figure 4.20 (Mir-Tutusaus, 2017b).

White-rot fungal pathway is similar to the human CBZ pathway (Jelic et al., 2012); and therefore WRF could generate the same transformation products. In fact, fungi were reported to produce 2- OH-CBZ and 3-3-OH CBZ when metabolizing CBZ, as both humans and fungus have similar cytochrome P450 systems (Mir-Tutusaus, 2017b).

In this work, the detection of CBZ and three of its degradation products were performed. The initial total amount of CBZ and the degradation products analyzed were 6292 $ng \cdot L^{-1}$. During the treatment, the fungal reactor removed 58% of CBZ and its metabolites and 45% of them were removed in the control reactor.

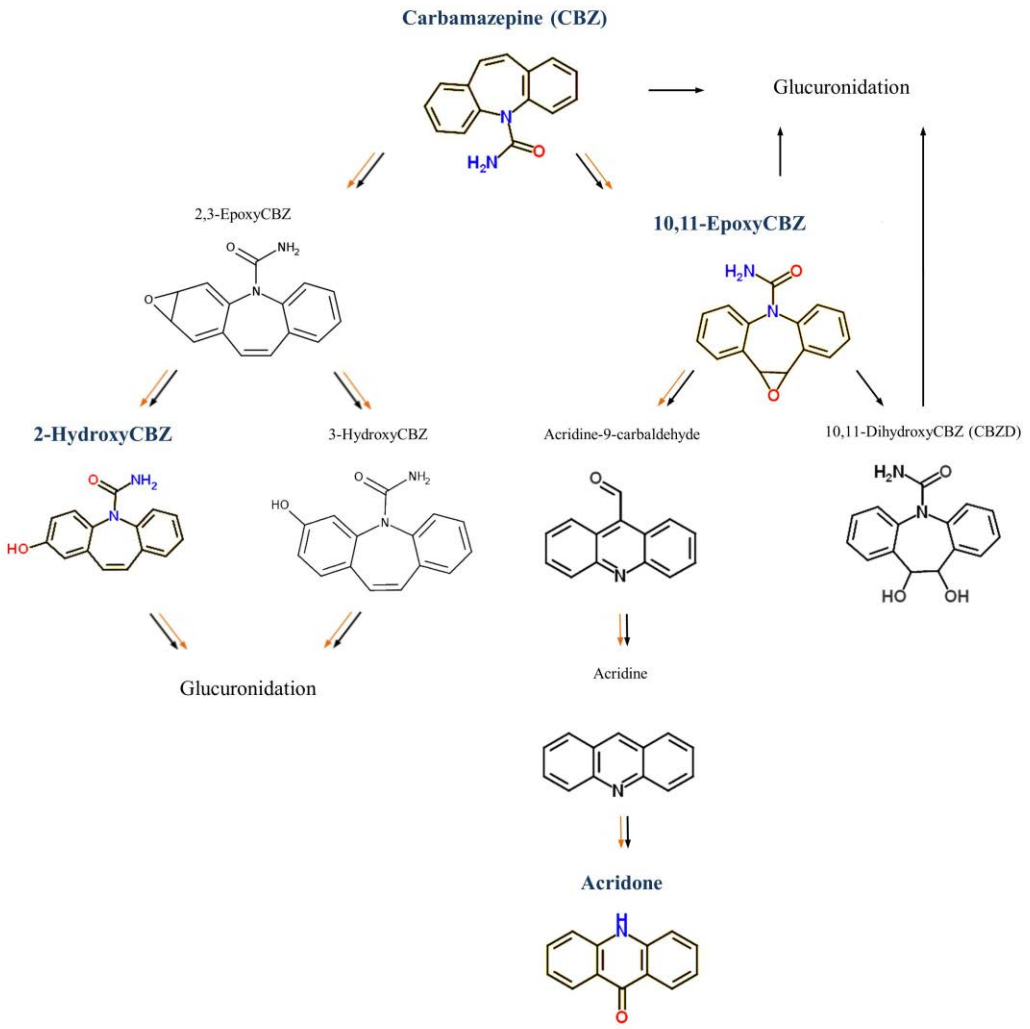


Figure 4.20. Transformation pathways of carbamazepine: in humans (black lines) and white-rot fungi (orange lines). Analyzed compounds are presented in bold. (Mir-Tutusaus, 2017b)

However, the removal profiles of the CBZ and its degradation products were different along the treatment. Figure 4.21 shows the concentration profiles of CBZ and its metabolites for the fungal reactor. The fungal reactor removed 40% of CBZ. Its metabolites 2-OH-CBZ and Epoxy CBZ were well removed during all treatment, achieving removal values of 79% and 74% respectively. Meanwhile, acridone concentration was increased on day 11 probably as a result from the Epoxy CBZ degradation. From this day to the end of the treatment, acridone concentration was the same. Taking into account that acridone is present in the HWW and also produced by the WRF, the equal concentration of acridone along the treatment suggest that the Epoxy degradation rate is similar to the acridone degradation rate because no accumulation of it was detected.

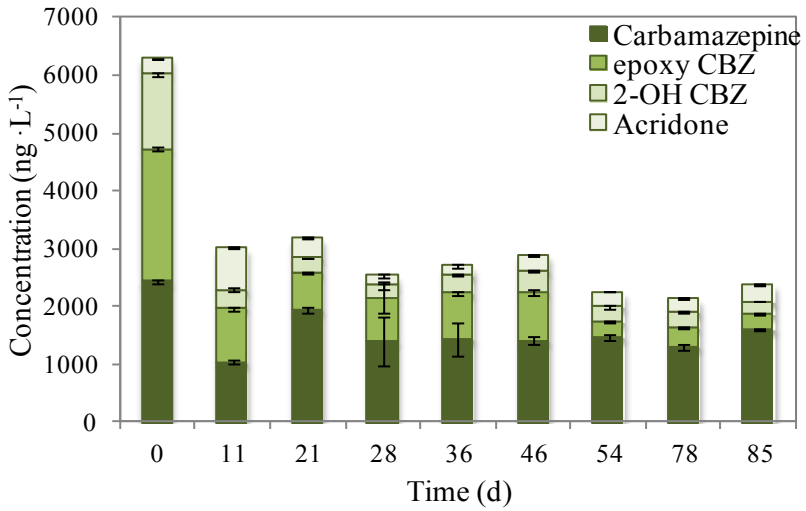


Figure 4.21. Concentration profile of CBZ and its transformation products in the fungal reactor.

The results obtained for the control reactor are presented in Figure 4.22; only 19% of CBZ was removed. Also, fewer removals were achieved for 2-OH-CBZ (69%) and Epoxy CBZ (58%). Acridone concentration was the main difference of the control reactor compared with the fungal reactor, as it was 57% removed in the control reactor. This removal is probably due to the adsorption and/or degradation by endogenous microorganisms present in the wastewater, so different degradation pathways occur in the control reactor and no increase in the acridone concentration was detected. Moreover, comparing with the fungal reactor, epoxy CBZ was less removed, which supposes less acridone production.

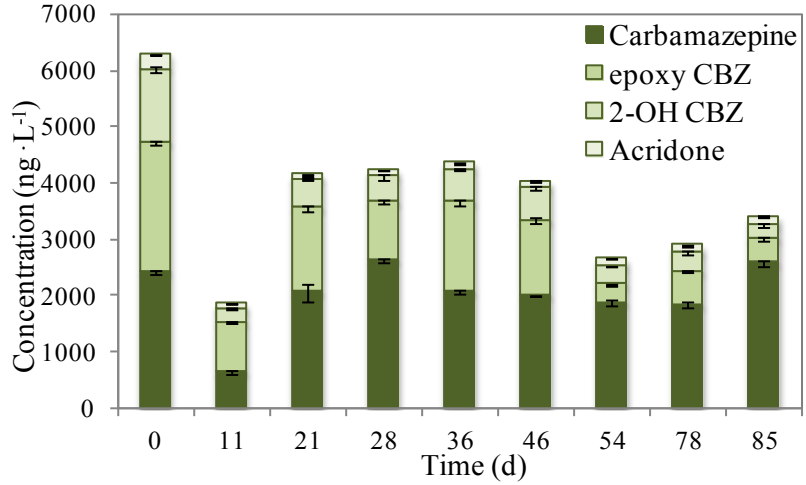


Figure 4.22. Concentration profile of carbamazepine and its transformation products in the control reactor.

- ***Venlafaxine removal and transformation products***

Venlafaxine (VLX) is an antidepressant increasingly used because its effectiveness in the treatment of melancholia and depression (Tzanakaki et al., 2000). VLX is not completely metabolized by human body after ingestion, and it is excreted as unchanged parent form and some transformation products. The major transformation products are O-desmethylvenlafaxine (ODV) and N-desmethylvenlafaxine (NDV) (Zucker et al., 2018).

Some studies demonstrated that VLX has negative to low removal efficiencies in WWTP (Escolá Casas et al., 2015; Ibáñez et al., 2016). In consequence, venlafaxine and its major transformation product, ODV, have been detected at various concentrations in sewage wastewaters, surface water, ground water, and even drinking water (Rúa-Gómez and Püttmann, 2012).

In our study, venlafaxine and its metabolites were detected in the HWW. VLX presented the higher concentration (501 $ng \cdot L^{-1}$), while ODV and NDV were detected at 162 and 222 $ng \cdot L^{-1}$ respectively. The total concentration of VLX and its degradation products was 887.41 $ng \cdot L^{-1}$, only 27% and 5% were removed by fungal and control reactor. Nevertheless, the removal profiles of the VLX and its degradation products varied along the treatment.

Figure 4.23 shows the removal for VLX and its metabolites in the fungal and the control reactors. Only 20% of the initial VLX concentration was removed in the fungal reactor and 8% in the control one. The poor removal is in agreement with other authors treating VLX with *T. versicolor* in fluidized-bed bioreactor in continuous mode under sterile conditions and non-sterile HWW (Mir-Tutusaus et al., 2017b). Instead, working in batch reactor with HWW, Cruz-Morató et al. (2014) reported 90% removal after 8 days and attributed it to the synergistic action of fungal and bacterial enzymes.

Regarding the VLX metabolites, in the fungal reactor ODV was almost completely removed (95%) until day 36, since when it started to increase. At the end, an increase of 360% of ODV concentration was detected. This metabolite could derive from the influent or from transformation of VLX. Since the VLX removal was stable along the treatment, the fungal reactor lost the capacity for the ODV removal from day 46. Also, the laccase activity diminished from day 30, but no information was found regarding ODV degradation by

laccase. The same trend was observed for NDV, it was totally removed until day 36 when started to increase. At the end of the treatment 50% of NDV was removed.

Mir-Tutusaus et al. (2017b) reported the same removal profile for VLX and its metabolites in a long-term reactor with *T. versicolor* pellets, with good removals at the beginning followed by an increase of the concentration´s compounds from the middle to the end of the treatment.

Meanwhile, in the control reactor, the ODV removal was very low (-28%) with a maximum removal on day 11 and 85. The NDV mean removal was 19% with a maximum value at day 21 (94%). No clear trend was found for the removal of VLX´s metabolites in the control reactor.

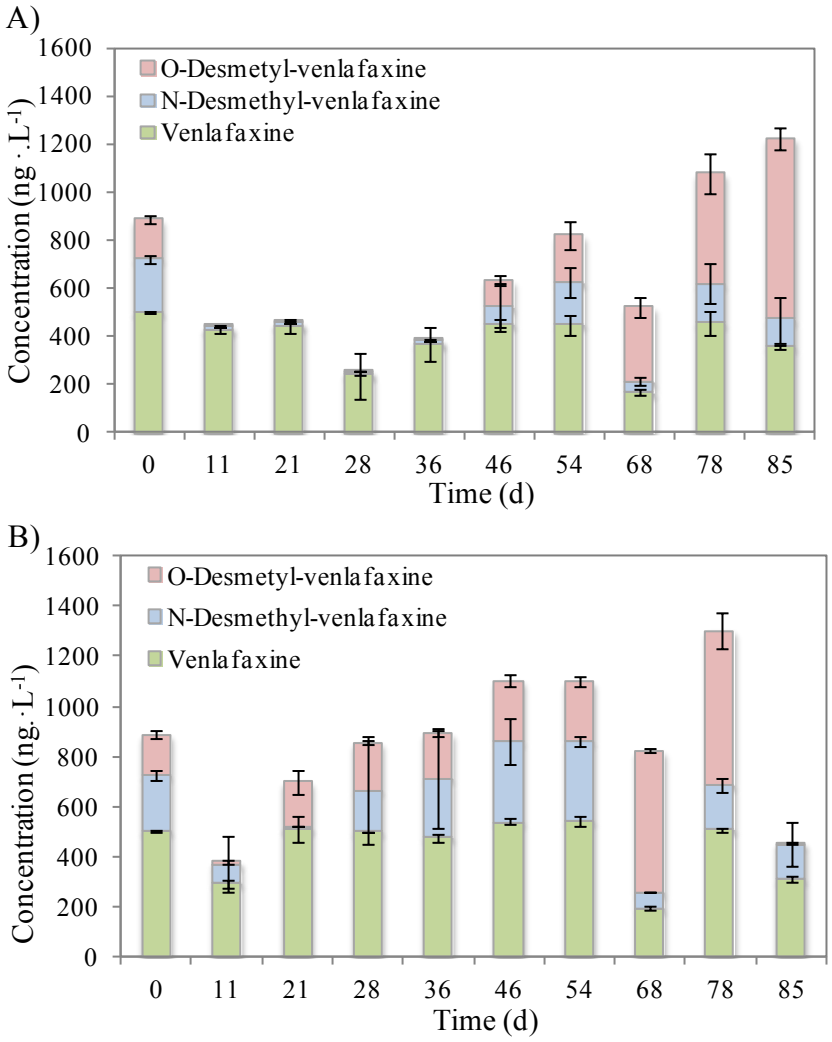


Figure 4.23. Concentration profile of venlafaxine and its transformation products in (A) fungal reactor and (B) control reactor.

4.2.4.3 Scale up of the trickle-bed bioreactor

Based on the good results obtained with the trickle-bed bioreactor in previous sections, it was scaled-up ten times to prove if the system could work with higher total volume since the final objective of this thesis is to develop a system for the treatment of wastewater for real applications (Figure 4.24).

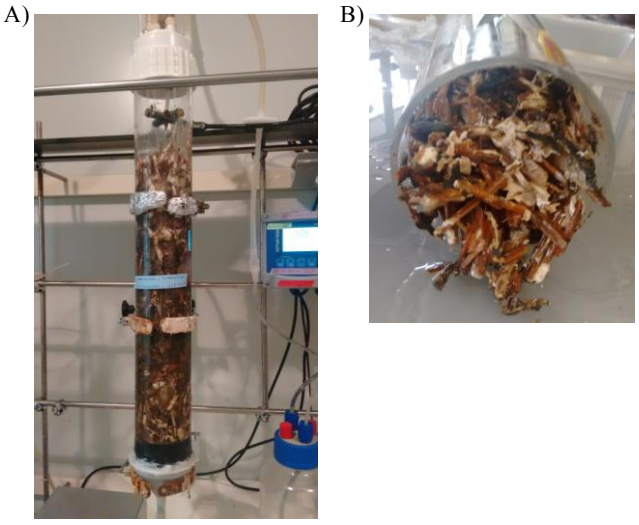


Figure 4.24. (A) Scale-up of the trickle-bed bioreactor. (B) Inoculated wood at the end of the treatment.

The same operational parameters were used, but in this case the total volume was 2.5L and 600 g of wet wood with *T. versicolor* immobilized on it was employed. In previous experiments the total volume was 0.25L and 60g of wet wood. The scale-up was made keeping the relation between the total volume and the amount of wood. Same HRT (3 days) and RR (877) were used. The bioreactor operated for 89 days in continuous mode, treating coagulated-flocculated HWW spiked with ibuprofen, ketoprofen and naproxen at a final concentration of 10 $mg \cdot L^{-1}$.

The results are presented in Figure 4.25; the average removal values obtained were 89% ibuprofen, 78% naproxen and 63% ketoprofen. Regarding ibuprofen, stable values were obtained during all the treatment, with high removal rates (more than 90%) until day 70. In the case of naproxen, a removal over 80% was obtained until day 60, and after that it decreased to 60%. The removal of ketoprofen was almost 80% during the first 40 days, after that it was decreased to 60% until day 70, since when the removal decreased to less than 40% at the end of the treatment.

The reactor exhibited a maximum laccase activity of 18 $AU \cdot L^{-1}$ at day 7, afterward the laccase production decreased to levels below 0.5 $AU \cdot L^{-1}$ from day 60 to the end of the experiments.

In general, same removal trend for the spiked compounds was observed until day 70 with high stable removals values. The stationary state of the bioreactor was maintained during 70 days and from that day to the end, the reactor loses its removal capacity in concordance with the lost of laccase activity. The scale-up of the system presented the same removal values compared with the previous reactor operated in this chapter.

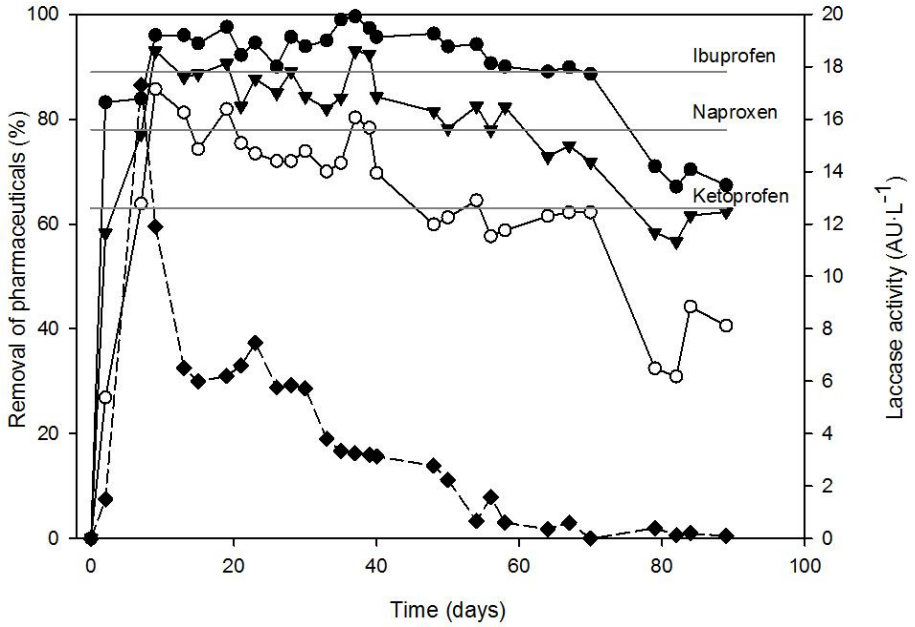


Figure 4.25. Removals of ibuprofen (●), naproxen (▼) and ketoprofen (○) in trickle-bed bioreactor inoculated with *T. versicolor* and (♦) laccase activity. The mean values for the removal is represented in a solid line for ibuprofen, naproxen and ketoprofen.

Figure 4.26 shows the values obtained for different parameters: heterotrophic plate count, total suspendid solids and COD during the fungal treatment in the trickle-bed bioreactor. Usually when working with *T. versicolor* an increase in the COD is observed, probably due to by-products or metabolites released during degradation (Palli et al., 2016). During all the treatment, the COD obtained was more than ten times higher than the initial one, only in the last period (from day 68) the COD decreased. The decrease is probably due to the losses of the fungal activity, since it matched with the decrease in the laccase activity and removal values. Hence, the measures of laccase and COD could be used as fungal viability

indicators. Also, as mentioned before, COD combined with the measures of TSS and heterotrophic plate count could be used as an indicator of the bacterial growth.

At the beginning of the experiment the heterotrophic plate count was $4.5 \cdot 10^5$ CFU·mL^{-1}. An increase in 3 orders of magnitude was observed until day 40, and then the bacterial concentration was maintained to 10^6 CFU·mL^{-1}. During the last 50 days of operation, the increase in the bacteria concentration was only 1 order of magnitude higher than the coagulated-flocculated influent.

Regarding to the total suspended solids (TSS), the initial value (5 mg·L^{-1}) was increased up to 50 mg·L^{-1} from day 20 to 40, after this initial period the values obtained were stable until the end of the treatment with a mean value of 7 mg·L^{-1}. This results are in agreement with the UFC and COD values obtained, taking into account the results obtained for this three parameters, we can conclude that the bacterial growth started to be limited.

As the bacteria overgrowth is a key issue in the reactor performance working with real HWW, in this system different strategies were combined to control it: the use of pallet wood as sole carbon source, the pH control at 4.5, the coagulation-flocculation pretreatment of the HWW inlet and the non-addition of nutrients.

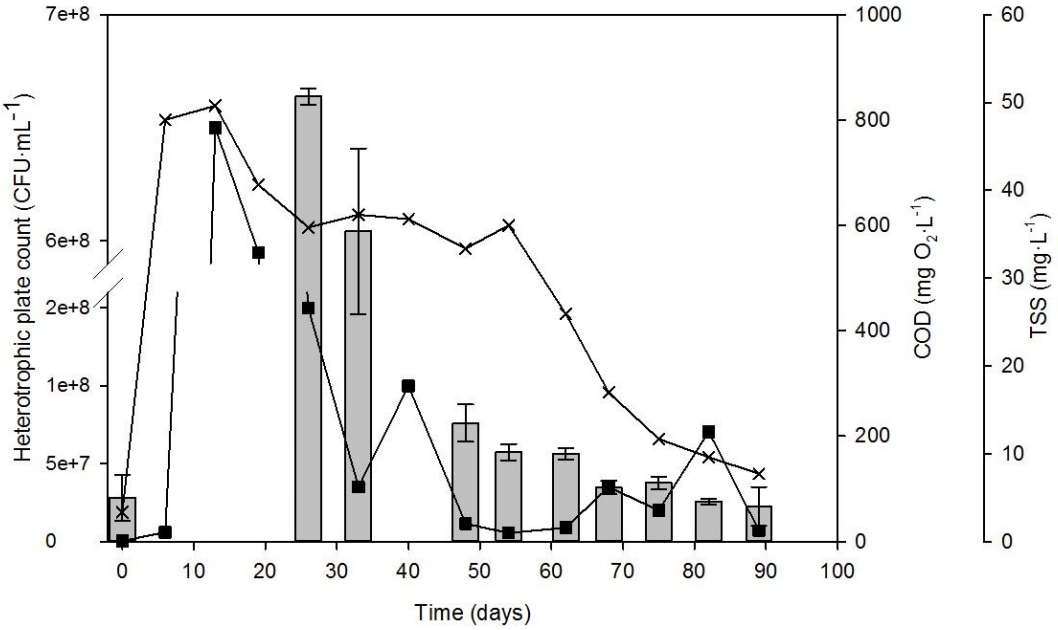


Figure 4.26. Scale-up reactor treating spiked HWW.
Symbols: (x) COD and (■) CFU. The bars lines represent the TSS.

4.2.4.4 Trickle-bed bioreactor treating non coagulated-flocculated HWW

In the trickle-bed bioreactors the wood is the only nutrient source available for *T. versicolor* and to the endogenous microorganisms (despite the nutrients presents in the HWW). So, maybe it is not necessary the application of a coagulation-flocculation pretreatment since no extra nutrients were added.

Based on that idea, a new trickle-bed bioreactor was performed with raw HWW spiked with ibuprofen, ketoprofen and naproxen to a final concentration of 10 $mg \cdot L^{-1}$. Same operational parameters were used for HRT (3 days) and RR (877) as in previous experiments. The total volume was 0.25 L.

The bioreactor was operated during 41 days with high removals values for ibuprofen (98%), naproxen (88%) and ketoprofen (76%). The removal profiles are presented in the Figure 4.27. Laccase activity profile is included showing a similar trend as in the previous trickle-bed bioreactor working with coagulated-flocculated HWW.

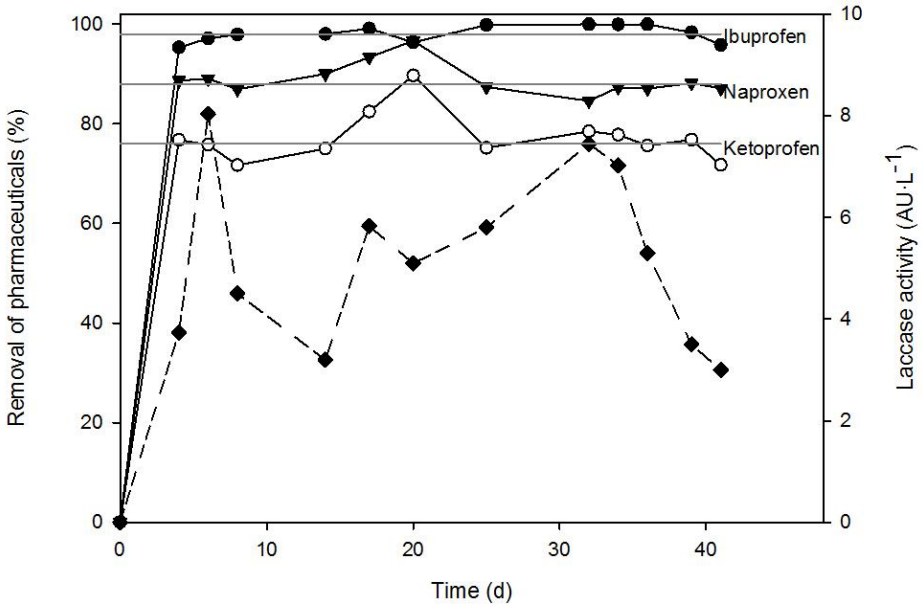


Figure 4.27. Removals of ibuprofen (●), naproxen (▼) and ketoprofen (○) from raw HWW and (♦) laccase activity. The mean values for the removal is represented in a solid line for ibuprofen, naproxen and ketoprofen.

Heterotrophic plate count, total suspended solids and COD were also monitored along the treatment (Figure 4.28). Raw wastewater was used in this reactor; the initial bacterial load was $1 \cdot 10^{8}$ $CFU \cdot mL^{-1}$. The bacterial concentration decreased during the fungal treatment and maintained stable at $6.7 \cdot 10^{6}$ $CFU \cdot mL^{-1}$, probably because no nutrients addition was

performed since the wood is the only carbon source. Also, the low pH (4.5) limited the bacterial growth as already been reported (Folch et al., 2013). Comparing with the same reactor working with coagulated-flocculated HWW, values in the same order of magnitude were obtained in all cases (10^6 CFU·mL^{-1}).

The total suspended solids profiles presented the same trend as the bacterial concentration, with maximum values at the begging of the treatment (67 mg·L^{-1}) and stable values at steady state (18 mg·L^{-1}). Different profile was obtained for the total COD, an increase was detected during all the treatment, which is in agreement with the previous results reported along this thesis working in reactor with *T. versicolor*.

