Removal of pharmaceuticals from WWTP streams by biological and physical processes

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GLÒRIA CAMINAL SAPERAS, Científica Titular del CSIC a la Unitat de Biocatàlisis Aplicada associada al IIQAB, i FRANCISCA BLÁNQUEZ CANO, Professora Titular del Departament d'Enginyeria Química, Biològica i Ambiental de la Universitat Autònoma de Barcelona,

CERTIFIQUEM:

Que el Llicenciat en Ciències Ambientals, Guillem Llorens Blanch ha realitzat sota la nostra direcció, als laboratoris del Departament d'Enginyeria Química, Biològica i Ambiental, el treball que amb el títol ***Removal of pharmaceuticals from WWTP streams by biological and physical processes***, es presenta en aquesta memòria, la qual constitueix la seva Tesi per optar al Grau de Doctor per la Universitat Autònoma de Barcelona.

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

Bellaterra, Setembre de 2016

Dra. Glòria Caminal Saperes Dra.Francisca Blánquez Cano

Li dedico aquest treball als meus pares,
al meu germa i a la meva neboda
Gràcies

Vull dedicar molt especialment aquesta
Tesi a la Sílvia i a la Lluna.
Moltíssimes gràcies.

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Si estic escrivint això vol dir que al final ho he aconseguit, no se com però ho he fet: HE ACABAT LA TESI! Si, pot semblar absurd, però hi havien moments en que això no es veia tant clar. És per això que vull agrair a les següents persones el fet d'haver-me ajudat en aquesta complicada i llarga empresa.

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Summary

Current human activities have led to the pollution of the environment, which has aroused as a global threat. In particular, the inefficient treatment of every-day products in wastewater treatment plants (WWTPs) is an important source of contaminants; especially when untreated sewage sludge and reclaimed water are valorised for agricultural porpoises. The present work describes the development of two bioremediation processes mediated with *Trametes versicolor* in order to treat different types of sludge. Additionally, one physical post-treatment has been proposed and studied so as to improve the final quality of a WWTP effluent. In both cases, the removal of pharmaceuticals (PhACs) in each stream has been assessed.

In the first section, the use of *Trametes versicolor* in biopiles at lab scale with dried sewage sludge from the WWTP of El Prat de Llobregat was studied, evaluating its capacity to remove Pharmaceutical and Personal Care Products (PPCPs) and assessing the evolution of the biopiles microbial communities.

- Appropriate lignocellulosic substrate for fungal biopiles was selected according to the review of published and unpublished experimental data.
- Total removal of drugs at real concentrations from sewage sludge was assessed for non-inoculated and fungal

inoculated biopiles, testing if the re-inoculation of the biopiles would improve the removal yields.

- The study of the bacterial and fungal communities revealed that fungal inoculated and non-inoculated biopiles evolved to similar communities.

In the second section, the growth of *Trametes versicolor* on Membrane Biological Reactor (MBR) sludge in bioslurry systems was assessed, and its capacity to remove PPCPs was evaluated.

- The ability of the fungus to remove a spiked compound in liquid medium cultures was assessed, testing different media and conditions for the bioslurry.
- In non-spiked Erlenmeyer fungal bioslurries under non-sterile conditions, the removal of a wide set of PhACs was assessed and microbial analysis were performed, showing that microbial diversity increased after 15 days of treatment.
- The fungal bioslurry treatment scale was improved to reactor scale and coupled to an anaerobic digestion process.
- Results showed that *Trametes versicolor* can remove PPCPs in bioslurry systems under non-sterile conditions in matrices as complex as an MBR sludge.

In the third section, the efficiency removal of PhACs in synthetic water was assessed in soil amended with low-cost

sorbents in order to determine if it was possible to improve the final quality of a WWTP effluent.

- The capacity of bare soil to adsorb the PhACs was assessed in 24h batch experiments in order to quantify the distribution coefficient of the drugs.
- The adsorption of three drugs with similar octanol/water coefficient at different soil:amendment ratios was assessed and carried out in 24h batch experiments, in order to determine which ratio would be the most suitable.
- The removal percentage of each pharmaceutical compound was measured in soil amended with two low-cost sorbents (biochar and NUA) during 21 days. The results showed high removal percentages for all the tested PhACs, proving the efficiency of sorbents to remove emerging pollutants from water by amending the soil.

The experiments of the two first sections were performed in the research group BioremUAB (Grup de Biodegradació de Contaminants Industrials i Valorització de Residus) from the Department of Chemical, Biological and Environmental Engineering in Universitat Autònoma de Barcelona (UAB). The aim of this group is the development of novel biological techniques to degrade emerging pollutants in different matrices. The experiments of the last section of this thesis were carried out in the centre Land & Water from the CSIRO. The

scope of this centre is to deliver innovative solutions to the complex challenges that arise from the demands and impacts of human activities.

Resum

Les actuals activitats humanes han comportat la contaminació del medi ambient, la qual cosa ha esdevingut una amenaça global. En concret, l'ineficient eliminació de productes d'ús diari a les plantes de tractament d'aigües residuals (EDARs) es una font de contaminació en si mateixa; especialment quan els llots no tractats i l'aigua resultant són valoritzats en activitats agrícoles. En el present treball s'han desenvolupat dues tècniques de bioremediació amb *Trametes versicolor* per tal de tractar diferents llots. A més a més, s'ha proposat i estudiat un post-tractament físic per tal d'incrementar la qualitat de l'efluent final d'una EDAR. En tots dos casos, l'eliminació de fàrmacs en cada corrent ha estat avaluada.

En la primera secció, es va estudiar l'ús de *Trametes versicolor* en biopiles a escala de laboratori per tractar llots d'EDAR secs; tot avaluant la seva capacitat per eliminar fàrmacs i l'evolució de les comunitats microbianes.

- Es va escollir un substrat lignocel·lulosic per les biopiles inoculades amb el fong en base a la revisió de dades experimentals publicades i no publicades.
- Es va avaluar l'eliminació total de fàrmacs a concentració real en llots de depuradora tractats en biopiles inoculades i no inoculades amb el fong, provant si la re-inoculació de les biopiles milloraria l'eliminació final.

- L'estudi de les comunitats bacterianes i fúngiques de les biopiles inoculades i no inoculades amb el fong va mostrar que totes dues comunitats van acabar evolucionant cap a poblacions similars.

En la segona secció, es va avaluar el creixement i la capacitat per eliminar fàrmacs de *Trametes versicolor* en llots provinents d'un reactor biològic de membrana (MBR) en sistemes de bioslurry.

- Es va avaluar la capacitat del fong per eliminar compostos en medis líquids dopats, provant diferents medis de cultiu i condicions.
- Es va avaluar l'eliminació d'un gran ventall de fàrmacs per part del *fong* en bioslurries no estèrils i no dopats. Les anàlisis microbiològics van demostrar un increment de la diversitat microbiana després de 15 dies de tractament.
- Es va incrementar l'escala dels bioslurries de Erlenmeyer a reactor de 5L i el seu corrent de sortida es va digerir anaeròbicament, per tal de comprovar si el tractament amb el fong podia ser re-valoritzat.
- Els resultats finals mostren que *Trametes versicolor* pot eliminar PPCPs en bioslurry en condicions no esterils.

En la tercera secció, es va avaluar l'eliminació de fàrmacs en aigua sintètica mitjançant l'esmena de sòls amb adsorbents de

baix cost, per tal de determinar si era posible millorar la qualitat de l'efluent d'una EDAR.

- Es va determinar la capacitat d'adsorció del sòl sense esmenar amb experiments en discontinu de 24h per tal de determinar els coeficients de distribució dels fàrmacs seleccionats.
- Per tal de determinar quina proporció d'esmena seria la mes adequada, es va estudiar l'adsorció en 24h de 3 compostos amb un coeficient octanol/aigua similar en experiments en discontinu.
- Es va mesurar el percentatge d'eliminació de cadascun dels fàrmacs provats per cada esmena de baix cost (biochar i NUA) utilitzada. Els resultats van mostrar alts percentatges d'eliminació per tots els fàrmacs, demostrant l'eficàcia dels adsorbents de baix cost com a esmenes de sòl.

Els experiments de les dues primeres seccions es va realitzar al grup d'investigació BioremUAB (Grup de Biodegradació de Contaminants Industrials i Valorització de Residus) del departament d'Enginyeria Química, Biològica i Ambiental de la Universitat Autònoma de Barcelona (UAB). L'objectiu d'aquest grup es el desenvolupament de nous processos biològics pel tractament de contaminants emergents en diferents matrius. Els experiments de l'última secció es van desenvolupar al centre Land & Water del CSIRO. L'objectiu d'aquest centre és el de

proporcionar solucions innovadores als complexos desafiaments que sorgeixen de les activitats humanes sobre el medi ambient.

Resumen

Las actuales actividades humanas han conllevado a la contaminación del medio ambiente, lo que se ha convertido en una amenaza global. En concreto, la ineficiente eliminación de productos de uso diario en las plantas de tratamiento de aguas residuales (EDARs) es una fuente de contaminación en sí misma; especialmente cuando los lodos no tratados y el agua resultante son valorizados en actividades agrícolas. En el presente trabajo se han desarrollado dos técnicas de biorremediación con *Trametes versicolor* para tratar diferentes lodos. Además, se ha propuesto y estudiado un post-tratamiento físico para incrementar la calidad del efluente final de una EDAR. En ambos casos, se ha evaluado la eliminación de fármacos en cada corriente.

En la primera sección, se estudió el uso de *Trametes versicolor* en biopilas a escala de laboratorio para tratar lodos de una EDAR secos; evaluando su capacidad para eliminar fármacos y la evolución de las comunidades microbianas.

- Se escogió un sustrato lignocelulósico para las biopilas inoculadas con el hongo en base a datos experimentales publicados y no publicados.
- Se evaluó la eliminación total de fármacos a concentración real en lodos de depuradora tratados en biopilas

inoculadas y no inoculadas con el hongo, probando si la re-inoculación de las biopilas mejoraría la eliminación final.

- El estudio de las comunidades bacterianas y fúngicas de las biopilas inoculadas y no inoculadas con el hongo mostraron que ambas comunidades acabaron evolucionando hacia poblaciones similares.

En la segunda sección, se evaluó el crecimiento y la capacidad para eliminar fármacos por parte de *Trametes versicolor* en lodos provenientes de un reactor biologico de membrana (MBR) en sistemas de bioslurry.

- Se evaluó la capacidad del hongo para eliminar compuestos en medios líquidos dopados, probando diferentes medios de cultivo y condiciones.
- Se evaluó la eliminación de un amplio número de fármacos por parte del hongo en bioslurries no estériles y no dopados. Los análisis microbiológicos demostraron un incremento de la diversidad microbiana después de 15 días.
- Se incrementó la escala de los bioslurries: de Erlenmeyer a reactor de 5L; y su corriente de salida se digerió anaeróbicamente, a fin de comprobar si el tratamiento con el hongo podía ser re-valorizado.
- Los resultados finales muestran que *Trametes versicolor* puede eliminar fármacos en bioslurry en condiciones no estériles.

En la tercera sección, se evaluó la eliminación de fármacos en agua sintética mediante la enmienda de suelos con adsorbentes de bajo coste, con el objetivo de determinar si era posible mejorar la calidad de un efluente de EDAR.

- Se determinó la capacidad de adsorción del suelo sin enmendar con experimentos en discontinuo de 24h para determinar los coeficientes de distribución de los fármacos seleccionados.
- Para determinar qué proporción de enmienda sería la más adecuada, se estudió la adsorción en 24h en experimentos en discontinuo de 3 compuestos con un coeficiente octanol / agua similar.
- Se midió el porcentaje de eliminación de cada uno de los fármacos probados por cada enmienda de bajo coste (biochar y NUA) utilizada. Los resultados indicaron altos porcentajes de eliminación para todos los fármacos, demostrando la eficacia de los adsorbentes de bajo coste como enmiendas.

Los experimentos de las dos primeras secciones se realizó en el grupo de investigación BioremUAB (Grupo de Biodegradación de Contaminantes Industriales y Valorización de Residuos) del departamento de Ingeniería Química, Biológica y Ambiental de la Universitat Autònoma de Barcelona (UAB). El objetivo de este grupo es el desarrollo de nuevos procesos

biológicos para el tratamiento de contaminantes emergentes en diferentes matrices. Los experimentos de la última sección se desarrollaron en el centro Land & Water del CSIRO. El objetivo de este centro es el de proporcionar soluciones innovadoras a los complejos desafíos que surgen de las actividades humanas sobre el medio ambiente.

Abbreviations

ACT: Methanogenit Activity
AD: Anaerobic Digestion
AEO: Alkylphenol Ethoxylates
AS: Activate Sludge Reactor
ASE: Accelerated Solvent Extraction
BMP: Biochemical Methane Potential
BOD: Biological Oxygen Demand
BQL: Below Quantification Limit
BTEX: Benzene, Toluene, Ethylbenzene, and Xylenes
CBZ: Carbamazepine
COD: Chemical Oxygen Demand
CP: Chlorophenols
CRT: Cellular Retention Time
DGGE: Denaturing Gradient Gel Electrophoresis
DMP: Dimethoxyphenol
DOC: Dissolved Organic Carbon
DON: Dissolved Organic Nitrogen
DW: Dry Weight
EDC: Endocrine Disrupting Chemicals
EP: Environmental Pollutants
ESI: Electrospray Ionisation
FAO: Food and Agriculture Organisation
FOG: Fat, Oil and grease waste
GHG: Green House Gasses
HRT: Hydraulic Retention Time
HZT: Hydrochlorothiazide
IBI: International Biochar Initiative
IBU: Ibuprofen
ITS: Internal Transcribed Space
IWC: Initial Water Content
KTP: Ketoprofen
LiP: Lignin Peroxidase
LME: Lignin-Modifying Enzymes
LMP: Lignin-Modifying Peroxidases
MBR: Membrane Biological Reactor
MLSS: Mixed Liquor Suspended Solids
MnP: Manganese Peroxidase
MWHC: Maximum Water Holding Capacity
ND: Non-Detected
NPEO: Nonylphenol Ethoxylates
NSAIDs: Non-Steroidal Anti-Inflammatory Drugs
NUA: Neutralised Used Acid
OFMSW: Organic Fraction of Municipal Solid Waste
OFX: Ofloxacin
PAE: Phthalic Acid Esters
PAH: Polycyclic Aromathic Hydrocarbon

PCB: Polychlorinated Biphenyl
PCE: Perchloroethylene
PCP: Personal Care Products
PCR: Polymerase Chain Reaction
PFC: Perfluorinated Compounds
PFOA: Perfluoro-n-Octanoic Acid
PFOS: Perfluorooctane Sulphonate
PhACs: Pharmaceutical Active Compounds
PPCP: Pharmaceutical and Personal Care Products
PRN: Propranolol
qPCR: quantitative PCR
SAT: Soil-Aquifer Treatment
SBR: Sequencing Batch Reactor
SD: Standard Deviation
SMX: Sulfamethoxazole
SPE: Solid Phase Extraction
SSF: Solid-State Fermentation
TAN: Total Ammonia Nitrogen
TC: Total Carbon
TCD: Thermal Conductivity Detector
TCE: Trichloroethylene
TOC: Total Organic Carbon
TP: Transformation Products
TPH: Total Petroleum Hydrocarbons
TRM: Trimethoprim
TSS: Total Suspended Solids
UASB: Up-flow Anaerobic Sludge Blanket
VFA: Volatile Fatty Acids
VOC: Volatile Organic Compound
VS: Volatile Solids
VSS: Volatile Suspended Solids
WD: Water Drained
WHC: Water Holding Capacity
WRF: White Rot Fungi
WWTP: Wastewater Treatment Plant

CHAPTER 1

General Introduction

1.1.Emerging pollutants

In general, water pollution occurs when a foreign substance is introduced into any water body, leading to a quality lost and/or a potential threat for the environment and the health of humans and animals (Hogan, 2014). Currently, 80% of the world population is exposed to contaminated water sources, being developing countries' habitants the most endangered (Vörösmarty *et al.*, 2010). The common diseases associated with water pollution are waterborne diseases (e.g. cholera, diarrhea, legionellosis and typhoid fever) (Schwarzenbach *et al.*, 2010); however, other less traditional and non-biological pollutants can lead to health disorders such us metal poisoning (caused by Ar, Be, Cd, Cr, Co, Pb, Hg, Mo, Se, Ag, TI and Zn) (Harada, 1995; Wittmann, 1981), endocrine and reproductive systems alterations (Damstra, 2002), obesity (Dirinck *et al.*, 2011; Elobeid and Allison, 2008), pregnancy complications (Damstra, 2002; Dewan *et al.*, 2013; Vafeiadi *et al.*, 2014; Vizcaino et al., 2014), cardiovascular problems, and cancer (Ljunggren *et al.*, 2014).

Emerging pollutants (EPs), also known as micro-pollutants, are a group of several man-made chemicals and some natural products, which are not yet regulated, have not been deeply studied, and are believed to be a danger to environmental ecosystems and for human and/or animal health. These pollutants can be detected in water bodies such as salt-water,

freshwater and wastewater, and in solid matrices, e.g. sludges and soils, with concentrations from a few $ng \cdot L^{-1}$ to thousands of $\mu g \cdot L^{-1}$. The concentration of these pollutants in wastewater treatment plants (WWTPs) depends on economic, geographic and seasonal parameters such as the size of the WWTP and it's general elimination efficiency, persistence of the pollutants, wastewater sources (i.e. industrial, urban, hospital or domestic origin) and potable water usage and consumption (Deblonde *et al.*, 2011; Farré *et al.*, 2008; Jiang *et al.*, 2013).

Although there is not a clear classification of EPs, they can be distributed according to its origin or source. The main compounds that comprise this heterogeneous group of pollutants are: pharmaceuticals products (PhACs), personal care products (PCPs), endocrine-disrupting chemicals (EDCs), and perfluorinated compounds (PFCs) (Farré *et al.*, 2008; Jiang *et al.*, 2013).

PhACs are a large class of chemical compounds that can be found in most wastewater streams. Briefly, this group is made up of prescribed and non-prescribed drugs for human and veterinary use, and drug abuse substances; however, antibiotics, anti-inflammatories, ß-blockers and X-ray contrast media are the most widely spread. All these compounds are biologically active, not easily biodegradable, and water-soluble (Jiang *et al.*, 2013).

PCPs are a wide and heterogeneous class of cosmetic and personal hygiene products. These compounds are discharged

into sewage collection and disposal systems through cleaning wastewater streams. Nonylphenol ethoxylates (NPEOs) and alkylphenol ethoxylates (AEOs) are the most spread groups of PCPs due to their extensive use in domestic and industrial tasks (Farré *et al.*, 2008). According to Jiang *et al.*, the possible destiny of PCPs in a WWTP are: (i) mineralization to CO_2 and water, (ii) retention into the biosolids if the compound is hydrophobic, (iii) pass all the treatments without further relevant transformation (Jiang *et al.*, 2013), and (iv) transformation into different compounds.

Some of the mentioned PhACs and PCPs (the two groups together are known as PPCPs – pharmaceutical and personal care products) would be transformed in WWTP by biological, physical or chemical processes into products quite different from the original or parent compound. These transformation products (TPs) can be equal, more or less toxic, persistent, mobile or accumulative than the parent compound itself, but it is known that the environmental exposure of organisms to these chemicals will be similar to the parental compounds (Fatta-Kassinos *et al.*, 2011b; Kolpin *et al.*, 2009).

EDCs are natural and synthetic chemicals that interfere with endocrine system functions of wildlife and humans. In fact, these compounds are PhACs that have been classified apart since their fate and effects are different and specific. As for PPCPs, humans and animals excrete these compounds, which can reach the environment through discharge of wastewater

or animal waste disposal. Estrone, 17b-estradiol (both natural compounds), and bisphenol A (synthetic) are the main inputs of EDCs into wastewater. The potential repercussions of EDCs in wildlife and humans may be accumulative and affect the reproduction and development of the exposed organisms (Manickum and John, 2014).

PFCs are a relevant class of emerging contaminants due to their toxicity, persistence, and bioaccumulation. PFCs are synthetic fluorinated hydrocarbons used for over 50 years in the manufacturing of many other products, e.g. surfactants, lubricants, paints, and fire retardants. Perfluoro-n-octanoic acid (PFOA) and perfluorooctane sulphonate (PFOS) are the most well-known and studied PFCs (He *et al.*, 2015).

In general, drugs' fate and distribution is related with their production and consumption, which differs from one country to another with an important seasonal fluctuation (Kaplan and Laing, 2005). In Spain, US$ 28,009,000,000 of PhACs were sold in 2011, representing a large amount of drugs put into circulation (IFPMA, 2012). In 2013, omeprazole was the most consumed drug in Spain with 54.4 millions of sold packages, followed by acetaminophen (32 millions of packages), simvastatin (24.7 millions of packages), salicylic acid (24.6 millions of packages) and atorvastatin (18.1 millions of packages) (Ministerio de Sanidad Servicios Sociales e Igualdad, 2014). PhACs can finally reach the environment through WWTP

discharges, industrial effluents, direct disposal and land application (Daughton and Ternes, 1999).

Nowadays WWTPs are only planned to eliminate certain impurities or pollutants, such as nutrients or heavy metals, but not PhACs. In consequence, large amounts of those products goes through all the treatment steps without any relevant change in their concentration in neither the final effluent nor the sludge (Deblonde *et al.*, 2011; Fatta-Kassinos *et al.*, 2011a; Gavrilescu *et al.*, 2014; Luo *et al.*, 2014). Briefly, some drugs will be removed by sorption in the primary treatment, and next they will be dispersed, diluted, biodegraded, and/or abiotic transformed during the secondary treatment. The best effectively removed PhACs during wastewater treatments are psychostimulants (ca. 97% removal). On the contrary, the less effectively removed are analgesics, anti-inflammatories and ß-blockers (Deblonde *et al.*, 2011; Luo *et al.*, 2014).

The principals concerns with PPCPs are that they were not traditionally considered as pollutants, they can have a point or diffuse origin, and they can be detected in environmental compartments and places where they were neither used nor produced. Because of that, traditional pollution prevention, control and attenuation techniques are out-dated (Gavrilescu *et al.*, 2014; Geissen *et al.*, 2015) and must be adapted to each environmental compartment, type of contaminant and source of pollution. For those reasons, the present thesis is focused on this type of pollutants – PhACs – in different WWTP's streams.