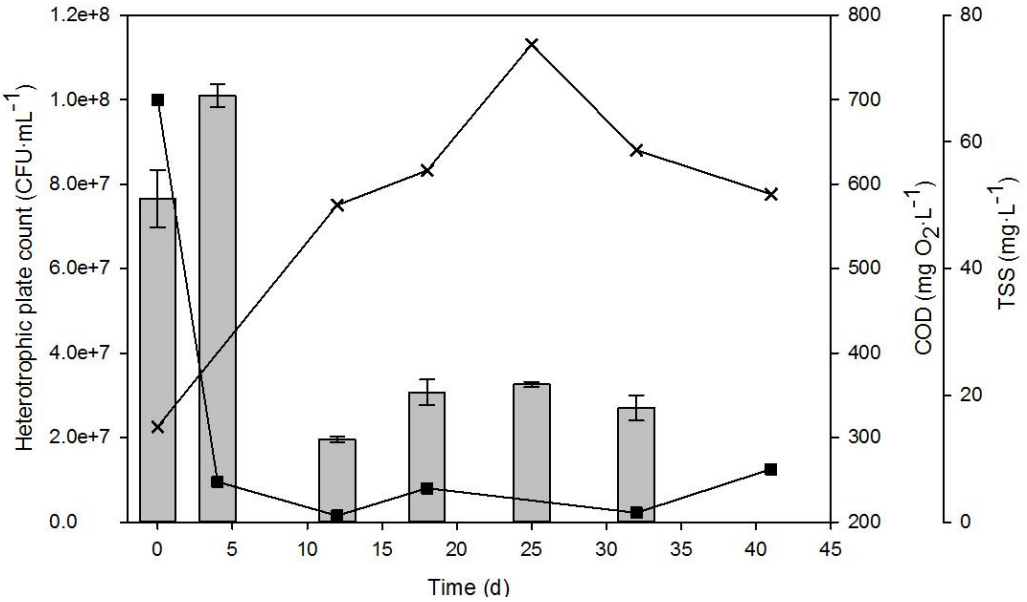


Figure 4.28. Trickle -bed bioreactor working with raw HWW. Symbols: (x) COD and (■) CFU. The bars lines represent the TSS.

Table 4.4 shows the comparison between the results obtained in this reactor working with raw HWW and previous reactors working with coagulated-flocculated HWW. In all cases the HWW was spiked with ibuprofen, ketoprofen and naproxen. Unexpected, higher removals values were obtained for ibuprofen and naproxen, and same value for ketoprofen working with raw HWW. These increases in the removal rates can be attributed to the synergistic action of fungal and bacteria present in the HWW. Therefore, based on these results, the additional step of coagulation-flocculation pretreatment of HWW is not necessary in the trickle-bed bioreactor.

Table 4.4. Comparison between the trickle-bed bioreactors performed in this thesis for the removal

HWW	Operation time (d)	Total volume (L)	Laccase activity ($AU \cdot L^{-1}$)		Removal (%)		
			Max.	Average	Ibuprofen	Ketoprofen	Naproxen
Raw HWW	41	0.25	17.7	4.8	98	76	88
CF HWW	49	0.25	8.1	5.0	90	80	60
CF HWW	89	2.5	17.4	3.6	89	63	78

CF HWW= flocculated-coagulated HWW

4.2.4.5 Fiber content in wood after reactor operation

The fiber determination was performed in order to study if the fiber content in wood changes after the reactor operation since wood is used as the only nutrient source.

The determination was performed in the wood of the bioreactors after treatment in the validation experiment (fungal and control reactor), the scale-up experiment (botton and top zone of the fungal reactor), the non-flocculated HWW experiment (fungal reactor) and the initial wood autoclaved not inoculated as wood control. The results obtained are presented in Table 4.5. No fiber degradation was detected, on the contrary the autoclaved wood presented fewer fibers content than the wood at the end of the different reactors. This difference is probably due to the use of different wood to perform the experiments. In all the cases the wood was from pallets waste, but obtained at different time.

The results indicated that wood is a good substrate for the fungus and it is not necessary to replace it during the operation, since after long time of treatment, the fiber content is maintained at high values.

Table 4.5. Fiber content in wood at the end of the experiments performed in the trickle-bed

Sample		Operation time	**Fibers ($\%\cdot DW^{-1}$)**			
			Cellulose	Hemicellulose	Lignin	Total
Initial autoclaved wood			16.19	52.47	19.81	88.47
Validation experiment	Fungal reactor	85	15.55	51.71	25.44	92.70
	Control reactor	85	15.17	52.99	25.16	93.32
Scale-up reactor	Top zone	89	16.88	51.87	21.49	90.24
	Botton zone	89	15.45	50.66	22.19	88.30
Non-floculated experiment	Fungal reactor	41	14.45	49.50	23.20	87.15

4.3 Comparison between operational strategies

The main objective of this thesis is to verify if working with the immobilized fungus on wood allows overcoming the problems involved in working with the fungus in pellet form, continuously during long periods of time treating non-sterile wastewater.

Next, the two systems are compared, the fluidized-bed bioreactor (FBR) by air pulses employed by Mir-Tutusaus (2017b) and the trickle-bed bioreactor (TBR) developed in this thesis. The characteristics of each system are presented in the Table 4.6.

	FBR	TBR
Nutrients addition	Yes	No
Initial biomass ($g \cdot L^{-1}$)	2.5-3	0.49
Biomass renovation	Yes	No
Support needs	No	Yes
Flocculation pre-treatment	Yes	No
HRT (d)	3	3

As a consequence of not adding nutrients in the TBR the process is economically more favorable and limits bacterial growth, which allows work with raw HWW without pretreating the hospital wastewater by flocculation processes, as can be deduced from the experiments presented.

In addition, the non-renewal of biomass in the TBR also favors the economics of the process, since the production of the pellets is one of the highest costs of the process in the FBR.

In the case of the TBR, an immobilization support is necessary, but pallet wood waste is used, so that at the same time an existing residue is being valorized, and the final disposition of the residue may be the same, such as composting, or use as fuel.

The experiments carried out with the FBR and the TBR present an important difference in the initial biomass concentration. In the case of the FBR, biomass in pellets form is greater than 2 g $DW \cdot L^{-1}$, however, in the case of the TBR, estimation of the initial biomass from the extraction of ergosterol is one order of magnitude inferior (0.49 g $DW \cdot L^{-1}$). Another important difference is that in the case of the FBR the biomass is completely

submerged and in the TBR case, the biomass is not submerged, but it is in contact with air that is the natural habitat of the fungus.

Having done all these clarifications, the results obtained in this thesis treating HWW with the TBR will be compared with the results obtained by Mir-Tutusaus (2017b) treating HWW of the same origin but different composition (different sampling data) with the FBR. In the case of the TBR, naproxen has been spiked in order to be able to monitor the reactor. Only the experimental reactor data is presented in both cases, the PhACs are sorted by families.

In both HWW the most abundant family is analgesics and anti-inflammatory drugs, and although, the total initial concentrations of these drugs are very different in both cases, 4000 and 35000 $ng \cdot L^{-1}$ in the TBR and FBR respectively, high removal rates are obtained in both cases (88% and 80% for TBR and FBR respectively) (Figure 4.29). It must be taken into account that when comparing these results with the controls without biomass of the two bioreactors, also high removal rates are obtained, so good degradation of these compounds is also obtained by the autochthonous microorganisms present originally in the HWW. Therefore, it can be deduced that removal is due to microbial biodegradation and non to adsorption on wood in the TBR, since the FBR control presents also good degradation (Mir-Tutusaus et al., 2017b) and no wood support is present. In addition, it is widely demonstrated that the fungus is able to degrade these compounds (Marco et al., 2009), so probably a synergetic effect of the fungus and the bacteria is the responsible for the biodegradation.

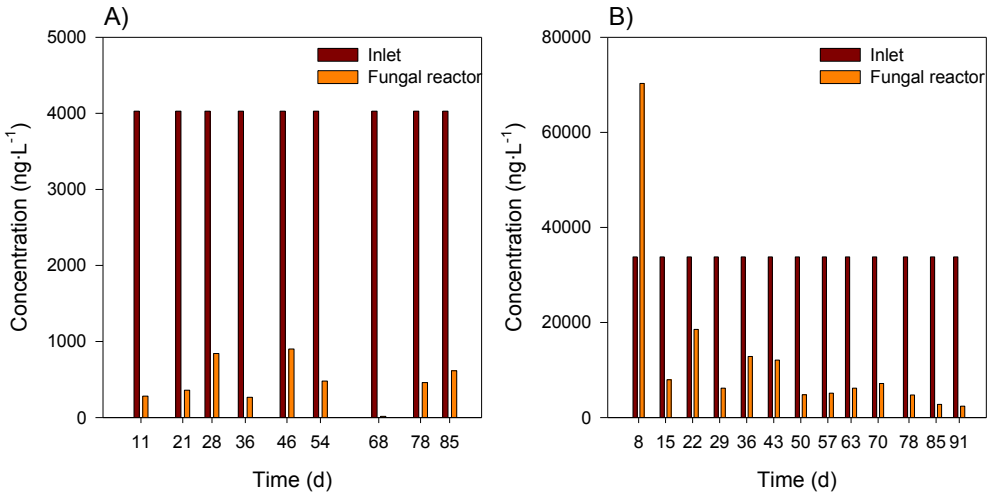


Figure 4.29. Analgesic and anti-inflammatories concentration in (A) TBR and (B) FBR.

Regarding to the antibiotics, in TBR the total initial concentration is ten times higher than in the FBR, but it can be observed that the behavior during the continuous treatment is very similar, with degradations of 38% and 42% respectively for TBR and FBR, and stable during the treatment (Figure 4.30). Comparing TBR with the non-inoculated control (20% removal) it can be seen that in the non-fungal control there was no removal for these drugs, therefore they are not absorbed in the wood or degraded by the autochthonous microorganisms of the HWW, and the same is observed for the FBR (Mir-Tutusaus et al., 2017b) so it can be concluded that antibiotics are mainly degraded by the fungus.

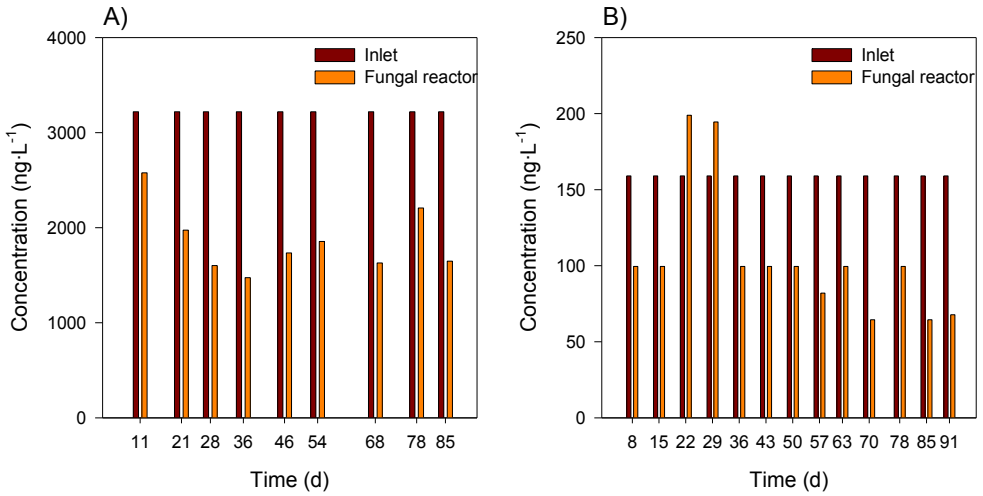


Figure 4.30. Antibiotics concentration in (A) TBR and (B) FBR.

Similar b- blockers total concentration in the HWW is present for both systems (Figure 4.31). The average degradation is slightly higher in the FBR (75%) than in TBR (68%). For the FBR it is demonstrated that autochthonous microorganisms are the responsible for the degradation of these drugs (Mir-Tutusaus et al., 2017b). Taking into account these previous results and the differences between concentrations of b-blockers in the experimental TBR and non-inoculated TBR (removal of 60%), it is suggested that b-blockers are degraded in the TBR by a synergetic effect of autochthonous microorganisms and the fungus rather than by adsorption onto the wood.

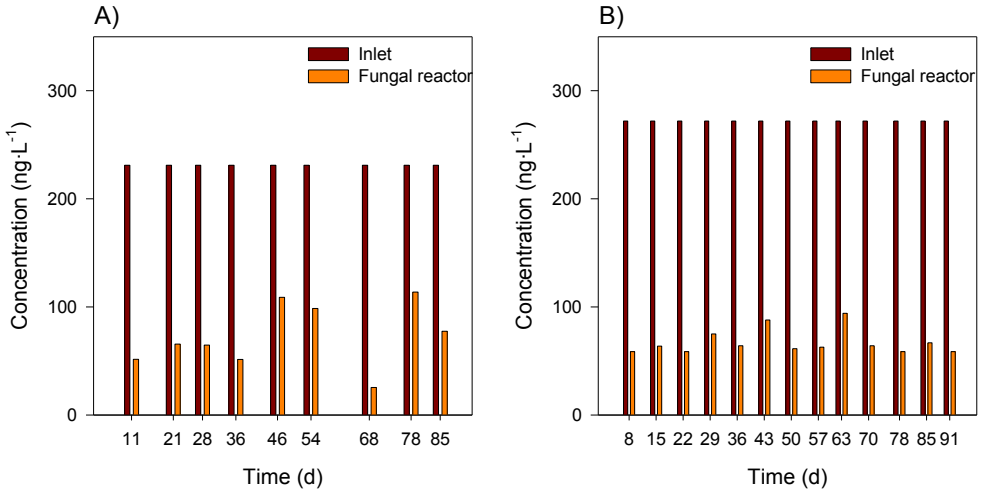


Figure 4.31. B-blockers concentration in (A) TBR and (B) FBR.

In relation to psychiatric drugs, the total concentration in the HWW is slightly higher for the TBR (Figure 4.32). The degradation is more stable in the TBR, whereas in the FBR when extracellular laccase is present in the liquid medium the average degradation is higher, but it worsens greatly after 53 days, when extracellular laccase activity is not detected. In the FBR it is demonstrated that autochthonous microorganisms are not able to degrade these drugs (Mir-Tutusaus et al., 2017b), therefore for the TBR the difference between the feed concentration and the control could be due to adsorption onto the wood.

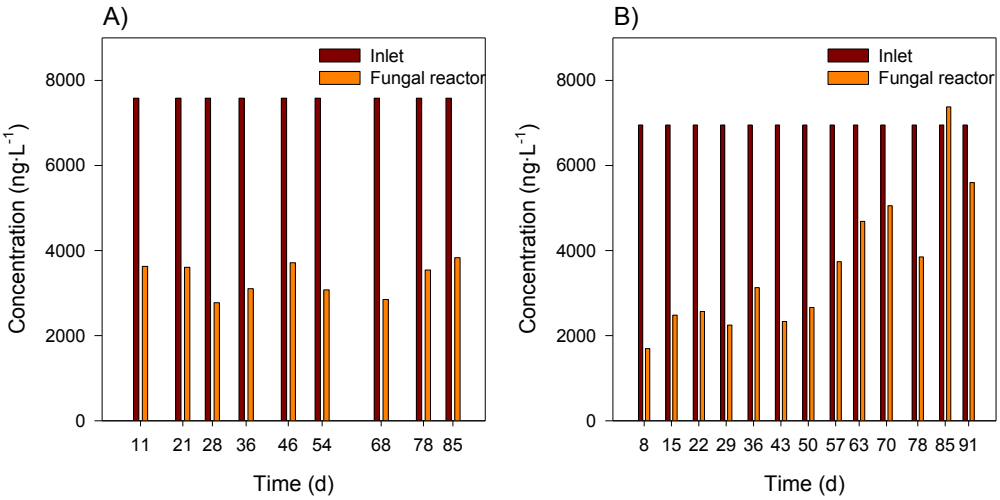


Figure 4.32. Psychiatric drug concentration in (A) TBR and (B) FBR.

In the HWW of the TBR the diuretics concentration is half the concentration present in the HWW feed of the FBR (Figure 4.33). The degradation in the FBR is clearly higher (45%), although the tendency to increase the concentration when the extracellular laccase activity is not detected occurs in both cases. Better removals in the FBR can be attributed to the higher amount of biomass in the bioreactor.

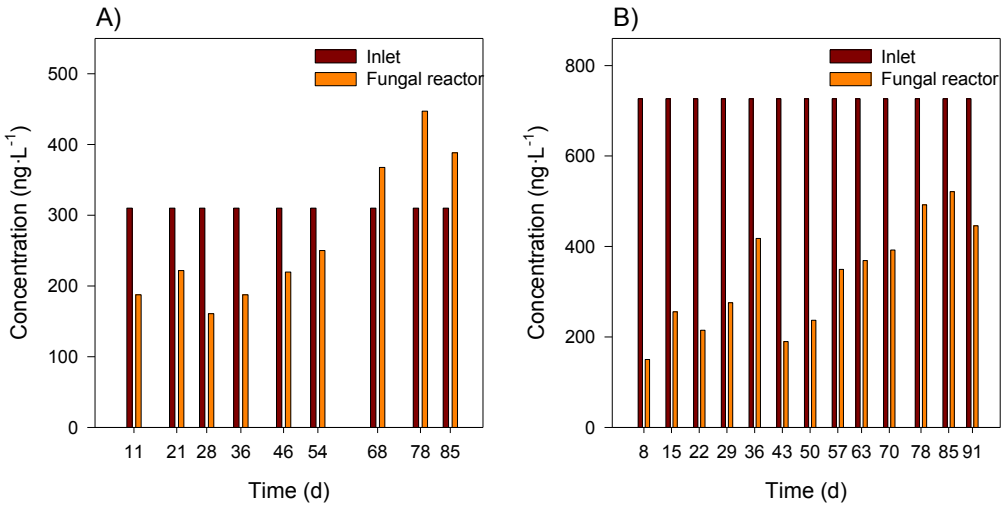


Figure 4.33. Diuretics concentration in (A) TBR and (B) FBR.

Regarding to lipids, in both systems almost total removal is observed, both in fungal and in non-inoculated controls, suggesting that can therefore be degradation by autochthonous microorganisms rather than adsorption onto the TBR wood (Figure 4.34).

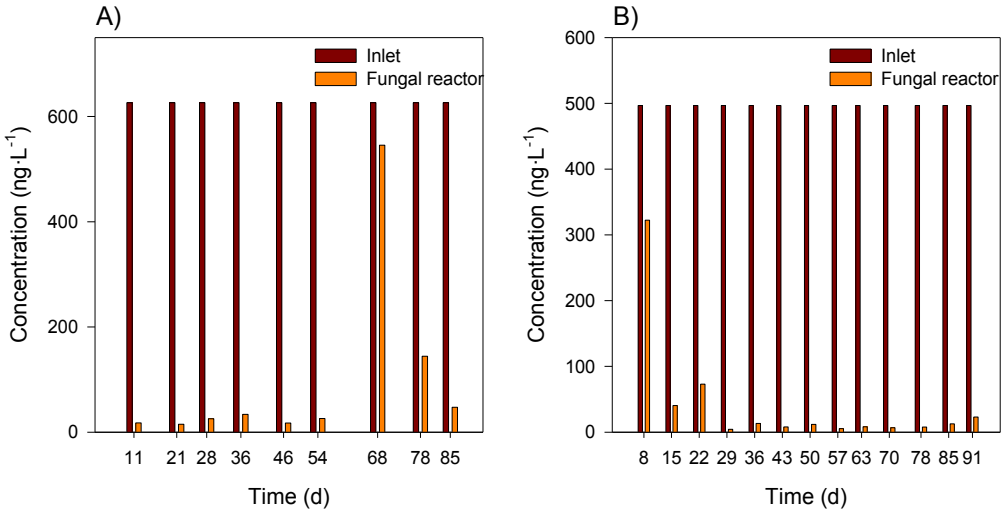


Figure 4.34. Lipids regulators concentration in (A) TBR and (B) FBR.

It can be concluded that TBR behaves very similar to FBR in the degradation of all the pharmaceutical families. Therefore the fungus acts the same, and probably more effectively in the TBR, because although there is less biomass in the system, the percentages of degradation are only slightly lower. All these results lead us to conclude that if the TBR is scaled, it is operated with larger columns, and the HRT is increased, the same results as with the FBR could be obtained at a much lower cost.

4.4 Conclusions

The immobilization of *T. versicolor* on wood is a good strategy to ensure fungus survival in HWW treatment. Complex wood pellets with a wood core were formed in a fluidized-bed bioreactor; these pellets exhibited high PhAC removal efficiencies when they treated coagulated-flocculated HWW for 28 days. However, the system was not scalable.

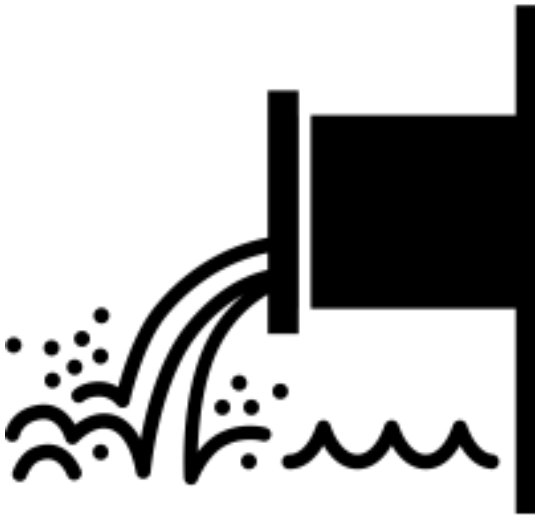
A trickle-bed bioreactor with *T. versicolor* that was immobilized on wood was successfully operated to remove ibuprofen, ketoprofen and naproxen from coagulated-flocculated HWW for 49 days. While, working at real PhACs concentrations, 61% removal was achieved during 85 days. Taking into account the small amount of biomass employed in this reactor, the results obtained are very promising. Increasing HRT or biomass amount, it was expected to improve the removal performance.

In all the experiments employing the trickle-bed bioreactor, same profiles for laccase activity, COD, heterotrophic plate count and TSS were obtained along the treatment. Also values in the same range were observed in all cases. An increase in all parameters was observed during the first 40-50 days of treatment, and then a decrease is observed. These observations suggest that in all cases the bioreactor inoculated with *T. versicolor* has the same behaviour.

In the trickle-bed bioreactor, different strategies were combined: immobilization on a lignocelullosic support, controlling the pH and using a coagulation flocculation pretreatment. The use of the last pretreatment is not necessary and this step can be removed for future applications. The scale-up of the system has been proved without operational problems.

CHAPTER 5

Humic acids



Food-processing companies are increasing over the world and result in environmental pollution characterized by the uncontrolled discharge of effluents. Some of the effluents contained a huge amount of humic acids, which are highly recalcitrant respect to bacterial biodegradation. In this chapter is presented the treatment of a rich-humic wastewater during long-term operation under continuous mode. Two types of bioreactors were employed: fluidized-bed bioreactor with *T. versicolor* pellets and trickle-bed bioreactor with *T. versicolor* immobilized on pallet wood.

5.1 Introduction

Humic substances (HS) comprise the most abundant portion of the natural organic matter in the environment, both aquatic and terrestrial (Hedges et al., 2000; Stevenson F., 1994). HS are produced during the decomposition of animal and plant tissue and are extremely resistant to biodegradation (Piccolo A., 2002). The HS involve a physically and chemically heterogeneous mixture of biogenic with a wide range of molecular masses that are built with mixed aliphatic and aromatic units (Steffen et al., 2002; Szymański et al., 2016).

Three main fractions of HS can be separated based on their solubility in acids or alkalis: humic acid (HA) (the major fraction of HS), which are soluble in alkali and insoluble in acid; fulvic acids (FA), which are lower-molecular-mass compounds with smaller number of total aromatic carbons compared to HA and are soluble in alkali and acid; and humins, which are insoluble in both acid or alkali (Stevenson F., 1994). An example of the structure of the humic acids is shown in Figure 5.1.

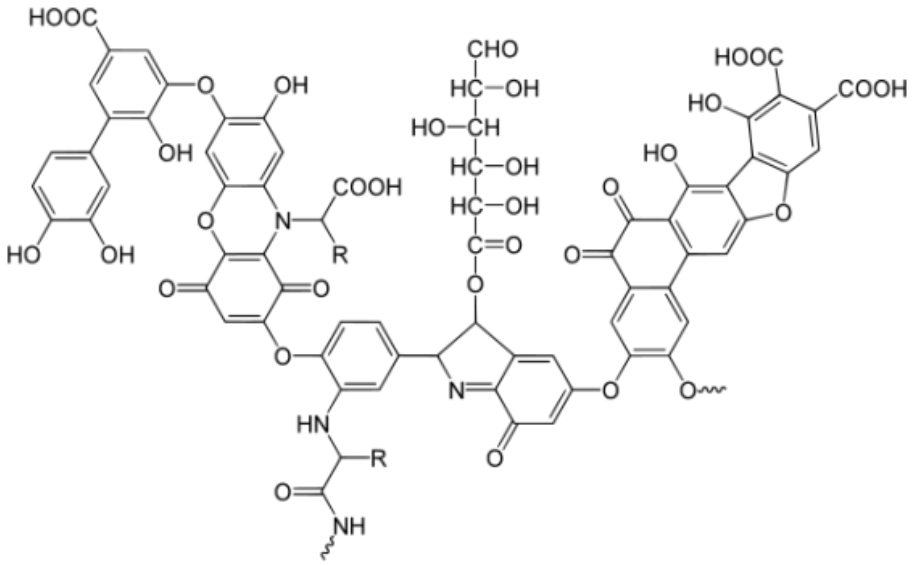


Figure 5. 1. Example structure of humic acids (Stevenson F., 1994).

Humic acids often cause environmental problems once released into the ecosystem. HA can carry heavy metal ions and other insoluble xenobiotics, increasing their solubility and mobility in soil and water (Tang et al., 2014). In addition, HA are precursors of trihalomethanes, which are carcinogenic compounds, formed during disinfection and chlorination of drinking water (Awad et al., 2016; Kim et al., 2007). In wastewater treatment plants, HA can cause membrane fouling (Šír et al., 2012; Sutzkover-Gutman et

al., 2010). Additionally, the presence of HA results in colored effluents (Wang et al., 2015a). Therefore, it is important to promote the degradation of humic acids in the wastewater before discharging the effluent (Esham et al., 2000).

HA are highly recalcitrant with respect to biodegradation and the ability of bacteria to degrade HA is limited. The removal of HA from waters by WRF has been demonstrated before (Steffen et al., 2002; Zahmatkesh et al., 2016, 2017a, 2017b). Figure 5.2 shows the decolorization in synthetic media with HA before and after the fungal treatment reported by Zahmatkesh et al. 2016.

Figure 5.2. HA synthetic media color before and after fungal treatment (modified from Zahmatkesh et al. 2016)

Most of the studies on degradation of HA by WRF have been conducted under sterile conditions in Erlenmeyer-scale. Under non-sterile conditions, fungal growth and enzyme activity are inhibited severely, resulting in the failure of HA removal (Sankaran et al., 2010). The application of WRF under non-sterile conditions has been reported to be the major hurdle in industrial application of these organisms. This is mostly attributed to bacterial proliferation, which results in severe competition for nutrients.

The main goal of this chapter is to apply a fungal reactor for continuous HA removal from humic-rich wastewater under non-sterile conditions. Two types of systems were employed in order to study the best system for the HA removal during long-term treatment: a fluidized-bed bioreactor and a trickle-bed bioreactor. Also the relationship between lacasse activity of *T. versicolor* with HA removal was studied.

First experiments were conducted in fluidized-bed bioreactors with *T. versicolor* pellets with periodic partial fungal renovation. The partial renovation of the fungal biomass has been reported to be effective in maintaining the fungal activity under non-sterile conditions,

with a cellular retention time of 21 days. This process was developed in our research group (Blánquez et al., 2006) for textile dyes removal from water and later successfully applied in different wastewater treatments (Badia-Fabregat et al., 2015b; Mir-Tutusaus et al., 2017a). The fungal biomass renovation facilitates the maintenance of a young fungal culture, which promotes the fungal activity and avoids operational problems, and therefore allow for continuous long-term operation of fungal reactor under non-sterile conditions.

Based on the previous good results working with trickle-bed bioreactors for the treatment of PhACs, in this chapter a trickle-bed bioreactor was operated for the HA removal from humic-rich wastewater

This chapter was performed in collaboration with Mostafa Zahmatkesh from the group led by Dr. Jules van Lier of the Delft University of Technology. They previously demonstrated the ability of *T. versicolor* to degrade HA, hence the objective of the collaboration was to run a continuous fluidized-bed bioreactor first with synthetic water and then with humic-rich wastewater from a food-processing industry. In this chapter, are presented only the results obtained with the humic-rich wastewater. Other results obtained in this collaboration are not included in this chapter, but a manuscript called “Continuous fungal treatment of humic-rich wastewaters under nonsterile conditions: application of a fluidized-bed bioreactor with partial renewal of fungal biomass” has been submitted to the Science of the Total Environment Journal including all of the results.

5.2 Results

5.2.1 Size exclusion chromatography

Since the HA comprise a broad range of molecules, each SEC chromatogram was sliced to three separate areas based on the MW distribution range, as it is shown in Figure 5.3. The large HA molecules having molecular weight of 6.5-1 kDa (Blue) comprise most of the HA complex. The lower molecular size acids and building blocks weighting between 1 and 0.5 KDa (Red), and small molecular size acids in range of 0.5-0.1 KDa (Green) cover the rest of the HA complex. This would help to not only qualitatively monitor the whole concentration of the HA as total area under the curve, but also determine the possible

changes in different portions of HA complex, which indicates changes in MW distribution of HA. The FA portion of the HA solution was also subjected to SEC analysis.

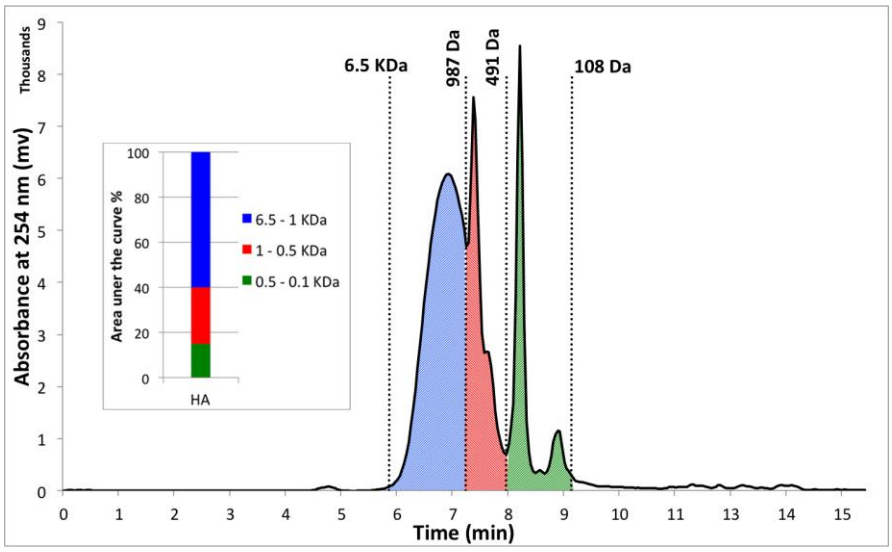


Figure 5.3. SEC chromatogram of HA and column presentation (Zahmatkesh et al. 2016).

5.2.2 Laccase *in vitro* experiments

In order to study the effect of laccase on HA degradation, experiments were carried out using purified laccase in absence and presence of mediators.

In the absence of mediators, only 20% reduction in the concentration of HA was observed after 24 h (Figure 5.4). Although, when comparing the results after 3 and 24 h, it becomes apparent that the degradation is very slow when there is no mediator present. ABTS proved to be the best mediator among the tested mediators. In the presence of ABTS, more than 60% of the humic acid was degraded after 24 h. The changes in the FA concentration was not as significant as in HA concentration. Although, in ABTS samples, a 40% increase in the FA concentration was observed. This simultaneous decrease in HA and increase in FA concentrations clearly shows the conversion of HA to FA by laccase in the presence of ABTS as the mediator. When VA was used as the mediator, 20% of HA was degraded after 24 h, without significant change in FA concentration. Results from this experiment indicate that laccase can degrade HA. However, the presence of mediators seemed to be crucial for enhancing the rate of the degradation.

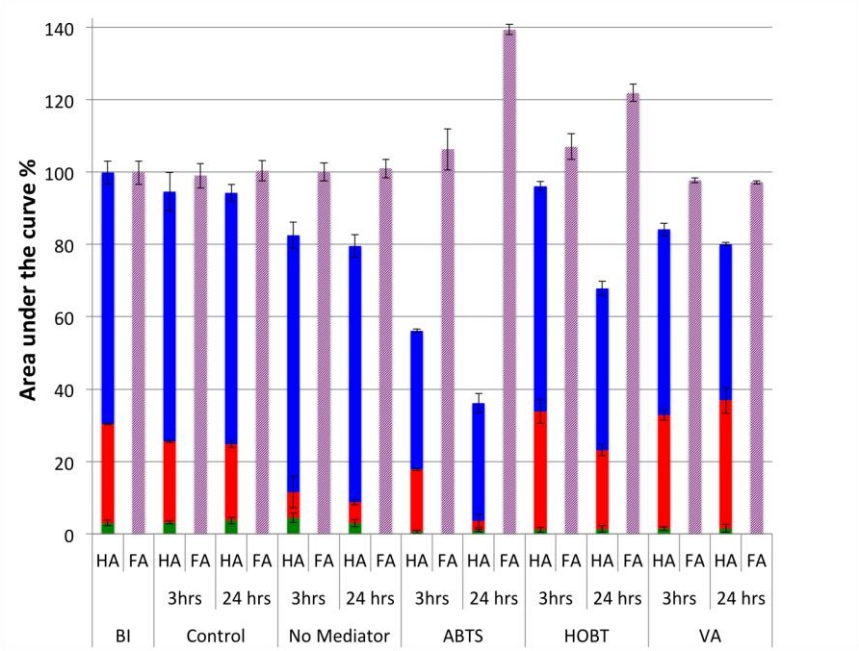


Figure 5.4. Effect of laccase with and without mediators on HA and FA portions of humic solution (BI: the Humic solution "Before Inoculation", Control: Humic acid without enzymes and mediators).

5.2.3 Continuous treatment of humic-rich wastewater

Industrial humic-rich wastewater was collected from the effluent of a wastewater treatment plant of a food-processing company (Eindhoven, The Netherlands) and stored at 4°C. Table 5.1 shows the wastewater characteristics.

Table 5.1 stewater.

Parameter	Humic rich-wastewater
pH	7
Conductivity ($mS \cdot cm^{-1}$)	4.32
Absorbance at 450 nm	0.23
HPC ($cfu \cdot mL^{-1}$)	$1 \cdot 10^3 \pm 5 \cdot 10^2$
Chloride ($mg\ Cl \cdot L^{-1}$)	819.43
Sulfate ($mg\ S \cdot L^{-1}$)	115.5
Nitrite ($mg\ N \cdot L^{-1}$)	14.4
Nitrate ($mg\ N \cdot L^{-1}$)	0.48
COD ($mg\ O_2 \cdot L^{-1}$)	294
TSS ($mg \cdot L^{-1}$)	6.5
VSS ($mg \cdot L^{-1}$)	5.5

5.2.3.1 Fluidized-bed bioreactor

A fluidized-bed bioreactor was used for the continuous treatment of humic-rich wastewater during 27 days. Two reactors were set-up in parallel: one inoculated with *T. versicolor* pellets (fungal reactor) and the other non-inoculated (control reactor). *T. versicolor* pellets were produced in Erlenmeyer flasks; 3.5 g $DW \cdot L^{-1}$ of simple pellets were added to the bioreactor and filled with 1.5 L of the wastewater. For the continuous treatment, the influent was fed with a HRT of 3 days. Every week, 1/3 of the biomass was replaced by fresh one, as determined by Blánquez et al. (2006). In both reactors, nutrients for maintenance (glucose as a carbon source and ammonia tartrate as a nitrogen source) were added to the reactors.

Figure 5.5 shows the color profile of the feeding tank, the fungal reactor and the control reactor. The color is presented in % and corresponded to the concentration over initial concentration (C/C_0). The difference at the time 0 is probably due the adsorption onto the fungal biomass.

The color of the wastewater in the feeding tank was stable (<10% change during the experimental period). However, the color of the control reactor was not stable, and increased to 55% higher than the initial color of the wastewater in the second week of treatment. Since the control reactor was also fed with the nutrient media, identical to the fungal reactor, this increase in the color could be due to growth of microorganisms in the media or fungal metabolites. The growth of bacteria, as well as fungi, can change the color of the media, usually resulting in an increase in the color (yellow to brown) of the media (Zahmatkesh et al., 2016, 2017a; Mert et al., 1977). Therefore, the changes in color of the media, resulting from fungal or bacterial growth could very well have masked the changes in color of the media resulted from HA removal.

As can be seen in Figure 5.5, the color of the wastewater in the fungal reactor was reduced to 60% (40% color removal) on day 5, but it started to increase slowly since then and reached 130% (40% higher than the initial color of the HA-rich wastewater) in the last week. Also in the last two weeks of the treatment, it was visually observed that the turbidity of the media was increased. Similar observation was made in the control reactor, but from the end of the first week onwards. The increase in turbidity is usually being considered as an indication of bacterial growth (Cruz-Morató et al., 2014; Gao et al., 2008). Overall, due

to the low initial color intensity of the humic-rich wastewater, it is not possible to evaluate the HA removal by monitoring the color of the media.

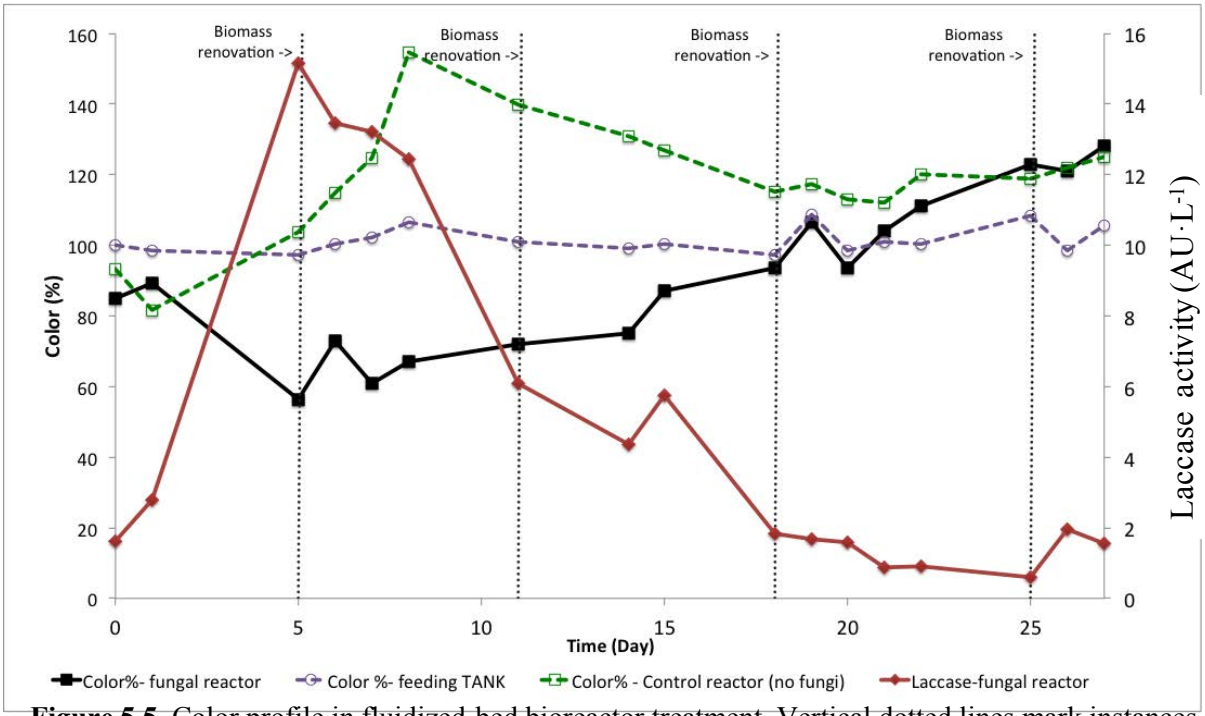


Figure 5.5. Color profile in fluidized-bed bioreactor treatment. Vertical dotted lines mark instances of partial fungal biomass renovations.

Therefore, the SEC analysis of the HA content of the wastewater during the fungal treatment becomes crucial for evaluating the HA removal.

In the humic-rich wastewater, large HA molecules comprised 57%, medium size HA covered 33% and small size molecules made up 10% of the total HA content (Figure 5.6). The average MW of the HA molecules in the humic-rich wastewater was 1.9 kDa. The SEC analysis of the feeding tank and the control reactor showed less than 15% change in the HA and less than 17% change in the FA content of the wastewater, indicating the stability of the humics (data not shown) .

In the fungal reactor after 5 days of treatment, almost all the large HA molecules were removed from the wastewater, and in total around 80% HA removal was achieved. It matches with the peak of laccase activity detected. It should be noted that on the same day that 80% HA removal was detected by SEC analysis, only 40% color removal was measured. However, by day 5 the stationary state of the bioreactor was not achieved

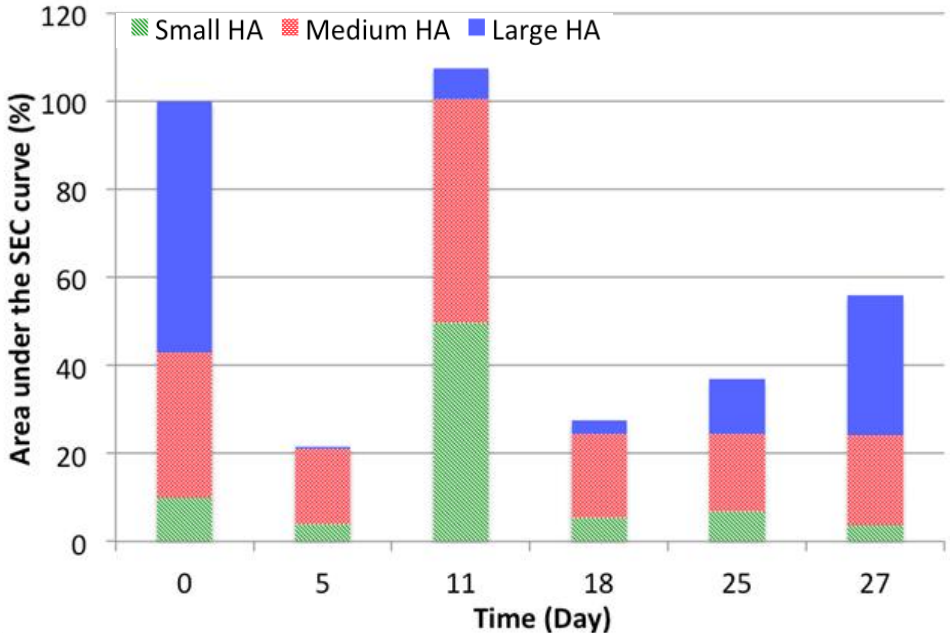


Figure 5.6. SEC analysis of the humic-rich wastewater during the fungal treatment in fluidized-bed bioreactor.

After 11 days of treatment, the total concentration (area under the SEC curve) of HA was higher than its initial concentration. This likely can be attributed to the vast increase in the small size HA molecules compared to their initial concentration (from 10% to 50%). The concentration of medium size HA molecules was also increased, but the concentration of large HA molecules was considerably reduced (from 57% to 7%). The average MW of the HA on day 11 was reduced to 0.65 kDa. The decrease in large HA and increase in small and medium size HA, suggest the incomplete degradation of large HA to smaller HA molecules. Corrales Escobosa et al. (2009) also demonstrated the degradation of HA with formation of lower MW soluble compounds by *Fusarium oxysporium*.

On day 18, the total concentration of HA was reduced to 27% of its initial value (73% removal). However, when compared to the decolorization results (Figure 5.5), the color of the wastewater was almost the same as its initial value (100%), indicating no color removal and no HA removal. This clearly demonstrate that the increase in the color of the wastewater is not related to the HA concentration. On day 25, there was an increase in the large HA molecules compared to day 18. This increase in large HA molecules continued more intensely until day 27, suggesting the decay in the fungal activity in the reactor.

The performance of the reactor along the wastewater treatment showed more than 90% removal of large HA molecules, until day 25. The results are comparable with other authors who also studied the HA removal from humic-rich wastewater applying different technologies. Zhai et al. (2016) have reported 66% HA removal efficiency by subcritical water catalytic oxidation technology with a batch reactor. Wang et al. (2016) reported the total removal of HA from concentrated leachates by ozonation. Biosorption of humic acid also has been studied using activated sludge (Feng et al., 2008) and *Rhizopus arrhizus* (Zhou et al., 1993).

During the fungal treatment of the humic-rich wastewater, the FA content of the wastewater was significantly increased (Figure 5.7). The FA concentration increased to 300% (3 times higher than its initial value) on day 11. The increase in the FA concentration continued with a lower rate (170% and 200% on day 18 and 25, respectively), but the FA concentration decreased to its initial value (100%) on day 27. The increase in the FA concentration suggests the incomplete enzymatic degradation of HA, which resulted in formation of FA molecules.

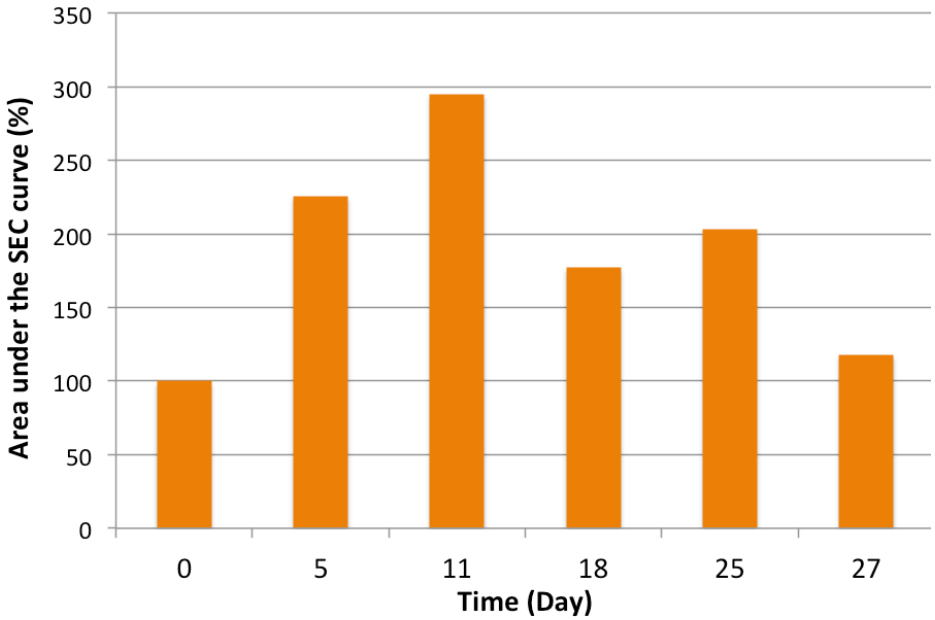


Figure 5.7. FA concentration of humic-rich wastewater during fungal treatment in fluidized-bed bioreactor (SEC analysis).

In addition, the decrease in the laccase activity is correlated with the decrease in the FA concentration after day 11, which suggests the involvement of laccase in conversion of HA to FA molecules. The conversion of HA to FA by laccase has been studied before in

previous section. Also, Fakoussa and Frost (1999) suggested the correlation between the decreased concentration of humic acids and the increased concentration of fulvic acid.

The concentration of FA, small and medium HA sometimes increased along the treatment. As indicated before, this increase results from the degradation of large HA into smaller molecules. The average MW of HA was decreased from 1.9 kDa to 0.6 kDa on day 11, and then it gradually increase to 1.8 kDa on day 27. The average MW of FAs in the wastewater was 0.6 kDa. This value started to increase from the second week of the treatment and reached 1.1 kDa on day 27.

The average laccase activity during the treatment was 5 $AU \cdot L^{-1}$. However, the laccase activity was used as a possible indicator of fungal activity. Working with real wastewater, detection of laccase activity confirms fungal activity; on the contrary the low level of the enzyme is not an indication of fungus inactivity as mentioned before along this thesis work.

Using the same fungal system (fluidized-bed bioreactor with *T. versicolor* pellets and weekly biomass renovation) treating synthetic tap water more than 75% HA removal was reported (Zahmatkesh et al., 2018 submitted). The lower level of removal obtained working with real wastewater is in accordance with previous studies (Hai t al., 2009). This performance deterioration is generally caused by the overgrowth of bacteria, which impose an inhibition on fungal growth and enzyme production (Sankaran et al., 2010; Yang et al., 2013).

Taking into account that the humic-rich wastewater was collected from a food processing wastewater treatment plant after going through multiple biological treatment units, it very likely contained more bacterial biomass than the synthetic wastewater.

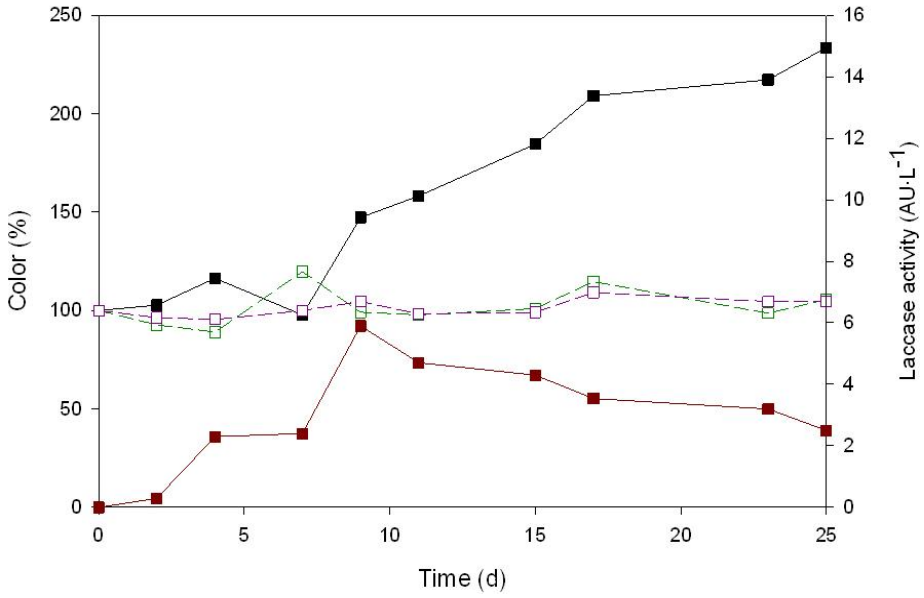
The observed decrease in efficiency of the reactor treating humic-rich wastewater from day 18 might be tackled by changing the process parameters in order to increase the fungal activity after week 3 of the treatment. Shortening the period of biomass renewal or increasing the portion of biomass that is getting renewed could be effective in increasing the fungal activity. Also, using a nutrient source that is more selective for fungi over bacteria could be helpful.

5.2.3.2 Trickle-bed bioreactor

Based on the observed decrease in efficiency of the fludized-bed reactor for the HA removal working with humic-rich wastewater, a trickle-bed bioreactor was proved with *T.versicolor* immobilized onto pallet wood. The total volume was 0.25 L and it was operated with a recycling ratio (RR) of 877. No additional experiments were performed in order to optimize this parameter, and the same RR as PhACs removal in chapter 4 was used. This election was based on the good results obtained for the hospital wastewater treatment and also due to limitation to get the humic acids humic-rich wastewater.

Two reactors were operated in parallel during 25 days: one filled with wood pre-inoculated with *T. versicolor* (fungal reactor) and the other with sterile wood (control reactor). No nutrients were added in both cases.

The color of the wastewater in the feeding tank and the control reactor were stable along the treatment, with less than <10% change as shown in Figure 5.8. In addition, the color profiles obtained were the same, suggesting that no modification in the color of the effluent occurs in the control reactor. This is a big difference compared with the control fluidized-bed reactor, when a color increase was detected, mainly due to the growth of bacteria because of the nutrient addition.



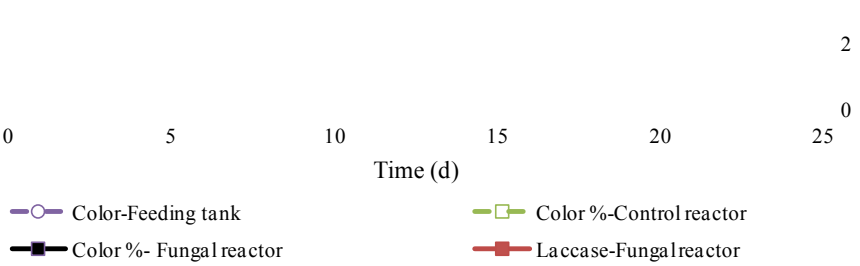


Figure 5.8. Evolution of the color in the trickle-bed bioreactor experiment.

In the fungal trickle-bed bioreactor, laccase activity was detected during all treatment in lower values compared with the fluidized-bed bioreactor. But in the same range of laccase activity when a trickle-bed bioreactor was employed for PhACs removal. Note that in the trickle-bed bioreactor, small amount of biomass was employed and no biomass renovation was performed. Figure 5.8 also shows the color profile of fungal reactor, a significant increase was obtained during the treatment. The final effluent was increased in 130% compared with the initial influent.

In one hand, when comparing the effluent color obtained along the fungal treatment in fluidized-bed bioreactor and the trickle-bed bioreactor (Figure 5.9), in both cases the color increase gradually during the experiment. 40% of color increase was detected in the fluidized-bed bioreactor and 130% in the trickle-bed bioreactor. Taking into account that in the first reactor partial biomass renovation was performed every week, it is probably that the adsorption played an important role in the decolourization.

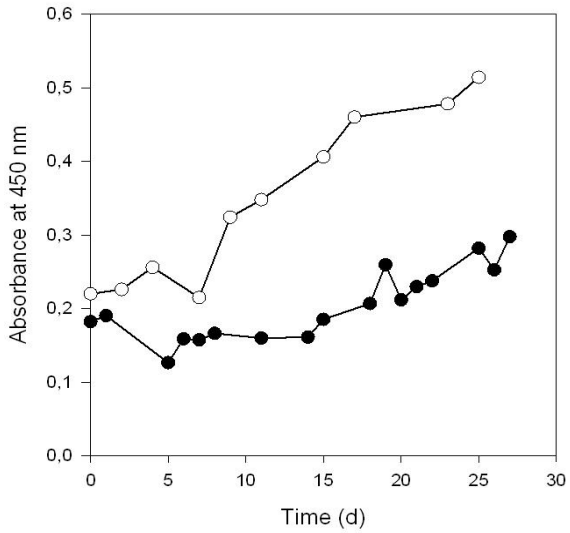


Figure 5.9. Effluent color obtained along fungal treatment in (●) the fluidized-bed bioreactor and (○) the trickle-bed bioreactor.

On the other hand, when comparing the use of the trickle-bed bioreactor for the treatment of different wastewaters: hospital wastewater, wastewater with pesticides (included in the next chapter) and humic acid (Figure 5.10), it is observed that the color only increases in

the case of humic acid treatment. This suggests that some interaction between the fungus attached to the wood and the wastewater increased the color, because the increase in the color is not observed B) ungi).