On one hand, the occurrence of PhACs in the aquatic environment, especially those with a hydrophilic behaviour, depends not only on the source and type of contaminant, but also in the water body itself (i.e. wastewater, surface water, groundwater and drinking water) (Luo *et al.*, 2014). First, the presence of drugs in wastewater is affected mainly by geographic and temporal factors – which are highly variable –; for instance, the local consumption of PhACs will determine the concentration of those reaching the WWTPs, and the operational of the plant will outline which amount is removed (Jiang *et al.*, 2013; Verlicchi and Zambello, 2015). Second, the removal achieved in upstream WWTPs, the water intake, and the season will define the occurrence of PPCPs not only in the effluent, but also in the subsequent drinking water; however, most of the contaminants detected in water purification plants have concentrations below the limits of quantification (Richardson, 2003; Vulliet and Cren-Olivé, 2011; Vulliet *et al.*, 2011; C. Wang *et al.*, 2011). Next, the presence of PhACs in surface water is also affected by the WWTPs effluents discharge, being the main source of pollution; but the occurrence of PPCPs can be naturally attenuated by dilution, sorption to sediments and biodegradation (Gómez *et al.*, 2012; Kasprzyk-Hordern *et al.*, 2009). Finally, although groundwater is the less polluted water body, landfill leachates, wastewater infiltration, ground-surface water interactions, artificial aquifer recharge systems, and septic tanks leaks can lead to its

contamination; but, as previously mentioned for surface water, dilution, adsorption and degradation processes could decrease the PPCPs concentration (Lapworth *et al.*, 2012; Loos *et al.*, 2013, 2010; Teijon *et al.*, 2010). Consequently, the key to a better pollution control for aquatic environments involves wastewater treatments that take into account not only the presence of PPCPs, but also its removal.

On the other hand, the application of organics wastes such as sewage and agro-industrial sludges in agricultural soils can also represent a sanitary and environmental risk due to the presence of EPs. The use of sludge as soil amendment helps to restore degraded soils, increasing the organic matter content and the water holding capacity of the soil; however, it also contributes to spread pollutants (Alvarenga *et al.*, 2015; Verlicchi and Zambello, 2015). As mentioned before, current WWTPs are not designed to treat these pollutants and, as a result, hydrophobic PPCPs will remain in the sludge. Once the drugs have arrived to the WWTP, they can be adsorbed, degraded, and transformed in the biosolids, or remain intact. Drugs can be sorb into sludge – especially in activated sludge reactors due to the large specific surface area –, being separated from the liquid phase, but not completely removed from the system. These pollutants can also be degraded via co-metabolism of the microbial communities during secondary treatment: the PhACs are used as secondary source of C, being consumed only if the primary C source is abundant, i.e.

biodegradable COD – chemical oxygen demand. Abiotic transformations can occur, but their impact in the general PPCPs removal is low (Semblante *et al.*, 2015). As a result, sewage sludge is a wide source of PPCPs when used as amendment for soils.

1.2. Bioremediation

Bioremediation techniques are procedures designed to eliminate pollutants using the abilities of certain microorganisms to degrade a wide range of compounds. Although physical and chemical processes have been developed to treat environmental pollutants with lower operational times (especially for polluted soils e.g. vapour extraction, solidification, incineration, venting/aeration, flushing and encapsulation), those techniques generate by-products and wastes that must be treated. In contrast, bioremediation systems do not generate useless by-products or wastes since the final product can be valorised, resulting in more sustainable and costless methods (Solanas, 2007). According to Adams *et al.* (2015), bioremediation can be performed with three different strategies: natural attenuation, biostimulation and bioaugmentation.

Natural attenuation (or intrinsic bioremediation) occurs when the pollution attenuation is mediated by the natural metabolic activity of the autochthonous microorganisms of the water, soil or sludge to be treated (Hooker and Skeen, 1996). In

biostimulation, the metabolic activity of the indigenous microorganisms is stimulated adding bulking material, water and nutrients or improving the aeration (Venosa, 2003). In comparison, in bioaugmentation procedures not only bulking material, water, nutrients or aeration are supplied, but also a microorganism (or a consortium) is inoculated, which has been previously demonstrated to be able to degrade the target compounds (Hairston *et al.*, 1997).

Despite these distinct strategies, any bioremediation technique can be performed in the same location were the polluted water, soil or sludge is located (in situ) or in especial treatment plants (ex situ) (Solanas, 2007). However, some factors must be considered before starting any bioremediation process in order to adapt the system, as the final results will be subjected to these factors. The most important factors to take into account are: the energy source (i.e. organic matter), the environmental factors (such as pH, temperature, moisture and presence of nutrients), the bioavailability (e.g. sorption/desorption, diffusion and dissolution of the pollutants), and the bioactivity (operational activity of the microorganisms) (Boopathy, 2000; M.Vidali, 2011).

1.2.1. Fungi in bioremediation: mycoremediation

Fungi are a wide heterogenic group of eukaryotic microorganisms that are spread all over the world, living in almost any habitat (Singh, 2006). However, it was not until 1977

that fungi were first proposed as suitable organisms to be used in the removal of environmental pollutants by Cerniglia and Perry (1973). These researchers isolated fungi from estuary mud that were capable to degrade crude oil, especially the non-ligninolytic fungus *Cunninghamella elegans*. Lately, in 1980s it was proposed to use a specific group of fungi for bioremediation: the white rot fungi (WRF) that are known for causing the white rot in trees (Bumpus and Aust, 1987; Tien, 1987). The WRF group is mainly composed of basidiomycetes, which are filamentous fungi formed of hyphae and with sexual reproduction – although some can reproduce asexually. These fungi were studied for pollutant attenuation because the enzymes that allow the wood decomposition can also drive the degradation of organic compounds (Singh, 2006; Webster and Weber, 2007).

In fact, ligninolytic fungi are capable to mineralize and depolymerize lignin under aerobic conditions because of their unspecific extracellular enzymatic system, which is utilized to break insoluble macromolecules into smaller and soluble molecules (Wallenstein and Weintraub, 2008). The extracellular enzymatic system of a WRF is composed of the so-called lignin-modifying enzymes (LMEs): laccases and lignin-modifying peroxidases (LMPs), which act at the same time during the lignin degradation, but with different electron acceptors: O_2 for laccases and H_2O_2 for peroxidases (Lundell *et al.*, 2010). The lignin degrading system generally takes place during the

secondary metabolism of the WRF, it is induced when the fungi are subjected to starvation (C and N), and it is affected by external factors such as agitation and temperature (Gao *et al.*, 2010). Additionally, it has been demonstrated that the intracellular cytochrome P450 also plays an important role in the degradation of organic compounds by WRF (Cerniglia, 1997; Marco-Urrea *et al.*, 2008).

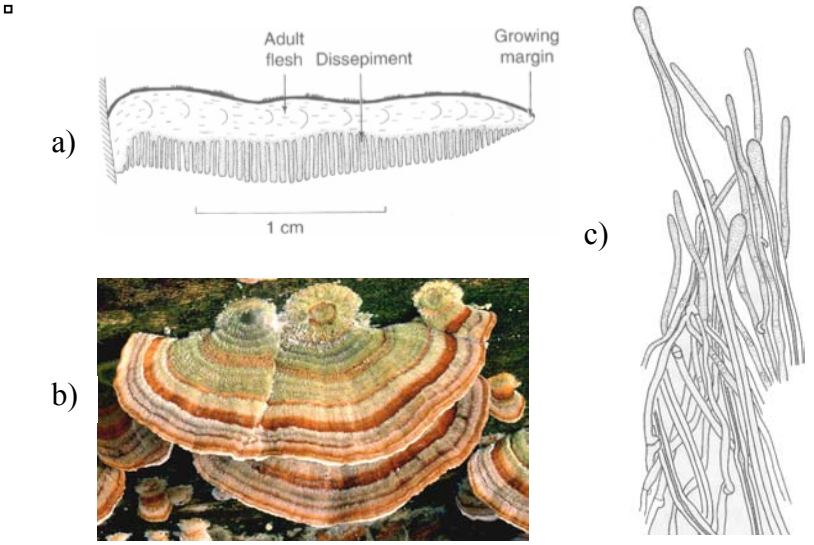


Figure 1.1. *Trametes versicolor:* a) vertical section of the basidiocarp, b) in the nature, and c) hyphae system (Webster and Weber, 2007)

In the present work, *Trametes versicolor*, which is a saprophyte basidiomicet from the Polyporaceae family commonly found all year-round on dead trees, has been used for further experiments. It is a common fungus in temperate forests of Europe, Asia and North America. The typical basidiocarp of *T. versicolor* is triangular or round, flat, tough

and shows concentric rings with different colours (images ***a*** and ***b*** in figure 1.1) (Webster and Weber, 2007). The ligninolytic system of this fungus is stimulated when subjected to N and C starvation, and it is capable to produce laccase, lignin peroxidases (LiP) and manganese peroxidases (MnP) (Wesenberg, 2003) depending on the medium composition (Acebes, 2008). Laccase is the most common expressed enzyme by *T. versicolor*, which can be produced constitutive or induced, (Sariaslani and Dalton, 1989) but also cytochrome P450 is part of *T. versicolor*'s enzymatic system (Ichinose *et al.*, 2002).

Fungal laccases (benzenediol:oxygen oxireductase, EC 1.10.3.2.) belong to the multicopper blue phenoloxidases, which can be (primarily) extracellular and (secondarily) intracellular. These laccases are glycoproteins with variable molecular weight (60 – 70 kDa), different forms, low redox potential (450 – 800 mV), isoelectric points ranging from 3.0 to 7.0, and low specificity for the electron-donating substrate (Baldrian, 2006; Svobodová *et al.*, 2008). The laccase enzyme encloses four copper atoms in diverse oxidation states (I, II, III), which plays an important role in the catalytic mechanism, and it oxidases the target compounds reducing O_2 to H_2O with a total reduction of four electrons (Giardina *et al.*, 2010). WRF in general and *T. versicolor* in particular are reported to be the most active laccase producers; however, it has been found that *Phanerochaete chrysosporium*, the most studied WRF, do not

have laccase genes and consequently do not produces this enzyme (Martinez *et al.*, 2004). Hence, the laccase role in the degradation of lignin has been questioned and is being investigated (Hatakka and Hammel, 2010; Lundell *et al.*, 2010).

The research group were this thesis has been developed has been studying and applying *T. versicolor* in both liquid and solid cultures for the degradation of synthetic dyes (Blánquez *et al.*, 2008, 2007, 2006, 2004; Casas *et al.*, 2013, 2009; Romero *et al.*, 2006); BTEX, TCE and PCE (Aranda *et al.*, 2010; Marco-Urrea *et al.*, 2009a, 2009b; Vilaplana *et al.*, 2008, 2007); PAHs (Borràs *et al.*, 2010; Sayara *et al.*, 2011); EPs (Badia-Fabregat *et al.*, 2015, 2012; Cruz-Morató *et al.*, 2014, 2013a; Marco-Urrea *et al.*, 2010a, 2010b, 2010c, 2010d, 2009c); and agrochemicals (Mir-Tutusaus *et al.*, 2014). Nevertheless, other researchers have been working with different WRFs in order to remove a wide range of contaminants from both soils (Chen *et al.*, 2015; Lladó *et al.*, 2013; Winquist, 2014) and liquid effluents (Xueqing Li *et al.*, 2015; Zhang and Geissen, 2012).

1.3. Wastewater Treatment Plants

As mentioned before, water pollution occurs when a foreign substance is introduced into any water body. In general, the traditional contaminants monitored and removed during the wastewater treatment are: organic matter, suspended solids, nutrients, metals, and pathogens (Conley *et al.*, 2009; Duruibe *et al.*, 2007; Eddy *et al.*, 1991; Lipp *et al.*, 2001;

Ramalho, 1996). A general flow diagram for municipal wastewater treatment is shown in figure 1.2. WWTPs are designed to treat these contaminants in order to obtain an effluent without hazardous impurities or compounds that can be returned into the water cycle. In general, a wastewater treatment is a sequence of physical and biological processes designed to remove the main pollutants from domestic, urban, agricultural or industrial wastewaters in 6 steps (Eddy *et al.*, 1991; Ramalho, 1996): pre-treatment, primary treatment, secondary treatment, tertiary treatment, disinfection and sludge treatment.

However, current municipal WWTP are not planned to remove xenobiotics – foreign substances that do not exist in nature before their industrial synthesis. Given that, large amounts of those contaminants goes through all the treatment steps without any relevant change in their concentration in neither the final effluent nor the sludge (Deblonde *et al.*, 2011; Fatta-Kassinos *et al.*, 2011a; Gavrilescu *et al.*, 2014; Luo *et al.*, 2014). Consequently, the present research has studied the removal of PhACs in different WWTP ellfuents: (i) the sewage sludge, (ii) the recirculation stream of a Membrane Biological Reactor (MBR) and (iii) the plant outlet stream or reclaimed wastewater.

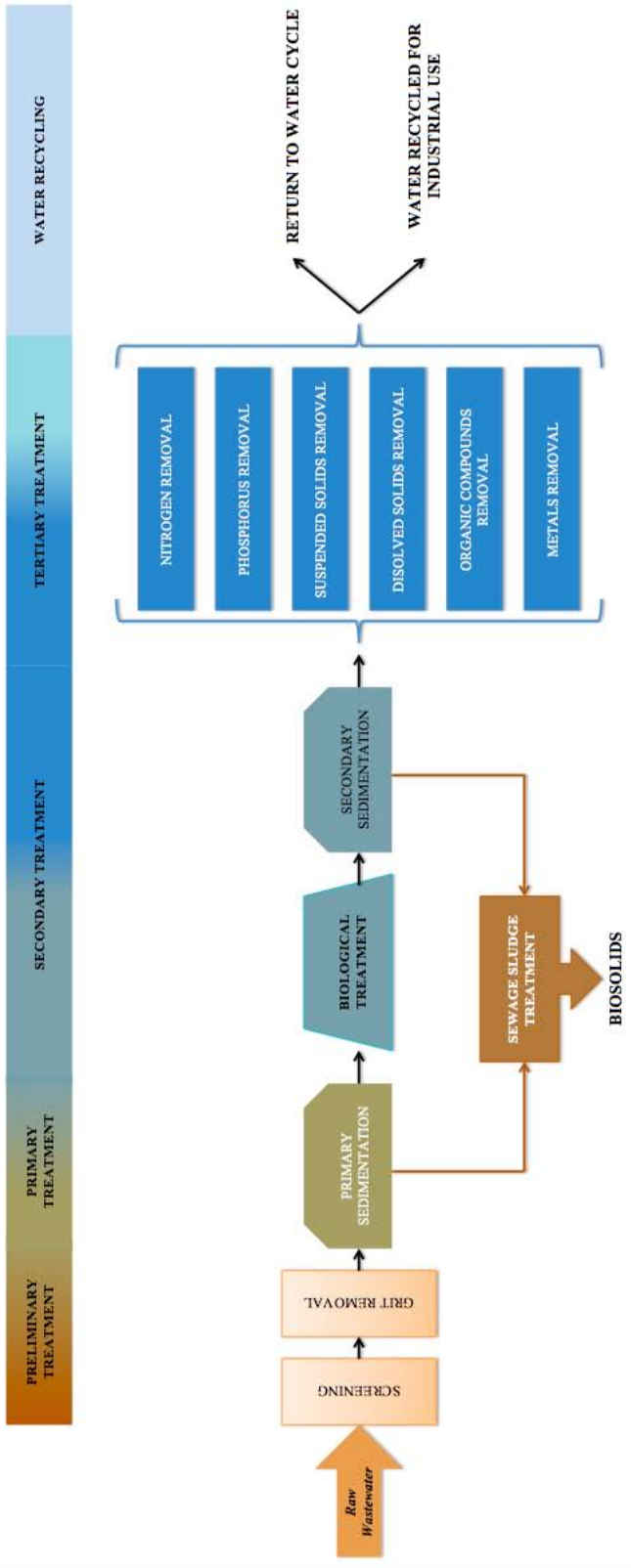


Figure 1.2. Generalized flow diagram for municipal wastewater treatment. Adaptet from Eddy *et al.* (1991) and Ramalho (1996).

1.3.1. Valorisation of sewage sludge

Sludge is an inevitable by-product in any WWTP that comprises all the insoluble wastes produced during the biological treatment of the wastewater. Since the sewage sludge is mainly liquid, before being stabilized it is subjected to treatments designed to increase the solid content in order to reduce the total volume (Eddy *et al.*, 1991; Ramalho, 1996). However, recent research has demonstrated that traditional dewatering techniques (e.g. centrifugation, filtration and evaporation) and stabilization methods (e.g. aerobic and anaerobic digestion, lime stabilization, composting and heat drying) do not efficiently remove the EPs in the sludge (Carballa *et al.*, 2007b; Clara *et al.*, 2004; Radjenovic *et al.*, 2007).

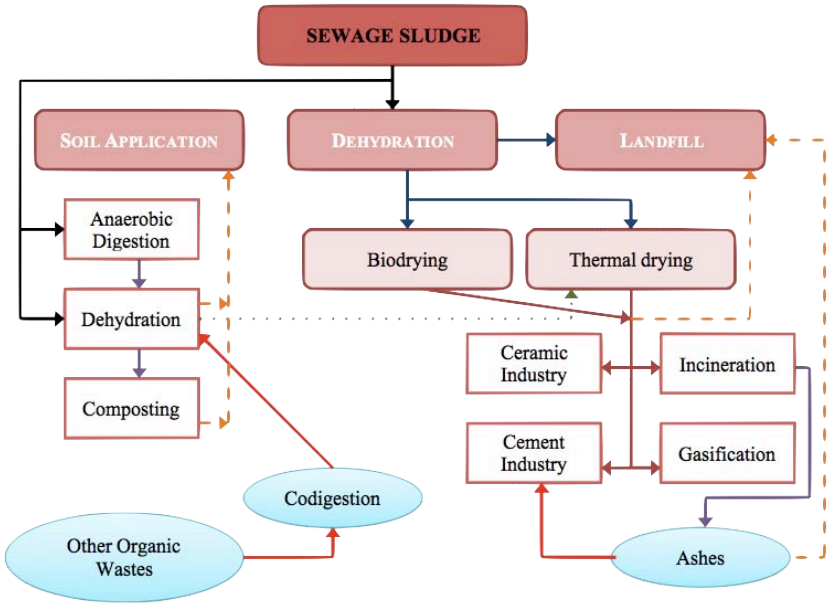


Figure 1.3. Main managing options use to manage sewage sludge in Catalonia. Adapted from Mata (2007)

Sewage sludge can be recycled, valorised or disposed using several routes. In the European Union the most common paths to stabilize, treat and valorise theses sludges are the energy recovery via biogas production or direct incineration, and the production of agricultural fertilizers and amendments via composting (Fytili and Zabaniotou, 2008; Gendebien *et al.*, 2010). The most common paths to manage sewage sludge in Catalonia are showed in figure 1.3. In this region the thermal drying of the sludge is done before further utilization, since it is economically feasible and the storage of wheat or liquid sludge in Catalonia is very limited. After this pre-treatment, the sewage sludge will be mainly incinerated in order to produce energy or revalorized in the ceramic industry to make bricks (Mata, 2007). However, as previously discussed, these methods for themselves do not avoid the environmental pollution by PhACs; in fact, they can be the cause of their dissemination. Of all the available methods to valorise WWTP sludge, only three of them will be revised since they are the most common and widespread: the energy recovery in anaerobic digesters, the production of compost and the soil application as amendment.

1.3.1.a. *Anaerobic digestion of sewage sludge*

The nowadays society is gaining interest in renewable sources of energy due to the depletion of traditional sources, which are based in fossil fuels, and the increased awareness on global warming (Hoel and Kverndokk, 1996; Höök and Tang,

2013; Kempton, 1993). The production of biogas from the anaerobic digestion (AD) of sewage sludge can be considered as a renewable source of energy that can generate both electricity and heat. Additionally, the AD is a widely practiced way to manage and valorise WWTP sludge since the mid 1900s. The AD of sludge, which can be carried out in one or two tanks/digesters, consists of two stages: (i) sludge from primary and secondary treatments are combined with already digested sludge, which acts as inoculum, and heated; (ii) and next, the mixture is allowed to digest without neither mixing nor additional external source of heat, as the process generates enough heat to maintain the process active. About 40% to 60% of the organic solids from the sludge will be converted into biogas, which is composed of CH_4 (60-65 %), CO_2 (30-35 %) and minor and trace elements such as H_2, N_2, H_2S and H_2O. The remaining organic matter of the process will be chemically stable and have fewer levels of pathogens than the initial sludge (Nazaroff and Alvarez-Cohen, 2001; Parkin and Owen, 1986; Ramalho, 1996).

Methane production is a complex process carried out by different microbial communities that can be divided in four phases according to the biochemical process performed in each step: hydrolysis, acidogenesis, acetogenesis/dehydrogenation and methanogeneis. First, polymers and monomers are initially degraded during the hydrolysis phase, producing acetate, hydrogen and volatile

fatty acids (VFAs). Second, acidogenic bacteria do further breakdown of the remaining organic matter, producing more VFAs, ammonia, CO_2 and H_2S. Next, acetogenic organisms produce acetic acid, CO_2 and H_2 digesting the simple molecules created during the acidogenesis step. Finally, the methanogenic bacteria produce methane, CO_2 and water converting the intermediate products of the previous step. For a balanced AD process, the degradation rates of all the steps must remain equal: an excessive degradation of organic matter will increase the acid concentration, dropping the pH level below 7, which will inhibit the methanogenic bacteria; and the methanogenic activity will be limited if the conversion rate of simple molecules is faster than their production in the hydrolysis step. Briefly, the AD process to produce biogas can be performed in batch or in continuous mode, and it can be classified according to the solids contents – wet or dry fermentation (solids content below or above 10% w/v, respectively) – and the temperature range – mesophilic (38 – 42 °C) or thermophilic (50 – 55 °C) (Weiland, 2010). The three main products of any AD process are biogas, digestate and liquid liquor (Eddy *et al.*, 1991; Ramalho, 1996):

- **Biogas:** it is the product with the major economical value of the AD process. Its compositions vary depending on the feedstock and the operational parameters. The methane can be burned to produce heat or electricity, but it must be refined for further

activities out of the WWTP, in order to remove mainly H_2S.

- **Digestate:** it is the remaining solid, which is composed of materials that can not be degraded and dead bacteria, and it can come in three forms: whole (solids content ranging 2 – 6 % and 18 – 23 % [w/v] for wet and dry fermentations, respectively), liquor (low solids content) and fibre (rough screened material, represents 10 – 40 % of the total solids).
- **Liquid liquor:** it is originated due to the moisture content of the input sludge and the water produced by the microbial activity. The water exiting the AD process has elevated levels of biochemical oxygen demand (BOD) and COD.

1.3.1.b. *Composting sewage sludge*

In the recent years, the numbers of regulations that impose minimum levels of recycling in different countries have increased. In order to achieve the recycling limits, waste management programs have been developed, with composting as the main methodology to stabilize and manage the organic fraction of municipal solid waste (OFMSW) (Diaz, 2007). In general, composting is the biologically decomposition and stabilization of organic matter where thermophilic conditions (35 – 75 °C) have been reached due to biological activity. The final product is known as compost, which is stable,

rich in nutrients and pathogens free, and it can be applied as fertilizer into soil. Composting has three main applications: the production of compost for land application, the production of selective substrates for mushroom growing, and the treatment of organic wastes (Miller, 1992).