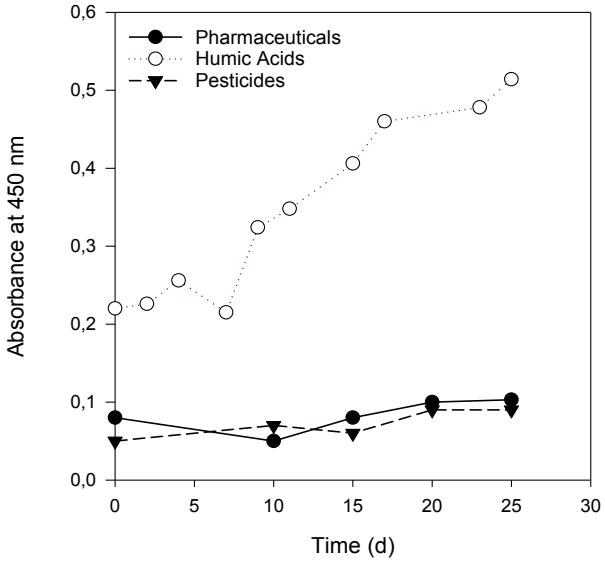


Figure 5.10. Effluent color obtained in trickle-bed bioreactor for the treatment of different humic-rich wastewater: pharmaceuticals, humic acids and pesticides.

As mentioned before, only the color is not a good indicator to study the humic acid removal from humic-rich wastewater. Hence, a SEC analysis was performed. The samples from the control reactor (with uninoculated wood) were not analyzed due to a precipitate formation in all samples, which prevented the HPLC injection.

In the humic-rich wastewater, large HA molecules comprised 51%, medium size HA covered 31% and small size molecules made up just 18% of the total HA content (Figure 5.11). The distribution is similar to the previous reported for the wastewater treatment in the fluidized-bed bioreactor.

For the trickle-bed bioreactor inoculated with *T. versicolor,* only two SEC analyses were analyzed. After 15 days, 90% of the humic acids were removed (only 10% of the Large HA remained) and the fulvic acids substantially increased up to 320% probably due to the transformation from HA to FA as has been previously explained. However, lower removal was obtained after 22 days of treatment, 50% of the humic acids were removed and consequently smaller concentrations of FA were detected at this time (only 17%).

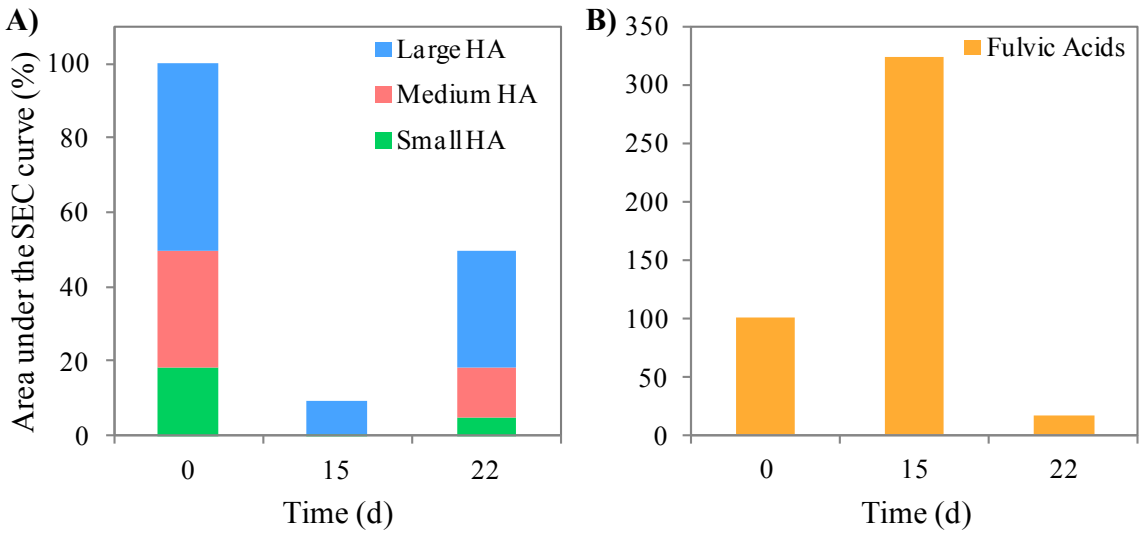


Figure 5.11. SEC analysis of trickle-bed bioreactor treatment.
A) HA concentration. B) FA concentration.

Based on the results from the SEC analysis, it can be concluded again that the color is not a good indicator of humic acid removal since in this case an increase in the fungal reactor color was detected meanwhile the SEC analysis shows the HA removal. However, due to the small amount of SEC samples and the differences obtained in the results suggesting that the stationary state was not achieved, it would therefore be premature at this stage to draw precise conclusions from this experiment. But, it is clear from the SEC results that the trickled-bed bioreactor inoculated with *T. versicolor* can remove the HA present in the humic-rich wastewater. More studies should be carried out for the optimization of the different operational parameters such as recycling ration and total volume in order to improve the HA removal.

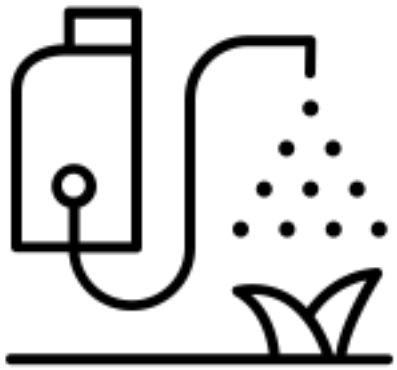
5.3 Conclusions

The use of a fungal bioreactor system is a good alternative for HA removal. In this study, the degradation of large HA molecules to smaller HA molecules and conversion of HA molecules to FA molecules were observed as result of the fungal treatment.

A fluidized-bed bioreactor with *T. versicolor* pellets was operated for 27 days and a trickled-bed bioreactor with *T. versicolor* immobilized on pallet wood for 25 days treating humic rich-wastewater. In both case, the color increased but the SEC analysis shows the HA removal. The global removal obtained in both bioreactors was 50% despite the difference in the biomass amount employed in each bioreactor. In the fluidized-bed bioreactor the fungal biomass was 3.2 $gDW \cdot L^{-1}$ and 1/3 of weekly biomass renovation was used; meanwhile, only 0.49 $gDW \cdot L^{-1}$ were employed in the trickle-bed bioreactor. Suggesting again the importance of the system conditions to ensure the viability and removal capacity of the fungus. However, in order to improve the HA removal and the wastewater decolorization, some operational parameters such as the recycling ratio and total volume should be optimized for continuous long-term treatment.

CHAPTER 6

Pesticides



Pesticides are widely used all over the world producing different environmental problems; therefore the study and develop of a technology for its elimination should be encouraged. In the first section of the chapter, the ability of *T. versicolor* to remove pesticides from treatment medium is presented. The second section presents the study and operation of a trickle-bed bioreactor and a packed-bed channel with *T. versicolor* immobilized on pallet wood for the continuous pesticides treatment.

6. 1 Introduction

Pesticides are widely used all over the world as they are necessary to sustain the agricultural sector, controlling the competition from weeds and the losses from insects and fungi (Bonnechère et al., 2012). However, their massive use has produced different environmental problems affecting animal and human health (Carter 2000; Köck-Schulmeyer et al., 2013; Zhang et al. 2011). A variety of pesticides are at this time included in the list of priority substances in the European Union regulations (Decision 2455/2001/EC), but many others are still unregulated.

The pesticides residues enter to aquatic environments mostly through surface runoff and wastewater effluents (Carvalho et al, 2002, 2017; Münze et al., 2017). The maximum pesticide concentration is achieved when it rains after the fumigation on the fields (Rabiet el al., 2010). These residues can be mutagenic, toxic and carcinogenic in low concentrations, representing a serious risk to human health and natural ecosystems (Qi et al., 2018).

Several methods have been developed for the pesticides removal divided into chemical, physical and biological. Some authors studied the biological treatment of pesticides using different systems and microorganisms: biomixture (Huete-Soto et al., 2017; Rodríguez-Rodríguez et al., 2018), rotary drum composter (Muntjeer et al., 2016), biopurification system (Diez et al., 2017), biosorption onto mycelium biomass (Behloul et al., 2017) and constructed wetlands (Gorito et al., 2017).

In bioremediation, different organisms are used for the pesticide degradation being the fungi a good alternative because they can degrade a wide range of pesticides. In particular, white-rot fungi can degrade different types of pesticides due to their unspecific oxidative enzymatic system (Mir-Tutusaus et al., 2014). Most of the published studies were conducted in soil, biomixtures or synthetic liquid medium spiked with pesticides (Mir-Tutusaus et al., 2014; Mori et al., 2017).

Working with real wastewater, a significant decline in the removal performance of the fungi commonly occurs; so many authors have studied some strategies to foster fungal growth and consequently maintain a good bioreactor performance (Mir-Turusaus et al., 2016; Yang et al., 2013). In chapter 4 of this work, a trickle-bed bioreactor with *Trametes*

versicolor immobilized over wood was successfully operated treating hospital wastewater, proving that the immobilization of the fungi on a lignocelullosic support is a good strategy to maintain the fungal activity.

Therefore, the objectives of this chapter are to study the ability of *T. versicolor* to degrade selected pesticides and then to apply a fungal reactor for continuous pesticides removal.

A sampling campaign in Llobregat River Basin (Catalunya, Spain) was performed in order to analyze the concentrations of pesticides. Based on the results obtained, the herbicide diuron was selected as the target compound for different experiments.

The first experiments were run at Erlenmeyer scale to study the ability of *T. versicolor* pellets to degrade the diuron at high concentrations ($mg \cdot L^{-1}$). Some additional experiments were performed to study the enzymes involved in the fungal degradation. Also, the capability of *T. versicolor* to degrade 3,4-Dichloroaniline, the main diuron transformation product, was studied.

Taking into account that pesticides in real wastewaters are usually present in the range of $ng \cdot L^{-1}$ to $\mu g \cdot L^{-1}$, new degradation experiments were run working at real environmental pesticides concentration. The experiments included diuron and 7 selected pesticides from different families of compounds.

In this chapter, a trickle-bed bioreactor with *T. versicolor* immobilized on wood was employed for the pesticides removal. Some operational parameters were optimized and then a long-term reactor was operated for the removal of pesticides from real wastewater.

Finally, a packed-bed channel bioreactor was developed and operated for the diuron removal in continuous treatment. The first experiments were run to study and identify the operational conditions that affect bioreactor performance. And secondly, a long-term bioreactor was operated for the diuron removal from synthetic tap water and real wastewater.

6. 2 Results

6.2.1 Characterization of real water from Llobregat River Basin

Real wastewater was collected from the Llobregat River Basin located in the northeast of Catalunya (Spain). The river extension is just over 170 km and its basin of 4,948 km², from the pre-Pyrenees mountains to its mouth in the Mediterranean Sea (Gónzalez et al., 2012).

The river has around 40 tributaries, but the main ones are the Cardener and Anoia rivers. Both rivers are a focus of pollution due to the agricultural activity in the zone (Gónzalez et al., 2012; Cespedes et al., 2005).

The first sampling campaign was performed during February 2017 and 51 pesticides were analyzed. Water was collected at 14 selected sites along the Llobregat River Basin.

Results obtained for the first sampling campaign are summarized in Table 6.1 (sum, maximum concentrations, average levels, and frequency of detection). 29 of the 51 pesticides analyzed were detected at concentrations over the detection limit (LOD) (approximately 56% of analytes). Organophosphorus, triazines, ureas, phenoxys, amides, carbamates, chloroacetanilides, neonicotinoids and nitriles were detected at concentrations ranging from <1 $ng \cdot L^{-1}$ to 2000 $ng \cdot L^{-1}$.

Terbutryn and diuron were the most frequent pesticides, occurring in 93% of the samples analyzed. Diuron presented the highest total value of all the sample points (2098 $ng \cdot L^{-1}$). It is a phenylurea herbicide applied to a wide variety of crops and frequently detected in surface waters.

On the other hand, terbutryn is a pesticide from the triazine family. It has been used as a selective herbicide on differents crops; and also terbutryn has been added to the list of priority substances of Commission Proposal 2011/876/EC.

Bromoxynil was the pesticide with the maximum value detected (1593 $ng \cdot L^{-1}$) in the sample point 4 (Figure 6.1), probably due to a specific single use since it was only detected in 2 points (14% of frequency). Bromoxynil is used for the control of broadleaved weeds in grain crop, classified as group C possible human carcinogens (U.S. EPA, 1998; Greene and Pohanish, 2005) and it has relatively high toxic effects on aquatic organisms.

Table 6.1. Sum, maximum and mean concentrations and frequency of detection of the studied

Family	Pesticides	09/02/2017				
		Concentration ($ng \cdot L^{-1}$)			Frequency	
		Sum	Max	Mean	N°	%
Amides	Diflufenican	279	145	140	2	14
Carbamates	Methiocarb	181	131	90	2	14
	Molinate	59	33	30	2	14
Chloroacetanilides	Alachlor	42	24	21	2	14
	Metolachlor	58	28	19	3	21
	Propanyl	19	19	19	1	7
Neonicotinoids	Imidacloprid	194	194	194	1	7
	Thiacloprid	47	31	24	2	14
Nitriles	Bromoxynil	1593	1519	797	2	14
Organophosphorus	Azinphos Ethyl	186	106	62	3	21
	Chlorfenvinphos	124	67	31	4	29
	Diazinon	163	71	23	7	50
	Dichlorvos	130	130	130	1	7
	Fenthion Oxon	37	37	37	1	7
	Fenthion Sulfoxide	32	32	32	1	7
	Malaoxon	48	24	24	2	14
	Malathion	57	32	28	2	14
Phenoxys	2,4- D	330	197	165	2	14
	Mecoprop	139	78	69	2	14
Triazines	Atrazine	52	21	17	3	21
	Cyanazine	29	29	29	1	7
	Irgarol	76	41	25	3	21
	Simazine	36	20	18	2	14
	Terbuthylazine	66	30	13	5	36
	Terbutryn	932	160	72	13	93
Ureas	Chlortoluron	206	67	41	5	36
	Diuron	2098	502	161	13	93
	Isoproturon	49	25	25	2	14
	Linuron	1140	524	380	3	21

Figure 6.1 shows the concentration of pesticides detected in water at each sampling point classified into family's compounds.

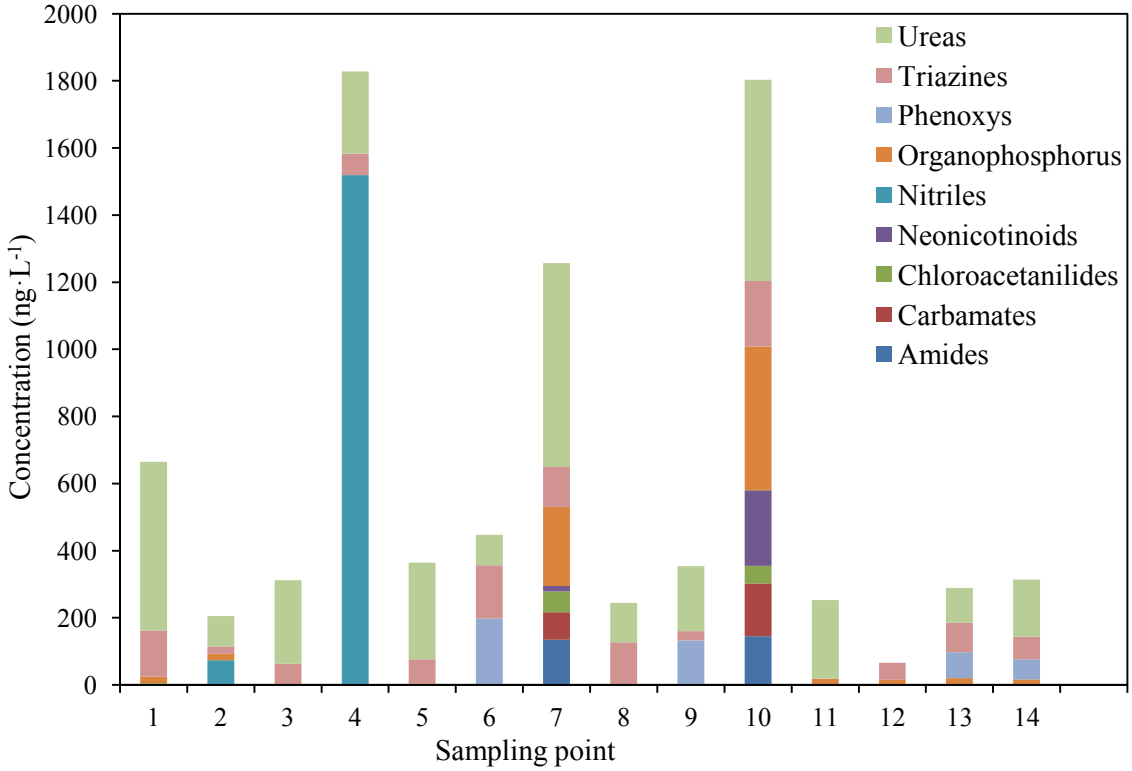


Figure 6.1. Pesticide families detected at each sampling point during the campaign at Llobregat River Basin. Sampling point = 1: Rubi stream. 2: Anoia River. 3: Anoia-Rubí confluence. 4: Infanta channel (end). 5: Infanta channel (before WWTP). 6: Sant Feliu WWTP. 7: Channel near highway. 8: Governor pipe. 9: Output Gavà WWTP. 10: Gavà Channel (point 1). 11: Gavà Channel (point 2). 12: Inlet Gavà WWTP. 13: Output Gavà IFAS. 14: Output Gavà MBR.

The points 4 and 10 were the sites with the higher total concentration of pesticides, in the case of point 4 due to the bromoxynil as mentioned before. The point 10 corresponds to an irrigation channel in Gavà zone, it presented a wide variety of pesticides from almost all the families analyzed. 24 of the 29 pesticides detected in all the sampling campaign were detected at this point. Based on these results obtained from the first campaign, the point 10 was chosen as the point for the water collection in future experiments and the pesticide diuron was chosen as the target compound for future experiments because it was the most frequently detected at higher concentrations.

6.2.2 Diuron degradation and enzymatic studies

Diuron (CAS 330-54-1) is widely used as a broad-spectrum herbicide for controlling weeds in sugarcane, citrus and coffee crops (da Silva Coelho-Moreira et al., 2013). The mean half-life of diuron in soil is around 320-330 days (Fontecha-Camara et al., 2007); from the soil the diuron arrives to the water bodies through leaching and runoff (Langeron et al., 2014; Louchart et al., 2001). Consequently, diuron is found in surface and groundwater at concentrations above the EU limit of 0.1 $\mu g \cdot L^{-1}$ (Lapworth and Gooddy 2006).

Diuron is included in the Priority Hazardous Substance list of the European Commission (Directive 2000/60/CE). It is classified as extremely harmful to aquatic ecosystems and as endocrine disruptor compound.

The structure of diuron is responsible for its recalcitrance and toxic potential in the environment. But, bioremediation is a promising alternative because it has been reported that the dissipation of diuron in the environment is caused by biological processes (Cameron et al., 2000).

Giacomazzi and Cochet (2004) pointed out the diversity of microorganisms involved in the diuron degradation and the accumulation of 3,4-Dichloroaniline (3,4-D) as a final degradation product. Figure 6.2 summarizes the main metabolic pathways for the total mineralization of diuron by fungi. It must be taken into consideration that not all the mechanisms involved are well known.

Degradation of diuron could lead to apparition of different transformation products and 3,4-D is the main metabolite (Tixier et al., 2002). However, fungal degradation of 3,4-D was also reported (Sandermann et al., 1998; Tixier et al., 2002). Another study reported the conversion of 3,4-D onto N-(3,4-dichlorophenyl)-b-ketoglutanyl-d-amide which was then mineralized (Sandermann et al., 1998).

In this section, experiments were done in order to evaluate the ability of *T. versicolor* to degrade diuron and its principal metabolite 3,4-D at high concentration. Enzymatic studies were also performed in order to know the involvement of the different enzymes.

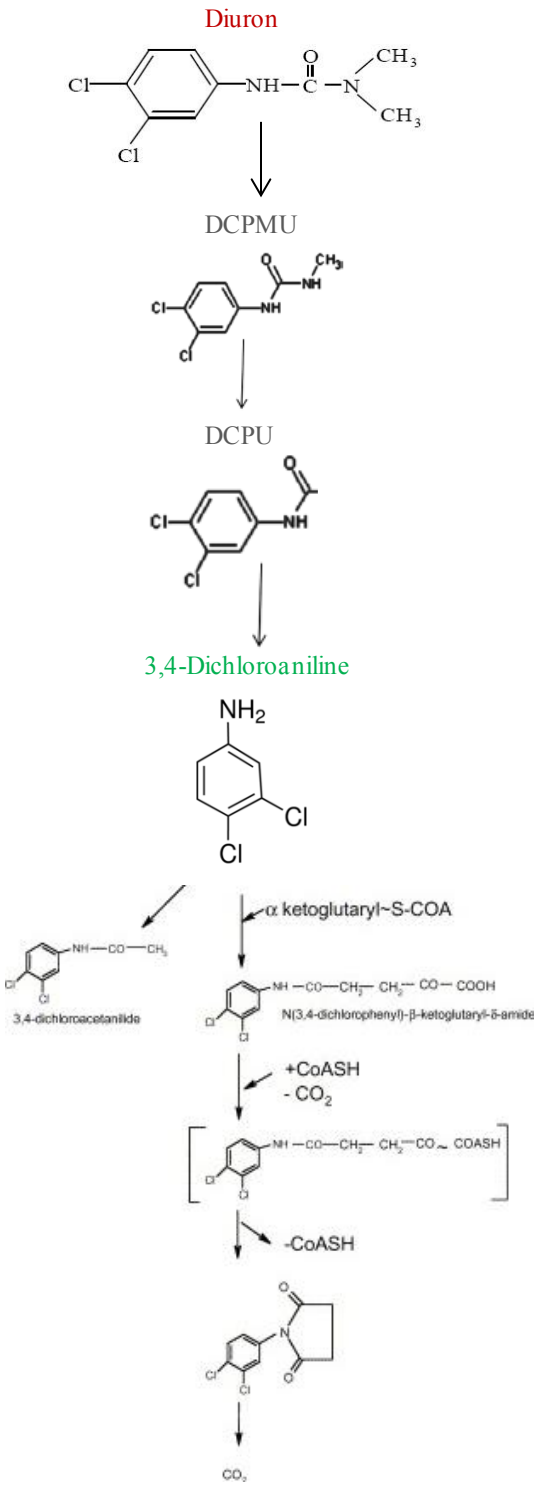


Figure 6.2. Fungal degradation pathways of diuron and 3,4-Dichloroaniline. DCPMU: [1-(3,4-dichlorophenyl)-3-methylurea], DCPU: [1-(3,4-dichlorophenyl) urea. (modified from Giacomazzi and Cochet, 2004)

6.2.2.1 Diuron removal by *T. versicolor* in treatment medium

Experiments were performed in Erlenmeyer flask with *T. versicolor* pellets in treatment medium spiked with diuron to a final concentration of 8 mg·L^{-1}. Two different biomass amounts were tested (1.4 g DW·L^{-1} and 3.2 g DW·L^{-1}).

In the experiments were included: experimental sets with *T. versicolor* pellets, killed controls with the same amount of heat-killed fungus to study the sorption, and abiotic controls to study the stability of the compounds.

T. versicolor was able to remove diuron, reaching an elimination of 72% in 12 days with 1.4 g DW·L^{-1} of pelletized biomass and 77% diuron removal in 6 days with 3.2 g DW·$L^{-1}$of pelletized biomass. In both cases, the abiotic losses and the sorption to the fungal biomass were negligible. The profiles of diuron removal cultures are shown in Figure 6.3.

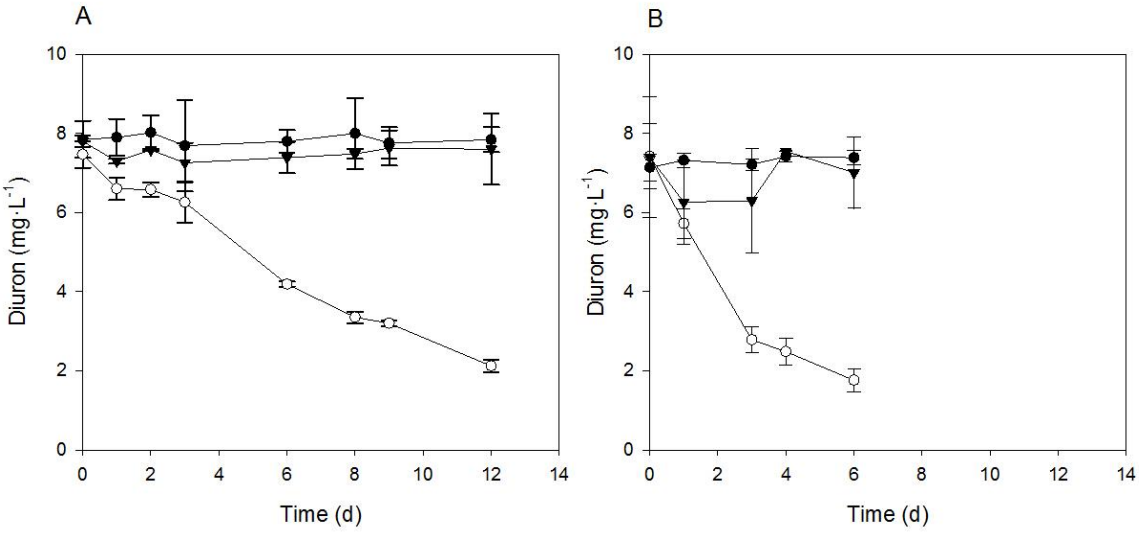


Figure 6.3. Diuron concentration profiles in the removal assays with *T. versicolor* pellets in Erlenmeyer flasks. Experiments with 1.4 g DW·L^{-1} of biomass (A) and 3.2 g DW·L^{-1} of biomass (B). Symbols: (●) uninoculated controls, (▼) heat-killed controls and (○) experimental assays. Values plotted are the means ± SD for triplicate cultures.

The diuron degradation rates were 0.45 and 0.97 mg diuron·d^{-1} employing 1.4 and 3.2 g DW·L^{-1} of biomass respectively. The difference in the degradation rates was due to the amount of pellets used in each experiment, demonstrating the correlation between the diuron removal and fungal concentration as other authors already reported (Ellegaard-Jensen et al, 2013). However, the same diuron degradation rate value was obtained taking into account the dry weight biomass employed, as it is shown in Table 6.2. In both cases, 0.3 mg diuron·[d·(g DW)]$^{-1}$ was obtained.

Other authors also studied the diuron degradation by fungi in liquid medium as it can be seen in Table 6.2. In some cases, they obtained higher degradation values (100%) probably due to longer contact time (Ellegard-Jensen et al., 2013). Only Bending et al. (2002) worked with *T. versicolor* and obtained good degradation values but employing a longer time of treatment (42 days). It was not possible to do a direct comparison since it was not clear the amount of biomass employed in that work.

However, the removal rates per unit of biomass obtained in this work are in the same order compared with others authors. Outstanding results were reported by Perissini-Lopes et al. (2016) and Wang et al. (2017) with removal rates almost 10 times bigger than the results obtained in this work, probably because they used strains with proven degradation capacity and isolated directly from soil with historical application of diuron.

Table 6.2. Diuron degradation experiments by fungi. The removal rate was calculated by the author based on the results reported.

Fungi	Time (d)	Initial diuron[a]	Biomass[b]	Removal (%)	Removal rate per biomass[c]	**Author**
A.brasiliensis	7	10	0.86	85	1.41	Perissini-Lopes et al., 2016
Dacrypionax elegans	7	10	4	40	0.14	Araraki et al., 2003
Ganoderma lucidum	10	7	5.2	55	0.07	da Silva Coelho et al., 2010
Mortierella sp.	28	5	1.9	100	0.09	Ellegard-Jensen et al., 2013
N. intermedia	3	50	4.8	95	3.28	Wang et al., 2017
P. chrysosporium	10	7	5	95	0.13	da Silva Coelho et al., 2013
P. chrysosporium	14	10	1.5	75	0.36	Fratila-Apachitei et al., 1999
T. versicolor	42	10	--	94	--	Bending et al., 2002
T. versicolor	6	7.5	3.2	77	0.30	This work
T. versicolor	12	7.5	1.4	72	0.32	

[a] Initial diuron units = $mg \cdot L^{-1}$

[b] Biomass units = $g\ DW \cdot L^{-1}$

[c] Removal rate per biomass units = $mg \cdot [d \cdot (g\ DW)]^{-1}$

Some authors optimized different parameters in the degradation experiments and consequently obtained more than 90% of diuron degradation, but the first aim in this work was to prove that *T. versicolor* was able to degrade diuron at high concentrations for future bioreactor applications. Based on that purpose no other experiments were carried out to increase the diuron removal in liquid medium employing pelletized biomass.

6.2.2.2 Experiments with purified laccase and cytochrome P450

- ***Purified laccase***

The capability of pure laccase extracts to degrade diuron *in vitro* was tested with and without the addition of different mediators (VA, HOBT, ABTS). No significant differences were observed between the control and the treatments containing enzymes and mediators as it is shown in Table 6.3. These results indicate that the laccase is not involved in the diuron degradation and they are in concordance with a previous study (da Silva Coelho-Moreira et al., 2013).

Table 6.3. Effect of laccase with and without mediators on diuron removal. (Control: without enzymes and mediators).

Time (h)	Diuron ($mg \cdot L^{-1}$)				
	Control	Laccase	VA	HOBT	ABTS
0	10.01 ± 0.41	9.69 ± 0.91	9.02 ± 0.31	9.41 ± 0.29	9.58 ± 0.72
8	9.83 ± 0.38	9.74 ± 0.59	8.96 ± 0.47	9.25 ± 0.71	10.35 ± 0.21
24	9.45 ± 0.75	9.40 ± 0.6	9.69 ± 0.49	10.60 ± 0.83	9.66 ± 0.35
72	9.32 ± 1.1	9.01 ± 0.89	9.54 ± 1.10	9.13 ± 1.47	10.74 ± 0.16

- ***Cytochrome P-450 inhibition***

The effect of 1-aminobenzotriazole (ABT), a cytrochrome P450 inhibitor, on the fungal degradation of diuron was also studied. As Figure 6.4 shows, inhibitor free cultures and those containing ABT presented similar percentages of diuron removal for the first 24h, probably due to sorption processes. After this period, degradation was inhibited in cultures with ABT whereas diuron in inhibitor free-cultures was almost totally degraded after 6 days.

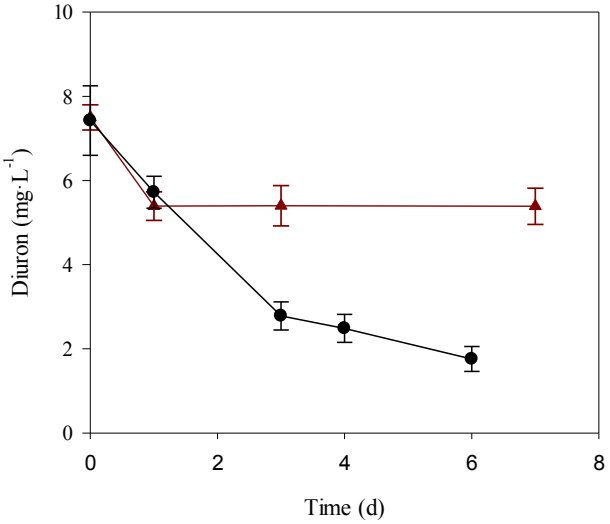


Figure 6.4. Influence of the cytrochrome P450 inhibitor (ABT) on the degradation of diuron. Symbols: (●) inhibitor-free controls and (▲) cultures containing ABT. Values plotted are the means ± SD for triplicate cultures.

These data combined with the data showing no participation of laccase in diuron degradation suggest the involvement of cytrochrome P450 in the degradation of this pesticide, which is in concordance with a previous study (da Silva Coelho-Moreira et al., 2013).

6.2.2.3 Removal of the transformation product 3,4-Dichloroaniline by *T. versicolor*

Degradation experiments were done in order to study the ability of *T. versicolor* to degrade 3,4-Dichloroaniline (3,4-D), the main metabolite formed by the diuron degradation. Experiments were performed in Erlenmeyer flask with *T. versicolor* pellets in treatment medium spiked with 3,4-D to a final concentration of 10 $mg \cdot L^{-1}$. Abiotic and killed-controls were included. In these experiments, 3.2 g $DW \cdot L^{-1}$ of *T. versicolor* pellets were employed.

The profiles of 3,4-D removal in liquid medium cultures are shown in Figure 6.5. The abiotic losses and the sorption to the fungal biomass were negligible. *T. versicolor* was able to completely remove 3,4-D in only 24 hours.

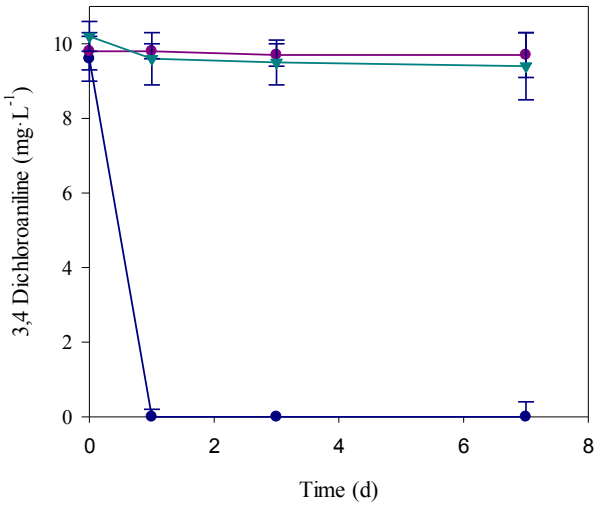


Figure 6.5. 3,4-D concentration profiles in the removal assays with *T. versicolor* pellets. Symbols: (●) experimental assays, (●) uninoculated controls and (▼) heat-killed controls. Values plotted are the means ± SD for triplicate cultures.

6.2.3 Degradation experiments of selected pesticides

In this section, the ability of *T. versicolor* to degrade selected pesticides was studied. The experiments were done in Erlenmeyer flasks containing treatment medium spiked with a mix of 8 pesticides. Since pesticides are found in the aquatic environment at low concentrations (range from $ng \cdot L^{-1}$ to $\mu g \cdot L^{-1}$), the experiments were carried out at 5 $\mu g \cdot L^{-1}$

for each pesticide to assess the capability of *T. versicolor* to degrade it at environmentally relevant concentrations. Hence, due to these low concentrations, the analyses were performed by the Water and Soil Quality Research Group from Institute of Environmental Assessment and Water Research (IDAEA-CSIC, Barcelona, Spain).

In the experiments were included: experimental sets with *T. versicolor* pellets, killed controls with the same amount of heat-killed fungi to study the sorption, and abiotic controls to study the stability of the compounds. More details of the methodology can be found in chapter 3.

Table 6.4 presents the pesticides used in this section and their characteristics. The selection was made with the aim of studying different family's substances. Two groups can be distinguished according to their solubility in water: hydrophobic and hydrophilic. The pesticides were divided into these groups for analytical purposes. The same division was made for the presentation of the results.

Table 6.4. Pesticides characteristics (Pesticide Properties Database, University of Hertfordshire).

Group	Pesticide	Family	Log P	Solubility in water ($mg \cdot L^{-1}$ at 20°C)
Hydrophobic	Chlorpyrifos	Organophosphorus	4.7	1.00
	Cypermethrin	Pyrethroids	5.55	0.01
	Dicofol	Organochlorines	4.3	0.80
Hydrophilic	Diuron	Phenylureas	2.87	35.60
	Irgarol	Triazines	3.95	7.00
	Malathion	Organophosphorus	2.75	148.00
	Simazine	Triazines	2.3	5.00
	Triallate	Thiocarbamates	4.06	4.10

For the hydrophobic group, samples were taken from the liquid medium and fungal biomass. Meanwhile, only liquid medium samples were analyzed for the hydrophilic group. Glucose concentration and laccase activity were also measured along the experiments.

The fungal transformation products (TPs) of the selected pesticides, which are listed in Table 6.5, have been previously reported by other authors. Only in the case of triallate, no fungal TPs were reported to the author's knowledge. In this work, TPs have not been

analyzed yet, but taking into account the previously reported products, the analysis of TPs will be carried out proximally.

Table 6.5. Pesticides and fungal degradation products.

Pesticide	Fungi	Degradation products	References
Chlorpyriphos	*Verticillum sp.*	3,5,6-trichloro-2-pyridinol	Yu et al., 2006.
	Acremonium sp.	3,5,6-Trichloropyridyl-2-phosphorothioate	Kulshresta and Kumari, 2011.
Cypermethrin	*T. versicolor*	3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid; 3-phenoxybenzoic acid	Mir-Tutusaus et al., 2014.
Dicofol	Cellulasa from *Trichoderma longbrachiatum*	4,4′-dichloro-dibenzophenone	Wang et al., 2015.
Diuron	*Mortierella sp.*	1-(3,4-dichlorophenyl)-3-methyl urea; 1-(3,4-dichlorophenyl) urea	Elleegard et al., 2014.
	P. chrysosporium	3,4-dichloroanliline; 3,4-dichloroacetanilide	da Silva Coelho et al., 2013.
Irgarol	*P. chrysosporium*	2-methylthio-4-tert-butylamino-s-triazine	Liu et al., 1997.
Malathion	*Aspergilus oryzae*	β-monoacid; malathion dicarboxylic acid	Singh et al., 2014.
Simazine	*Rhodococcus Sp.*	Deisopropylatrazine	Behki et al., 1994.
Triallate	--	--	

- ***Glucose consumption and laccase activity***

Glucose was the only carbon source available. As it shows Figure 6.6, glucose was totally consumed after 7 days of experiment. Same glucose consumption rate (0.3 g ·g $DW^{-1}·d^{-1}$) was previously reported in our research group (Blánquez, 2005).

In an attempt to monitor the fungal activity, the laccase extracellular activity was analyzed during the experiments. Laccase activity increased during the first week to a maximum value at day 7 (48 $AU·L^{-1}$) and then it was maintained at the same value to the end of the experiments. At Erlenmeyer flask scale, a peak in laccase activity is usually observed in the day 1 or 2 (Castellet, 2017) for similar experiments treating another pollutants. In our case the peak was not detected probably because the samples were taken

only at times 0 h, 12 h, 3 days and 7 days, and maybe the peak was between these times. However, the laccase activity obtained is in the same range of the results previously reported (Castellet 2017; Cruz-Morató, 2014).

Glucose consumption rate and laccase activity observed are in agreement with previous works from our research group, concluding that the fungus was active during the experiments and that the pesticides chosen are not toxic for *T. versicolor* at low concentration.

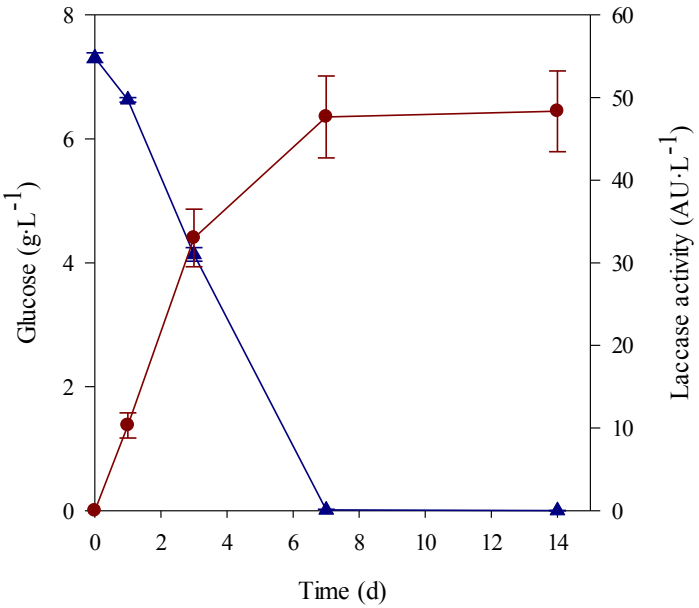


Figure 6.6. (▲) Glucose concentration and (●) laccase activity in experimental sets.

6.2.3.1 Degradation experiments of hydrophobic pesticides.

As mentioned before, the hydrophobic pesticides tested were: chlorpyrifos, dicofol and cypermethrin.

Chlorpyrifos is one of the most widely used broad-spectrum organophosphorus pesticide. It is effectively used against a various insect pests of crops with high economic importance (Singh et al., 2012a). The continous application of chlorpyrifos causes accumulation in soil affecting the microorganisms present in soil and insects (beetles, bees and wasps) and aquatic organisms (Jabeen et al., 2015). Chlorpyrifos has been detected in different environmental samples with a half-life from days to 4 years (John and Shaike, 2015).

Dicofol is a low-toxicity pesticide used globally in many countries (Zahm and Ward, 1998). Dicofol is synthesized by dichlorodiphenyltrichloroethane (DDT), a pesticide that has been restricted by the Stockholm Convention (Wang et al., 2015b), and both pesticides present similar structures. Due to its structure, the dicofol degradation is difficult in the environment (Hoekstra et al., 2006). The use of dicofol is prohibited in most of the developed countries due to its toxicity and environmental persistence (Li et al., 2015c).

Cypermethrin is a synthetic pyrethroid pesticide and it is used to control pests in cotton and vegetable crops (Akbar et al., 2015). It is highly toxic to fish and aquatic invertebrates (Bradbury and Coats 1989). Cypermethrin has been classified as a possible human carcinogen by US Environmental protection agency (EPA).

For this pesticides group, samples from liquid media and fungal biomass were analyzed. The results are presented divided into the different trials performed: abiotic control, killed control and experimental sets.

- ***Abiotic pesticide stability assessment***

Abiotic controls were performed only with the treatment medium and the pesticides in order to study the compound stability. Erlenmeyer flasks were spiked to a final concentration of 5 $\mu g \cdot L^{-1}$ of each compound. Figure 6.7 presents the mean detected concentration of each pesticide along the experiment. In all cases, no statistical differences were detected in the concentrations along the experiment, demonstrating the compound stability.

Dicofol was detected at low concentration in all samples, probably due to the adsorption onto the Erlenmeyer glass; since it is a pesticide with a high affinity to be adsorbed onto different materials (Aboulfadl et al., 2001; Beltran et al., 1995; Oliveira et al., 2012). No desorption from glass was proved.

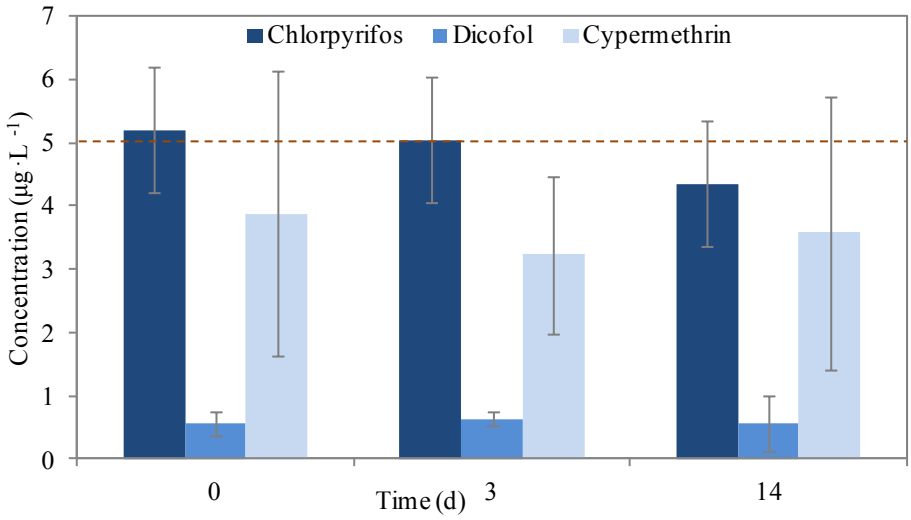


Figure 6.7. Pesticides concentrations in abiotic controls. Dotted line represents the theoretical initial concentration. Error bars represent the SD for triplicate cultures.

In the case of cypermethrin, the mean initial concentration was 3.88 $\mu g \cdot L^{-1}$ (instead of 5 $\mu g \cdot L^{-1}$), probably due to the errors in the samples analysis if we take into account the standard deviation between samples as it shows Figure 6.7.

In summary, for the hydrophobic pesticides, no removal was detected in abiotic controls since same pesticide concentration was detected at each sample point.

- ***Sorption contribution in pesticides removal***

In order to study the sorption onto the fungal biomass, killed controls were performed with the treatment medium spiked with the pesticides and heat-killed *T. versicolor* pellets.

Figure 6.8 shows the results obtained from liquid medium samples. In the case of chlorpyrifos and cypermethrin, statistical differences were obtained in the pesticide concentration during the experiment. In contrast, for dicofol same concentration was detected in all samples. In all cases, the initial concentration was lower than the theoretical one. At the end of the experiment, chlorpyrifos was detected at 0.15 $\mu g \cdot L^{-1}$, dicofol at 0.003 $\mu g \cdot L^{-1}$ and cypermethrin was not detected.

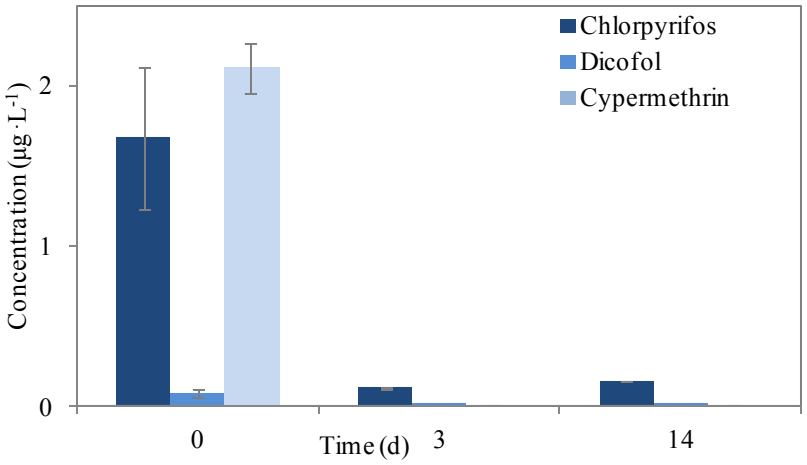


Figure 6.8. Pesticides concentration in liquid samples from killed control. Error bars represent the SD for triplicate cultures.

In order to study if the pesticides were adsorbed, samples from fungal biomass were also analyzed. The results are presented in Figure 6.9. In all cases, no statistical differences were detected during the treatment. It means that the pesticides are adsorbed onto the *T. versicolor* pellets from the beginning and the same absorbed amount is maintained along the 14 days. In the case of dicofol, higher concentration was detected in fungal biomass compared with liquid medium sample since as mentioned before it has high affinity to be adsorbed onto different materials.

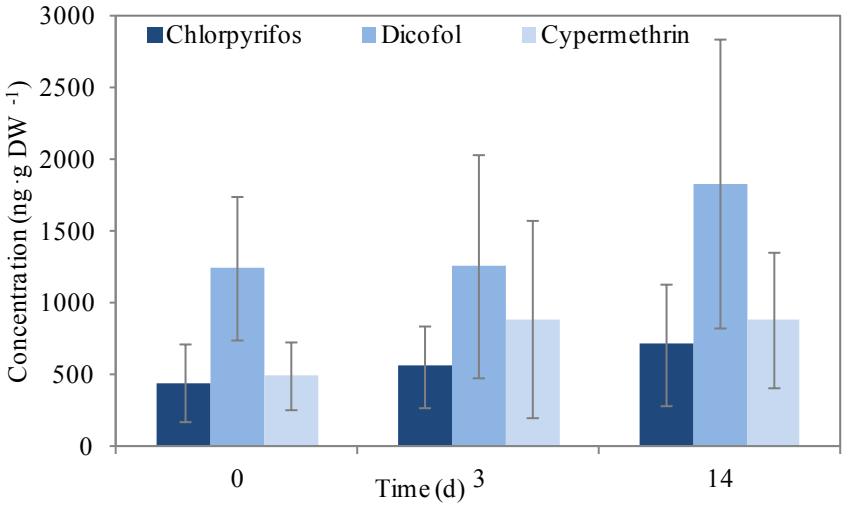


Figure 6.9. Pesticides concentration in fungal biomass samples from killed control. Error bars represent the SD for triplicate cultures.

Finally, the mass balance is presented in Figure 6.10 where the results obtained from liquid medium and biomass samples are combined. Pesticides weight is the sum of

pesticides weight in the liquid medium and in the biomass. At time 0, in the case of chlorpyrifos and cypermethrin, the initial amount did not achieve the theoretical spiked amount (500 ng), probably due to experimental errors and also because samples were not acquired at time 0 since the protocol for samples extraction takes at least 30 minutes (section 1.7.3.2). For the three pesticides, no statistically differences were obtained along the experiments. That means that the same pesticide amount was adsorbed from the beginning of the treatment to the end. The great sorption of these pesticides is due to its high partition coefficients indicating its hydrophobic character.

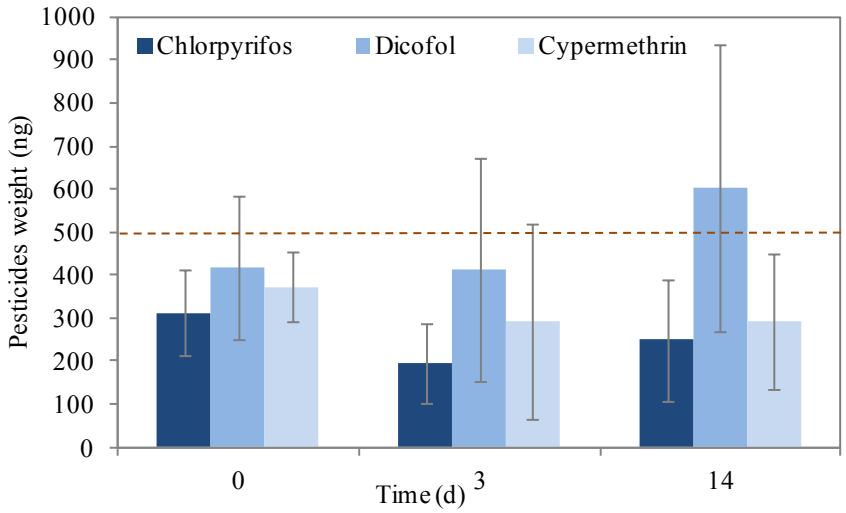


Figure 6.10. Pesticides mass balance for killed controls experiments from liquid medium samples and biomass samples. Dotted line represents the theoretical initial amount. Error bars represent the SD for triplicate cultures.

- ***Removal of pesticides by T. versicolor***

In the experimental set, the Erlenmeyer flasks contained the treatment medium with the pesticides and *T. versicolor* pellets. Samples were acquired from the liquid medium and fungal biomass.

Figure 6.11 shows the results obtained for the liquid medium samples. In all cases, statistical differences were obtained for the concentration along the experiment. It is remarkable that any of the pesticides is presented at the theoretical concentration (5 $\mu g \cdot L^{-1}$) at time 0, probably due to the high affinity to be adsorbed onto fungal pellets and glass.

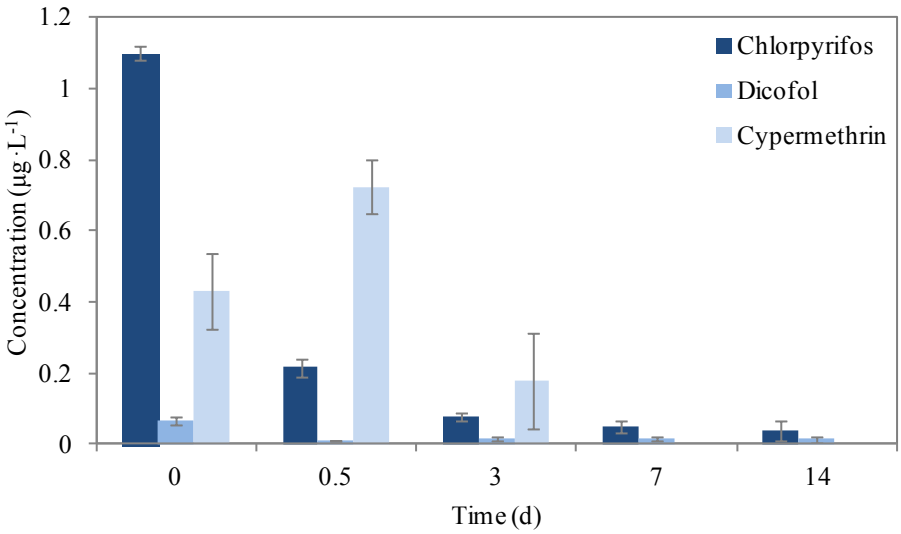


Figure 6.11. Pesticides concentration in liquid medium samples from experimental set. Error bars represent the SD for triplicate cultures.

Therefore, with the aim to study if the pesticides were degraded or adsorbed, the fungal biomass was analyzed. Results are presented in Figure 6.12. For all pesticides, statistical differences were obtained along the treatment. Pesticides concentration changed during the experiment, in contrast with the killed control results, where no differences in pesticides concentration were detected. These results suggest that chlorpyrifos, dicofol and cypermethrin are biodegradated by *T. versicolor*.

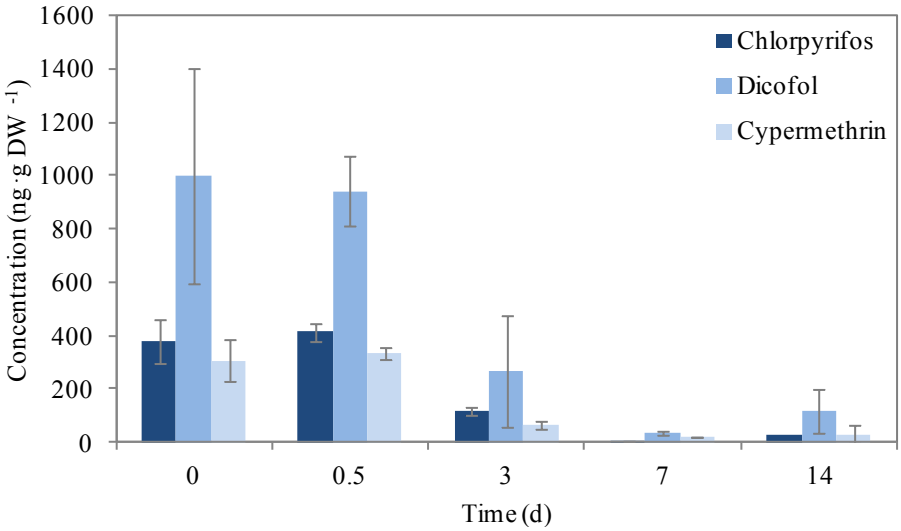


Figure 6.12. Pesticides concentration in fungal biomass samples from experimental set. Error bars represent the SD for triplicate cultures.