Composting is the aerobic process in which thermophilic microorganisms transform organic products into more stable materials. There are many methods to perform the composting, but the microbial processes that drive any composting system are the same for all of them, with temperature variations resulting from the microbial activity. Firstly, mesophilic bacteria degrade the easily decomposable material, working in an optimal temperature range of 20 – 50 °C. Secondly, the thermophilic bacteria began to decompose when the temperature exceeds 40°C. The thermophilic range of temperatures (50 – 65 °C) remains during the first stage of the process, which is characterized for high decomposition rates, and it is maintained for 3 – 15 days; being necessary in order to eliminate pathogenic bacteria, weed seeds and parasites eggs. Next, the temperatures decrease and mesophilic organisms continue the process at slower rates. Finally, the composting system enters in a stage called curing period, in which the temperature decrease and the final compost is stabilized. The main factors that can affect the composting are: the C:N ratio, the water content, the porosity,

the pH and foreign substances such as PhACs (Schaub and Leonard, 1996).

1.3.1.c. *Application of sewage sludge in soils*

The application of sewage sludge in agricultural soils can improve the crops growth and yields. The amendment with WWTP sludge improves the soil fertility by recirculating nutrients with low cost investments. Additionally, the organic matter of the sludge improves the soil structure, which is higher in composted sewage sludge (Schowanek *et al.*, 2004). Furthermore, soils amended with sewage sludge present higher water infiltration rates and porosity, and lower erosion potential (Chambers *et al.*, 2003). The N content in sewage sludge is also an important factor that will determine its feasibility as fertilizer, but its availability is affected by the operational parameters of the WWTP (e.g. temperature, moisture and pH) and the stabilization techniques used (Mantovi *et al.*, 2005).

Nevertheless, the application of raw sewage sludge as soil amendment in agricultural activities may represent important health risks if the pollutants have not been efficiently removed. For instance, it has been reported that sewage sludge can contain PAHs, PCBs, pesticides, heavy metals and xenobiotics (Eriksson *et al.*, 2008; Hua *et al.*, 2008; Stevens *et al.*, 2003), which can be highly toxic and can move through different environmental compartments (Nadal *et al.*, 2009). These sludge

pollutants has been documented to be transferred into soils when sewage sludge has been used as amendment (Sposito *et al.*, 1982; Wang, 1997; Wild *et al.*, 1991), and from there to crops and livestock (Wild and Jones, 1992).

Anaerobically digested sewage sludge (digestate) can be used and valorised as biofertiliser in agricultural activities given its high nutrient content. The main nutrients in the sewage sludge are transferred to the digestate – except for some N lost as NH_3 and H_2S –, which can be recirculated into soils improving crops yields. Furthermore, the application of the digestate into land does not imply additional costs for farmers, since it can be applied with the same agricultural machinery as the traditional synthetic fertilizers (Holm-Nielsen *et al.*, 2009; Thomson, 2012). Although it has been demonstrated that EPs do not affect the AD process, these pollutants are not removed at all, showing diverse concentrations depending on the compound (Hernandez-Raquet, 2012). Consequently, the EPs must be removed before or after the AD process.

Both, raw sewage sludge and anaerobically digested sludge can be composted (Parr *et al.*, 1978). In fact, the composting of sewage sludge has been proposed as a way to solve two problems at the same time: the reduction of waste disposal into landfills and the restoration of agricultural soils with low organic matter. It has been assessed that composted sewage sludge improved soil properties, i.e. soil water content and retention capacity, compactibility, aggregation, porosity and

pore size distribution, and penetration resistance (Aggelides and Londra, 2000). In addition, composting has been described as a suitable method to remove organic pollutants and pharmaceutical antibiotics (Kumar, 2012; Petersen *et al.*, 2003). However, not all the sewage sludge can be composted as not all the sludges have the optimal factors for the composting process (Hassouneh *et al.*, 1999), and they must be conditioned before composting.

1.3.2. Removal techniques for EPs in WWTPs effluents

Given that sewage sludge is a by-product continuously produced in any WWTP, which contains PhACs – among other pollutants –, and it can be used in further applications; it is necessary to implement new techniques so as to remove EPs, preventing their spread. Sustainable techniques has been studied and developed, and fungal bioremediation has arisen as one of them. However, the feasibility of the treatment is restricted by the fungi ability to survive and to colonize the sewage sludge. Additionally, only a few bioremediation processes can be applied for the sewage sludge treatment according to its special characteristics, origin and production. For instance, in solid matrices fungal remediation could be used to increase the removal of PPCPs from raw sewage sludge or digestate, preparing the sludge before being composted; or in liquid matrices the fungus could be inoculated in a bioreactor to treat the effluent of an AD. So, this section is focused in

three treatments that can remove EPs in WWTPs effluents: two treatments lead by the fungus on solid phase effluents (Solid-phase bioreactor and Slurry-phase bioreactor) and one abiotic treatment of the liquid effluent (adsorption into valorised wastes).

1.3.2.a. *Solid-phase bioreactor*

A solid-phase bioreactor (or biopile) is an engineered process that uses the biochemical mechanisms of microorganisms to degrade pollutants from solid matrices, transforming these contaminants into more simple compounds such as CO_2 or water (mineralisation). Biopiles are part of the so-called ex-situ bioremediation techniques, as the sewage sludge will not be treated in the same place or stage where it is produced. The sludge to be treated is mixed with a substrate or a bulking material, which improves the aeration, gives structure, and it is also used as co-substrate by inoculated fungus. Biopiles also include the following complementary systems: aeration, irrigation – where nutrients can be also supplied – and leachate collection; and the typical monitored parameters are: moisture, heat, nutrients, oxygen and pH (Environment Protection Authrority, 2005; Juwarkar *et al.*, 2010; Khan *et al.*, 2004). Globally, these systems require minimum maintenance and inputs (i.e. energy and water), making them cost-effective processes, even for long time

treatments (Gomez and Sartaj, 2014; Jørgensen *et al.*, 2000; Nano *et al.*, 2003).

Biopiles mediated by WRF imitate the natural habitat of the fungus, providing the minimum growth conditions. Indeed, the lignocellulosic substrate provides the essential lignocellulosic nutrients and promotes the LMEs production (Gadd, 2001; Rodríguez Couto and Sanromán, 2005; Singh, 2006). Biopiles at different scales have been used not only in the degradation of recalcitrant organic pollutants in soils such as total petroleum hydrocarbons (TPHs) (Coulon *et al.*, 2010; Mao *et al.*, 2009; Whelan *et al.*, 2015), PAHs (Lors *et al.*, 2010; Lladó *et al.*, 2013; Pelaez *et al.*, 2013), chlorophenols (CPs) (Laine *et al.*, 1997; Namkoong *et al.*, 2002; Zeng *et al.*, 2011) and pesticides (Gavrilescu, 2005; Madrigal-Zúñiga *et al.*, 2015; Ruiz-Hidalgo *et al.*, 2014), but also in the degradation of emerging contaminants from WWTP sludges (Rodríguez-Rodríguez *et al.*, 2014, 2012b, 2011).

In comparison to composting piles, which are not inoculated, are typically 2-3 m high and are usually covered with plastics in order to prevent run-off and evaporation, and to increase and maintain the heat temperature (Haug, 1993); fungal biopiles have lower heights (0.6 – 0.7 m) and are not covered (Winquist, 2014). This is due to the fact that higher heights and plastic covers will increase the temperature of the biopiles up to 60°C, killing the inoculated fungus and,

consequently, stoping the fungal mediated bioremediation process (Sayara *et al.*, 2011).

1.3.2.b. *Slurry-phase bioreactor*

Slurry-phase bioreactors (or bioslurry) are an ex-situ technology mainly used when the pollutants are absorbed in solid particles, resulting in a low bioavailability. The system consists of the suspension of a solid phase in water to a concentration between 5% and 40% (w/v), with agitation and aeration. Because of the increased mass transfer from the solid phase to the liquid phase, where the degradation is assumed to occurs, high molecular weigh pollutants will be degraded (Admassu and Korus, 1996). The pollutants removal can be led by either indigenous microorganisms of the sludge or inoculated enriched microbial consortium from the same sludge or exogenous specialized microorganisms. Additionally, nutrients, neutralizing agents, surfactants and co-metabolites may be added to improve the microbial degradation. Also, temperature, pH and dissolved oxygen are monitored, increasing the global efficiency of the treatment (Robles-González *et al.*, 2008).

Generally, slurry bioreactors inoculated or non-inoculated with bacteria have mainly been studied and applied to treat polluted soils (Łebkowska *et al.*, 2011; Robles-González *et al.*, 2012; Smith *et al.*, 2015; Tomei *et al.*, 2013; Venkata Mohan *et al.*, 2008, 2007) and oily sludges (Hu *et al.*, 2013). Nevertheless,

fungal mediated bioslurries to treat sewage sludge have not been deeply studied and only a few studies have been performed (Rodríguez-Rodríguez *et al.*, 2010a); although, these studies were made by adding water to dried sewage sludge as the watered sludge – prior its thermal drying – contained coagulant compounds, being unfeasible to treat the sludge in bioslurry.

1.3.2.c. *Adsorption*

Adsorption techniques are considered to be very efficient removing both organic and inorganic pollutants from wastewater. In general, the adsorption is a surface phenomenon that occurs when a liquid that contains a solute, which must be absorbable, contacts a porous solid. Then, some solute molecules will be retained into the solid's porous due to liquid-solid intermolecular attraction forces. The retained solute is called adsorbate, the solid where it is retained is the adsorbent, and the general surface accumulation of solute is the adsorption itself. A solid can act as an adsorbent when its surface atoms are not completely surrounded by other adsorbent atoms, attracting adsorbate molecules because of electrostatic attraction or chemical (i.e. covalent bonding – chemisorption) or physical (i.e. weak Van Der Waals forces – physicsorption) mechanisms. The adsorbents can be classified as natural (e.g. charcoal, clay minerals and zeolite) or synthetic (made up of e.g. agricultural, industrial and house wastes, sewage sludge, fruit and forestry wastes) (Nageeb, 2013).

At industrial scale, activated carbon has been extensively used as main adsorbent to treat water. This adsorbent not only has high adsorption capacities, but also it has shown to have great removal efficiencies for certain organic pollutants, reaching efficiencies up to 100%. Nowadays, water is treated via adsorption mechanisms using activated carbon in column mode; however, wastewater is not treated with this specific adsorbent due to its expensive costs. Giving that, alternative less expensive adsorbents are being tested (Biswas and Mishra, 2015; Putra *et al.*, 2009; Tamilselvi and Asaithambi, 2015). For instance, dyes in wastewaters from textile, pulp, paper and cosmetic industries has been removed using low cost sorbents (Nageeb, 2013) such as activated rice husk, cedar sawdust, wood-shaving bottom ash, sewage sludge (to prepare activated carbon) and fly ash (Gupta *et al.*, 2006; Hamdaoui, 2006; Martin *et al.*, 2002; Monsalvo *et al.*, 2012; Wang *et al.*, 2005). Also, phenolic compounds, a wide spread group of pollutants, has been removed from aqueous solutions by different costless adsorbents: bagasse ash, wood charcoal, natural clay and industrial wastes (Ahmaruzzaman, 2008; Djebbar *et al.*, 2012; Jain *et al.*, 2004; Larous and Meniai, 2012; Mukherjee *et al.*, 2007). Furthermore, agrochemicals, which are intentionally released into the environment, extensively used worldwide and highly toxic (Gilliom, 2007; IARC, 1987; Mir-Tutusaus *et al.*, 2014), has also been removed from water and wastewater streams using low-cost sorbents including rice

bran and husk, fruit shells, fly ash, saw dust and clay minerals (Akhtar *et al.*, 2007; Cara and Jităreanu, 2015; Gopal *et al.*, 2004; Memon *et al.*, 2007). Finally, EPs has been removed from the wastewater streams via adsorption with both expensive commercial adsorbents (i.e. activated carbon, clays and minerals) and low-cost sorbents (i.e. agricultural and industrial wastes) (Baccar *et al.*, 2012; Grassi *et al.*, 2012; Gupta *et al.*, 2009).

Even though the utilisation of low-cost sorbents to amend soils has been studied in the past (Adriano *et al.*, 1980; Dechene *et al.*, 2014; Douglas *et al.*, 2012; Filiberto and Gaunt, 2013), and they have also been implemented to treat water and wastewater (Bhatnagar *et al.*, 2015; Kurniawan *et al.*, 2006; Mohan *et al.*, 2014); the use of low-cost sorbents to remove organic pollutants from wastewater streams via soil amendment has not been deeply studied, which is an interesting and costless method to improve the effluent's quality of any WWTP; especially for those plants that discharges into underground basins or aquifers.

CHAPTER 2

Objectives

The main objective of the present thesis is to develop novel biological and physical processes to remove PPCPs in different streams of WWTPs. In order to achieve this general purpose, the work has been divided in specific goals:

- To find the best conditions to colonize sewage sludge by *Trametes versicolor* using lignocellulosic substrate.
- To assess the strategies to remove PhACs in bioslurry and biopiles systems inoculated with *Trametes versicolor* under non-sterile conditions.
- To determine the applicability of the anaerobic digestion as a valorisation method for the fungal biomass.
- To study the microbial communities evolution in fungal mediated biopiles.
- To investigate the utilization of low-cost sorbents to adsorb EPs as post-treatment of a WWTP's effluent.

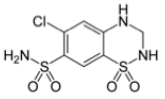
CHAPTER 3

Materials and Methods

3.1. Chemicals

Hydrochlorothiazide (HZT) (6-Chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide) (table 3.1a) was obtained from Sigma-Aldrich Co (St. Louis, MO, US).

Table 3.1a Selected target compound for degradation studies

Compound	CAS Number	pKa*	Molecular Structure
Hydrochlorothiazide	58-93-5	7.9	

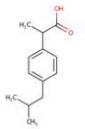
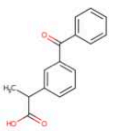
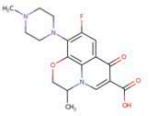
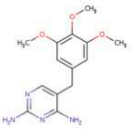
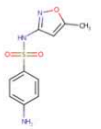
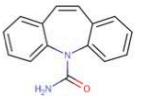
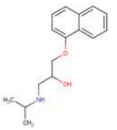
* (Law et al., 2014)

Ibuprofen (IBU) (2-[4-(2-methylpropyl)phenyl]propanoic acid), carbamazepine (CBZ) (benzo[b][1]benzazepine-11-carboxamide), ofloxacin (OFX) (9-Fluoro -3-methyl -10-(4-methyl-1-piperazinyl)-7-oxo-2,3-dihydro -7H-[1,4]oxazino [2,3,4-ij]quinolone -6-carboxylic acid), trimethoprim (TRM) (5-[(3,4,5-trimethoxyphenyl) methyl] pyrimidine-2,4-diamine), ketoprofen (KTP) (2-(3-benzoylphenyl) propanoic acid), propranolol (PRN) (1-naphthalen-1-yloxy-3-(propan-2-ylamino) propan-2-ol), and sulfamethoxazole (SMX) (4-amino-N-(5-methyl-1,2-oxazol-3-yl) benzenesulfonamide) (table 3.1b) were obtained from Sigma-Aldrich Pty Ltd. (NSW, Australia).

Internal isotopically labeled standard compounds, methanol and acetonitrile were of high purity grade (> 90%) and were purchased from Sigma-Aldrich (Steinheim, Germany), US Pharmacopeia USP (MD, USA), Europea Pharmacopeia EP (Strasbourg, France), Toronto Research Chemicals TRC (Ontario, Canada) and CDN isotopes (Quebec, Canada). Further

information can be consulted in Gros *et al.* (2012). The individual standard solutions as well as isotopically labeled internal standard solutions were prepared according to Gros *et al.* (2012).

Table 3.1b Selected pharmaceuticals for adsorption experiments

Compound	CAS Number	pKa*	log K_{OW}*	Polarizability* (Å^3)	Molecular Structure
			Analgesics and anti-inflammatory drugs		
Ibuprofen (IBU)	15687-27-1	4.85	3.84	23.76	
Ketoprofen (KTP)	22071-15-4	3.88	3.61	26.56	
			Antibiotics		
Ofloxacin (OFX)	82419-36-1	5.45	0.65	36.69	
Trimethoprim (TRM)	738-70-5	17.33	1.28	29.71	
Sulfamethoxazole (SMX)	723-46-6	6.16	0.79	24.99	
			Psychiatric drug		
Carbamazepine (CBZ)	298-46-4	15.96	2.77	25.00	
			ß- Blocker		
Propranolol (PRN)	525-66-6	14.09	2.58	29.98	

* (Law et al., 2014)

3.2. Microorganisms

The strain *T. versicolor* ATCC 42530 was obtained from the American Type Culture Collection, and maintained by subculturing every 30 days on 2% malt extract agar slants (pH 4.5) at 25°C.

Three different anaerobic inoculums were collected from distinct anaerobic digesters of WWTPs: one from Terrassa's WWTP digester (Catalonia), another from the anaerobic digester of a WWTP placed in Sabadell (Catalonia) and another one from a WWTP located in Blanes (Catalonia). Terrassa WWTP was designed to treat 75,000 $m^3 \cdot d^{-1}$ of urban wastewater and planned for an equivalent population of 400,000 inhabitants. Sabadell WWTP was designed to treat 33,000$m^3 \cdot d^{-1}$ of urban wastewater with physical-chemical and biological processes and planned for an equivalent population of 200,000 inhabitants. Blanes WWTP was designed to treat 23,500$m^3 \cdot d^{-1}$ of urban wastewater for an equivalent population of 109.985 inhabitants. The three inoculums were collected in 5L opaque plastic tanks equipped with caps that allowed the gases output, and they were subjected to starving conditions for 7 days at 36°C prior the commencement of the experiments.

Homogeneous sludge samples were taken from the recirculation stream of the MBR unit of Terrassa's WWTP. Samples used for experiments under sterile conditions were autoclaved twice at 120°C for 30min. For non-sterile

experiments, samples were collected the same day. The MBR, which has an internal configuration and was equipped with ZENON microfiltration membranes (nominal porosity: 0.10μm), was designed to treat a 7,200$m^3 \cdot d^{-1}$ stream with a Hydraulic Retention Time (HRT) of 0.79d and a concentration of 4-5 $g \cdot L^{-1}$ of Total Suspended Solids (TSS).

3.3. Low-cost sorbents

3.3.1. Biochar

The Biochar used was prepared with eucalyptus leafs from Sydney as feedstock. First, eucalyptus leafs were dried in a convention oven and then it was grounded. Then, the resulting dried leafs were pyrolysed at 550°C under a flow of N_2 gas.

3.3.2. NUA

Table 3.2 Properties and composition of NUA. Excerpt from Wendling *et al.* (2012).

pH	EC ($mS \cdot cm^{-1}$)	Mineralogical composition	Major geochemical composition	Trace elements (C≥10ppm)
7.7	2.4	80% gypsum, 7% magnetite/maghemite, 7% quartz, 5% bassanite	35.3% SO_3, 24.7% CaO, 21.9% Fe_2O_3, 5.3% SiO_2, 3.9% MgO, 3.0% TiO_2, 1.6% MgO, 0.5% Al_2O_3, 0.1% Na_2O, 0.1% K_2O, 0.1% P_2O_5	Ba (30), Br (12), Ce (156), Co (193), Cr (121), Cs (13), Cu (39), Ga (21), La (59), Nb (53), Nd (73), Ni (54), Pb (55), Sm (17), Se (5), Sr (959), Th (142), V (110), Y (23), Zn (68), Zr (148) [0.24% in total]

Neutralised Used Acid (NUA) was obtained from Iluka Resources Ltd. at their synthetic rutile upgrading facilities in Capel (Western Australia, Australia). NUA is the processing residue resulting of upgrading ilmenite into synthetic rutile via

the Becher process and the subsequent neutralization of the sulphuric acid leach with CaO. Additional information given by Wendling *et al.* (2012) is summarised in table 3.2.

3.4. Sludge, lignocellulosic substrate and soil

20L of dry sewage sludge was collected from the final stage of the sludge processing system at El Prat de Llobregat WWTP – i.e. the thermal drying – the 29th January of 2014. The initial water content of the sludge was 16.71 ± 0.03 %, and its water holding capacity was 1.19 ± 0.06 $gH_2O \cdot gDW^{-1}$. At the arrival to the laboratory the sludge was frozen until its use.

10 L of commercial decorative pine bark (*pinus halepensis*) were bought from a local supplier and used as lignocellulosic substrate for the biopiles systems. The initial size of the pine barks ranged from ca. 2.5cm to 10cm. The initial water content was 21.27 ± 0.43 %, and its water holding capacity was 1.28 ± 0.01 $gH_2O \cdot gDW^{-1}$. The substrate was kept at room temperature until its use.

Soil from basin E of the Alice Springs SAT (Soil Aquifer Treatment) (Northen Territory, Australia) was collected from the 0 to 10cm surface layer and used for all the adsorption experiments.

3.5. Mediums

In the degradation study of HZT in liquid cultures, *T. versicolor* was grown in a liquid media prepared according to Marco-Urrea *et al.* (2009c): the components listed in tables 3.3a

and 3.3b were added to sterile distilled water and mixed until its completely dissolution at room temperature.

Table 3.3a. Components of the growing medium.

Component	Quantity
Glucose	8 $g \cdot L^{-1}$
Ammonium Tartrate	3.3 $g \cdot L^{-1}$
Dimethyl Succinate	1.168 $g \cdot L^{-1}$
Macronutrients	100 $mL \cdot L^{-1}$
Micronutrients	10 $mL \cdot L^{-1}$

Table 3.3b. Macro and micronutrients composition of the growing medium according to Kirk (1978).

Macronutrient	Quantity ($g \cdot L^{-1}$)	Micronutrient	Quantity ($g \cdot L^{-1}$)
KH_2PO_4	20	Nitrilotriacetic acid	1.5
$MgSO_4 \cdot 7H_2O$	5	$MgSO_4 \cdot 7H_2O$	3.0
$CaCl_2$	1	$MnSO_4 \cdot H_2O$	0.5
		NaCl	1.0
		$FeSO_4 \cdot 7H_2O$	0.1
		$CoSO_4$	0.1
		$ZnSO_4 \cdot 7H_2O$	0.1
		$CaCl_2 \cdot 2H_2O$	0.1
		$CuSO_4 \cdot 5H_2O$	0.01
		$AlK(SO_4)_2 \cdot 12H_2O$	0.01
		H_3BO_3	0.01
		$NaMoO_4$	0.01

For the bioslurry experiments, three different mediums were prepared: (i) defined media, (ii) glucose media, and (iii) no-nutrient media. First, defined media was prepared adding the components listed in tables 3.3a and 3.3b to MBR sludge

and mixing until its completely dissolution at room temperature. Next, glucose media was made dissolving 8g·L^{-1} of glucose into MBR sludge. Finally, no-nutrient media was prepared with only MBR sludge, not containing any external nutrient or component.

The nutrients solution for the methanogenic activity test was prepared according to Field *et al.* (1988). The nutrients listed in table 3.4 were added to the anaerobic digesters.

Table 3.4. Composition of the nutrients solution used for the methanogenic activity test according to Field (1988).

Macronutrients (g·L^{-1})			
NH_4Cl	*170*	$CaCl_2·2H_2O$	*8*
KH_2PO_4	*37*	$MgSO_4·4H_2O$	*9*
Micronutrients (mg·L^{-1})			
$FeCl_3·4H_2O$	*2000*	$(NH_4)_6Mo_7O_{24}·4H_2O$	*90*
$CoCl_2·6H_2O$	*2000*	$Na_2SeO_3·5H_2O$	*100*
$MnCl_2·4H_2O$	*500*	$NiCl_2·6H_2O$	*50*
$CuCl_2·2H_2O$	*30*	EDTA	*1000*
$ZnCl_2$	*50*	HCl 36%	*1*
H_3BO_3	*50*	Resazurin	*500*
Sulphur (g·L^{-1})			
$Na_2S·9H_2O$	*100*		

3.6. Experimental procedures

3.6.1. Fungal mycelial and pellet suspensions

Blended mycelial suspension and pellet suspension were prepared according to Blánquez *et al.* (2004). Mycelial

suspension was made as follows: Erlenmeyer flask (V=500mL) with 150 ml of malt extract medium (2%) were inoculated with 1cm^2 plug from agar cultures in Petri dishes and shook (orbital shakers: 135rpm and r=25mm) for 5 days at 25°C; resulting fungal mass was homogenized (X10/20, Ystral GmbH) and stored in sterilized saline solution (0.85% NaCl) at 4°C. The pellet suspension was obtained inoculating Erlenmeyer flasks (V=1000mL; 250ml 2% malt extract medium, pH 4.5) with 1mL of the previous mycelial suspension, followed by an incubation period of 6–7 days in orbital shakers (135 rpm, r=25 mm) at 25°C. The resulting pellets were stored in sterilized saline solution (0.85% NaCl) at 4°C.

3.6.2. Water Holding Capacity and moisture

The initial moisture of the sewage sludge from El Prat de Llobregat WWTP, the pine bark and the soil from Alice Springs SAT was determined weighing homogeneous volumes of sludge or substrate, and drying it for 24h at 105°C in an oven. It was expressed as percentage of moisture.

The water holding capacity (WHC) of the sewage sludge and the pine bark was determined as described by CEN (1999): metal cylinders with one of its ends covered with paper filter were filled (up to 2/3 parts of their volume) with sludge or substrate and placed in trays; water was added until it fully covered the sludge or substrate, without exceeding it; next, after two hours in contact with water, the cylinders with sludge or substrate were dried by capillarity for 30 min and weighted

(wet weight); and finally, the cylinders were dried for 24h at 105°C in an oven and weighted (dry weight – DW). The WHC was calculated as the difference between the dry and the wet weights and expressed as grams of water by gram of dry sludge/substrate ($gH_2O \cdot gDW^{-1}$).

The soil was dried in trays for 48h at 40 °C (initial moisture 17.0 ± 0.2 %) and sieved manually at <1.0mm. The maximum water holding capacity (MWHC) was 11.9 ± 0.7 %, and it was assessed according to Jenkinson and Powlson (1976): 50 g of soil (in triplicates) were placed in a funnel coated with filter paper and 50mL of water were added, after 30 minutes the water was drained into a graduated cylinder for 30 minutes or until it stops draining, and the amount of drained water was measured. The MWHC value was calculated as:

$$MWHC(\%) = \left(\frac{(50mL - WD - 7.3 + IWC)}{50g\ of\ soil} \right) \cdot 100 \qquad \text{(Eq1)}$$

Where WD is the measured drained water, IWC is the initial water content of the soil, and 7.3 is the water held in the filter paper (7.3 ± 0.6 sd mL).

3.6.3. Solid-phase experiments

Cultures were performed in Schott bottles (250mL, 95 x 105mm; GLS 80; Duran, Inc) equipped with 4-port screw caps (GL 18; Duran, Inc). Three ports of the caps were hermetically

closed, while one was kept opened, using a 0.45µm filter as passive air intake. First, 6g of sterile lignocellulosic substrate (20min at 120°C) were placed in each bottle and inoculated with 2mL of mycelial suspension, setting humidity to 60% of the water holding capacity. After 7 days of static incubation (25°C), biopiles were prepared adding 14g of non-sterile dry WWTP sludge in every single pre-grown fungal culture and then homogenised; the moisture level was adjusted to 60%. Biopiles cultures were incubated at 25°C in static conditions until sampling. After 22 days of incubation, half of the biopiles were re-inoculated with concentrated mycelial suspension, while same volume of water was added to the other half. Additionally, control cultures were performed with 6g of sterile lignocellulosic substrate (20min at 120°C) and 14g of non-sterile dry WWTP sludge at 60% of the water holding capacity, but without *T. versicolor* inoculation. Triplicate cultures were sacrificed for analysis in every sampling time.

3.6.4. HZT degradation study

Erlenmeyer flasks (V=500mL) were used to perform liquid cultures. Each flask was filled with 100 mL of sterile growing medium (120°C, 30min), inoculated with *T. versicolor* pellets (0.55gDW) and spiked with HZT (10mg·L^{-1}). Incubation was carried out in orbital shakers (130rpm, r=25 mm) at 25°C. Experiments also included abiotic controls, which contained 20mL of water instead of pellets, and killed controls, that consisted of inoculated cultures with sodium azide (0.2g·L^{-1}) or

autoclaved cultures (120°C, 30min) depending on the experiment. 2.5mL of each culture was sampled at every sample point-time. All the experiments were run in triplicate.

3.6.5. Spiked Bioslurry at Erlenmeyer scale

Different kinds of experiments were carried out: cultures with defined medium, cultures with glucose media, and cultures without any nutrient. The experiments were conducted under sterile conditions (120 °C for 30 min), except for cultures without nutrients where sterile and non-sterile conditions were tested. In general, Erlenmeyer flasks (V=500mL) were filled with 100 mL of MBR sludge and nutrients or glucose when required.

Flasks were inoculated with 0.55gDW of pellets, and spiked with 10mg·L^{-1} of HZT. Initial pH (7.0 – 8.1) was adjusted to 4.50, and cultures were incubated on orbital shakers (130 rpm, r=25 mm) at 25°C. Degradation experiments included abiotic controls, containing 20 mL of water instead of pellets, as well as heat-killed controls or autoclaved cultures (120°C, 30min). Samples of 2.5mL from the cultures were collected at each sampling point for the analysis. All experiments were run in triplicate.

3.6.6. Non-spiked Bioslurry at Erlenmeyer scale

Erlenmeyer flasks (0.5 L) were filled with 100 mL of non-sterile MBR sludge and initial pH adjusted to 4.5; afterwards no further pH control and adjustment was carried out. Experimental cultures were inoculated with 0.55gDW of

pellets; killed controls were inoculated with 0.55gDW of heat-killed pellets (120°C, 30min); and abiotic cultures were made with water (20mL) replacing the pellets volume. All the cultures were incubated at 25°C on orbital shakers (130rpm, r=25 mm). All the experiments were made in triplicates.

3.6.7. Bioslurry at reactor scale

According to Rodríguez-Rodríguez *et al.* (2012a), 8L steel stirred bioreactor with a working volume of 6L was used. The tank was equipped with a pH controller (InPRo 325X probe, M300 controller, Mettler Toledo, Switzerland) and an anchor stirrer – 115rpm – (OST 20 digital, Yellow line, IKA, Germany). The reactor was filled with 5L of raw MBR sludge without additional nutrients and initial pH adjusted to 4.5. At the beginning of the experiment, 1.8g·L^{-1} of fungal biomass was inoculated and the temperature maintained at 25°C. Aeration was supplied in order to keep the dissolved oxygen level ca. 40%. pH was measured every day, and concentrated HCl provided to maintain the pH level between 4 and 4.5 units. Every day, 1.5mL were sampled directly from the reactor and laccase activity was measured. After five days, the final Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS) of the bioslurry were measured.

3.6.8. Anaerobic Digestion

Biochemical Methane Potential (BMP) tests of the bioslurry were performed in reactors made from commercial aluminium bottles (total volume of 1L) with a modified cap that included a

manual valve for biogas measurements (Ferrer *et al.*, 2004). Three anaerobic inoculums from different WWTP digesters (i.e. Terrassa, Sabadell and Blanes WWTPs) were selected and their methanogenic activities were evaluated. The entire anaerobic batch tests were based on Field *et al.* (1988), modified by both Angelidaki *et al.* (2007) and Martín-Gonzalez *et al.* (2010). In order to determine the methanogenic activity of the inoculums, a concentration of 1.5gVS·L^{-1} – in a volume of 600mL with a headspace of 400mL – was chosen. The initial volatile fatty acids (VFA) concentration was set to 4.0 gCOD-VFA·L^{-1} (C^2:C^3:C^4 ratio 73:21:04 gCOD), 2mL·L^{-1} of nutrients (table 3.4) were added, and pH was adjusted to 7 with NaOH.

Table 3.5 Total Solids (TS) and Volatile Solids (VS) at BMP tests.

Sample	TS (g·L^{-1})	VS (g·L^{-1})
Terrassa inoculum	47.67 ± 1.60	28.44 ± 0.99
Sabadell inoculum	8.87 ± 0.25	5.41 ± 0.13
Blanes inoculum	6.34 ± 0.14	4.34 ± 0.08
Raw MBR sludge	3.99 ± 0.19	2.25 ± 0.16
Fresh fungal biomass	33.31 ± 1.93	31.52 ± 1.79
Bioslurry	4.63 ± 0.04	2.58 ± 0.05

BMP test were carried out with the MBR sludge after 5 days of treatment with *T. 51ersicolour* in bioslurry reactors under non-sterile conditions. Additionally, the following controls were included: (i) blank, with only inoculum; (ii) a sludge control, with raw MBR sludge and inoculum; and (iii) fungus control, with fungal biomass and inoculum;. All the cultures were prepared with a substrate:inoculum ratio of 0.5 and an initial

volatile solids (VS) concentration of 3gVS·L^{-1} in a volume of 600mL.

All the anaerobic reactors were maintained in static conditions at 36ºC for the whole experiment. After the preparation of each culture, the bottles were sealed and flushed with nitrogen gas in order to remove all the air content. Biogas production was measured as the pressure increase in the headspace of the digestion bottles with a SMC Pressure Switch (1bar, 5% accuracy) manometer. Accumulated volumetric biogas production was calculated from this pressure increase and expressed at standard temperature and pressure conditions.

3.6.9. Sorption experiments

Two different sorption experiments were carried out in order (i) to determine the capacity of the soil to adsorb a mixture of the PhACs described in Table 3.1b and their distribution coefficient (Kd), and (ii) to assess how the soil:amendment ratio would affect the adsorption of 3 PhACs (TRM, PRN and SMX). In both cases, a 50ppm mixture working solution with the mentioned PhACs (water:methanol 90:10 %) was prepared and used. The two experiments followed the OECD/OCDE Guideline for the testing of chemicals (OECD, 2000).

Glass tubes were filled with the appropriated solid matrix and 0.01 M $CaCl_2$ stock solution. Next, the tubes were shook (10rpm) for 12h prior the commencement of the experiment, in

an end-over-end shaker. At the beginning of the experiments, all the experimental tubes were spiked with 50-ppm PhACs mix solution, reaching an initial concentration of 500ppb in each, and shook for 24h in an end-over-end shaker at room temperature (ca. 20ºC). For both experiments, triplicate cultures were sacrificed for analyses at every sampling time: 0, 1, 2, 4, 6, 8 and 24h.

Table 3.6 Cultures distribution for the assessment of soil capacity to adsorb PhACs.

Group	Solid Matrix	Ratio	Total Volume (mL)	$CaCl_2$ (mL)	PPCP's solution (mL)	Soil (g)
Blk	*n.a.*	*n.a.*	15	15	0	0
CC	*n.a.*	*n.a.*	15	14.85	0.15	0
SC	Soil	1:5	15	15	0	3
G1	Soil	1:1	3	2.97	0.03	3
G2	Soil	1:5	15	14.85	0.15	3
G3	Soil	1:50	25	24.75	0.25	0.5

Blk: Blank control; **CC:** Chemical control; **SC:** Soil control

In order to ensure that 60% of the chemical compounds remained in the aqueous phase, 3 soil:solution ratios were used (1:1, 1:5 and 1:50 g:mL) when determining the capacity of the soil to adsorb the selected drugs. For each ratio, the following compounds had been analysed according to its octanol-water partition coefficient's (log K_{OW}) value: for 1:1 (G1) IBU, CBZ, and KTP (log $K_{OW} \geq 2.60$); for 1:5 (G2) TRM, PRN, and SMX ($2.60 > \log K_{OW} \geq 0.70$); and for 1:50 (G3) OFX (log $K_{OW} < 0.70$). Additionally, 3 controls were prepared: a chemical control without soil, but with the compounds mixture in $CaCl_2$ solution; a soil control

with $CaCl_2$ solution, but without the PhACs mixture; and a blank control with only $CaCl_2$ solution. Table 3.6 summarizes the groups' distribution.

Table 3.7 Cultures distribution for ratio selection.

Group	Amendment			Soil (g)	Sand (g)
	Type	Ratio	g		
Blk	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*
CC	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*
NUA+Sand 0.1	NUA	0.1	0.003	*n.a.*	2.997
NUA+Sand 0.5	NUA	0.5	0.015	*n.a.*	2.985
NUA+Sand 1	NUA	1	0.03	*n.a.*	2.97
NUA+Sand 2	NUA	2	0.06	*n.a.*	2.94
NUA+Sand 5	NUA	5	0.15	*n.a.*	2.85
NUA+Soil 0.1	NUA	0.1	0.003	2.997	*n.a.*
NUA+Soil 0.5	NUA	0.5	0.03	2.97	*n.a.*
NUA+Soil 1	NUA	1	0.06	2.94	*n.a.*
NUA+Soil 2	NUA	2	0.15	2.85	*n.a.*
NUA+Soil 5	NUA	5	0.3	2.7	*n.a.*
BIO+Sand 0.1	Biochar	0.1	0.003	*n.a.*	2.997
BIO+Sand 0.5	Biochar	0.5	0.015	*n.a.*	2.985
BIO+Sand 1	Biochar	1	0.03	*n.a.*	2.97
BIO+Sand 2	Biochar	2	0.06	*n.a.*	2.94
BIO+Sand 5	Biochar	5	0.15	*n.a.*	2.85
BIO+Soil 0.1	Biochar	0.1	0.003	2.997	*n.a.*
BIO+Soil 0.5	Biochar	0.5	0.015	2.985	*n.a.*
BIO+Soil 1	Biochar	1	0.03	2.97	*n.a.*
BIO+Soil 2	Biochar	2	0.06	2.94	*n.a.*
BIO+Soil 5	Biochar	5	0.15	2.85	*n.a.*

Blk: Blank control**; CC:** Chemical control**; SC:** Soil control

In order to determine the best amendment ratio, only three compounds with similar log K_{OW} were selected (TRM, PRN, and SMX), and only one matrix:solution ratio was used,

1:15 g:mL. First, experimental cultures were prepared with NUA or biochar mixed with soil at different ratios: 0.1, 0.5, 1, 2, and 5%. Second, control cultures were made with NUA or biochar in order to determine their adsorption capacity, mixing the amendments with washed sand instead of soil but at the same ratios (0.1, 0.5, 1, 2, and 5%). Finally, a chemical control and a blank control were prepared. Table 3.7 shows cultures distribution for the ratio screening experiments.

3.6.10. Removal experiments in amended soil

Glass tubes were filled with soil/sand and the corresponding amendment at a fix ratio of 1% (w/w) and a solution of 0.01 M $CaCl_2$. As previously described when determining the capacity of the soil to adsorb selected compounds, 3 soil:solution ratios (1:1, 1:5 and 1:50 g:mL) were used in order to ensure that 60% of the chemical compounds would remain in the aqueous phase. Tubes were shook at 10rpm (end-over-end shaker) for 12h before the addition of 50-ppm PhACs mix solution. After spiking all the cultures (initial concentration of 500ppb), all the tubes were shook for 21 days in an end-over-end shaker at room temperature (ca. 20°C). Experimental cultures of soil amended with NUA or biochar, control cultures with NUA or biochar with washed sand, chemical and soil controls, and blank tubes were prepared (Table 3.8). Triplicate cultures were sacrificed at each sampling time-point: 0, 1, 3, 7, 14, and 21d. Additionally to drugs concentration analysis, pH and EC determinations were conducted.

Table 3.8 Cultures distribution for removal experiments.

Group	Amendent		Solution	Soil	Sand
	Type	g	Ratio	(g)	(g)
Blk	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*
SC	*n.a.*	*n.a.*	1:5	3	*n.a.*
CC-G1	*n.a.*	*n.a.*	1:1	*n.a.*	*n.a.*
CC-G2	*n.a.*	*n.a.*	1:5	*n.a.*	*n.a.*
CC-G3	*n.a.*	*n.a.*	1:50	*n.a.*	*n.a.*
BIO+Sand G1	Biochar	0.03	1:1	*n.a.*	2.97
BIO+Sand G2	Biochar	0.03	1:5	*n.a.*	2.97
BIO+Sand G3	Biochar	0.005	1:50	*n.a.*	0.495
BIO+Soil G1	Biochar	0.03	1:1	2.97	*n.a.*
BIO+Soil G2	Biochar	0.03	1:5	2.97	*n.a.*
BIO+Soil G3	Biochar	0.005	1:50	0.495	*n.a.*
NUA+Sand G1	NUA	0.03	1:1	*n.a.*	2.97
NUA+Sand G2	NUA	0.03	1:5	*n.a.*	2.97
NUA+Sand G3	NUA	0.005	1:50	*n.a.*	0.495
NUA+Soil G1	NUA	0.03	1:1	2.97	*n.a.*
NUA+Soil G2	NUA	0.03	1:5	2.97	*n.a.*
NUA+Soil G3	NUA	0.005	1:50	0.495	*n.a.*

Blk: Blank control**; CC:** Chemical control**; SC:** Soil control

3.7. Analytical methods

3.7.1. MBR sludge and anaerobic digestion inoculums characterization

TS were assessed drying 25mL of homogeneous samples at 105°C for 24h. Then, dried samples were burned at 550°C during 30min to assess the VSS (Rice *et al.*, 2012). On one hand, Total Carbon (TC) and Total Organic Carbon (TOC) were analysed using a 1020A Total Organic Carbon Analyser (O·I·Analytical, TX, USA). On the other hand, Total Ammonia Nitrogen (TAN) and Chemical Oxygen Demand (COD) were assessed using the LCK

303 and the LCK 114 cuvette test kits (HACH-Lange, UK), respectively.

3.7.2. Glucose quantification

Homogeneous samples of both the liquid media cultures and the bioslurries were centrifuged at 15,000g for 15min (Heraeus Pico21 Centrifuge, Thermo Electron Corporation, USA), and 200µL of the supernatant was analysed using an YSI 2700D Selecta (YellowSprings Instruments, UK). The glucose content was expressed as grams of glucose per litter ($g \cdot L^{-1}$).

3.7.3. Laccase activity

Laccase from biopiles systems was extracted according to a modified method by Lang *et al.* (1998): 30mL sodium acetate buffer (0.16M, pH 5) were added to 3g of homogenized sample and shaken for 30 min at 4°C.

Culture's extracts from the biopiles systems and homogeneous samples from both the liquid media and bioslurry cultures were taken and centrifuged at 15,000g for 15min. Enzymatic activity was measured using a modified version of the method for manganese peroxidase determination (Wariishi *et al.*, 1992). The reaction mixture consisted of 200µL sodium malonate (250mM, pH 4.5), 50µL 2,6-dimethoxyphenol (DMP, 20mM), and 600µL sample. DMP is oxidized by laccase even in the absence of a cofactor. Changes in the absorbance at 468nm were monitored for 2min at 30°C. Results were expressed as activity units per litter ($U \cdot L^{-1}$). One U

was defined as the number of DMP micromoles oxidized per min. The DMP extinction coefficient was 24,800$M^{-1}cm^{-1}$.

3.7.4. HZT quantification

Based on the work of Wankhede *et al.* (2007), a method to extract and quantify HZT from liquid samples was developed. Homogeneous samples (1mL) were collected into glass vials, shook at 35Hz for 2min (ZX3, VELP Scientifica, Spain), and filtered (Millipore Millex-GV 0.22 μm) prior their analysis.

HZT analyses were performed using a Dionex 3000 Ultimate HPLC equipped with a UV detector (271nm), a GraceSmart RP 18 5μ column (250x4.6 mm) and an Altima C18 5μ pre-column (7.5x4.6 mm). An isocratic mixture of 0.05M KH_2PO_4 (pH 3) and acetonitrile (70:30 v/v) was used as mobile phase, with a 1.2mL·min^{-1} flow rate. 20μL of each sample was injected into a 200μL injection loop.

3.7.5. Total PPCPs quantification for biopiles and bioslurries

Bioslurries cultures were frozen in thin layer (CHRIST cooling bath CB 18-40, Wiegand International, Hamburg, Germany) and then maintained at -80°C for 24h prior lyophilization (Virtis Sentry freeze-drying equipment, Gardiner, NY), while biopiles cultures were frozen (-80°C) for 24h without further pre-treatment. 1.0g of grounded biopiles and 0.2g of bioslurry samples were extracted using an accelerated solvent extraction (ASE) system (Jelić *et al.*, 2009). The concentrated extracts were diluted in 500mL of water and filtered through

0.45µm nylon membrane filters (Whatman, UK) in order to retain the suspended solids. A certain volume of the chelating agent EDTA was added to all of the samples to a final concentration of 3% (ml solute · ml^{-1} solution), which is well known to enhance the extraction of some PhACs. The clean up of the samples was performed by Solid Phase Extraction (SPE) using a Baker (J.T. Baker®) system and Oasis HLB 3cc, 60mg, extraction cartridges (Waters Corp. Mildford, MA, USA). The cartridges were conditioned using 5mL of methanol, followed by 5mL of HPLC grade water at 1mL·min^{-1}; then 50mL of each sample were loaded at 1mL·min^{-1}. Elution of the samples was performed by passing 6mL of pure methanol at a flow rate of 2mL·min^{-1} through the cartridges. The extracts were evaporated under nitrogen stream using a Reacti-Therm 18824 system (thermo Scientific) and reconstituted with 1mL of methanol:water (10:90 v/v). Finally, 10µL of a standard of the internal standard mix at 10ng·μL^{-1} were added in the extracts for internal standard calibration and to compensate for a possible matrix effect, if necessary.