Figure 6.13 shows the mass balance of the experiments with *T. versicolor* pellets. Pesticides weight in the sum of pesticides weight in the liquid medium and in the biomass.

In all cases, statistical differences were obtained along the treatment suggesting the biodegradation of the compounds by the fungus. The initial amount of pesticides was not the theoretical one (500 ng) in all cases probably due to the losses by the sorption onto the glass, taking into account the results from abiotic control.

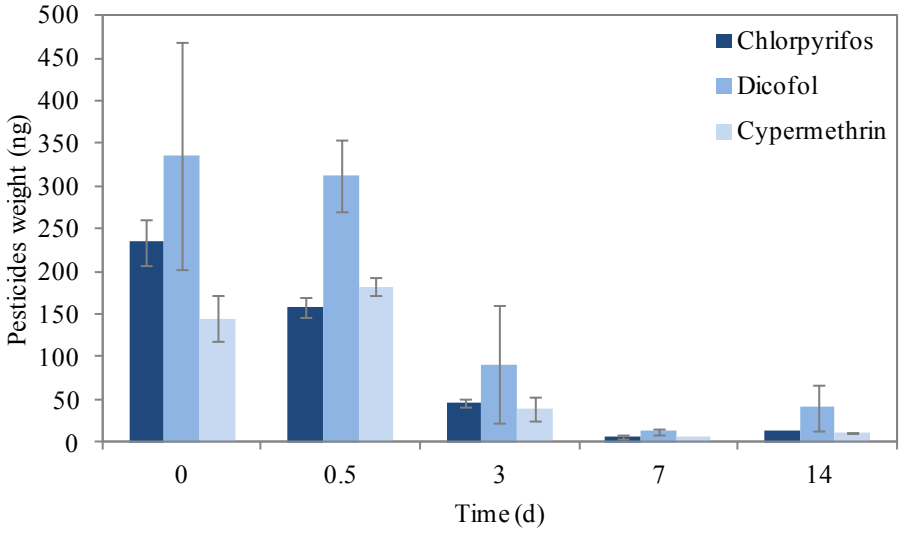


Figure 6.13. Pesticides mass balance for experimental set from liquid medium and biomass samples. Error bars represent the SD for triplicate cultures.

- ***Fungal biodegradation? Comparison between experimental and killed control removals***

Concentration profiles for killed control and experimental sets are presented in Figure 6.14. In all cases comparing the results obtained for the killed control with the living fungus, statistically differences were obtained.

Chlorpyrifos was almost 95% removed by *T. versicolor*. In contrast, in killed controls 20% removal was obtained with big standard deviation. Minimum biodegradation was calculated by subtracting the killed control removal value to the experimental removal value, resulting in 75% degradation by *T. versicolor*. There are several reports on chlorpyrifos biotransformation by bacteria, but only few works reported fungus biodegradation of chlorpyrifos (Supreeth and Raju, 2017). Sivagnanam and Jayanthi (2013) reported the biodegradation of chlorpyrifos (300 $mg \cdot L^{-1}$) and its major metabolite 3.5.6-trichloro-2-pyridinol by *Aspergillus terreus* in 24 h of incubation in a mineral medium. No

previous works studied the chlorpyrifos degradation by *T. versicolor* in liquid medium at concentrations in the range of $\mu g \cdot L^{-1}$.

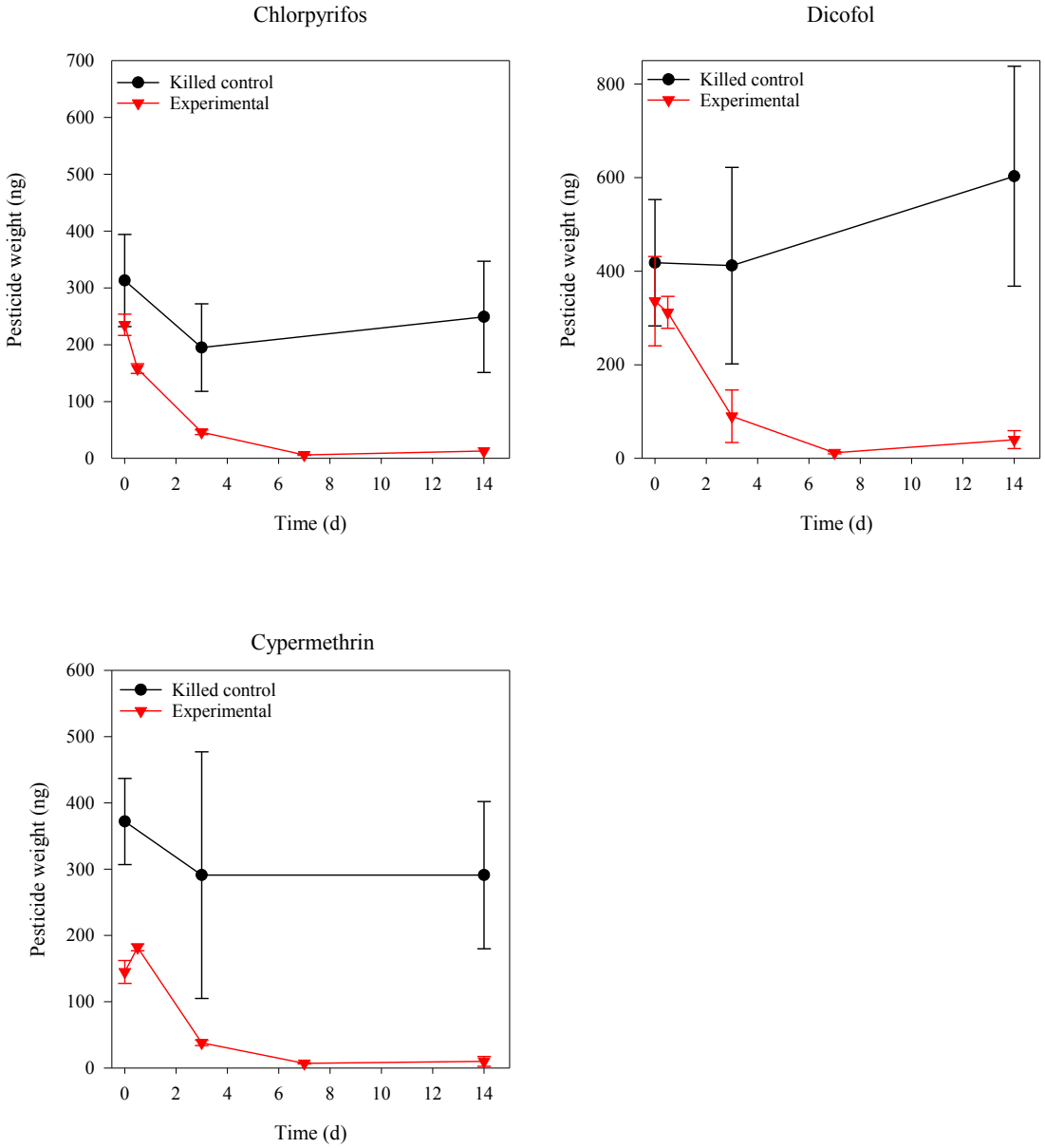


Figure 6.14. Mass balance profile of experimental and killed control for hydrophobic pesticides in Erlenmeyer flasks. Error bars represent the SD for triplicate cultures.

Regarding dicofol, 88% of the initial amount was degraded by *T. versicolor* since no removal was detected in the killed control. Bumpust and Aust (1987) already reported the mineralization of dicofol by the white rot fungi *Phanerochaete chrysosporium* in liquid medium.

Finally, 93% of cypermethrin was removed by the fungus meanwhile the 20% was removed in the killed control, resulting in a minimum 73% of biodegradation. The same biodegradation value was reported by Mir-Tutusaus (2014) with *T. versicolor* pellets, but with a higher initial concentration (10 $mg \cdot L^{-1}$). In the same study, cypermethrin degradation was confirmed by the detection of two transformation products which appear as a result of the breakdown of the molecule.

6.2.3.2 Degradation experiments of hydrophilic pesticides.

The second pesticides group includes: diuron, irgarol, malathion, simazine and triallate. The pesticides were spiked to a final concentration of 5 $\mu g \cdot L^{-1}$. However, as it was shown with the hyprophobic pesticides, the initial concentrations detected were not the theoretical one due to sorption, abiotic losses and experimental errors. In addition it must be taken into account that the samples for the time 0, are obtained at least after 30 minutes due to the time for sampling preparation. In Table 6.6 are presented the initial concentrations of each pesticide.

The initial concentration of diuron was almost the theoretical one in all cases. In the case of irgarol, it was detected at higher concentration in the experimental and killed-controls cultures, but not in the abiotic controls suggesting experimental errors instead of sorption or abiotic losses. Opposite case is the malathion, in which higher concentration was detected in abiotic controls; and smaller concentration for experimental and killed-controls suggesting the sorption onto the biomass. Regarding the simazine, the lowest initial concentration was measured in the experimental trial suggesting the active sorption process onto fungal biomass. Triallate is not presented because due to analytical errors, the removal was calculated with the areas instead of concentration.

For this pesticides group, only samples from the liquid medium were analyzed. The results from the experimental set, killed controls and abiotic controls are presented together for each pesticide.

Table 6.6. Initial concentration of hydrophilic pesticides in degradation experiments.

	Initial concentration µg·L^{-1}		
	Experimental	Killed-control	Abiotic-control
Diuron	5.63 ± 0.15	5.43 ± 0.57	5.93 ± 0.76
Irgarol	8.27 ± 0.25	6.23 ± 0.45	5.9 ± 0.01
Malathion	4.43 ± 0.35	3.83 ± 0.21	5.97 ± 0.23
Simazine	3.7 ± 0.1	4.5 ± 0.21	4.53 ± 0.21

- ***Diuron removal***

In the case of diuron, no abiotic losses were detected during the experiment (Figure 6.15). Diuron was totally removed by *T. versicolor* in only 12 hours with a removal rate of 3.4 µg·g DW of biomass^{-1}·d^{-1}. The killed control removed 56% of the initial diuron concentration, but the removal rate was slower (0.065 µg·gDW^{-1}·d^{-1}). Probably the combination of sorption and biodegradation allows the complete diuron removal in the experimental sets in only 12 h.

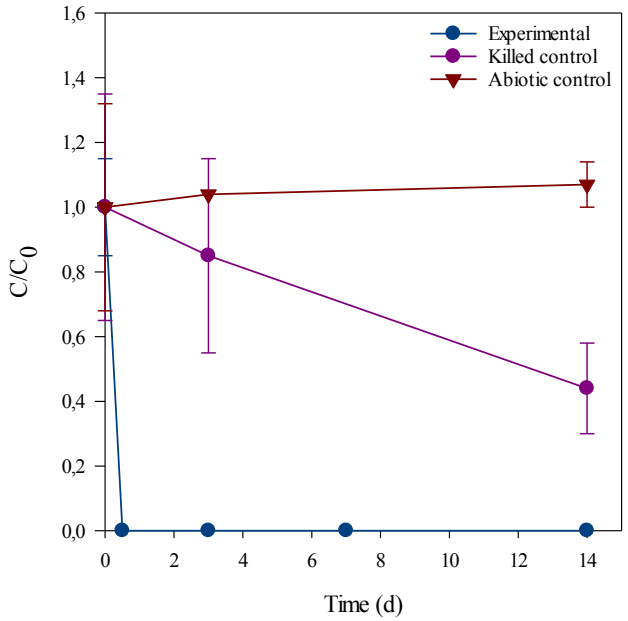


Figure 6.15. Removal of diuron added at 5 µg·L^{-1} by *T. versicolor* pellets in Erlenmeyer flask. Error bars represent the SD for triplicate cultures.

In previous section, it was demonstrated the ability of *T. versicolor* to remove diuron at a removal rate of 0.3 mg·gDW^{-1}·d^{-1}, in this case the removal rate obtained is 100 times lower. This difference is probably due to the initial amount of diuron in the first experiments, that

was 7.5 mg·L^{-1} and in this case is 5 µg·L^{-1}, so it is expected higher consumption rate with higher initial concentration. Otherwise, in this case the total diuron removal was detected at the first sample point (12 h), but it is probably that the fungus completely eliminates the diuron in less time, which would influence on the removal rate.

- ***Irgarol removal***

Irgarol is an active biocide to prevent biofouling in materials submerged in water (Maragou et al., 2011). Due to the nature and finality, irgarol is highly stable. Ranke (2002) estimated that irgarol can be present in the sea during more than 10 years. If so, irgarol can be considered as a persistent organic pollutant (Zhou, 20089. Irgarol was detected in the sea at concentration of µg·L^{-1} (Biselli et al., 2000; Sargent et al., 2000).

No abiotic losses were detected along the experiments (Figure 6.16). The killed control removed 86% of the initial concentration with a removal rate of 0.11 µg·gDW^{-1}·d^{-1}. Meanwhile, *T. versicolor* was able to totally remove irgarol in 3 days showing a removal rate of 0.83 µg·gDW^{-1}·d^{-1}.

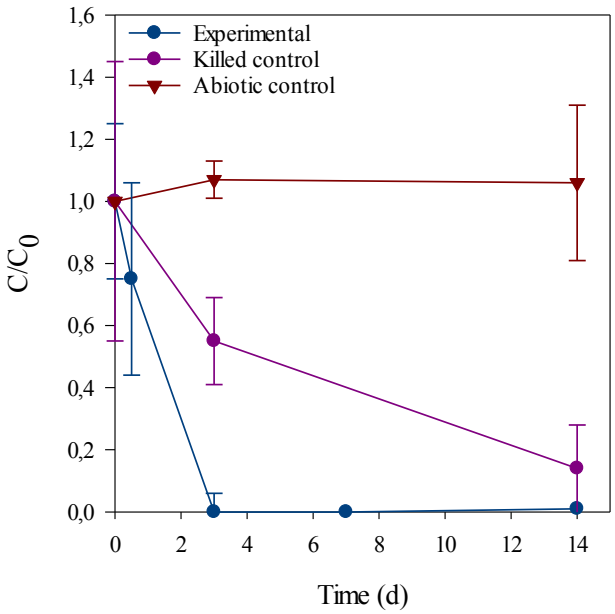


Figure 6.16. Removal of irgarol added at 5 µg·L^{-1} by *T. versicolor* pellets in Erlenmeyer flask. Error bars represent the SD for triplicate cultures.

Liu et al. (1997) reported the ability of *Phanerochaete chrysosporium* to degrade irgarol and its degradation products resulting from N-dealkylation. Later, Ogawa et al. (2004)

demonstrated the involvement of the enzyme manganese peroxidase prepared from *P. chrysosporium* in the irgarol biodegradation.

- ***Malathion removal***

Malathion is a nonsystemic, wide-spectrum organophosphorus pesticide used in public health and agricultural activity (Singh et al., 2012b). Malathion is used for the control of insects of fruits and vegetables, mosquitoes, flies, household insects, animal parasites and head and body lice (Kamrin, 1997). Malathion is used to control the vector (mosquitoes) in some diseases such as malaria, dengue and yellow fever (Chareonviriyaphap et al., 2013). Due to the excessive use of malathion, it is recognized as recalcitrant and a hazardous material (Singh et al., 2012b).

Figure 6.17 shows the pesticide removal profiles. Malathion was totally removed in 12 h, showing a removal rate of 2.68 $\mu g \cdot gDW^{-1} \cdot d^{-1}$. No abiotic losses were detected along the experiment. 46% of malathion was removed in the killed control, showing a slower removal rate than the experimental one (0.03 $\mu g \cdot gDW^{-1} \cdot d^{-1}$).

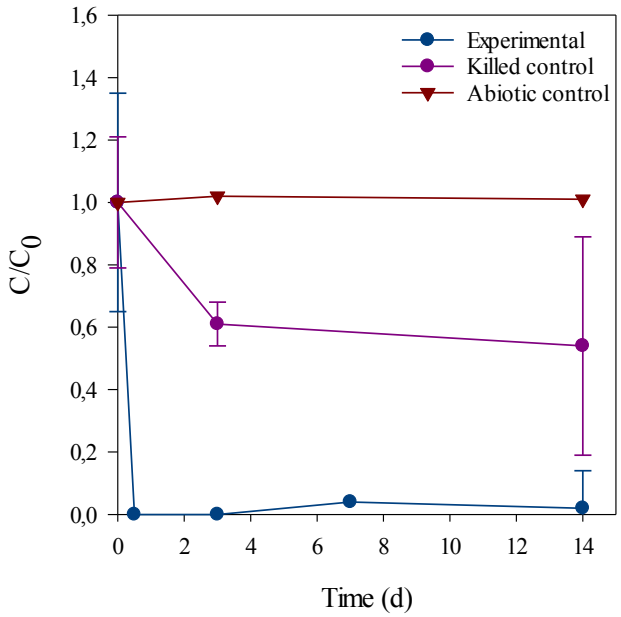


Figure 6.17. Removal of malathion added at 5 $\mu g \cdot L^{-1}$ by *T. versicolor* pellets in Erlenmeyer flask. Error bars represent the SD for triplicate cultures.

Shing et al. (2014) reviewed the malathion biodegradation by several bacteria and fungi, although *T. versicolor* and WRF were not included in that study. They reported that the predominant biodegradation pathway for malathion involves formation of mono and diacid

metabolites through carboxylesterase activity. Recently, Zhang et al. (2017) studied the enzymes involved in lignocelluloses degradation by *T. versicolor* employing different carbon sources; they reported one gene which expresses the carboxylesterase and its expression depends on the carbon source. The malathion degradation could be done by this enzyme or other.

- ***Simazine removal***

Simazine is one of the most applied *s*-triazine herbicides and it present mutagenic and carcinogenic potential (Dinamarca et al., 2007). Hence, the commercialization of simazine has been prohibited in EU community (Morgante et al., 2010). However, simazine is actually used in North America, Australia and South America (Seeger et al., 2010).

As Figure 6.18 shows, simazine was completely removed in the killed control at the end of the experiment, presenting a removal rate of 0.09 $\mu g \cdot g^{-1} DW \cdot d^{-1}$. Meanwhile, *T. versicolor* removed 76% of the simazine initial concentration with a removal rate of 0.06 $\mu g \cdot g^{-1} DW \cdot d^{-1}$. Since no analysis was made from the fungal biomass, we cannot distinguish between biodegration and sorption. No abiotic losses were detected along the treatment.

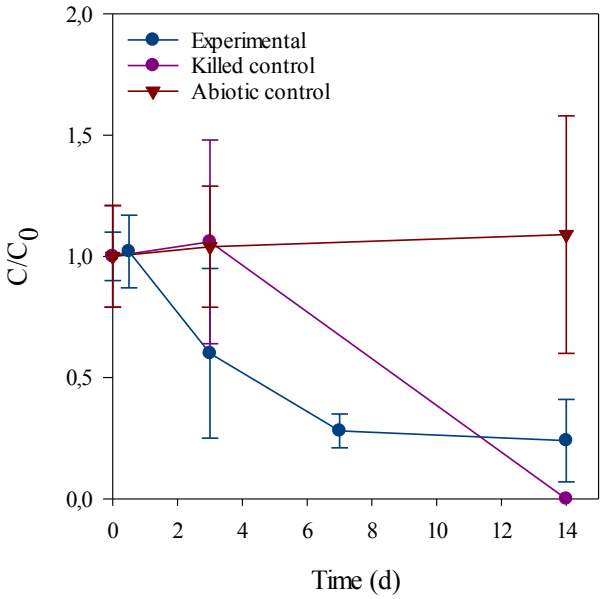


Figure 6.18. Removal of simazine added at 5 $\mu g \cdot L^{-1}$ by *T. versicolor* pellets in Erlenmeyer flask. Error bars represent the SD for triplicate cultures.

Fragoeiro and Magan (2008) reported 89% simazine removal by *T. versicolor* in soil microcosms with wood chips after 6 weeks of incubation. No other studies in liquid medium were found.

➢ ***Triallate removal***

Triallate is a selective herbicide to control annual weeds in wheat, barley, legumes and other crops (Atienza et al., 2001). It is extensively used in Europe, some studies indicated than more than 500 tons per year of triallate were used in some countries during the last decade (Volpe et al., 2004).

Triallate removal was calculated with the areas instead of concentration due to errors in the quantification methods (Figure 6.19). In this case, no abiotic losses were detected. The killed control and the experimental sets presented same removal profiles. Since no analysis from the fungal biomass was performed, we cannot distinguish between sorption and biodegradation. The great sorption of triallate might be due to its high partition coefficient (log P = 4.06) indicating its hydrophobic character.

There are few studies about triallate degradation; Anderson (1984) reported the triallate degradation in soil by a fungal consortium which includes more than 40 fungal species, but *T. versicolor* was not included in that study.

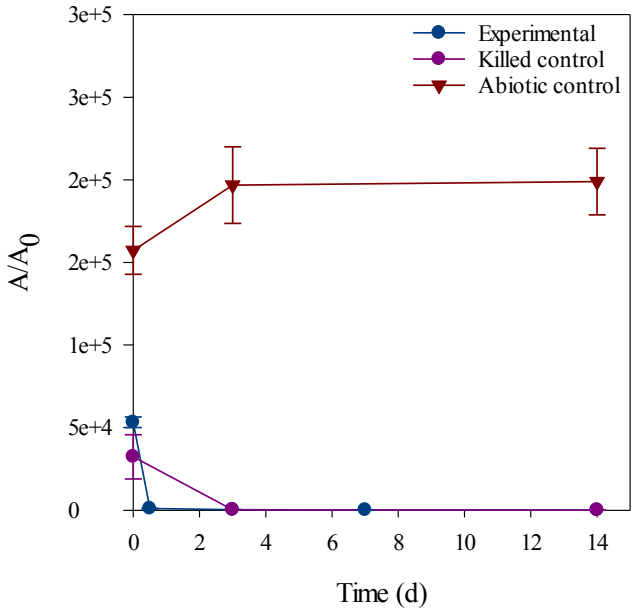


Figure 6.19. Removal of triallate added at 5 µg·L^{-1} by *T. versicolor* pellets in Erlenmeyer flask. Error bars represent the SD for triplicate cultures.

6.2.3.3 Pesticides degradation by *T. versicolor* at real concentration

T. versicolor was able to remove all the selected pesticides at low concentrations (range of µg·L^{-1}) with high removal values (Table 6.7). In all cases, no abiotic losses were

detected along the experiment, demonstrating the stability of the compounds. The sorption onto fungal biomass represented a key issue; hence samples of fungal biomass are necessary for hydrophobic and hydrophilic pesticides.

In this experiment, biomass samples were only analyzed for the hydrophobic group, so in the case of chlorpyrifos, cypermethrin and dicofol we can conclude that more than 75% of the initial concentrations were degraded by *T. versicolor*.

Regarding the hydrophilic group, the fungal biomass was not analyzed because the sorption of the pesticides onto the biomass was not expected, so we cannot distinguish if the pesticides were degraded or not. However, in all the cases higher removal rates were obtained in experimental cultures than in killed-control cultures suggesting minimal biodegradation of 44% for diuron, 14% for irgarol and 53% for malathion.

Table 6.7. Pesticides removal for experimental, killed control cultures and abiotic control cultures in Erlenmeyer flasks with 5 $\mu g \cdot L^{-1}$ of each pesticide.

Group	Pesticide	Removal after 14d (%)		
		Experimental	Killed control	Abiotic control
Hydrophobic	Chlorpyrifos	95 ± 1	20 ± 44	13 ± 70
	Cypermethrin	93 ± 0.5	21 ± 42	4 ± 70
	Dicofol	88 ± 8	0	7 ± 55
Hydrophilic	Diuron	100 ± 0	56 ± 3	0
	Irgarol	100 ± 1	86 ± 2	0
	Malathion	99 ± 2	46 ± 9	0
	Simazine	76 ± 5	100 ± 0	0
	Triallate	100 ± 0	100 ± 0	0

6.2.4 Continuous treatment for pesticides removal

Two types of bioreactors were employed for the continuous treatment of wastewater containing pesticides. In both cases, *T. versicolor* immobilized onto pallet wood was used.

6.2.4.1 Trickle-bed bioreactor: optimization and validation in continuous treatment

In this section, a trickle-bed bioreactor described in chapter 3 was employed for the pesticides removal experiments (Figure 6.20). The cylindrical fixed-bed is filled with the wood inoculated with the fungus. The wastewater is continuously feed from the influent tank to the reservoir bottle and then to the effluent tank with an HRT of 3 days. From the reservoir bottle, the wastewater is recirculated throw the fixed-bed. The total volume includes the volume of the reservoir bottle and the volume recirculated into the fixed-bed. In the reservoir bottle, the pH is controlled to be 4.5.

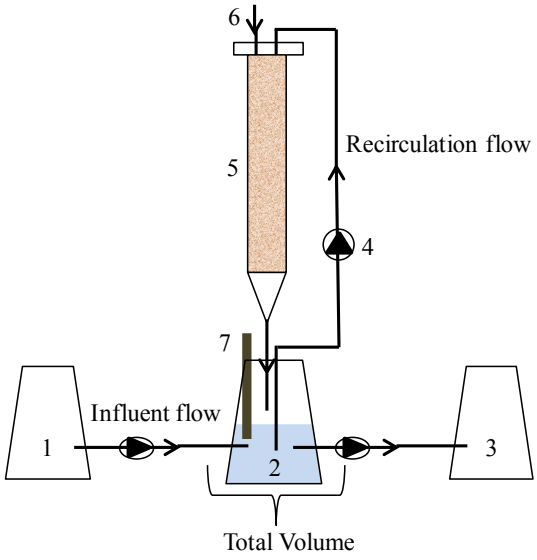


Figure 6.20. Schematic diagram of the trickle-bed bioreactor. 1: influent tank. 2: reservoir bottle. 3: effluent tank. 4: recycling pump. 5: fixed-bed. 6: air supply. 7: pH controller.

- ***Optimization of fungal treatment conditions for pesticide removal***

For the optimization experiments, the bioreactors were filled with 60 g wet wood (29 g DW) pre-inoculated with *T. versicolor* during 9 days. The continuous process was carried out with synthetic tap water spiked with diuron to a final concentration of 10 $mg \cdot L^{-1}$.

An experimental design was performed using a Central Composite Design (CCD) with the objective to determine the recycling ratio (RR) and the total volume to obtain the highest diuron removal.

The RR can be defined as the ratio of recirculated flow rate to that of the main influent flow rate. The recirculation flow, as well as the total volume, determines the contact time between the water and the fungus attached to the wood in the bioreactor since the same hydraulic retention time (HRT) was employed in all cases. For example with the same RR, if the total volume increases a shorter contact time is obtained.

In order to exclude the effect of the longevity of the fungus and the adsorption onto wood, a new reactor was started with fresh pre-inoculated wood at each experimental condition. Each bioreactor was operated for 18 days under continuous mode and the steady state was considered from day 9 forward.

- ***Central Composite Design***

As mentioned before, the recycling ratio (RR) and the total volume were optimized for maximum diuron removal using a Central Composite Design (CCD). Each factor was evaluated at 3 levels with 4 replicates at the point with the higher removal in 12 different trials (Table 6.8). The RR was fixed at 400, 700 and 1000 (normalized values -1, 0, 1, respectively), and the total volume (mL) was fixed at 200, 500 and 1000 (normalized values -1, 0, 1, respectively). Diuron removal (%) at steady state was the response (Y).

The values for RR and total volume were selected based on previous experiments with the trickle-bed bioreactor for hospital wastewater treatment, in which a RR of 800 and 250 mL of total volume were used.

The removal of each reactor used for the CCD was the mean removal value obtained during the steady state. In all cases the standard deviation was less than 3%. The maximum and minimum diuron removal obtained were run 1 (83%) and run 9 (26%), respectively (Table 6.8). The low variability (<3%) of the higher removal points (runs 1, 10, 11, 12) is an indicator of the reproducibility of the experimental data. In experiments with the higher total volume (800 mL) a larger difference between the experimental and the predicted

values was observed, probably because the contact time is shorter, increasing the variability in the diuron removal.

Table 6.8. Central Composite Design (CCD) matrix for the optimization of the RR and total volume for diuron removal with the coded and real values for the variable. Experimental and predicted values for the response.

RUN	RR		Total volume (mL)		Y: Diuron removal (%)	
	Coded	Real	Coded	Real	Experimental	Predicted
1	-1	400	-1	200	83.0	81.2
2	-1	400	0	500	64.6	68.2
3	-1	400	1	800	62.6	55.2
4	0	700	-1	200	74.4	74.1
5	0	700	0	500	56.7	56.1
6	0	700	1	800	29.5	38.2
7	1	1000	-1	200	67.3	66.9
8	1	1000	0	500	44.7	44.1
9	1	1000	1	800	26.5	21.3
10	-1	400	-1	200	84.0	81.2
11	-1	400	-1	200	82.0	81.2
12	-1	400	-1	200	78.0	81.2

The experimental data was evaluated using analysis of variance (ANOVA). The combined effect of the normalized recycling ratio (A) and the normalized total volume (B) on the diuron removal (Y, %) was determined, yielding the following equation with two interaction factor (2 IF) :

$$Y = 56.15 - 12.06\ A - 17.90\ B - 4.91\ AB \qquad \text{Eq. (1)}$$

The proposed model is significant with an F value of 59.29, obtained by Fisher's F-test, along with a very low probability value, which is significant at a 95% confidence interval (Table 6.9). The R^2 value of 0.9570 indicates that the model explains 95.70% of the total diuron removal variability. The predicted R^2 of 0.7997 is in reasonable agreement with the adjusted R^2 (0.9408), which is close to R^2, the difference is less than 0.2. The lack of fit was found to be non-significant (p-value 0.1079) which indicates that Eq. (1) is adequate to predict the diuron removal between the ranges studied.

Table 6.9. Analysis of variance of the fitted model obtained from CCD.

Source	Sum of squares	Degrees of freedom	Mean Square	F-value	p-value
Model	4303.38	3	1434.46	59.29	< 0.0001*
A-Recycling ratio	1042.09	1	1042.09	43.08	0.0002*
B-Volum	2293.63	1	2293.63	94.81	< 0.0001*
AB	127.19	1	127.19	5.26	0.005*
Residual	193.54	8	24.19		
Lack of Fit	172.79	5	34.56	5	0.1079 NS
Pure Error	20.75	3	6.92		
Total	4496.92	11			

*Statically significant (95% confident interval) // NS Statically not significant (95% confident interval)

Figure 6.21 shows the response surface obtained from Eq. (1). Both parameters resulted significant for the diuron removal; and the interaction between them had a significant impact on the results obtained. The decreasing in the recycling ratio and the total volume had a positive effect on the diuron removal.

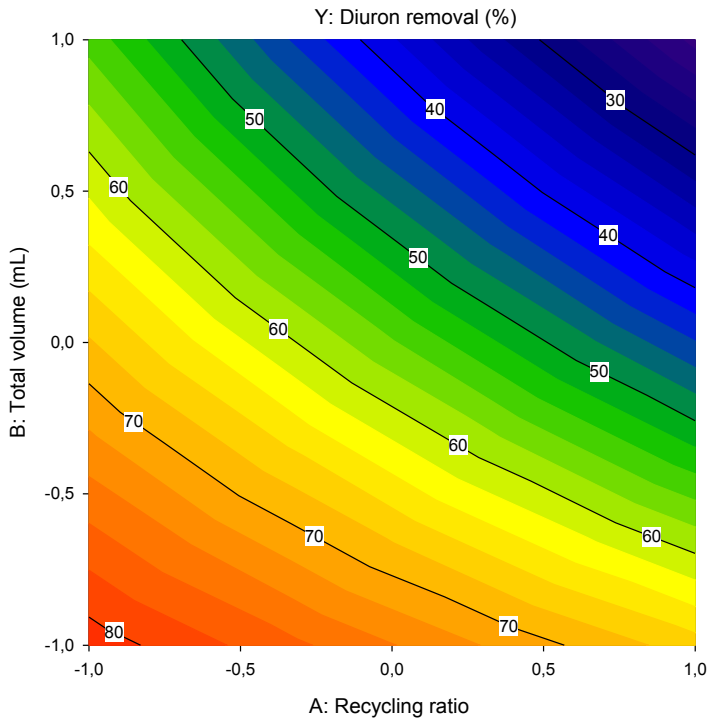


Figure 6.21. Response surface for diuron removal (%) obtained at different recycling ratio: 400 (−1), 700 (0) and 1000 (1); and the different total volume: 200 (−1), 500 (0) and 800 (1) mL.

The optimal recycling ratio and total volume were the lowers ones (RR: 400 and 200 mL), which imply more contact time between the fungus attached to the wood and the

diuron present in the water. Since, cytochrome P450 is involved on the diuron degradation instead of extracellular enzymes, diuron removal is increased with higher contact time because the removal takes place when the pesticide is in contact with the fungus.

Additional experiments were carried out with the best volume obtained and decreasing the recycling ratio to 200 in order to study if the diuron removal was increased, but only 72% of diuron removal was obtained; therefore a recycling ratio of 400 was selected for further experiments. Small total volumes were not performed since the objective of this work was to study a system for the pesticide treatment suitable to be applied at real scale.

- ***System validation for pesticides removal from real wastewater***

Monitoring of bioreactors

A new experiment was carried out in order to validate the treatment of real wastewater containing pesticides at real concentrations. The wastewater was collected on September 2017 from an irrigation channel of Gavà (Catalunya, Spain) based on the results obtained in the first sampling campaign.

A scale-up of the system was carried out because it was necessary a bigger volume of sampling for analytical purpose. The analyses were made at the end of the experiment by Water and Soil Quality Research Group from Institute of Environmental Assessment and Water Research (IDAEA-CSIC, Barcelona, Spain).

The scale-up was carried out keeping the relation between the amount of wood inoculated with the fungus and the total volume. A 3-fold scaling was applied, for this experiment the total volume was 600 mL and 180 g of wet wood were employed. Meanwhile, in the optimization 60 g of wet wood and 200 mL as total volume were used. The RR was the one obtained in the optimization experiments (RR=400) and the HRT was the same as before (3 days).

Two bioreactors were set up in parallel, one packed with wood inoculated with the fungus and other with sterile wood as a control. The bioreactors were continuously operated for 26 days treating real wastewater under non-sterile conditions.

In the inoculated reactor, the laccase profile showed a peak on day 7, and decreased to the end of the experiment as it is shown in the Figure 6.22. The laccase average obtained

was 2.5 $AU \cdot L^{-1}$ along the treatment, it is a low value compared with other studies working with *T. versicolor* in fluidized bed reactors (Badia-Fabregat et al, 2015b; Mir-Tutusaus et al., 2017a), but in accordance with the previous studies in this bioreactor without nutrient addition (laccase average of 4 $AU \cdot L^{-1}$). However, the detection of laccase activity during all the continuous treatment indicates fungal activity.

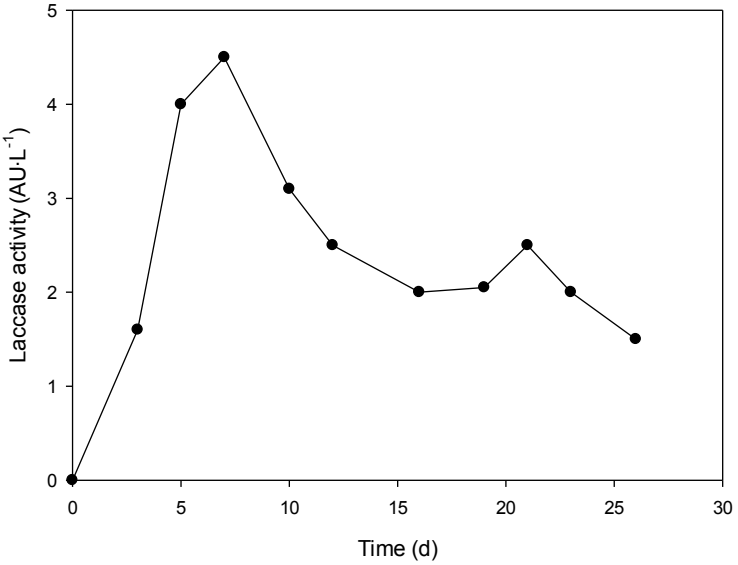


Figure 6.22. Evolution of laccase activity in the trickle-bed bioreactor inoculated with *T. versicolor* treating real wastewater.

Table 6.10 shows the physicochemical parameters and heterotrophic plate count of the real wastewater influent and the average values obtained along the treatment in the experimental and control trickle-bed bioreactors. Based on the wastewater characterization, it was decided not to apply any pretreatment to diminish the bacterial concentration.

In both reactors, the pH was 4.5 and no significant differences in ammonia amount, number of bacteria and TSS were detected. An increase in the COD concentration was observed in both treatments, probably due to by-products or metabolites released during fungal wood degradation (Palli et al., 2016). However, the COD averages in both reactors were maintained at 70 mg $O_2 \cdot L^{-1}$ which did not represent deterioration in water quality.

The absorbance, number of bacterial colonies and TSS values obtained did not change along the treatment. Values obtained were in accordance with the legislation enacted for the water treatment from agriculture reuse (Jeong et al., 2016; Real Decreto 1620/2007). These parameters allow concluding that bacterial growth did not increase during the treatment and did not represent a problem in the reactor performance, in contrast with other studies, where

bacterial overgrowth is the main cause for the reactor deterioration because of the inhibition of fungal growth and enzyme production (Yang et al., 2013).

Table 6.10. Physicochemical characterization of the real wastewater, average values of experimental and control trickle-bed bioreactor.

	Real wastewater	**Experimental**	**Control**
		Average	Average
pH	7.91	4.5	4.6
Absorbance at 650 nm	$8 \cdot 10^{-3}$	$5.1 \cdot 10^{-3} \pm 1 \cdot 10^{-3}$	$5.2 \cdot 10^{-3} \pm 2 \cdot 10^{-3}$
Heterotrophic plate count ($CFU \cdot mL^{-1}$)	$1.2 \cdot 10^{5} \pm 4 \cdot 10^{4}$	$5 \cdot 10^{5} \pm 4 \cdot 10^{5}$	$4.5 \cdot 10^{5} \pm 9 \cdot 10^{4}$
Ammonia (mg $N \cdot L^{-1}$)	0.0	0.01 ± 0.001	0.03 ± 0.002
TSS ($mg \cdot L^{-1}$)	11.5 ± 2.1	13.4 ± 5	10 ± 2
COD (mg $O_2 \cdot L^{-1}$)	30.7	68 ± 5	72 ± 3

Fungal treatment: pesticides removal

Only 6 pesticides of the 51 pesticides analyzed were detected, in contrast with the first sampling campaign when 24 pesticides were found with a final concentration of 2000 $ng \cdot L^{-1}$. As it is shown in the Table 6.11, the total amount of pesticides was 115.77 $ng \cdot L^{-1}$, one order of magnitude less than in the previous sampling campaign. Previous studies indicated that the pesticides concentration varied depending on the time of the year (Ginebreda et al., 2010; Proia et al., 2013). Furthermore, the collection of the wastewater was performed after a dry summer, this situation probably affected the less concentration of pesticides obtained because this compounds mostly enter into surface waters from diffuse sources after rainfall episodes (Rabiet el al., 2010).

Table 6.11. bioreactor.

	Date Sampling 30/10/17
Pesticides	Real Wastewater ($ng \cdot L^{-1}$)
Diazinon	1.48
Diuron	24.69
Irgarol	6.88
Terbutryn	28.09
Chlorpyriphos	27.33
Dicofol	27.31
Total	**115.77**

The wastewater was divided and frozen into 500 mL PET bottles until use. Every day during the experiment, one PET bottle was thawed to fill the inlet tank in order to always use fresh wastewater. During the experiment, samples were obtained from the fungal reactor, the control reactor and from the inlet tank.

Only chlorpyrifos (CPF) was detected in the inlet tank and both trickle-bed bioreactors. None of the other pesticides were detected in the inlet samples; therefore they were not detected in the reactors either. The pesticides were probably adsorbed onto the PET bottles or other physicochemical losses occurred. The small initial amount of pesticides in real wastewater was a problem for the analysis. In Figure 6.23 are presented the CPF concentrations in the inlet tank, the fungal trickle-bed bioreactor and the control trickle-bed bioreactor. The inlet concentration was not stable along the treatment. However, in the trickle-bed bioreactor lower CPF concentrations were detected compared with the control reactor during all the treatment, suggesting the CPF biodegradation by *T. versicolor*.

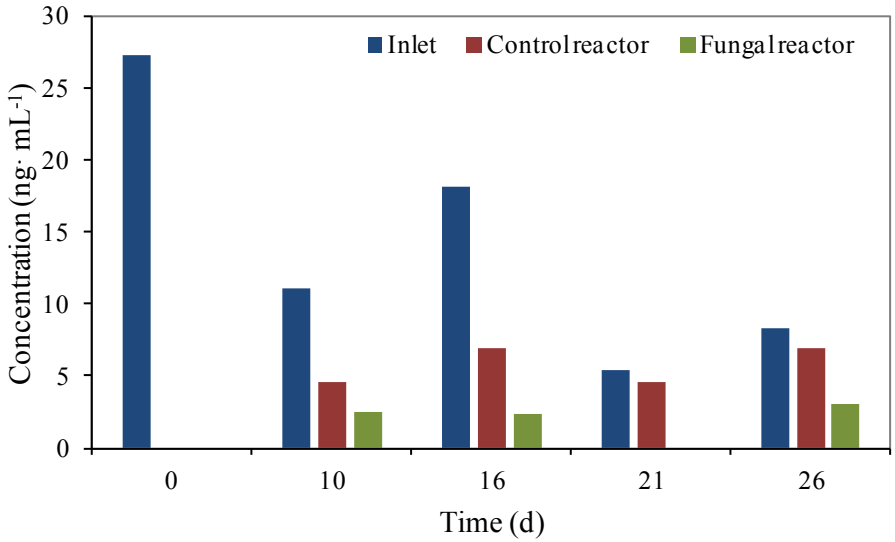


Figure 6.23. Chlorpyriphos concentration in trickle-bed bioreactor validation experiment for the pesticides removal from real wastewater.

Due to the lack of pesticides in the inlet, it would therefore be premature at this stage to draw precise conclusions from this experiment. In the future, new experiments should be performed in order to validate the fungal reactor for the pesticides removal from real wastewater. But, it is clear that better removals were obtained with the fungal trickle-bed bioreactor than in the control one during all treatment.

6.2.4.2 Packed-bed channel for diuron removal

In previous sections, good results were obtained for the wastewater treatment employing a trickle-bed bioreactor with *T. versicolor* immobilized on pallet wood. Based on these results, a packed-bed channel bioreactor was developed in this section for the diuron removal using *T. versicolor* immobilized on pallet wood.

The new system configuration was tested considering that a packed-bed channel bioreactor would be more easily installed in a rural area, which is the target location. Meanwhile, for the application of the trickle-bed bioreactor, it is necessary to install additional structures to support the column fixed-bed.

More details of the packed-bed channel bioreactor can be found in section 3.5.3 from materials and methods. The channel body contains the wood and the water (Figure 6.24). For these experiments, the total volume was 1 L and the HRT was fixed at 3 days.

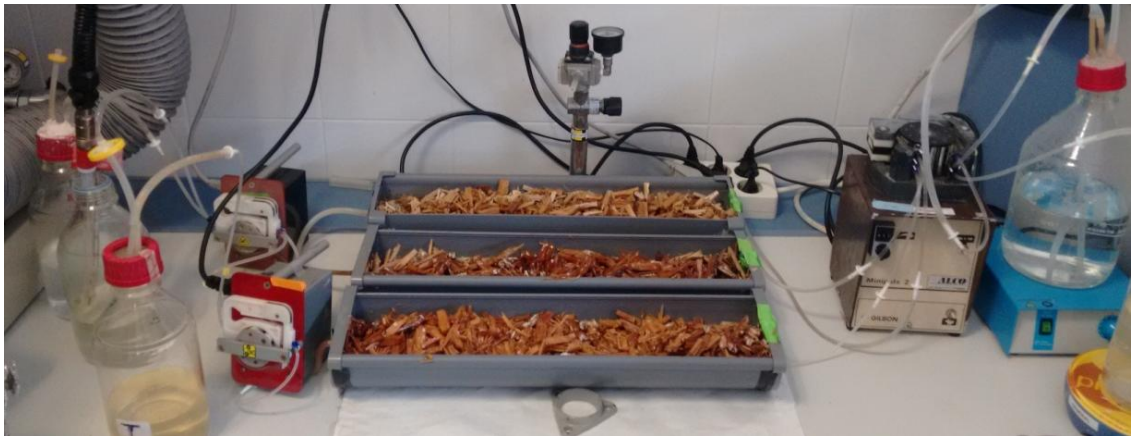


Figure 6.24. Packed-bed channel bioreactors.

The first experiments carried out in the packed-bed channels were done in order to study different operational conditions to maximize the diuron removal. For this purpose, four channels were operated varying the amount of wood inoculated with the fungus, adding or not aeration in the reactor and operating at immerged condition in totally or partially time. The best operational conditions were selected to perform a long-term reactor with synthetic tap water and real wastewater. Table 6.12 summarizes the experiments performed. The term “Mixed” in the table is referred to the cases when wood is partially submerged in water; hence the mixing was performed to ensure that all the wood is in contact with the

water during the experiment. In all the experiments, the water was spiked with diuron to a final concentration of 10 $mg \cdot L^{-1}$.

Table 6.12. Summary of experiments.

	Channel code	Wood (Kg wet)	Biomass (g DW·L^{-1})	Submerged	Water	Mixed*	Observation
First experiments	C1	0.6	1.2	Totally	SW		
	C2	0.6	--	Totally	SW		
	C3	0.6	1.2	Totally	SW		Air pumped
	C4	1	2	Partially	SW	Every 2-3 days	
Long-term operation	C-I	1	2	Partially	SW	Every day	
	C-NI	1	--	Partially	SW	Every day	
	C-IR	1	2	Partially	Real WW	Every day	

SW= synthetic tap water // Real WW= real wastewater

* Manually mixing of the wood

- ***Packed-bed channel bioreactors: first operational studies***

Four channels were operated in parallel proving different operational conditions in order to compare their performance on diuron removal. The bioreactors were fed with synthetic tap water spiked with 10 $mg \cdot L^{-1}$ of diuron. As it is shown in Table 6.12, channels C1 and C3 were filled with 0.6 kg of wet wood inoculated with *T. versicolor*. Channel C2 was packed with 0.6 kg of non inoculated wet wood to study the adsorption. Finally, channel C4 was packed with 1 kg of wet wood inoculated with the fungus. The reactors were operated for 25 days.

For the channels C1, C2 and C3 the wood was totally submerged in water. C3 was aerated by 2 aquarium air diffusers located along the channel in order to study the effect of air supply. In the case of C4, the amount of wood inoculated with *T. versicolor* was increased 40% and it was manually mixed every 2-3 days. Part of the wood (60%) was submerged in water and the extra wood (40%) was not submerged, so it was in contact with the air. The mixing was implemented in order to ensure that all the wood was in contact with the water. Besides, it allows the fungus to recover, since it facilitates its contact with air during partial time.

Figure 6.25 shows the diuron removal profiles of each channel. In the case of the experimental (C1), the control reactor (C2) and the reactor with pumped air (C3), similar diuron removal profiles were obtained probably due to the adsorption on wood. At the end of the operation period in C1, C2 and C3, the removals were only 30% and the stationary state was not achieved in any case. In the case of the reactor C3, it was expected a better performance due to the air supplying, taking into account that the fungus is an aerobic microorganism. Other authors obtained higher diuron removal rate when dissolved oxygen was not limiting, but employing a microbial community able to degrade diuron obtained from a contaminated soil instead of *T. versicolor* and also adding nutrients to the medium (Castañón-González et al., 2016).

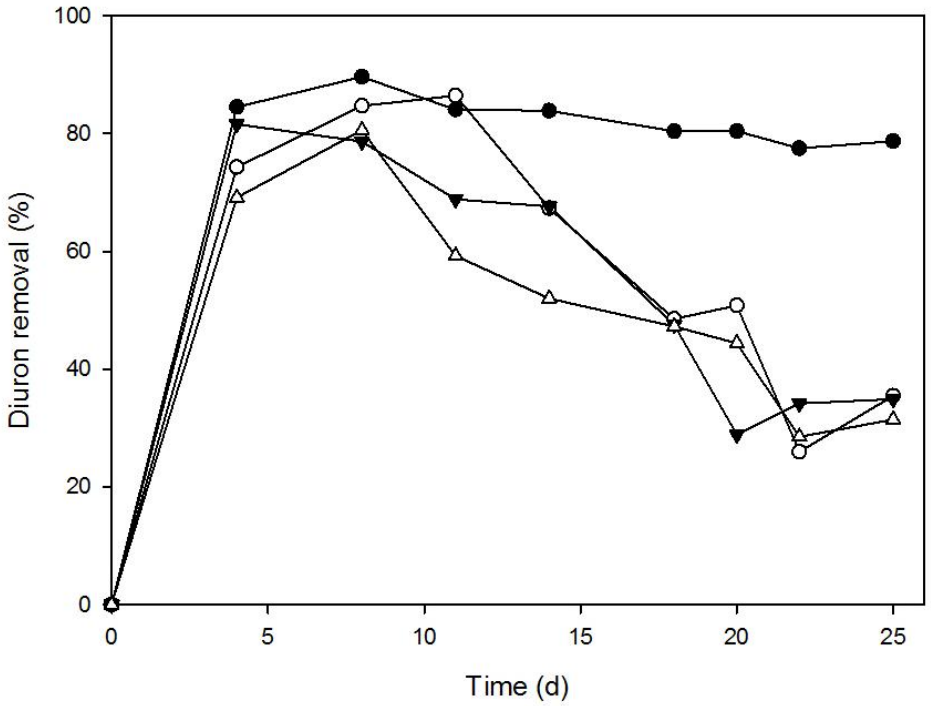


Figure 6.25. Diuron removal profile in packed-bed channels: (▼) experimental C1, (Δ) non inoculated control C2, (○) with pumped air C3 and (●) with 40% more biomass C4.

Furthermore, the reactor with more wood (C4) presented 80% of removal during all the treatment achieving the stationary state. In this reactor more inoculated wood was employed and consequently more biomass was available for the treatment of the same water volume with the same HRT compared with the other channels. In channels C1, C2 and C3 the amount of biomass employed was 1.2 g $DW \cdot L^{-1}$, while in channel C4 the biomass used was 2 g $DW \cdot L^{-1}$. These results corroborate previous one (section 3.1.1)

demonstrating again that the amount of biomass had a significant impact on the diuron removal rate. Moreover, extra wood was available for adsorption.

Every 2-3 days, the wood was manually mixed to guarantee the total contact between the water and the wood. It was also a way to maintain the fungus active since *T. versicolor* is an aerobic microorganism.

In previous section, a trickle-bed bioreactor with *T. versicolor* immobilized onto wood was studied for the diuron removal. Similar removal values were obtained in the channel and the trickle-bed bioreactor, but in the last one using 70% less biomass. The treatment was carried out during 20 days achieving 84% of diuron removal employing only 0.49 g $DW \cdot L^{-1}$. From these results it can be concluded that the contact with air is an important factor for diuron removal, as well as the biomass amount, so these parameters should be taken into account for future experiments.

- ***Continuous long-term operation of a packed-bed channel reactor***

Based on previous results, a channel with 1 kg of wood inoculated with *T. versicolor* and mixed every 2-3 days was chosen for the continuous long-term diuron removal.

The continuous long-term treatment was proved increasing the frequency of the mixing to every day in order to improve the fungal activity. Two channels were operated working at the same conditions with synthetic tap water spiked with diuron (10 $mg \cdot L^{-1}$): one inoculated with *T. versicolor* (C-I) and other uninoculated as a control (C-NI). The C-NI was performed to study the adsorption of the diuron onto the wood. The differences obtained between the C-I and C-NI can be then attributed to biodegradation by the fungus.

The reactors were operated for 49 days, the mean removal for the inoculated reactor C-I was 89 % (±4) and 69 % (±11) for the control reactor C-NI (Figure 6.26). For the C-I the removal presented low variation along the treatment, in contrast with the control C-NI which achieved 80% of removal during the first 14 days and then started decreasing to the end of the experiments probably because the adsorption onto the wood started to be limited, although the saturation point was not achieved. Comparing the mean values obtained in both reactors, 20% higher removal was obtained when the wood was inoculated with *T. versicolor*, this removal difference can be attributed directly to the biodegradation by the

fungus. At the end of the treatment, 30% more removal was achieved with the reactor filled with wood inoculated with *T. versicolor*. 3,4-Dichloroaniline (3,4-D) was not detected during the experiment. The ability of *T. versicolor* to completely degrade 3,4-D in less than 24 h was demonstrated in previous results, so these results suggest that 3,4-D was degraded at the same time that it was produced.

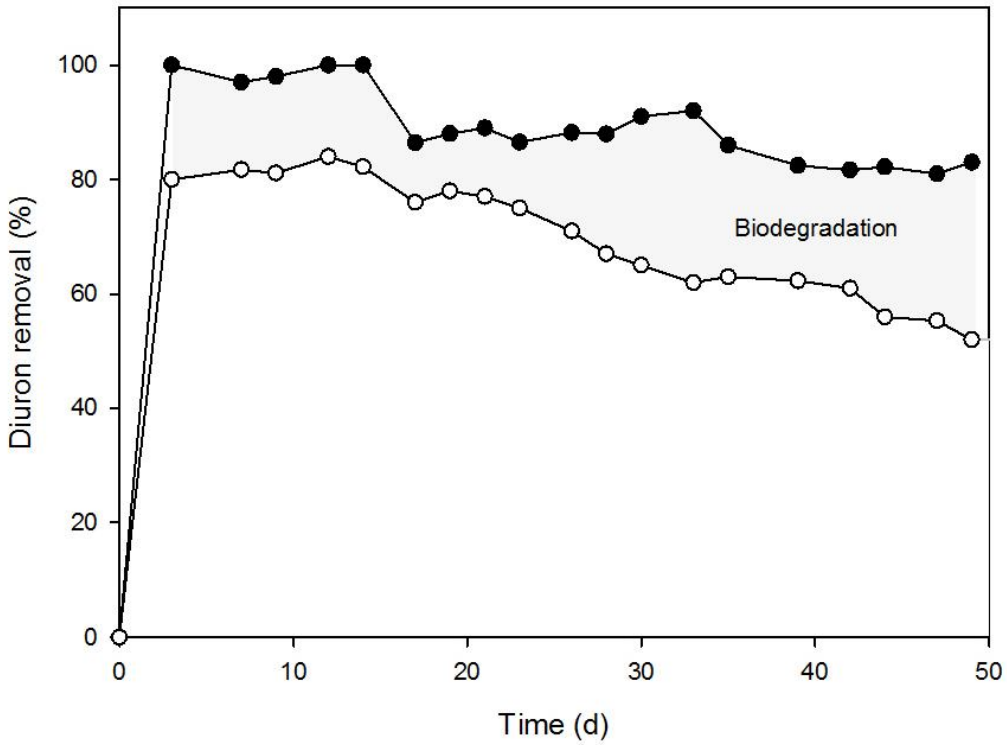


Figure 6.26. Diuron removal profile in packed-bed channels (●) inoculated with *T. versicolor* (C-I) and (○) non inoculated control (C-NI).