Chromatographic separation was performed using an Ultra-Performance liquid chromatography (UPLC) system (Waters Corp. Mildford, MA, USA) equipped with a binary solvent system (Mildford, MA, USA) and a sample manager equipped with an Acquity HSS T3 column (50mm × 2.1mm, 1.7µm particle size; Waters Corp. Mildford, MA, USA) for the compounds analysed under positive electrospray ionization and with an

Acquity BEH C18 column (50mm × 2.1mm, 1.7µm particle size) for the ones analysed under negative electrospray ionization, both purchased from Waters Corporation. The UPLC instrument was coupled to a 5500 QqLIT, quadrupole–linear ion trap mass spectrometer (5500 QTRAP, Applied Biosystems, Foster City, CA, USA) with a Turbo V ion spray source. All of the transitions were recorded by using the Scheduled MRM™ algorithm, and the data were acquired and processed using Analyst

Elimination rates were calculated comparing initial and final concentration of each pharmaceutical compound and expressed as removal percentage. Those PhACs that were non-detected (ND) at the end of the assay in the experimental cultures, but detected in the control samples, were considered as fully degraded in those experiments. In addition and just for removal calculations, those compounds detected below quantification limit (BQL) were considered to have a concentration equal to their detection limit divided by two (Gilliam and Wiles, 1996; Hopke *et al.*, 2001).

3.7.6. PhACs quantification in soil amended experiments

All the culture tubs were centrifuged at 1.500 rpm for 30min, and the supernatants were transferred into amber vials and kept frozen until further analysis. Samples were analysed in a Thermo-Finnigan TSQ Quantum Discovery Max (Thermo Electron Corporation) LC-MS/MS. 10µL of the supernatant were injected into a 100 µL injection loop and then driven into a

Phenomenex 3µ C18 110A (100x2mm) column at a temperature of 40°. The mobile phase used was a mixture of (A) acetonitrile and a (B) solution of 0.1M-ammonium formate and 0.1% formic acid with the following gradient parameters: 90% A / 10% B (0.0-2.0min), 5% A / 95% B (2.1-6.0min) and 90% A / 10% B (6.1-10.0min). MS/MS analyses were performed using atmospheric pressure electrospray ionisation (ESI) in both positive and negative ionisation modes. Compounds determinations were based on retention time and multiple reactions monitoring of two transition ions.

3.7.6.a. *Adsorption and distribution coefficient determinations*

Adsorption (A) is the percentage of a compound that is adsorbed onto a solid matrix related to its initial quantity. The adsorption can be calculated as follows if the compound is stable and it is not significantly adsorbed onto the matrix:

$$A_{t_i} = \frac{m_s^{ads}(t_i)}{m_0} \cdot 100 \qquad \text{(Eq2)}$$

Where A_{ti} is the adsorption percentage at time t_i (%), $m^{ads}{}_s(t_i)$ is the mass of the compound adsorbed onto the matrix at time t_i (g), and m_o is the mass of the compound at the initial time (g).

Distribution coefficient (K_d) is the ratio between the content of the compound in the matrix (solid phase), and its mass concentration in the aqueous phase when the adsorption

equilibrium is reached. K_d is related with the adsorption at the equilibrium (A_{eq}) by:

$$K_d = \frac{A_{eq}}{100 - A_{eq}} \cdot \frac{V_0}{m_{soil}} \quad \text{(Eq3)}$$

Where K_d is the distribution coefficient ($cm^3 \cdot g^{-1}$), A_{eq} is the adsorption at the equilibrium (%), V_o is the initial volume of the aqueous phase in contact with the soil (cm^3), and m_{soil} is the mass of solid phase (g – dry weight).

3.7.7. Biogas quantification

Methane and carbon dioxide content in the biogas was analysed by injecting 100µL (injector at 150°C) of the accumulated gas in the headspace of each bottle into a Hewlett Packard Chromatograph (HP 5890) equipped with a thermal conductivity detector (TCD) at 180°C and a Supelco Porapack Q 3 m × 1/8" column (oven at a constant temperature of 70°C), and helium as carrier gas (338kPa) (Martín-González *et al.*, 2010).

3.8. Microbial community analysis

3.8.1. Biopiles systems

Total DNA was extracted from lyophilized samples with PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc) following the procedure described by the company. Additionally, PCR inhibitors were removed with OneStep™ PCR Inhibitory Kit (Zymo Research, Inc). Fragments of bacterial 16S

and fungal Internal Transcribed Spacer (ITS) region of 18S rDNA were PCR amplified by Taq DNA polymerase (invitrogen). For bacteria, the primers 341F-GC (5' CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCC TAC GGG AGG CAG CAG 3') (Muyzer *et al.*, 1993) and 907R (5' CCG TCA ATT CMT TTG AGT TT 3') (Muyzer *et al.*, 1995) were used. For fungi, two sets of primers were used in two rounds: first, the primers EF4 (5' GGA AGG GRT GTA TTT ATT AG 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3'); and second, the primers ITS1F-GC (5' CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCT TGG TCA TTT AGA GGA AGT AA 3') and ITS2R (5' GCT GCG TTC TTC ATC GAT GC 3') (Gardes and Bruns, 1993). The PCR programs for bacteria and fungi are shown in table 3.9.

Table 3.9 Thermocycler programs used for the amplification of bacteria and fungi DNA in biopiles systems.

Bacteria				**Fungi**			
Step	*T (°C)*	*t (min)*	*Cycles*	*Step*	*T (°C)*	*t (min)*	*Cycles*
Denaturing	94	5		Denaturing	94	5	
Denaturing	94	1		Denaturing	94	0.5	
Annealing	65*	1	20	Annealing	55	0.5	35
Extension	72	3		Extension	72	0.5	
Denaturing	94	1		Final extension	72	5	
Annealing	55	1	15	Maintain	15	-	
Extension	72	3					
Final extension	72	7					
Maintain	15	-					

*-0.5°C/cycle
T: Temperature
t: Time

Denaturing Gradient Gel Electrophoresis (DGGEs) was performed in a Dcode Universal Mutation Detection System (Bio-Rad). Urea gradients were adjusted in order to optimize the bands separation, being the final gradients 30-70 % for bacteria and 15-55 % for fungi, and 6% of acrylamide gel for both. Electrophoreses were performed during 16 hours at 75V in 1x TAE buffer at 60ºC. Gels were stained with ethidium bromide (1µg/ml). Selected DGGE bands were excised and re-amplified. Purification and sequencing were performed by a commercial service (Macrogen Inc., South Korea) with the primers: 341F-GC for bacteria, and ITS1F for fungi. Dendograms were calculated with InfoQuest FP 4.50 (Bio-Rad, Inc) and elaborated with FigTree v1.4.2.

3.8.2. Bioslurry systems

3.8.2.a. *Nucleic acid extraction, PCR-DGGE, sequencing and phylogenetic analyses*

Total DNA was extracted from 50-100 mg of lyophilized bioslurry samples with FastDNA SPIN Kit for Soil (MP Biomedicals) following the procedure described by the company. Fragments of bacterial 16S and fungal ITS region of 18S rDNA were PCR amplified by DreamTaq polymerase (Thermo Scientific). Universal primers were used in both reactions: ITS1F forward (5' CT TGG TCA TTT AGA GGA AGT AA 3')(Gardes and Bruns, 1993) and ITS2 reverse (5' GCT GCG TTC TTC ATC GAT GC 3')(White *et al.*, 1990) for fungi and F1055 forward (5' ATG GCT GTC GTC AGC T 3') and R1378 reverse (5'

CG GTG TGT ACA AGG CCC GGG AAC G 3') for bacteria. A GC clamp (5' CCC CCC CCC CCC CGC CCC CCG CCC CCC GCC CCC GCC GCC C 3') was attached to the primers ITS1F and R1378 at the 5' end. The PCR programs for bacteria and fungi are shown in table 3.10. The length and amount of PCR products were estimated in 1% agarose gel with DNA ladder and labeled with ethidium bromide.

Table 3.10 Thermocycler programs used for the amplification of bacteria and fungi DNA in bioslurry systems.

Bacteria				**Fungi**			
Step	*T (°C)*	*t (min)*	*Cycles*	*Step*	*T (°C)*	*t (min)*	*Cycles*
Denaturing	95	5.0		Denaturing	95	5.0	
Denaturing	95	0.5		Denaturing	95	0.5	
Annealing	56	0.7	40	Annealing	55	0.7	40
Extension	72	1.0		Extension	72	1.0	
Final extension	72	5.0		Final extension	72	5.0	
Maintain	15	-		Maintain	15	-	

T: Temperature
t: Time

DGGEs were performed in an INGENYphorU (Ingeny, The Netherlands) machine. Urea gradients were adjusted in order to optimize separation of the bands, being the final gradients 40-80 % for bacteria and 25-60 % for fungi, and 7.5% acrylamide/bisacrylamide (37:5:1) both of them. Electrophoreses were performed during 16 hours at 75V in 1x TAE buffer at 60°C. Gels were stained with SYBR Gold (Invitrogen, Life Technologies). Selected DGGE bands were excised, reamplified (22 cycles) and run in a DGGE gel until the

Table 3.11 Fungal sequences obtained with the primers ITS1F and ITS2.

Sequence identification	Lenght	Sequence 5'→3'
F1	183	AATGTTTCGTGGCTGTAGAGGATATAACGCGAGTTGTTGAATCTCAGTTACTT TAGCCCACTCCCGAAAGGGAGATGGACAGCACGCATTAGCTTTGCTTTTGTGT GTGACATAGACTTTAAATCATGACTTATTTCATTATTAAAATAACCAAAACTT TTAACAATGGATCTCTTGGCTCTT
F2	194	GATCATTACAAAATGTTAAAGGGGTGCAGTTGCGTGTTTGCGTGAGCGACTG TGATTGCACTCTGTAAGCCCACTCCCGAAAGGGAGATGGACACGTGTGCGCTT TCGAGCGTGCATGACATAGACTTTAAATCATGACTTAACCAACCATTTAATTA AAACCAAAACTTTTAACAATGGATCTCTTGGCTCCT
F3	170	AGGATCATTACAAATCATTGCAAGCCCAGTCAGCTTATTACTTACTGGGCGAG CACATTGGCACAGGGTTGCTGCTGCTGCAGCGACCTTGATGTTTACGCTTGTA TGTTTGACAGAGTAAATTGTGACTAAAATATAGACAACTTTTAACAATGGATC TCTTGGCTCTT
F4	164	ATCATTACAAATCATTGCAAGCCCAGTCAGCTTATTACTTACTGGGCGAGCAC ATTGGCACAGGGTTGCTGCTGCAGCGACCTTGATGTTTACGCTTGTGTGTTTG ACAAAGTAAATTGTGACTAAAATATAGACAACTTTTAACAATGGATCTCTTG GCTCTT
F5	224	AGGATCATTAACGAGTTTTGAAACGAGTTGTAGCTGGCCTTCCGAGGCATGT GCACGCTCTGCTCATCCACTCTACCCCTGTGCACTTACTGTAGGTTGGCGTGG GCTCCTTAACGGGAGCATTCTGCCGGCCTATGTATACTACAAACACTTTAAAG TATCAGAATGTAAACGCGTCTAACGCATCTATAATACAACTTTTAGCAACGGA TCTCTTGGCTCTC

bands were clear enough (3-6 cycles). Purification and sequencing were performed by a commercial service (Macrogen Inc., South Korea) with the ITS1F without GC tail and F1055 primers. Partial fungal and bacterial DGGE-derived sequences were aligned with sequences retrieved from databases of GenBank/EMBL/DDBJ with Blastn algorithm. Bacterial sequence data have been deposited to GenBank database under Accession Numbers from KJ599735 to KJ599740. Fungal sequences could not be deposited because

their length was less than 200bp. Their sequences can be seen in table 3.11.

3.8.2.b. *Quantitative PCR*

Quantitative PCR (qPCR) were performed for total fungi and specific for *T. versicolor.* The primers used were the same described in the last section, but without GC clamp for total fungi (ITS1F and ITS2) and those described by Eikenes *et al.* (2005) in the ITS1 region for *T. versicolor.* The 20µL of the reaction mixture contained 10µL of Maxima SYBR Green qPCR Master Mix (Fermentas), 0.375µM of each primer and 1µL of DNA. The reactions were carried out on a Rotor-gene 6000 (Corbett Research) apparatus using the temperature program described in the article of Eikenes *et al* for *T. versicolor* and the program described at Rajala *et al.* (Rajala *et al.*, 2013) for total fungi. Standard curves were performed with known amounts of *T. versicolor* (CT = -3.126*log(conc)+32.221, efficiency 1.089) and *Heterobasidion annosum* (CT= -3.748*log(conc)+36.037, efficiency 0.848), respectively.

3.9. Statistical analysis

Descriptive statistics were calculated using Microsoft Excel 2011 and SOFA Statistics (v. 1.4.3.). One-factor ANOVA procedure with $p<0.05$ was utilised, and Dixon's Q-test was used in order to identify and reject outlier values.

CHAPTER 4

Removal of pharmaceutical products by solid-state fermentation

Summary

EPs can reach the environment through the sludge of WWTPs. In this work, the use of *Trametes versicolor* in biopiles at lab scale was studied, evaluating its capacity to remove the most hydrophobic PPCPs and assessing the evolution of the biopiles microbial communities. First, an appropriate lignocellulosic substrate for the fungal biopiles was selected according to the review of published articles – from our research group and from other authors – and un-published experimental data of the research group. Second, the total removal of drugs at real concentrations from sewage sludge was assessed for non-inoculated and fungal inoculated biopiles, testing if the re-inoculation of the biopiles after 22 days of treatment would improve the removal yields. It was assessed that 2 out of the 15 initially detected pharmaceuticals were totally degraded after 22 days, and re-inoculated fungal biopiles achieved higher removal rates than non-re-inoculated fungal biopiles for single compounds and for all the drugs simultaneously: 66.45% and 49.18% re-inoculated and non-re-inoculated biopiles, respectively. Finally, the study of the bacterial and fungal communities revealed that fungal inoculated and non-inoculated biopiles evolved to similar communities adapted to the presence of those drugs.

4.1. Introduction

The main residue of any WWTP is the sludge, which is originated during the solid-liquid separation (Fytili and Zabaniotou, 2008) performed in primary, secondary and tertiary treatments, and its composition and quantity depend on several factors such as the general operational methods and the geographic situation of the plant (Eddy *et al.*, 1991). Furthermore, the wastewater source had an important role not only in the formation of the sludge, but also in its final composition and physical-chemical properties. The most common receiving wastewaters for a WWTP have an urban, domestic and/or hospital origin (Harrison *et al.*, 2006).

Although the available methods and techniques to storage and eliminate WWTP's sludge, the possible hazardous compounds – i.e. bacteria and viruses, organic compounds, and heavy metals – contained in it must be treated and removed. In Europe, 40% of the sludge produced in WWTPs is landfilled; 37% and 12% are used in agriculture and forestry activities, respectively; and the remaining 11% is incinerated (Fytili and Zabaniotou, 2008). The landfilling is the most common and widespread way to dispose sludge; however, it is inadvisable given the environmental, social and economical impacts (Calvo *et al.*, 2005). Thermal processes to eliminate WWTP sludge, such as combustion, pyrolysis and gasification, could be an

interesting revalorization method, as local communities can use these residues to generate energy (Fytili and Zabaniotou, 2008; Wall *et al.*, 1984); however, thermal treatments are not a final disposal method as the ash produced must be landfilled.

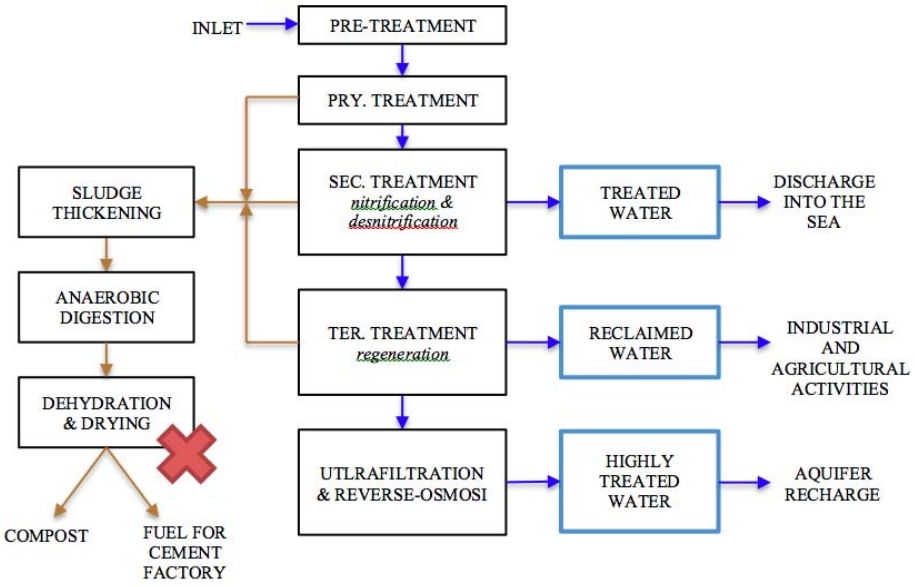


Figure 4.1. Flow-chart for the WWTP of El Prat de Llobregat. Brown arrows represent solid streams and blue arrows represent liquid streams. The red X shows the sludge collection point.

The use of WWTP's sludge in agricultural and forestry activities has become an interesting valorisation method, because of its ability to fertilise soils and the low economic impact of the operation. These actions improve the physical-chemical properties of the land and can increase the crops yield (Singh and Agrawal, 2008). Nevertheless, the application of

untreated sludge into soils can increase the potential risks for human and animal health (Dean and Suesst, 1985), as it can contain different types of pollutants. Consequently, sludge must be treated before its application into soil in order to remove micro-pollutants.

In order to study the removal of PPCPs in solid systems, a thermal dried sludge from a WWTP located in El Prat de Llobregat (Catalonia), which is designed to treat 419,000 $m^3 \cdot d^{-1}$ of wastewater for an equivalent population of two million inhabitants, was selected. It is a typical Activated Sludge (AS) plant that uses anaerobic digesters, followed by dehydration and thermal drying techniques, in order to treat the produced sludge. The flow chart for this WWTP can be observed in figure 4.1. Note that the WWTP produces three different water outlet streams, depending on the treatment, and each one is used for one specific purpose. Additionally, the produced sludge is employed in two different activities: in agriculture as compost and in cement industry as fuel. The plant has been widely studied by other researchers in order to assess the impact of reclaimed water and sludge in natural water bodies (Köck-Schulmeyer *et al.*, 2011), fate and occurrence of PPCPs in wastewaters and sludges (Badia-Fabregat *et al.*, 2012; García-Galán *et al.*, 2011; Rodríguez-Rodríguez *et al.*, 2014), and to test new analytical methods for emerging pollutants (Negreira *et al.*, 2013).

4.1.1. Solid-phase bioreactor: an alternative treatment for WWTP's sludges

The sludge produced in a WWTP is usually a liquid-solid with a high concentration of solids, between 0.25 to 15 % in weight; which is mainly composed of organic matter. As previously discussed, its treatment and disposal is one of the most complex and expensive problems during wastewater treatment. In general, sewage sludge must be stabilized, thickened and disinfected before its disposal out of the plant. Common techniques to stabilize the sludge are: anaerobic and aerobic digestion, lime stabilization, composting and heat drying; and the general thicken treatments are: centrifugation, filtration and water evaporation (Eddy *et al.*, 1991; Ramalho, 1996). However, it has been proved that these traditional treatments are not capable to remove EPs from the sludge (Clarke and Smith, 2011; Semblante *et al.*, 2015; Stasinakis, 2012).

On one hand, expensive tertiary or advanced treatments have been developed in the last years in order to decrease, partially or totally, the presence of EPs in wastewater and sludge: adsorption into activated carbon, advanced oxidation (e.g. ozone and ultra-violet), UV photolysis, ion exchange and membrane filtration (e.g. reverse osmosis and nanofiltration) (Bolong *et al.*, 2009; Gavrilescu *et al.*, 2014). Nevertheless, these

technologies imply high implementation, operational and maintenance costs (Heal the Ocean, 2001; USEPA, 2015).

On the other hand, fungal bioremediation has arisen as an economical and sustainable alternative. Fungi are known to degrade a wide variety of compounds and have been deeply studied in the removal of EPs produced by human activities. In this regard, in biopile systems the sludge to be treated is mixed with a bulking material, which improves the aeration, gives structure (Environment Protection Authrority, 2005; Juwarkar *et al.*, 2010; Khan *et al.*, 2004), and it is used as co-substrate by the fungal inoculum (Gadd, 2001; Singh, 2006). Furthermore, these systems require minimum maintenance and inputs (i.e. energy and water), making them cost-effective processes even for long time treatments (Gomez and Sartaj, 2014; Jørgensen *et al.*, 2000; Nano *et al.*, 2003).

The substrate plays an important role in biopiles inoculated with WRF, being one of the key factors for a successful mycoremediation application (Leštan *et al.*, 1996). In those cases, a lignocellulosic waste from agriculture, forestry and/or food industry is supplied as substrate, providing the essential lignocellulosic nutrients that the fungus needs to growth, and promoting the production of the LMEs (Rodríguez Couto and Sanromán, 2005). The most studied and used lignocellulosic substrates are: sawdust, wood chips and barks, wheat straw, corn cobs, grape stalks, and olive oil waste (Kassaveti, 2008;

Rigas *et al.*, 2007; Stahl and Aust, 1998). The initial fungal lignocellulosic inoculum is prepared inoculating fungal biomass grown in liquid phase (i.e. mycelia suspension) to the substrate, and letting the fungi enough time to growth at constant temperature in static conditions (the so-called pre-grow phase) (Leštan *et al.*, 1996). In general, the fungi is inoculated during its vegetative phase of the life cycle, as the mycelium will be able to penetrate solid matrices and will produce the extracellular enzymes involved in the degradation of complex compounds such as lignin (Singh, 2006; Webster and Weber, 2007). In conclusion, the correct selection of a ligninolytic substrate will lead to better pollutant removal by the inoculated WRFs, with low operational time and minor investments.

4.1.2. Aim of the chapter

Previously to this work, Rodríguez-Rodríguez *et al.* (2014) treated sewage sludge from the same WWTP in biopiles systems; however, the authors used a lignocellulosic substrate that was already valorised as commercial pet's food and was unable to maintain the biopile structure. As a result, the aim of this chapter was to determine if bioaugmentation could be used in order to treat dry WWTP sludge in biopiles systems with inexpensive lignocellulosic substrates, removing emerging pollutants from it. To begin with, a review of different

lignocellulosic substrates was made so as to establish which one would be the most suitable, using unpublished studies of the research group and articles from other authors. Both, the capability of the fungus to colonize the substrate (assessed by means of laccase production, ergosterol quantification or other indirect measurements of fungus activity), and its PPCPs removal performance were weighed; but also economical aspects, origin and availability, and its function inside the biopile were taken into account before choosing a substrate. Next, biopiles systems under non-sterile conditions were constructed with the selected substrate and non-spiked sludge, treating it for 42 days. Also, a re-inoculation was carried out for half of the experimental biopiles after 22 days of treatment. Finally, PPCPs removals were assessed and microbial analysis were performed in order to determine the feasibility of a biopile system with *T. versicolor* to treat thermal dried WWTP sludge.

4.2. Results and Discussion

As already mentioned, solid bioremediation techniques, i.e. biopiles, with fungal inoculation are an eco-friendly alternative to remove EPs from solid matrices. All the works with biopiles have been focused to treat soils contaminated with recalcitrant compounds (Abdul Salam *et al.*, 2013; Lladó *et al.*, 2013; McErlean *et al.*, 2006; C. Wang *et al.*, 2014; Wu *et al.*, 2008),

while only a few researchers have studied this system for the treatment of EPs in sewage sludge (Rodríguez-Rodríguez *et al.*, 2014, 2012b). The goal was to improve the knowledge in this field while assessing if it was feasible to treat WWTP sludge with *T. versicolor* inoculated in non-tested lignocellulosic substrates.

4.2.1. Substrate selection: a review

As previously discussed, choosing an adequate substrate was an important factor, since the correct fungal inoculation of the biopile would depend on this. It was also necessary to ensure both that the selected material would act as bulking agent and the lignocellulosic substrate should be extensively available and locally produced, in order to maintain the process economically viable and sustainable. Although the treatment of contaminated soils in biopiles systems was largely studied and applied for decades, treating sewage sludge in biopiles inoculated with *T. versicolor* was new and few or non literature was available. However, it was possible to collect enough experimental data and information to select an adequate lignocellulosic substrate for the treatment of thermal dried WWTP sludge in biopiles systems at lab scale.

4.2.1.a. *Agro-industrial wastes*

Agro-industrial wastes encompass all those residues and by-products generated during the growing, processing and treatment of foods in the agricultural and food industry (i.e.

vegetables, meat, diary products and crops), which can be in the form of solids, liquids or slurries. These wastes not only represent an important source of valuable substances that can be recovered (i.e. nutrients), but also a source of inexpensive substrates for solid-state fermentations (SSF). Generally, agro-industrial wastes consist of high amounts of diverse organic matters with great BOD and COD values (Petruccioli *et al.*, 2011; Prasertsan *et al.*, 2007). Among all the available by-products, only rich lignocellulosic wastes were of interest for our research, since WRF needs the lignocellulosic nutrients that only these materials can provide, and their composition will also prevent the growing of less adapted microorganisms.

The most common lignocellulosic substrates from the agricultural and food industry used for fungal growth in SSF are palm fibers and fruits, other fruit seeds and shells, and wastes from brewery and fishery activities (Cheirsilp and Kitcha, 2015; Gassara *et al.*, 2010; Kitcha and Cheirsilp, 2014; Xu *et al.*, 2015). Nevertheless, these materials are not suitable to construct biopiles, as their physical properties (i.e. structure, shape and resistance to mechanical forces) could lead to systems with low internal porosity; implying an extensive use of external inputs, such as energy – in order to keep a correct structure by mechanical means – and aeration – more complex aeration systems. However, other agricultural wastes can be used as

lignocellulosic substrates, although their production is lower than the previously mentioned, such as straw (Sánchez, 2009).

Table 4.1. Summary of the tested lignocellulosic substrates by Borràs *et al.* and some of their physic-chemical characteristics.

Substrate	WHC ($gH_2O \cdot g\ DWsub^{-1}$)	C/N (w/w)	pH_{water}
Wheat straw pellets	3.13	40.15	5.65
Rabbit feedstock 1	3.33	11.75	5.35
Rabbit feedstock 2	3.37	15.98	5.31
Maize stalks	1.03	79.67	3.99
Wheat straw	3.66	51.39	6.96
Pine stardust	2.3	121.32	3.96
Rice Husk	7.2	1075.32	5.33

During the very first stage in the research group, seven different agro-industrial wastes (table 4.2) were tested as lignocellulosic substrates, so as to assess their applicability in soil bioremediation with *T. versicolor* (Borràs *et al.*, 2011). According to CO_2 production, biomass content (as ergosterol) and laccase activity, three of the initial wastes were selected for biopiles made of sterile and non-sterile soil. Next, the fungal degradation ability was tested using the ND24 test (Rodríguez-Rodríguez *et al.*, 2010b).

All the tested substrates showed a low pH, which should facilitate the fungal growth (Stoilova *et al.*, 2010; Tavares *et al.*, 2006), and C/N higher than 10, which should also contribute to high ligninolytic activities (Fakoussa and Frost, 1999; Yu *et al.*,

2006). However, CO_2 production demonstrated that the substrates with the higher C/N ratio and low pH values, i.e. pine stardust and rice husk, were no able to maintain the fungal inoculum, showing low rates of CO_2 production. Furthermore, maize stalk and wheat straw, which also had high C/N ratios, not only achieved low laccase activity (maximum values below 0.3U·gDW^{-1}), but also a low biomass content after 17 days of fungal growth; ranging less than 0.05mg of ergosterol per gram of dry substrate. Given that, only the processed agro-industrial wastes were tested to colonize soil in biopiles systems: the wheat straw pellets and the two rabbit feedstock (a mix of different non-commercial agricultural by-products).

With the three processed agro-industrial wastes was possible to colonize both sterile and non-sterile soils with *T. versicolor* using biopiles systems; achieving the higher laccase activity under non-sterile conditions: maximums ranging ca. 9 and 13 U·gDW^{-1} for rabbit feedstock 1 and wheat straw pellets, respectively. Additionally, it was proved that the fungus was able to maintain its degradation ability under non-sterile conditions, reaching naproxen degradation removal rates (test ND24) higher than 20% within a time period of 24h. Nevertheless, although these three processed agricultural wastes were locally produced and available, they were already valorised wastes, being used and commercialised for home animals as feedstock and litter. So, their economical cost

discouraged using them in biopiles at higher scale. Additionally, during the experiments it was observed that these substrates were not able to maintain the biopile structure for long periods, resulting in a loss of volume due to a collapse of the system structure.

Thus, it was necessary to search other lignocellulosic substrates capable to maintain the biopile structure – allowing fluids transport and avoiding anaerobic conditions –, while providing the essential habitat for the fungi. Previously to Borràs *et al.* (2011) work, Rodriguez *et al.* (1996) studied de lignin degradation caused by diverse fungi in different natural lignocellulosic substrates, and their later applicability in the removal of dyes in liquid media. These researchers found that the tested ligninolytic organisms were able to degrade milled wood lignin from wheat straw and pine, reaching a higher lignin mineralization for wheat straw (22-28%) than for pine (>19%). The researchers pointed that it was easier to degrade grass lignin than wood lignin, because of the intrinsic differences between them, such as the presence of phenolic acids. Indeed, it was proved that wood materials could be used for SSF with WRF. So, wood by-products were studied as lignocellulosic substrates for biopiles systems, assessing if they could provide the essential lignocellulosic nutrients and biopile structure.

4.2.1.b. *By-products from forestry activities*

Nowadays, the uses that humankind gives to forests have changed in the recent decades: from being a wide source of products (e.g. energy storage, construction materials, foods and remedies) to a place that must be kept undisturbed (Elliott and FAO, 2014; Pearce, 2001; Petrov, 2003). Nonetheless, some management must be done in order to achieve the desired sustainable objectives. Modern sustainable forestry activities are designed to manage forests with the lower impact and maximum economical benefit, taking advantage of all the extracted materials (Hahn and Knoke, 2010; Sample, 2004); however, some non-commercial wood by-products are produced: sawmill, sawdust, trimmings and bark. According to the annual Forest Products report that the Food and Agriculture Organisation (FAO) elaborates, 178,829m^3 of wood wastes were produced in the year 2012 – with an increasing tendency from 2009 –, of which 61,134 m^3 were generated in Europe (FAO, 2014). So, large amounts of wood by-products could be supplied and used as lignocellulosic substrate for biopiles systems inoculated with WRF.

Bark and small wood fractions from pruning are considered as undesirable materials for most of the wood industries (Biermann, 1996), using them as bulking material in composting activities, as fuels or as decorative amendments for gardens (Harkin and Rowe, 1971). However, these by-products are

highly applicable in bioremediation techniques with WRF, since the wood composition not only provides the essential nutrients for fungal growth, but also avoids the system colonization by other organisms (Steffen *et al.*, 2007). Cellulose, hemicellulose and lignin are the main biopolymers of the plant cell walls, with different contents between species. Lignin, which is an amorphous and hydrophobic polymer, protects cellulose and hemicellulose, and it is responsible for the rigidity and strength of the plant (Lebo *et al.*, 2001). However, WRF developed an extracellular unspecific system of oxidases and ligninolytic enzymes capable to decompose these biopolymers (Floudas *et al.*, 2012; Martínez *et al.*, 2005; Ruiz-Dueñas *et al.*, 2013).

Olive oil production has experienced an increasing tendency in the last years, with an estimated production of $2.8 \cdot 10^6$Ton per year. The Mediterranean area is the largest olive oil producer, being the most extended agricultural activity in the zone, with a dedicated cultivation area of $5.4 \cdot 10^6$ha that produces 3000kg of lignocellulosic residues per year (García Martín *et al.*, 2010; ICO, 2014). Olive by-products such as olive cake, olive mill wastes and olive husk, have been studied and used in the bioremediation of soils polluted with pesticides, CP, PAH and heavy metals; however, only composting techniques have been employed (Chen *et al.*, 2015).

In order to assess if it was possible to use olive pruning in biopiles, a screening research was performed using dried

sludge from the MBR of Terrassa WWTP under sterile conditions (unpublished work). Biopiles in tubes, as described by Borràs *et al.* (2011), with dried sludge and olive pruning from a particular garden as lignocellulosic substrate were constructed. *T. versicolor* was able to growth in this substrate and colonizes the sludge; however, the laccase activity was low and only a maximum of 0.113 ± 0.001 $U \cdot gDW^{-1}$ was achieved after 15 days of treatment. Additionally, 10ppm of HZT were initially spiked into the cultures, so as to determine the fungi degradation capability; reaching a total removal of 86% after 15 days. Although *T. versicolor* was able to degrade the target compound, the colonization of the biopiles was quiet, with a low laccase production and without appreciable fungi biomass. So, other lignocellulosic substrates were searched.

Another important wood by-product is bark, which has been already studied in bioremediation processes. For instance, Valentín *et al.* (2009) studied the degradation of soil organic matter by different fungus with pine bark as co-substrate; achieving both good enzymatic activity and degradation of organic matter. Later, the same researchers studied the suitability of pine barks as co-substrate for fungal bioremediation, testing two different organisms: *Phanerochaete velutina* and *Stropharia rugosoannulata*. After 90 days of treatment under sterile conditions, the researchers found that pine bark was a suitable substrate for fungal

bioremediation processes, as the wood material provided the essential nutrients that the fungus need (Valentín *et al.*, 2010). More recently, Winquist (2014) was able to treat soils polluted with PAHs at field scale (up to 2Tn of soil) with fungi inoculated on pine bark. Although the degradation in both experimental and control biopiles were similar, it was proved that fungal inoculation of wood by-products could effectively be applied in the bioremediation field.

4.2.1.c. *Valorised wastes: the compost*

The current society produces large amounts of municipal solid wastes, which comprises not only everyday products – such as packaging products, clothing, glass and appliances –, but also food waste and small-sized plant waste – the so-called green waste. This last is known as the OFMSW, characterised for having high water content (ca. 80% in weigh) and for being biodegradable. This organic matter can be aerobically degraded, resulting in the well-known compost; which can be used as fertilizer for agricultural soils (BRA, 2012; Christian *et al.*, 2009). Compost has been studied and utilised as amendment for polluted soils with PAHs, pesticides, heavy metals and petroleum derivatives, since it can increase both the fertility and the organic matter content of the soil; improving the microbial communities (Chen *et al.*, 2015). However, it has not been studied as possible co-substrate in fungal bioremediation

processes, although it has a middle-high content of lignocellulosic residues and a low cost.

In order to determine the applicability of compost as growing substrate for WRF in biopiles systems, a screening was performed with dry sludge from El Prat de Llobregat's WWTP, and compost made of olive pomace, wood chips and stone, and raw wool; following the biopiles in tubes described by Borràs *et al.* (2011). The compost was produced in bags and watered with olive mill wastewater at the TIRSAV Plus Composting Research Centre (Facolt *et al.*, 2013). This research was performed jointly with an international PhD student from the TIRSAV Centre, which aimed to search new applications for the compost produced in the research plant. A sequence of different biopiles tubes were prepared following a central point design with only two factors: substrate:sludge ratio (0.09 – 0.5) and moisture (20 – 60 %); as shown in fig 4.2. Although the relation between Laccase expression and degradation of some compounds is not clear, its activity was used as presence indicator of *T. versicolor*, since is the main enzyme produced and the determination method was simple. Also, the degradation ability of *T. versicolor* was assessed spiking 10ppm of CBZ in each culture; determining the percentage of removal.

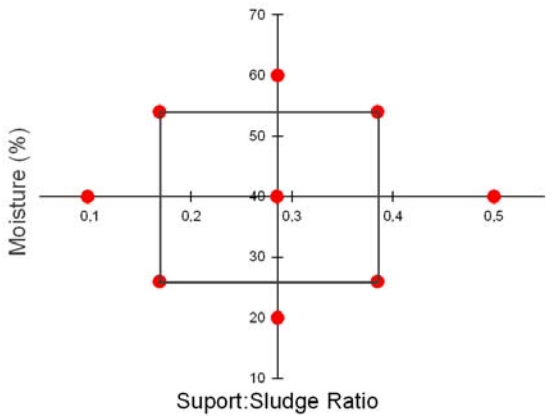


Figure 4.2. Representation of the tested preparation conditions for each biopile made of compost as substrate and dry WWTP sludge.

During the pre-growth phase it was observed a low fungal growth compared with previous experiments, and after the addition of the sludge these observable growth was decreased. Additionally, no laccase activity was measured during the whole experiment, indicating that the enzymatic activity of the fungus was null. In contrast, CBZ degradation was observed for all the cultures, with a maximum removal of 76.7 ± 1.0 % (day 14; humidity: 54%; substrate:sludge ratio: 0.17) and a minimum removal of 7.2 ± 2. 4% (day 14; humidity: 20%; substrate:sludge ratio: 0.3). However, no clear removal tendency was achieved, since constant bouncy removal percentages were observed for all the cultures and conditions. Additionally, it was observed that the physical structure of the biopile was not maintained during the experiments and finally collapsed – ca. 50% of

volume loss at the end of the experiments. Although the use of compost as co-substrate in the fungal bioremediation of spiked WWTP sludge achieved some middle-high removal percentages under certain conditions, its use for further large fungal inoculated biopiles systems was not recommended; as the structure of the biopile was not maintained – leading to a poor fluids transport – and the fungus was not able to colonize the substrate.

4.2.1.d. *Final remarks*

It has been demonstrated that different lignocellulosic substrates can be used in order to growth WRF in bioremediations systems; however, not all the described substrates were able to act as bulking material inside fungal biopiles systems. Given the results obtained by Valentin *et al.* (2009), we decided to use and examine pine bark as co-substrate, as this material was proved to be an effectively bulking material in SSF, and it was already reported that fungi could growth in this type of wood. The selected barks were of *Pinus halepensis*, since in our region (Catalonia) the most common forests are of Aleppo pine (CREAF, 1998), and it has the lowest economical value (CTFC, 2015).

4.2.2. Total drugs removal

Once the lignocellulosic substrate was chosen, biopiles were constructed with pre-grown fungi onto sterile pine barks and non-sterile thermal dried WWTP sludge. The fungus activity

was monitored by means of laccase activity, as *T. versicolor* has been reported to produce large amounts of this enzyme (Couto *et al.*, 2002; Lorenzo *et al.*, 2002; Pazarlıoğlu *et al.*, 2005). The maximum enzymatic activity was achieved by day 10 with 0.007 ± 0.002 $U \cdot g^{-1}$, which decreased to nil by day 22. After re-inoculating half of the experimental cultures, laccase activity was maintained without any fluctuation for these systems, but it was null for non-re-inoculated biopiles. These laccase activities were low if compared with other studies, for instance activities 1 and 4 orders of magnitude higher were achieved for small biopiles with soil (Borràs *et al.*, 2010) and for biopiles with sewage sludge (Rodríguez-Rodríguez *et al.*, 2010a); however, these researchers used wheat straw as ligninolytic substrate, which has been proved to be more easily degradable by the fungus. Also, the phenolic groups of the pine barks could inhibit the laccase expression. Although the laccase activity was low, the degradation pathway not only involved the fungal co-metabolism (ligninolytic enzymes), but also its metabolism and detoxification mechanisms (cytochrome P450) (Badia-Fabregat *et al.*, 2014; Corvini *et al.*, 2006; Reddy, 1995). Additionally, the competition with the sludge's autochthonous populations could lead to a lower laccase expression. So, it was necessary to perform a microbial community analysis in order to establish the potential mechanisms of interaction between indigenous populations and *T. versicolor*.

Table 4.2. Detected PhACs in Biopiles systems and its removal yields before/after re-inoculation.

Pharmaceutical	Initial concentration ± RSD ($ng \cdot g^{-1}$)	Removals (%) ± SD		
		Before re-inoculation (22d)	*Non-re-inoculated (42d)*	*Re-inoculated (42d)*
Analgesics and anti-inflammatory drugs				
IBU	19.38 ± 13.57	[a]	14.33 ± 0.14	35.96 ± 0.36
Oxycodone	4.45 ± 0.82	[a]	11.81 ± 0.12	70.29 ± 1.00
Codeine	0.34 ± 0.22	[a]	0.00 ± 0.00	[a]
Anthelmintic				
Levamisol	5.95 ± 0.35	22.77 ± 0.23	21.90 ± 0.22	57.08 ± 0.57
Antibiotics				
SMX	6.43 ± 0.95	87.48 ± 1.00	87.48 ± 1.00	87.48 ± 1.00
Antihypertensive drugs				
Amlodipine	18.18 ± 5.43	43.45 ± 0.43	49.68 ± 0.50	82.78 ± 0.83
Calcium channel blockers				
Diltiazem	6.91 ± 1.17	100.00 ± 1.00	100.00 ± 1.00	100.00 ± 1.00
Diuretic				
HZT	8.98 ± 0.16	47.25 ± 0.47	82.30 ± 9.12	82.30 ± 9.12
Tamsulosin	7.09 ± 0.51	31.95 ± 0.32	15.71 ± 0.16	53.93 ± 0.54
Lipid regulators and cholesterol lowering statin drugs				
Gemfibrozil	36.52 ± 11.32	60.74 ± 0.61	54.03 ± 0.54	69.13 ± 0.69
Atorvastatin	20.79 ± 3.23	83.01 ± 0.83	75.44 ± 0.75	93.80 ± 0.74
Psychiatric drugs				
Citalopram	75.55 ± 10.46	42.76 ± 0.43	19.69 ± 0.20	35.45 ± 0.35
Sertraline	52.58 ± 5.69	92.97 ± 1.00	92.97 ± 1.00	92.97 ± 1.00
Fluoxetine	51.14 ± 8.39	81.53 ± 0.82	72.79 ± 0.73	75.16 ± 0.75
Paroxetine	42.13 ± 5.51	87.38 ± 0.87	79.32 ± 0.79	87.57 ± 0.88
Trazodone	34.91 ± 5.97	75.00 ± 0.75	53.79 ± 0.54	83.88 ± 0.84
Venlafaxine	29.64 ± 1.72	3.34 ± 0.03	[a]	16.69 ± 0.17
CBZ	5.05 ± 0.69	[a]	[a]	0.42 ± 0.00
Olanzapine	4.79 ± 1.31	100.00 ± 1.00	100.00 ± 1.00	100.00 ± 1.00
Total	430.79 ± 103.26	56.89 ± 0.57	49.18 ± 0.52	66.45 ± 0.96

[a] Removal not assessed, final concentration was higher than the initial

Table 4.2 shows the detected PhACs at the initial biopiles, and their removal rates before the re-inoculation (22d) and after for both cultures, re-inoculated and non-re-inoculated, at the end of the treatment (42d). Out of the 45 PhACs analysed, 19 were detected in the initial cultures at day 0 giving a total drugs amount of 430.79 ± 103.26 $ng \cdot g^{-1}$, and the rest showed concentrations below the quantification limits. As can be seen, some standard deviations (SD) were high (up to 70% of the measured concentration value), which has been previously described (Radjenović *et al.*, 2009) as in solid matrices the heterogeneity of the samples and the extraction procedures are limiting steps, implying great dispersion and variability between replicates.

The highest initial concentrations were found for the psychiatric drugs citalopram (75.55 ± 10.46 $ng \cdot g^{-1}$), sertraline (52.58 ± 5.69 $ng \cdot g^{-1}$) and paroxetine (42.13 ± 5.51 $ng \cdot g^{-1}$); and the lowest initial concentration was for the analgesic codeine (0.34 ± 0.22 $ng \cdot g^{-1}$), followed by another analgesic, oxycodone (4.45 ± 0.82 $ng \cdot g^{-1}$), and one psychiatric drug: olanzapine (4.79 ± 1.31 $ng \cdot g^{-1}$). Rodríguez-Rodríguez *et al.* (2012b) studied this sludge twice in biopiles systems with wheat straw as lignocellulosic substrate (trays with a total content of 350-370g of sludge and bulking material [62:38% w/w]), evaluating the total PPCPs removal with the same analytical method as in the present work, detecting only 9 PPCPs in the sludge, 3 of which have

been also detected in the present experiment, but at different concentrations: atorvastatin (13.7 ± 1.8 $ng \cdot g^{-1}$), CBZ (10.5 ± 0.7 $ng \cdot g^{-1}$) and IBU (161.0 ± 21.4 $ng \cdot g^{-1}$).

In the second study of Rodríguez-Rodríguez *et al.* (2014), the authors found 21 pharmaceutical compounds, 8 of them detected in the current biopiles: amlodipine (13.66 $ng \cdot g^{-1}$), CBZ (5.13 $ng \cdot g^{-1}$), citalopram (309.52 $ng \cdot g^{-1}$), codeine (9.28 $ng \cdot g^{-1}$), gemfibrozil (86.17 $ng \cdot g^{-1}$), IBU (203.93 $ng \cdot g^{-1}$), olanzapine (19.58 $ng \cdot g^{-1}$) and venlafaxine (134.05 $ng \cdot g^{-1}$). Therefore, although the sludge was produced in the same WWTP and collected from the same point, the pollutants content and concentrations in the sewage sludge varied among time (this can be appreciated comparing the results found by other authors – listed in the two paragraphs above – and the values found in the present study – table 4.2.); implying that EPs treatment systems should not be specific for certain compounds and concentration ranges. In this regard, fungal biopiles were proved to remove a wide variety of PPCPs without any or minimal changes in their configuration.