The use of a lignocellulosic support has been reported as a good strategy to favor the fungal activity. In this reactor, the wood chips were used as a sole nutrients source since the diuron degradation is probably a co-metabolic process (Ellegard-Jensen et al., 2014). In addition, the wood was used as a substrate for the fungus immobilization.

The removal obtained in the channels (>90%) was higher than the removal obtained in submerged cultures with *T. versicolor* pellets (74%) maybe due to the adsorption onto the wood.

- ***Channel treating diuron from real wastewater***

Working at the same conditions as in previous experiments, a new reactor (C-IR) was filled with inoculated wood and real wastewater spiked with diuron (10 $mg \cdot L^{-1}$) in order to study how the real matrix influences in the reactor performance. The real wastewater was obtained from an irrigation channel in Gavà (Llobregat River, Barcelona, Spain).

As Figure 6.27 shows, a peak of laccase (62 $AU \cdot L^{-1}$) was obtained at day 5, then the activity was reduced to around 30 $AU \cdot L^{-1}$ until day 17 and from this day the laccase activity started to decrease up to 4 $AU \cdot L^{-1}$ at the end of the experiment. Since the laccase is not involved on the diuron removal, the measurement of this enzyme was employed to confirm *T. versicolor* activity along the treatment.

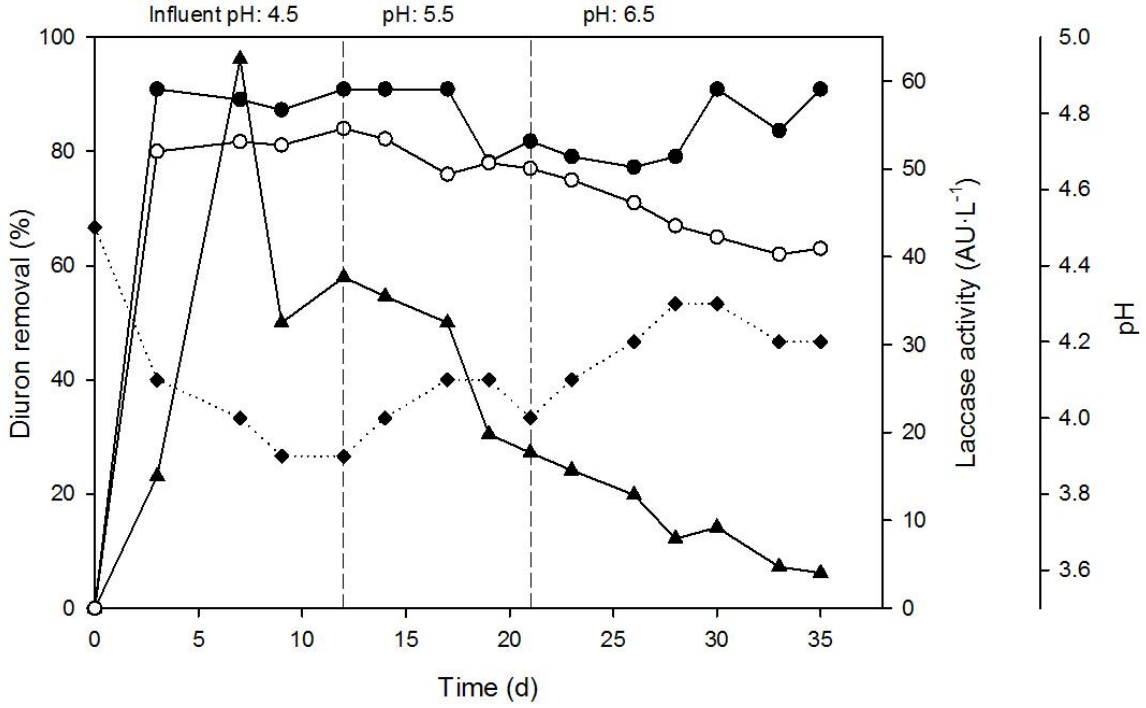


Figure 6.27. Channel C-IR treating diuron from real wastewater. Symbols: (●) diuron removal in C-IR, (▼) laccase activity profile in C-IR, (○) diuron removal in control reactor working with synthetic tap water (C-NI) and (♦) pH profile Vertical dashed lines mark changes in the pH influent.

The mean diuron removal value obtained was 94 % (±5) during 35 days as shows the Figure 6.27. Since no control reactor was performed to compare the adsorption onto wood, the diuron removal profile for the control channel with tap water (C-NI) is represented also in Figure 6.27. During all the treatment the differences between the C-IR and the C-NI were attributed to the biodegradation of the diuron. The wood had a high adsorption

capacity, as can be already observed in previous results, but at the end of the treatment it started to be limited. Meanwhile the C-IR maintains stable good removal levels during all the treatment. Also, in this case 3,4-Dichloroaniline (3,4-D) was not detected during the experiment.

The removal for this channel C-IR was slightly higher than for the channel C-I working with synthetic tap water, probably due to the presence of microorganisms in the real wastewater that are able to degrade the diuron. Other authors reported the capacity of the microorganisms communities from soil or water contaminated with diuron to degrade it (Ellegaard-Jensen et al., 2014).

Sanchis et al. (2014) obtained 66% of diuron removal after 145 days of operation of a SBR with activated sludge, but it was necessary an acclimation period of 100 days when the removal obtained was only 30%. So, the acclimation of the microorganisms is a key issue in this kind of treatments, in our case *T. versicolor* and the endogenous bacteria worked in a synergistic way without a need for an acclimation period.

The optimal pH for *T. versicolor* is in the range of 4.3 to 4.7. Because of that, the influent pH was adjusted at the beginning of the experiments from the real value (7.9) to the optimal one (4.5) (Tavares et al., 2006). During the treatment, the pH was monitored directly just before the reactor outlet but it was not controlled in the system.

The pH started decreasing up to 3.8 at day 11 because *T. versicolor* usually acidifies the medium (Tavares et al., 2006), it is a problem because pH lowers than 4 often kills the fungus. In order to increase the pH in the channel, the pH of the influent tank was increased to 5.5 from day 11. Consequently an increase of the pH was obtained up to day 19, since when the pH started to decrease again. A new increase in the influent pH was performed up to 6.5 in the final period of the treatment from day 21 (Figure 6.27).

The control of pH is an important issue in systems working with *T. versicolor* as already has been reported by other authors (Folch et al., 2013). The operation of the channels with the real pH of the influent (7.9) results in an advantage because it would not be necessary to reduce the pH of the influent to the optimal pH for the fungus (4.5).

Some authors indicated that the efficiency of a packed-bed biofilm reactor depends on the microbial community employed, the support material selected and the environmental conditions (Marrón-Montiel et al., 2014; Biswas et al., 2013; Stephenson et al., 2013). In

addition, in aerobic bioprocesses, the oxygen mass transfer is a key factor. The results obtained with the packed-bed channel inoculated with *T. versicolor* mixed every day indicate that it is a good strategy for the reactor operation. It is remarkable that the system was able to operate in a continuous mode with high diuron removal and without operational problems for 35 days treating diuron from real wastewater.

Physicochemical parameters

Ammonia concentration, CFU, COD, TSS and absorbance were monitored in the channel C-IR. Table 6.13 shows the mean values obtained along the fungal treatment for these parameters and the initial values for the real wastewater showing no significant differences.

The increase of bacterial communities usually represented a big problem working with fungal reactors with real wastewater because it limits the fungal activity. In this work no increase in the bacterial content was detected, probably because of the nutrient limitation. CFU number, absorbance, COD and TSS values suggest that bacterial growth was not increased during the treatment and does not represent a problem in the reactor performance.

Table 6.13. Physicochemical characterization of real wastewater and average values obtained along the reactor treatment.

	Real water	**Channel C-IR Average**
Absorbance at 650 nm	0.008	0.005 ± 0.001
Heterotrophic plate count ($CFU \cdot mL^{-1}$)	$1.2 \cdot 10^5 \pm 4 \cdot 10^4$	$9.4 \cdot 10^5 \pm 1.3 \cdot 10^6$
Ammonia ($mg\ N \cdot L^{-1}$)	0.04	0.06 ± 0.001
TSS ($mg \cdot L^{-1}$)	11.5 ± 2.1	14.2 ± 2
COD ($mg\ O_2 \cdot L^{-1}$)	31	36 ± 3

- ***Effect of the low temperature***

Another reactor was performed outdoor to study the effect of the temperature since in a real application the reactor would work at environmental temperature. Monitoring data was collected from December 2th (2017) to December 22th (2017). During this period outside temperature ranged from 5±2°C to 14.5±2°C as it is shown in Figure 6.28.

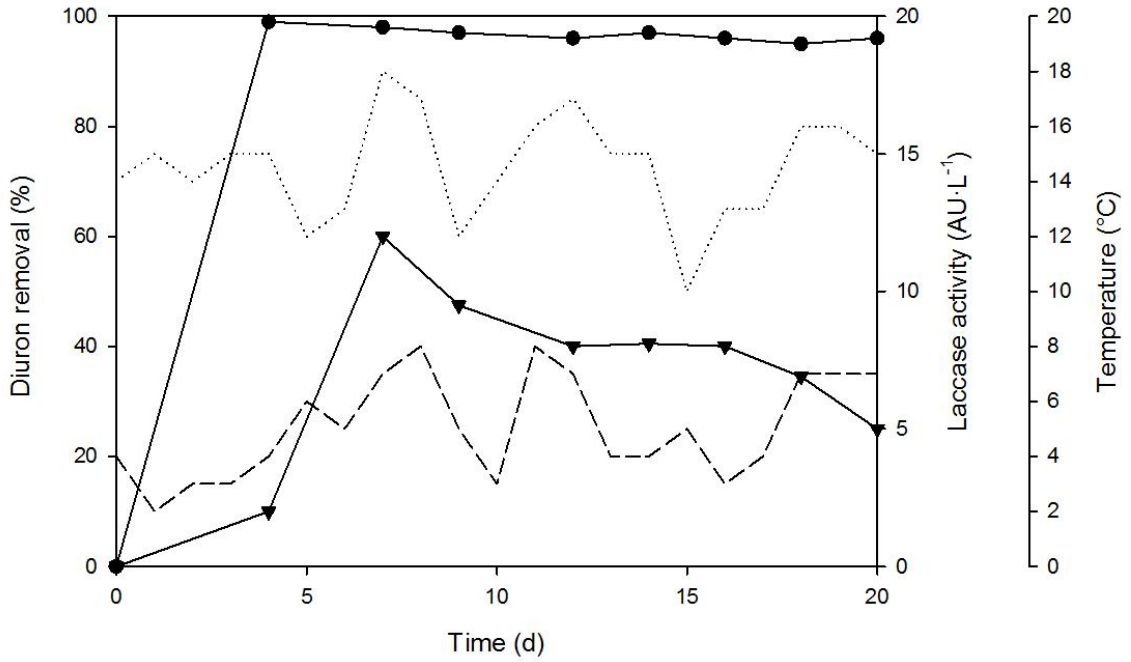


Figure 6.28. Packed-bed channel working with real wastewater spiked with diuron at environmental temperature. Symbols: (●) diuron removal, (▼) laccase activity, (···) maximum outside temperature profile and (---) minimum outside temperature profile.

The reactor was operated during 20 days with high diuron removal values (97%). No differences in the removal were obtained if compared with the channel performed at room temperature (99% removal). So, the low temperature did not affect the diuron removal taking into account that the adsorption and biodegradation act in a synergic way. More studies would be necessary to study the effect of the temperature during a long-term treatment.

However, the laccase activity seems to be affected by the low temperature. Working at environmental temperature, the maximum laccase activity obtained was 12 $AU \cdot L^{-1}$ on day 7, in contrast more than 60 $AU \cdot L^{-1}$ were obtained in the channel working at room temperature. It has been reported that laccase activity increase with the increase of the temperature, with a maximum of 50°C (Bucić-Kojić et al., 2017). Despite the differences in the laccase activity values, in both cases *T. versicolor* was active during all the treatment.

- ***Economic considerations***

The reactor was constructed with a polyvinylchloride gutter and filled with *T. versicolor* inoculated on wood chips. The wood employed was from pallets waste from a company that builds and repairs pallets, so in this system wood waste is reused and does not represent a cost.

Regarding the fungus inocula, Gabarrell et al. (2012) studied the cost of the mycelial production, which is usually expensive because it is necessary to work under sterile conditions. In this system the mycelial is only required one time for the inoculation of the wood before starting the treatment because it is not necessary to re-inoculate the fungus.

Operational costs are a major parameter determining the technology feasibility usually including the cost of chemicals and power (Sasidharan Pillai et al., 2016). In this system, no chemicals are used for the reactor operation and power is only necessary for the feeding pump. The major cost is probably derived from the necessity of the mixing of the wood, which would represent a daily task in this system. It should be necessary to try to automate the system.

Another cost is associated with the pH, first for the addition of some acid to decrease the initial influent pH and then at the final step to increase the pH before discharging the effluent. More studies are necessary about this point, because it was demonstrated that the system can work at initial pH around 6.5, so probably it would be feasible to work at real pH. One alternative for future experiments is to decrease the pH of the wastewater to the optimal fungal pH (4.5) only to fill the reactor, and then use the wastewater influent at real pH for the continuous treatment (around 7).

Taking into account the points mentioned before and the simplicity of the system, it is concluded that the packed-bed channel results in a low-cost technology for its construction and operation that could made this application feasible for wastewater treatment.

6. 3 Conclusions

T. versicolor was able to completely remove diuron, the selected target compound, at high concentrations ($mg \cdot L^{-1}$) and at real concentrations ($\mu g \cdot L^{-1}$) at Erlenmeyer scale. The implication of the cytochrome P450 instead of extracellular enzyme laccase on the diuron degradation was demonstrated. In addition, *T. versicolor* was able to completely remove 3,4-Dichloroaniline, the main degradation product, in less than 24 hours.

Furthermore, *T. versicolor* was able to remove other pesticides from different families at real concentrations (range of $\mu g \cdot L^{-1}$) with high removal values for all cases (>80%). The pesticides sorption onto fungal biomass represented a key issue. Hence, the fungal biomass samples are necessary for hydrophobic and hydrophilic pesticides, in order to distinguish the biodegradation from the sorption phenomena.

Two bioreactor systems were successfully operated for the diuron removal using *T. versicolor* immobilized onto pallet wood. The optimization of a trickle-bed bioreactor was performed and 84% diuron removal was obtained with the best operational conditions. A validation experiment with non-spiked wastewater was carried out, but due to the small pesticides amount detected in the real wastewater, no conclusion about the removal reactor performance can be drawn. However, higher removal values were obtained in the trickle-bed bioreactor inoculated with *T. versicolor* in comparison with the non-inoculated control.

Finally, a packed-bed channel bioreactor was developed. The new system was operated treating synthetic tap water during 49 days, with a result of 89% diuron removal. The system presented high diuron removal values (94%) working with real wastewater during 35 days.

Based on the results obtained in this chapter, it can be concluded that *T. versicolor* is a good alternative for the treatment of wastewater containing pesticides. Its ability to degrade a wide range of pesticides from different families was proved. In addition, two types of bioreactor with *T. versicolor* immobilized on wood were successfully operated during more than 30 days with good removal values and without operational problems.

CHAPTER 7

General conclusions

The main conclusion derived from this thesis is that bioreactors with *T. versicolor* immobilized on pallet wood are a good alternative for the long-term treatment of different wastewaters in continuous mode.

The wood pallet is a suitable support and substrate for fungal immobilization. *T. versicolor* can grow and colonize it without nutrient addition. Moreover, the pallet wood used in this work is an industrial waste, which decreases both economical and environmental impacts of the process.

The bacterial overgrowth, the main problem working with real wastewater in fungal bioreactors, was successfully controlled with the use of wood pallet as a selective carbon source.

A trickle-bed bioreactor system was developed and successfully operated for the treatment of wastewater. The optimization of some operational conditions such as recycling ratio and total volume is essential to increase the reactor performance. Good removals values were obtained, despite the small amount of biomass employed, suggesting that the fungus is more active working at this conditions.

High removal values were achieved for the treatment of wastewater from different sources:

- 61% PhACs removal from hospital wastewater during 85 days.
- 50% humic acid removal from industrial wastewater for 26 days.
- 84% diuron removal from synthetic tap water during 18 days.

The trickle-bed bioreactor was employed for the pesticide removal from real wastewater, but due to the small pesticides amount detected, no conclusion about the removal reactor performance can be drawn.

A packed-bed channel bioreactor was developed and successfully operated for pesticide removal. The aeration, pH, wood sorption and biomass amount are key issues in the performance of the bioreactor. Promising results were obtained for diuron removal (> 90% removal during 50 days).

In both type of bioreactors, no fungal biomass renovation was performed during continuous long-term treatment. *T. versicolor* was active during all the treatment process, probably because wood support provides to the fungus an environment similar to its natural habitat.

In the future, in-depth study and optimization of operational conditions which directly affect the fungal viability should be done. The use of higher biomass amount and/or the increase of the system HRT (fixed in all experiments at 3 days) are possible alternatives to improve the removal.

In general, in this work, new low-cost alternatives for the wastewater treatment with *T. versicolor* are proposed. The removal efficiency of both fungal bioreactor systems were proved for hospital wastewater (pharmaceutically active compounds), food-processing industrial wastewater (humic acid) and rural area wastewater (pesticides) during long-term treatments working in continuous mode. These results proved the capacity of *T. versicolor* to remove a variety of pollutants, which is a good feature since pollutants from different families are detected together in many real wastewaters.

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Submitted manuscript

- Zahmatkesh M., **Torán J.,** Spanjers H., Blánquez P., Caminal G. and van Lier J.B. Continuous fungal treatment of humic-rich wastewaters under nonsterile conditions: application of a fluidized bed bioreactor with partial renewal of fungal biomass" at Science of the Total Environment Journal (under revision).

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- **Torán J.,** Llorca M., Villagrasa M., Barceló D., Rodriguez-Mozaz S., Blánquez P. and Caminal G. Long-term treatment of hospital wastewater by *Trametes versicolor* in a trickled-bed bioreactor: validation and scale-up.

- **Torán J**., Peris A., López de Alda M., Eljarrat E., Blánquez P., Caminal G. and Vicent T. Degradation of selected agrochemicals by *Trametes versicolor* pellets and identification of some transformation products.

- **Torán J**., Blánquez P. and Caminal G. Biodegradation of pesticides from agriculture wastewater in immobilized reactors using a white-rot fungus on wood chip.

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ACADEMIC EDUCATION

2013 – 2014 **Master in renewable energies** – Jaén University.
Final work: Ethanol fuel from rapeseed straw.

2011 – 2014 **Degree in Health and Safety in work** - University of Salvador (Bs.As., Argentina).

2006 – 2010 **Environmental sciences degree -** University of Salvador (Bs. As, Argentina).

PREVIOUS WORK

Jun/14 – Aug/14 **Intelec Energy Engineering** (Jaen, Spain).
Training period of the Master Degree: Industrial energy audits. Design of autonomous photovoltaic system.

Jul/12 – Oct/14 **SIM Consulting Company and Enviroment Services** (Bs. As., Argentina).
Account coordinator: Consulting and advisory services in the areas of Environment and Safety and Hygiene.

Ago/10 – Mar/12 **Repsol YPF** (Bs. As., Argentina).
Analyst: in Hygiene, Security and Environment Area of the Commercial Executive Office. Tasks: legal compliance, remediation follow-up, development of indicators, risk analysis, training, SAP.

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Environmental Analyst: environmental monitoring, integrated management of hazardous waste, noise measurements.

May/09 – Oct/09 **Senate of the Argentine Nation** (Bs. As., Argentina).
Professional Practice at the Federal Institute of Parliamentary Studies: joint consulting in mining.

TEACHING EXPERIENCE

Universitat Autonoma de Barcelona (Barcelona, Spain)

2015-2016//2016-2017	Laboratorio integrado IV – Grado en Biotecnologia.
2015-2016	Procesos de separación y purificación - Grado en Biotecnologia.
2014-2015	Fundamentos de Ingeniería Química - Grado en Química.

University of Salvador (Buenos Aires, Argentina)

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OTHER PROFESSIONAL EXPERIENCES

Co-supervisor of the **Master Thesis** of Ana Vázquez Fernandez, titled "Study of bioethanol production by fermentation of glucose and xylose with different strains of white-rot fungi" Màster en Enginyeria Biològica i Ambiental. September 2017 – January 2018.

LIST OF PUBLICATIONS

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PARTICIPATION ON SCIENTIFIC CONFERENCES

XIX Reunión de la Red Temática Española Lignocel. Madrid, Spain. October 2017.
"Degradación de fármacos en agua real de hospital utilizando T. versicolor: estrategias para su tratamiento en continuo" (Oral presentation).

10th Micropol and Ecohazard Conference 2017. Vienna, Austria. September 2017.
"Continuous treatment of hospital wastewater by Trametes versicolor immobilized on lignocellulosic support" (Póster).

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I Congreso de Microbiología Agrícola y Ambiental – Asociación Argentina de Microbiología. Buenos Aires. Argentina. Septiembre 2010.
"Estudio del Sulbactam como agente estresante sobre aislamientos nosocomiales de *Acinetobacter baumannii*" (Póster)

Annexes

Analytical methods

1. Analysis of pharmaceutically active compounds in non-spiked wastewater

Samples were filtered through 0.45 μm PVDF filters (Millipore, Barcelona, Spain) and kept in PET containers at -20ºC until PhAC analysis. These analyses were performed by the chemical analytical group of Institut Catala de Recerca de l'Aigua (ICRA), led by Dra. Sara Rodríguez and Dr. Damià Barcelò.

The analytical procedure performed is based on Gros et al. (2012). Briefly, samples were filtered through 1 μm glass fiber followed by 0.45 μm PVDF membrane filters (Millipore; Billerica, MA, USA) and an appropriate volume of Na_2 EDTA was added to obtain a final concentration of 0.1% (w/w). Then, the sample 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.

2. Pesticides analysis from real wastewater

- Analysis of hydrophobic pesticides

The analysis of dicofol, chlorpyriphos and cypermethrin were made by the group from IDAEA-CSIC led by Dra. Ethel Eljarrat.

In the liquid samples, 15 ng of the Isotope Solution are added to 30 mL of sample and left to stand for 2h. Ultrasonic is used for the extraction. 1 mL of chloroform is added to the sample and mixed during 5 min. Then, the samples are centrifuged during 5 min at 3500 rpm. The organic phase is extracted. The extraction process is repeated once more and the extracts are combined. The obtained extract is evaporated with Nitrogen in a GC vial and reconstituted with 50 µL ethyl acetate.

The fungal biomass samples are first lyophilized. Copper is added in a scintillation vial. Then, 15 ng of Isotope Solution are added and left to stand overnight in the fridge. The extraction is made by pressurized liquid in ASE equipment. The cell of ASE is prepared with 6 g of Florisil, the sample mixed with 2 g of Florisil, and finally the hydromatrix until complete filling the cell. The extraction is carried out with a mixture of hexane and dichloromethane (1: 1), temperature of 100 °C, pressure of 1650 psi and two cycles of 10 min are applied. The extracts obtained are evaporated with nitrogen (Turbovap) to an approximate volume of 1 mL. Then, the concentrated extract is evaporated with nitrogen in a GC vial to dryness and reconstituted in 50 µL of ethyl acetate.

For the pesticides analysis, a 7890B GC system (Agilent Technologies, Shanghai, China) coupled to triple quadrupole 7000C (Agilent Technologies, Santa Clara, CA, USA) has been used.

A DB-5MS column (30 m x 250 µm x 0.25 µm) was used for the chromatographic separation. The carrier gas was helium at a flow of 1 $mL \cdot min^{-1}$. The injection volume was 2 µL and the temperature of the injector was 280°C. The chromatographic ramp was 80°C initial (maintained for 2 min) up to 180°C at 25°C·min^{-1} (maintained for 6 min), then at 240°C at 5°C·min^{-1} (maintained for 5 min), 280°C at 10°C·min^{-1} (maintained 5 min) up to 325°C at 30°C·min^{-1} (maintained for 2 min).

MS-MS conditions: work has been done in electronic impact mode (EI), with a temperature of the ionization source of 280°C and transfer line of 300°C, at 70 eV of collision energy.

- **Analysis of hydrophilic pesticides**

The analysis of diuron, irgarol, malathion, simazine and triallate were made by the group from IDAEA-CSIC led by Dra. Miren López de Alda.

Analysis of pesticides in the water samples was performed with a fully automated method based on isotope dilution on-line SPE sample processor (Prospekt-2, Spark Holland, Emmen, The Netherlands) connected in series with the LC–MS/MS instrument, using HySphere Resin GP and PLRP-s trace enrichment polymeric cartridges (Spark Holland), as described by Köck Schulmeyer et al. (2014).

The HySphere Resin GP cartridge was used for extraction of pesticides measured in negative ionization (NI) mode, and the PLRP-s cartridge was used for extraction of the pesticides measured by positive ionization (PI). After sample loading, cartridges were washed with 1 mL of LC-grade water and eluted with the chromatographic mobile phase.

Chromatographic separation was performed with a binary HPLC pump model 1525 from Waters using a Purospher STAR RP-18e column (125 × 2 mm, 5 μm particle diameter, from Merck, Darmstadt, Germany) and a gradient elution with acetonitrile and water as the mobile phase. The 40 min gradient started with ACN/H2O (10:90), increased to 50:50 in 5 min, then to ACN/H2O 80:20 in another 20 min, and finished with 100% ACN at 26 min. During the following 5 min the column was cleaned with 100% ACN, adjusted to the initial conditions in 1 min, and finally equilibrated for an additional 8 min.

MS/MS detection was performed in the selected reaction monitoring (SRM) mode acquiring 2 SRM transitions per compound (the first, and more abundant, is used for quantitation and the second for confirmation), and 1 SRM transition per surrogate using a TQD triple- quadrupole mass spectrometer from Waters equipped with an ESI interface (Köck-Schulmeyer et al., 2013).

3. Fiber content of lignocelulosic substrates

These analyses were performed by the Chemical Analysis Service of the Universitat Autónoma de Barcelona (Bellatera, Spain). The fibers content was determined using the method reported by Van Soest et al. (1991). The cellulose content is the result of the difference between neutral detergent fibers (NDF) and acid detergent fibers (ADF). Also, the hemicelluloses content corresponds to the difference between ADF and ligning content (ADL)

a) Neutro detergent fibers (NDF): its determination is based on the solubility of the fiber components in dedecyl sodium sulphate at neutral pH. The soluble components of the cell wall such as starch and simple sugars are solubilized by the detergent, while recalcitrant components such as cellulose, hemicelluloses and lingning reamin in the solid matrix.

Cellulose (%) = NDF –ADF

b) Acide detergent fibers (ADF): its determination is based on the cellular component solubility in a centhylmethyl ammonioum bromide solution. The residue obtained after the treatment contains mainly cellulose and lignin.

Hemicellulose (%) = ADF – ADL

c) Lignin (ADL): the dry solid obtained as a result for ADF determination is used for the ADL determination.

Lignin (%) = ADL

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