It can be said that, in the present study, partial or total PhACs removals have been achieved. Olanzapine and diltiazem were the only drugs completely removed, both after 22 days of treatment. For non-re-inoculated biopiles, 5 drugs (not taking into account the compounds completely removed) were eliminated with removal efficiencies higher than 75%:

atorvastatin, paroxetine, sertraline, SMX and HZT; whereas for re-inoculated cultures 8 PhACs experienced removals higher than 75%: amlodipine, atorvastatin, paroxetine, sertraline, SMX, trazodone, fluoxetine and HZT. On the contrary, 5 compounds showed removal yields lower than 20% for non-re-inoculated biopiles: IBU, oxycodone, codeine, tamsulosin and citalopram; while in re-inoculated cultures 2 drugs experienced removals lower than 20%: venlafaxine and CBZ. Furthermore, 4 drugs showed higher concentrations after 22 days of treatment (i.e. IBU, oxycodone, codeine and CBZ), 2 for non-re-inoculated cultures at 42d (i.e. venlafaxine and CBZ) and 1 in re-inoculated biopiles at 42d (i.e. codeine). This can be explained by the occurrence of conjugates, which could be formed by some organisms in order to increase the solubility of the compounds. Although conjugation/deconjugation processes of PPCPs have mainly been observed in liquid environments, it has been described that it could also take place in river sediments and sewage sludge (Andersen *et al.*, 2003; Gomes *et al.*, 2004; Labadie and Hill, 2007; Matejícek *et al.*, 2007; Petrovic *et al.*, 2002; Ternes *et al.*, 2002).

The general removal trends are displayed in figure 4.3, where it can be observed that re-inoculated biopiles achieved the lowest final drugs concentration (144.51 ± 4.06 $ng \cdot g^{-1}$) and the higher removal rate (66.45 ± 0.96 %). On the contrary, non-re-inoculated cultures achieved a higher final total drugs

concentration (218.92 ± 49.71 ng·g^{-1} ~ 49.18 ± 0.52 %), and non-inoculated biopiles (control group) got similar final concentration (207.22 ± 47.15 ng·g^{-1} ~ 51.90 ± 0.54 %). Moreover, it can be noticed that further addition of *T. versicolor* at 22d caused a low PhACs concentration decrease in re-inoculated biopiles, while this concentration in non-re-inoculated cultures remained nearly constant, considering the error bars.

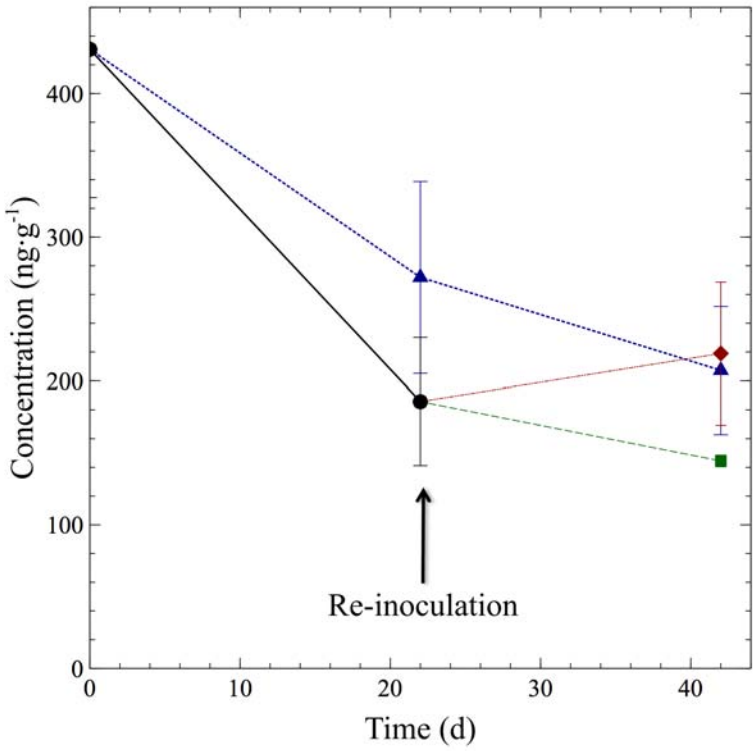


Figure 4.3. Evolution of the total PPCPs concentration for non-inoculated biopiles (▲) and for inoculated biopiles before re-inoculation (●) and after: re-inoculated (■) and non-re-inoculated (◆).

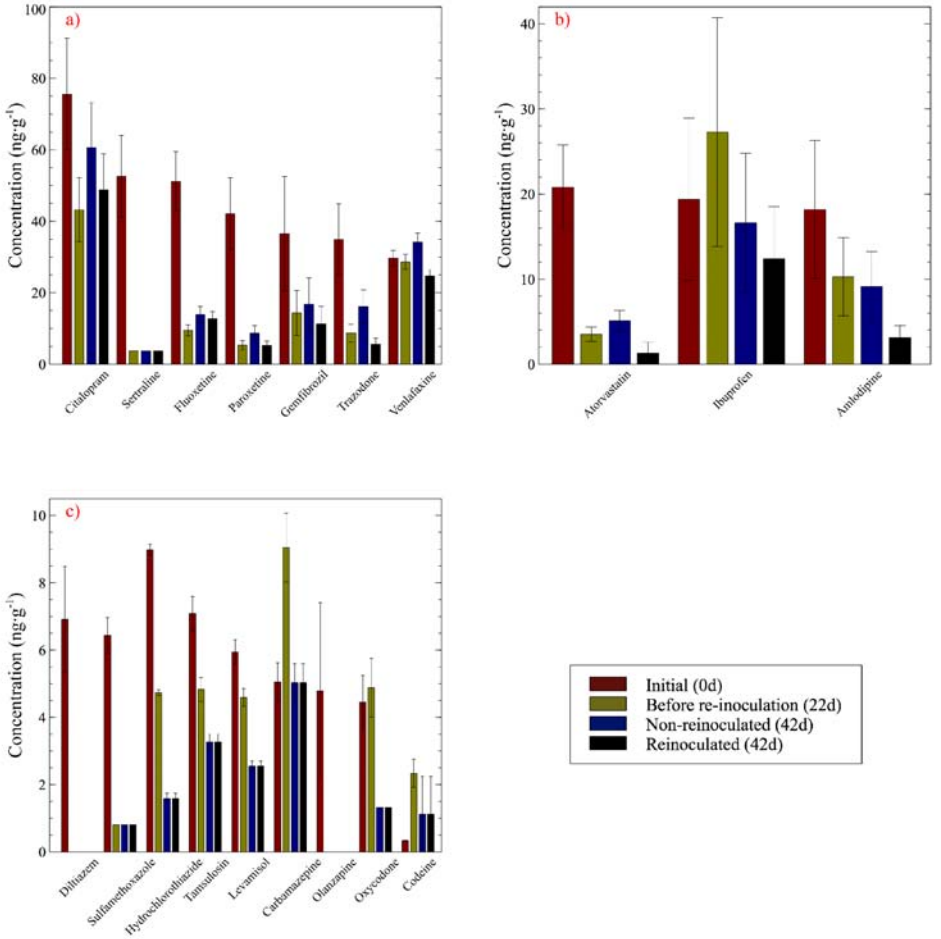


Figure 4.4. Evolution of each compound in the biopiles. Pharmaceutical compounds have been classified in three groups according to their initial concentration: a) more than 25ng·g^{-1}, b) between 25 and 10 ng·g^{-1} and c) less than 10ng·g^{-1}.

The concentrations of each pollutant in the biopiles are shown in figure 4.4. In general, important concentrations decreases were obtained for all the detected compounds

except for citalopram, venlafaxine (both psychiatric drugs; plot ***a*** in figure 4.4) and IBU (plot ***b*** in figure 4.4). Citalopram is a largely consumed antidepressant that can interfere both the behaviour regulation and neuro-endocrine signals of aquatics organisms. Since only a moderate biodegradability (ca. 50%) has been achieved in traditional WWTP, further tertiary treatments are being studied in order to increase its removal (Calisto *et al.*, 2014; Hörsing *et al.*, 2012). Similarly, venlafaxine is a widely prescribed antidepressant that affects the brain serotonin levels of fishes. This drug has been poorly removed in conventional WWTP, so advanced treatments such as ozonation are being studied (Bisesi *et al.*, 2014; Xiang Li *et al.*, 2015; Rúa-Gómez and Püttmann, 2013). Finally, IBU is an extensively used analgesic worldwide that is inadequately removed in regular WWTP, and its degradation in sewage sludge, wastewater, surface and ground water has been broadly studied (Collado *et al.*, 2012; Evgenidou *et al.*, 2015; Kosma *et al.*, 2014).

Rodríguez-Rodríguez *et al.* (2014) observed similar removal patterns for single and overall compounds while studying re-inoculation strategies for larger fungal biopiles. Briefly, the highest concentrations decreases were observed before the re-inoculation of *T. versicolor* after 22 days of treatment, with 17 out of 21 PPCPs 100% removed. After re-inoculation, the non-completely degraded compounds that remained in the biopiles

experienced a concentration decrease. These researchers noted that the re-inoculation of the system lead to an increase of the global removal. However, these authors did not assessed how the microbial communities of the sludge evolved and affected both the removal percentage and the biopile colonization by *T. versicolor*. So, the influence of the autochthonous WWTP sludge populations during the fungal bioremediation strategy was studied.

4.2.3. Microbial community evolution

PCR-DGGE fingerprints and phylogenetic affiliations were done in order to determine the microbial diversity in the biopiles system. Fungal and bacterial characterisation was done in 5 different time-points for inoculated cultures (experimental group): 0d, 10d, 22d, 23d (only for re-inoculated biopiles) and 42d; and for non-inoculated cultures (control group) only times 0d, 22d and 42d were examined. Although 10d samples for control group were collected and frozen, they were not analysed because of the previous experience of the research group with this sludge (Rodríguez-Rodríguez *et al.*, 2014), where an important shift in the microbial community of the control group was not shown.

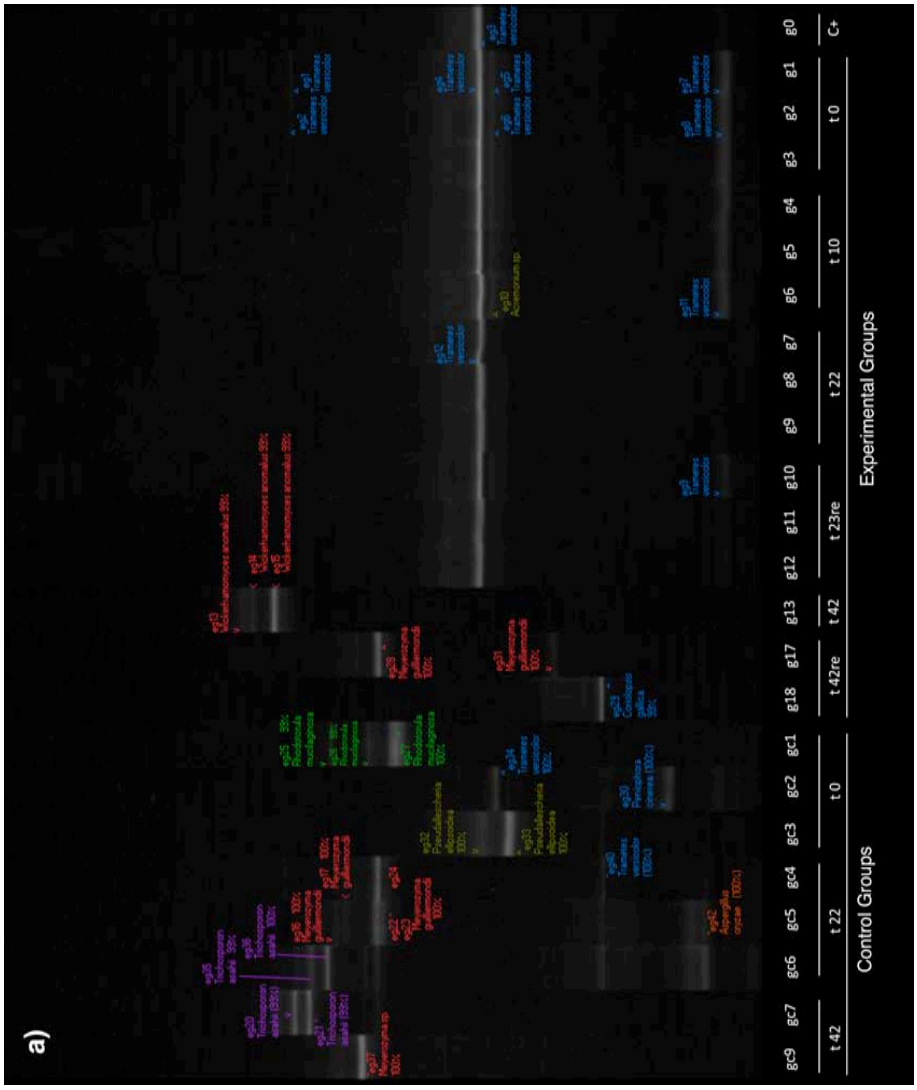
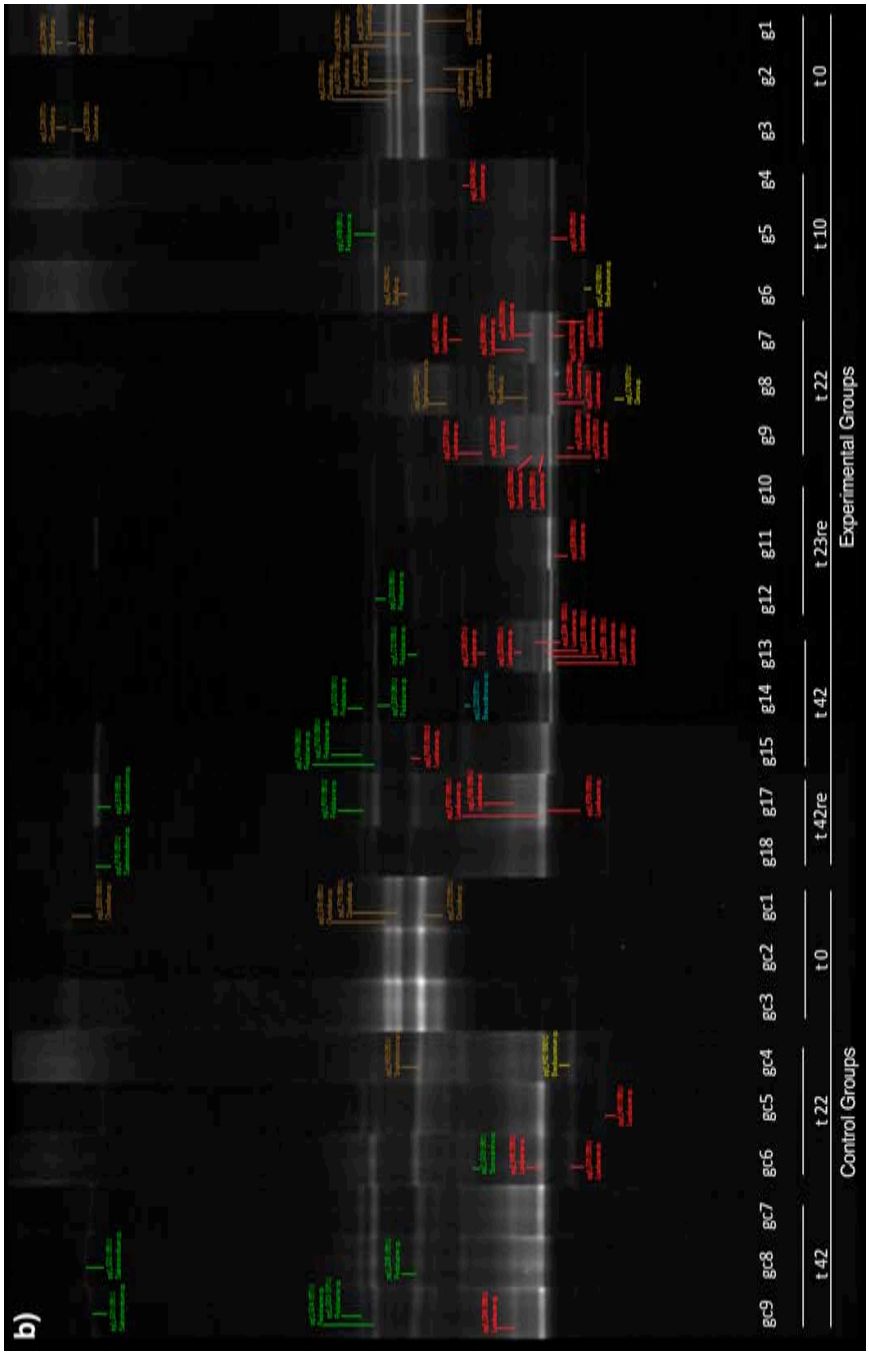


Figure 4.5. PCR-DGGE fingerprints of (a) fungal and (b) bacterial populations. For inoculated biopiles (experimental group): g0 was *Trametes versicolour* inoculum control; g1, g2 and g3 were biopiles sampled at time 0d; g4, g5 and g6 at time 10d; g7, g8 and g9 at time 22d (before re-inoculation); g10, g11 and g12 were re-inoculated biopiles sampled at 23d; g13, g14 and g15 were non-re-inoculated biopiles sampled at 42d; and g17 and g18 were re-inoculated biopiles sampled at 42d. For non-inoculated biopiles (control group): gc1, gc2 and gc3 were control biopiles sampled at time 0d; gc4, gc5 and gc6 at time 22d; and gc7, gc8 and gc9 at time 42d. *Note: Higher resolution images can be displayed in the CD*


b)
gc9 gc8 gc7 gc6 gc5 gc4 gc3 gc2 gc1 g18 g17 g15 g14 g13 g12 g11 g10 g9 g8 g7 g6 g5 g4 g3 g2 g1
t 42
t 22
t 0
t 42re
t 42
t 23re
t 22
t 10
t 0
Control Groups
Experimental Groups

4.2.3.a. *Community diversity and dynamics*

DGGE profiles are presented in figure 4.5, showing different communities and structures along time. It can be observed that *T. versicolor* was the predominant organism in the biopiles after the inoculation at time 0d, and it remained as the prevailing one at least until day 23 – the day after half of the experimental biopiles were re-inoculated. In general, it can be said that the fungus inoculation of the sludge changed both fungal and bacterial populations initially; however, should be noted that the PPCPs contained in the sludge could have affected the microbial populations dynamics. Nonetheless, at the end of the experiment similar communities were observed in both experimental and control cultures. This could be due to the origin of the sewage sludge: AD sludge from the WWTP was thermal dried prior its treatment in the biopiles, so spores from anaerobic microorganisms could remain until the present experiments

Previously to this work, the increase in the number of microorganisms and the change of the microbial population in soils due to bioremediation processes, such as bioaugmentation and biostimulation techniques, was reported in polluted soils. For instance, Lin *et al.* (2010) noticed that, while treating soil polluted with diesel and fuel oils in biopiles, the microbial presence not only increased, but also changed to other microorganisms, which were more adapted to degrade

recalcitrant contaminants. Wang *et al.* (2011) observed similar microbial communities tendencies when composting polluted soil with hydrocarbons followed by a rhizodegradation process. Betancur-Corredor *et al.* (2015) found similar microorganism increase during the biostimulation of soil with pesticides, and Kao *et al.* (2015) noted a microbial community shift when polluted groundwater with chlorinated products was also biostimulated. These observations corroborate the community change experienced not only in the biopiles without fungal inoculation, but also the cultures with *T. versicolor*.

Table 4.3 Sequence information for the DGGE bands obtained analysing the fungal community.

Band code	Accession number	Closest related sequence	Similarity (%)	Order
gc5	KP975532	*Aspergillus oryzae*	100	Eurotiales
g6	KF669512	*Acremonium sp.*	99	Hypocreales
gc3	KP132722	*Pseudallescheria ellipsoidea*	100	Microascales
g18	AY684172	*Coriolopsis gallica*	99	Polyporales
g1-g12; gc2	KP761168	*Trametes versicolor*	100	Polyporales
gc2	LN808982	*Peniophora cinerea*	100	Russulales
gc9	KR054629	*Meyerozyma sp.*	100	Saccharomycetales
g13	KJ451713	*Wickerhamomyces anomalus*	99	Saccharomycetales
g17; gc4-gc5	KR085964	*Meyerozyma guilliermondii*	100	Saccharomycetales
gc1	LN833560	*Rhodotorula mucilaginosa*	100-99	Sporidiobolales
gc6-gc7	KP658861	*Trichosporon asahii*	100-99	Tremellales

On one hand, dry sludge in non-inoculated biopiles was stimulated by adding water and pine bark, which offered a better fluid transport and an additionally carbon source, resulting in a fungal and microbial community adapted to the presence of PPCPs and to the cultivation conditions, leading to the assessed PPCPs removal. On the other hand, in biopiles where *T. versicolor* was inoculated, the sludge was not only stimulated by the addition of a bulking material and water, but also with an organism that mineralised the lignocellulosic substrate and certain pollutants of the sludge (Dehorter and Blondeau, 1992; Marco-Urrea *et al.*, 2008; Sack *et al.*, 1997; Tuomela *et al.*, 1998), making them a more accessible source of C and N for other organisms. Furthermore, the addition of the substrate and the fungus changed the aerobic/anaerobic conditions of the sludge, exposing anaerobic organisms to O_2. This would explain the rapid shift of the bacterial community, as obligate anaerobes would have died, have produced spores or were less than 1% of the community population. In the fungal community, the prevalence of *T. versicolor* during the first half of the experiment was due to three main reasons: (i) it was inoculated in high amounts, (ii) the normal fungi strategy to prevent the colonisation by other fungi (Cano and Bago, 2005), and (iii) the different colonisation strategies of each fungus (Mucha *et al.*, 2014). The demise of the fungus could be due to

Table 4.4. Sequence information for the DGGE bands obtained analysing the bacterial community.

Band code	Accession number	Closest related sequence	Similarity (%)	Order
g6	LN774422	*Bacillus humi*	94	Bacillales
*	JQ415989	*Bacillus niacini*	71	Bacillales
g8	KP670289	*Bacillus sp.*	97	Bacillales
gc6	HQ603002	*Sporosarcina sp.*	98	Bacillales
g8	KT261256	*Staphylococcus nepalensis*	99	Bacillales
gc4; *	KR732655	*Staphylococcus stepanovicii*	95-84	Bacillales
*	KC787352	*Staphylococcus warneri*	78	Bacillales
*	KT693286	*Alcaligenes sp.*	93	Burkholderiales
g14; gc4	LC082101	*Brevibacterium siliguriense*	100-83	Caulobacterales
g6	LC068966	*Brevibacterium sp.*	100	Caulobacterales
g14	KP895785	*Brevundimonas vesicularis*	97	Caulobacterales
gc1	AB971795	*Clostridium celatum*	98	Clostridiales
g1-g2; gc1	KF528156	*Clostridium cellulovorans*	99-94	Clostridiales
g1	KJ722507	*Clostridium glycolicum*	98	Clostridiales
g3	EU089965	*Clostridium maritimum*	97	Clostridiales
g1; gc1	KJ722512	*Clostridium ruminantium*	99-98	Clostridiales
g1-g2; gc1	AB610575	*Clostridium sp.*	99	Clostridiales
g2	FJ424481	*Clostridium sp.*	100	Clostridiales
g2	NR_027573	*Intestinibacter bartlettii*	97	Clostridiales
g8	KR181931	*Dietzia sp.*	98	Corynebacteriales
g17-g18; gc8-gc9	HG008896	*Salinimicrobium sp.*	95	Flavobacteriales
g5; g12-g15; g17; gc9	NR_117231	*Pedobacter bauzanensis*	97-96	Sphingobacteriales
g4-g5; g7-g9; g11; g15; g17; gc6; gc9	DQ490982	*Lysobacter sp.*	100-77	Xanthomonadales

* Bands not marked in the DGGE profiles due to the low quality of the sequence

the interaction with the autochthonous organisms of the system (Ejechi and Obuekwe, 1994).

4.2.3.b. *Phylogenetic assessment and characteristics of the microbial communities*

The closest phylogenetic affiliations from fungal and bacterial DGGE bands have been summarized in tables 4.3 and 4.4, respectively.

(i) Fungal community

As can be seen in figure 4.5a, the predominant band in fungal DGGE belonged to *T. versicolor* in inoculated cultures during the first 23 days. In contrast, non-inoculated biopiles did not show any predominant band, and different fungi were observed in every time-point; being *Trichosporon asahii* and *Meyerozyma sp.* the only fungi observed twice in these cultures (times 22d and 42d for both). In fact, all fungi detected in control cultures at initial time (0d) existed also in inoculated cultures, but they were not found during the microbial characterisation as *T. versicolor* was in a far greater concentration (>99% of the community population); being almost the only one amplified during the DGGE. Moreover, and given the source of the sewage sludge, the biopile's fungi came from the ligninolytic substrate. In particular, 5 out of the 11 detected fungi are known to decompose or take advantage of lignocellulosic materials: *Acremonium sp.* (species from Hypocreales order are saprophyte organisms that decompose

plants), *Pseudallescheria ellipsoidea* (Microascales are saprophytic fungi that lives in soils rotting vegetation), *Peniophora cinerea* (Russulales order are plant parasites, saprophytes or symbiotic organisms), *Rhodotorula mucilaginosa* (which can grow in water, soil and air), and *Coriolopsis gallica* (from the same order of *T. versicolor*, this fungus also decomposes decaying wood), which was the only one found at time 42d (Maddison *et al.*, 2007; Malloch, 2013; Nordberg *et al.*, 2014; Robert *et al.*, 2013).

Furthermore, 8 of the identified fungi have been reported to be able to degrade and/or to live in habitats with EPs (Ahalya *et al.*, 2003; Ertuğrul *et al.*, 2009; Huang and Huang, 1996; Lakshmi and Das, 2013; Lakshmi *et al.*, 2013; Matheus *et al.*, 2000; Moreira Neto *et al.*, 2011; Pointing, 2001; Viegas *et al.*, 2014), but only 3 of them have endured until the end of the experiment: *C. gallica*, which has been studied in the degradation of organic pollutants in liquid media (Bressler *et al.*, 2000; Yagüe *et al.*, 2000), and *Meyerozyma spp and Wickerhamomyces anomalus*, two Saccharomycetales that have been reported to degrade oil products (Goulart *et al.*, 2014).

Additionally, the 4 fungi that had mycostatic abilities persisted until the end of the experiment: *Trichosporon asahii* (from the Tremellales order) is a yeast that parasites other fungi (El-Tarabily, 2004), and *Wickerhamomyces anomalus*, *Meyerozyma sp.* and *Meyerozyma guilliermondii*, which can

produce mycocin killer toxins (Coda *et al.*, 2013; Druvefors *et al.*, 2005; Parafati *et al.*, 2015; Walker, 2010).

(ii) Bacterial community

At the beginning of the experiment (0d), both cultures mainly consisted of organisms from the Clostridiales order (*Clostridium spp* and *Intestinibacter bartlettii*); which are obligate anaerobes, gram-positive and have heat and desiccation resistant endospores (Gupta, 2015). *Clostridium spp* has been frequently used as faecal pollution indicator in wastewater and sewage sludge (Marín *et al.*, 2015; Nikaeen *et al.*, 2015), but it has also been studied for the production of hydrogen in anaerobic environments polluted with organic contaminants (Ho *et al.*, 2010; Liu *et al.*, 2015; Zacharof *et al.*, 2015). The demise of those organisms was due to the inoculation of *T. versicolor* in inoculated biopiles, and the addition of the ligninolytic substrate in control cultures. The two perturbations, which are essentially the same, caused a similar effect in both systems: the input of O_2. While facultative anaerobes were able to survive, obligate anaerobes such as *Clostridium spp* and *Intestinibacter bartlettii* were not able and died (or less than 1% of the population).

As can be seen in figure 4.5b, both inoculated and non-inoculated biopiles showed a similar bacterial community along all the time, being *Lysobacter sp.* the predominant band in bacterial DGGE. This bacterium is from the Xanthomonadales

order, which are characterised for being phytopathogens, gram-negative and catalase-positive, non-sporulating, and having a rod shape (Gupta, 2015). *Lysobacter spp* have been found during the treatment of synthetic effluents with PPCPs in experimental sequencing batch bioreactor (SBR) inoculated with activated sludge from a WWTP in Portugal (Amorim *et al.*, 2014); and in a anoxic/oxic-MBR at lab scale inoculated with activated sludge from a WWTP in China (Gao *et al.*, 2014). Additionally, *Lysobacter spp* have been identified in the bioremediation of soils polluted with pesticides (Betancur-Corredor *et al.*, 2015), and in the biodegradation of surfactants in river sediments (Z. Wang *et al.*, 2014).

Next, eleven of the detected bacteria were from the Bacillales order; similar to the Clostridiales order as both are encompassed in the Firmicutes phylum (Gupta, 2015). These bacteria were only detected during the firsts days and until time 22d, after which no more species of this order were identified. The species from the Bacillales order have been found as one of the dominant orders in mixed liquor suspended solids (MLSS) of a lab scale SBR inoculated with anaerobic sludge (Jena *et al.*, 2015), and they have also been detected in compost piles made of anaerobic sludge and wheat residues as bulking material (Li *et al.*, 2014). Further, it has been reported that not only some Bacillales in compost piles degraded cellulose, solubilized lignin and colonized the

lignocellulosic bulking material, but also they could have increased the degradation of organic pollutants (Li *et al.*, 2013; Martins *et al.*, 2013).

Further, four organisms of single bacterial orders were detected at intermediate and final stages: *Alcaligenes sp.*, an aerobic gram-negative from the Burkholderiales order; *Dietzia sp.*, a gram-positive bacteria of the Corynebacteriales order; *Salinimicrobium sp.*, a gram-negative Flavobacteriales; and *Pedobacter bauzanensis*, a gram-negative from the Sphingobacteriales order (Madigan and Martinko, 2005). All these orders and species have been found in WWTP (Wagner and Loy, 2002), and they have also been reported to help in the degradation of organic pollutants in wastewaters, sludges or soils (Jin *et al.*, 2012; Mao *et al.*, 2012; Maqbool *et al.*, 2012; Pérez *et al.*, 2015; Satheesh Babu *et al.*, 2015; Sun *et al.*, 2015).

At the end of the experiment (42d) and in addition to *Lysobacter sp.*, five more organisms were detected in both inoculated and non-inoculated cultures: *Alcaligenes sp.*, an aerobic gram-negative from the Burkholderiales order; *Salinimicrobium sp.*, an aerobic gram-negative from the Flavobacteriales order; *Pedobacter bauzanensis*, an aerobic soil gram-negative organism from the Sphingobacteriales order (Madigan and Martinko, 2005); and *Brevibacterium siliguriense* and *Brevibacterium vesicularis*, aerobic soil gram-negative non-sporulating microorganisms from the Caulobacterales order

(Bhatawadekar and Sharma, 2011; Collins, 2006; Dass *et al.*, 2002), which are also the only two not detected in non-inoculated cultures at 42d. In general, all these species have been found in WWTP (Beale *et al.*, 2015; Friha *et al.*, 2014; Li *et al.*, 2011; Wagner and Loy, 2002) and reported to degrade organic pollutants in wastewaters, sludges or soils (Jin *et al.*, 2012; Mao *et al.*, 2012; Maqbool *et al.*, 2012; Pérez *et al.*, 2015; Satheesh Babu *et al.*, 2015; Sun *et al.*, 2015), except for the two Caulobacterales, which contribution in the removal of environmental pollutants has not been described yet.

(iii) Final remarks

First, it can be said that the initial fungal communities of the biopiles were drastically disturbed by the inoculation event, even though *T. versicolor* was not the predominant organism at the end of the experiment. Next, the re-inoculation of the fungus led to lower levels of drugs. In fact, the reintroduced *T. versicolor* had higher degradative capabilities than the initial one, but the re-inoculated was not able to colonize the biopile. So, *T. versicolor* was no further identified in the biopiles after time 23d. Finally, inoculated cultures shifted from one predominant specimen to three prevalent species: one lignocellulosic decomposer (*C. gallica*) and two fungi with mycostatic abilities (*W. anomalus* and *M. guilliermondii*). All three had been reported to degrade organic pollutants in solid systems. Moreover, the degradation of the substrate by *C.*

gallica could have resulted in a C and N source for the other organisms of the biopile (Lang *et al.*, 2000). Furthermore, the use of fungi in bioaugmentation processes has been described to improve the removal capacities of the autochthonous fungal and bacterial populations (Covino *et al.*, 2015), which normally shift into communities adapted to degrade the main pollutants of the biopile (Grace Liu *et al.*, 2011).

Second, non-inoculated biopiles evolved from a diverse community into a community of two predominant species (*T. asahii* and *Meyerozyma sp.*), both with fungal inhibitory systems and pollutant removal abilities, which would explain the degradation pattern of those biopiles: low PhACs removal at the beginning of the experiment and high at the end. It is known that overall pollutants removal of contaminated soils is improved when applying lignocellulosic waste as amendment, since it stimulates the indigenous populations (Federici *et al.*, 2012), explaining why non-inoculated biopiles achieved removals similar to inoculated biopiles.

Third, the inoculation of *T. versicolor* did not affect the bacterial community. In contrast to the fungal community, the initial bacterial community was shocked by the addition of the lignocellulosic substrate (with or without pre-grown fungi), as the anaerobic conditions were modified and changed to aerobic conditions. Although the bacterial population of non-inoculated biopiles was able to degrade PPCPs, the fungal

inoculated biopiles achieved higher removal rates with a more rapidly decrease. As seen in figure 4.3, inoculated biopiles showed a fast removal tendency, due to the fact that *T. versicolor* was already the predominant organism, and it did not require additional time to grow and colonize the biopile before degrading the organic compounds. As a consequence, the use of *T. versicolor* to remove PPCPs from thermal dried WWTP sludge in biopiles systems has been proved as more effective than treating the same sludge by stimulating the autochthonous organisms with lignocellulosic substrate and water.

4.3. Conclusions

It has been proved that *T. versicolor* was able to degrade drugs from thermal dried sewage sludge in biopiles systems under non-sterile conditions with pine barks as lignocellulosic substrate. This lignocellulosic material offered the necessary nutrients for *T. versicolor* and acted as a bulking material in the biopiles systems. Moreover, it has been proved that the fungus led to a higher PPCPs removal: the percentage in fungal inoculated biopiles was 66.45%, while for non-inoculated biopiles was 49.18%.

Although the laccase activity was low, further microbial analysis demonstrated that the fungus was still present, at least, until day 23. The bacterial and fungal community analysis

demonstrated how the populations evolved. In both cases, the introduction of lignocellulosic substrate – inoculated or non-inoculated with *T. versicolor* – drastically changed the initial communities of the sludge, because not only *T. versicolor* directed the evolution of the fungal community during the first days of the experiment, but also the addition of the substrate implied the alteration of the anaerobic conditions by adding O_2. Moreover, fungal inoculated and non-inoculated biopiles reached a similar final microbial structure and diversity, which were described to be able to degrade organic pollutants in different matrices.

Finally, the re-inoculation of the biopiles after 22 days of treatment has led to an enhanced PPCPs removal, as the removal rates were improved globally and for each single compound. In fact, the re-inoculated fungus decreased the global drugs' concentration, but it was unable to colonise the biopiles. In particular, the initial fungus – the one pre-growth on lignocellulosic substrate – was adapted to growth in this matrix, but the re-inoculated fungus was not acclimated, since it came from a liquid media. So, further research should be carried out in order to: (i) evaluate the degradation rate of each compound, and (ii) determine if the re-inoculation of the biopiles should be performed at an early stage while the fungus is not only still active, but also the predominant organism.

CHAPTER 5

Removal of pharmaceuticals in bioslurry systems

Summary

EPs can reach the environment through the effluent of WWTPs. The growth of *Trametes versicolor* on MBR sludge in bioslurry systems at both Erlenmeyer and reactor scales were assessed, and its capacity to remove PhACs was evaluated. First, the ability of the fungus to remove HZT from liquid medium cultures was assessed, testing different bioslurry media (i.e. complete nutrient, glucose and no-nutrient addition) and conditions (sterile and non-sterile). Second, the highest spiked HZT removal at Erlenmeyer scale was assessed under non-sterile conditions without nutrient addition (93.2%). Third, the removal of a broad set of pharmaceuticals was assessed in non-spiked Erlenmeyer bioslurries under non-sterile conditions. *T. versicolor* was able to completely degrade 12 out of the 28 drugs initially detected in the MBR sludge, reaching an overall degradation of 66.9%. Subsequent microbial analysis showed that the microbial diversity increased after 15 days of treatment, but there was still some *T. versicolor* in the bioslurry bottles. Finally, the bioslurry treatment scale was improved to reactor scale and coupled to an AD. The removal yields after the fungal bioslurry reactor decreased (40%) if compared with the Erlenmeyer scale bioslurries, but the anaerobic digestion of the biomass increased the overall final removal yield (78%); however, the methane yield was low. Results showed that *T. versicolor* can remove drugs in bioslurry systems under non-sterile conditions, without extra nutrients in the medium, and in matrices as complex as an MBR sludge.

5.1. Introduction

A MBR system combines a suspended biomass reactor with a filtration process, avoiding the need for a settler. Two configurations are possible for an MBR unit: (i) an internal/submerged configuration where the filtration membranes are submerged into the reactor; and (ii) an external/side-stream configuration where the membranes are out of the reactor and an additional pump system is required. MBR units became an interesting way to improve the existing WWTPs, because of the technological improvements and the cost reduction. High cellular retention times (CRT) and high biomass concentrations are typical operational parameters for these units, promoting and enhancing the biodegradation of organic contaminants (Sipma *et al.*, 2010).

Using MBRs to treat wastewater has become common in many countries since the outlet stream can be recycled to recharge groundwater basins and for indirect potable reuses (Melin *et al.*, 2006). However, it is necessary to ensure that this process would effectively remove not only nutrients and dissolved carbon, but also organic pollutants. Boonyaroj *et al.* (2012) demonstrate that MBR were able to eliminate phenolic compounds and phthalic acid esters (PAEs) from landfill leachate (removal rates from 77% to 96%), concluding that biodegradation and adsorption were the main mechanisms involved in the

elimination process. Similarly, Komesli *et al.* (2015) examined the occurrence and removal of 7 EDCs in 5 WWTP from Turkey, one of which was equipped with an MBR. These researchers observed that only 3 compounds were not totally removed (i.e. CBZ, diltiazem and acetaminophen) from the water stream in the plant equipped with the MBR unit. While CBZ and diltiazem by-passed all the process without any change in their concentrations – since the two drugs were not detected in the sludge –, acetaminophen was adsorbed into the MBR sludge. Meanwhile, Trinh *et al.* (2015) studied the removal of trace organic chemical compounds (i.e. hormones, estrogens, pesticides and PPCPs) when hazardous events (i.e. salinity, ammonia and organic carbon shocks, feed starvation, loss of power supply and physical membrane damage, among others) affected MBR units. These researchers found that removal rates of hydrophilic chemicals were affected, especially under shock load conditions; however, the overall removal of hydrophobic compounds was not or only slightly affected, indicating that these chemicals were highly adsorbed into the MBR biomass. Indeed, MBR sludge could contain emerging contaminants even when the operational conditions were affected.

The excessive sludge production and its disposal are a rising challenge for WWTPs due to economical and environmental factors. MBR units has high sludge yields – although the concentration of suspended solids is lower than 1mg·L^{-1} – and the treatment and disposal costs are similar to traditional

activated sludge systems (Hai *et al.*, 2014). Some years ago, WWTP sludge was only treated in order to remove heavy metals and, in some cases, eliminate some priority pollutants (Eriksson *et al.*, 2008). However, the current scenario of better analytical methods, increasing population, reutilization of water and extensive agriculture systems, implies that new treatments must be used in order to face the current problems. Bioremediation could help to mitigate the problems derived from emerging pollutants. Additionally, in the case of mycoremediation, the fungal biomass from the treatment could be valorised to improve energy production in those WWTP where sludge is anaerobically digested.

5.1.1. The WWTP of Terrassa

The selected WWTP is located in Terrassa (Catalonia), and it was designed to treat 75,000 $m^3 \cdot d^{-1}$. The flow chart for this WWTP is as follows (figure 5.1): the wastewater flow enters the plant via a roughing filter followed by a sand-trap, and then its sent to the primary settler – the sludge produced in this treatment is centrifuged, anaerobically digested and dehydrated; the resulting effluent is divided into two streams, one is sent to the MBR, and the other to an AS reactor; while the stream driven to the MBR is discharged directly into the river, the second stream is sent to a secondary settler after the AS and before its final discharge into the river.

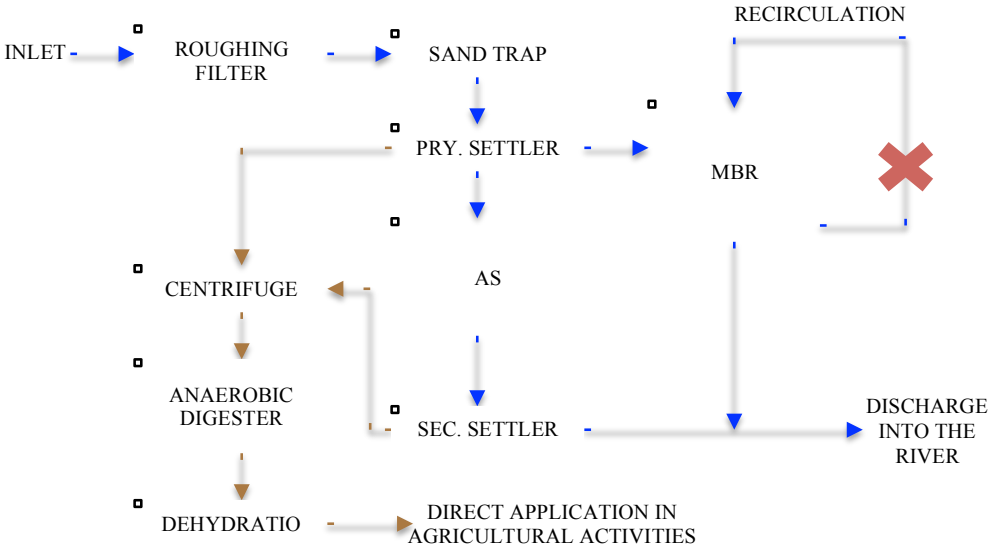


Figure 5.1. Flow-chart for Terrassa's WWTP. Brown arrows represent solid streams and blue arrows represent liquid streams. The red X shows the sludge collection point.

5.1.2. Sludge digestion

The nowadays society is gaining interest in renewable sources of energy due to the depletion of traditional sources, which are based in fossil fuels, and the increased awareness on global warming (Hoel and Kverndokk, 1996; Höök and Tang, 2013; Kempton, 1993). The production of biogas from anaerobic digestion (AD) can be considered as a renewable source of energy that can generate both electricity and heat. Additionally, the AD is a widely practiced way to manage and valorise WWTP sludge since mid 1900s. The AD of sludge, which can be carried

out in one or two tanks/digesters, consists of two stages: (i) sludge from primary and secondary treatments are combined with already digested sludge, which acts as inoculum, and heated; (ii) and next, the mixture is allowed to digest without neither mixing nor additional external source of heat, as the process generates enough heat to maintain the process active. About 40% to 60% of the organic solids from the sludge will be converted into biogas, which is composed of CH_4 (60-65 %), CO_2 (30-35 %) and minor and trace elements such as H_2, N_2, H_2S and H_2O. The remaining organic matter of the process will be chemically stable and have fewer levels of pathogens than the initial sludge (Nazaroff and Alvarez-Cohen, 2001; Parkin and Owen, 1986; Ramalho, 1996).

It has been suggested that AD could be used in order to remove PPCPs from WWTP sludge. Carballa *et al.* (2007b) worked with two lab-scale digesters, one in the mesophile range and the other one in the thermophile range of temperatures, in order to eliminate 13 spiked drugs and assess if the presence of these compounds would affect the normal operation of the digesters. These authors found that not only the AD was not affected by the presence of the 13 drugs, but also the drugs were removed in both temperature ranges; however, the removal range was wide: from less than 20% removal for iopromide to more than 85% removal for naproxen, SMX, roxithromycin and oestrogens. In contrast, it was found that

some compounds such as EDCs were not eliminated during AD of WWTP sludge at pilot plant scale, and they were highly adsorbed onto the biomass (Muller *et al.*, 2010). In order to improve PPCPs removals during AD, some authors have suggested treating the sludge before entering the digester. One of these possible pre-treatments is ozonation, which was evaluated by Carballa *et al.* (2007a). The researchers found that this advanced pre-treatment lead to improved COD solubilisation and better biogas production; however, the elimination of PPCPs was not influenced by the ozonation, and the removal rates remained constant.

Treating WWTP sludge with *T. versicolor* before AD would solve these problems. The fungal biomass after the bioremediation of WWTP sludge could be used as co-substrate in AD. Co-digestion is performed when two or more (co-) substrates are homogeneously mixed and simultaneously digested, improving biogas yields due to substrates synergisms in the digestion medium. Some of the most remarkable advantages of co-digestion are: improved nutrient balance and digestion, optimization of sludge qualities, and more effective use of digesters volumes (Braun *et al.*, 2002; Desai *et al.*, 1994; Mata-Alvarez *et al.*, 2000). The use of *T. versicolor* biomass – or from any other organism used to bioremediate effluents with EPs – as co-substrate for AD has not been deeply documented (Passos *et al.*, 2016); however, the biomass digestion of other white-rot fungi (WRF) has been previously reported, as the

treatment of lignocellulosic residues with WRF, in order to improve its biodegrability on industrial AD, is more economically and environmental friendly than other physical and chemical treatments. For instance, Lalak *et al.* studied how the pre-treatment of agricultural residues (tall wheatgrass) at different moisture ratios with WRF (*Flammulina velutipes*) affected the subsequent AD. The authors found that treating lignocellulosic biomass before digestion processes lead to improved biogas production: 120% more biogas production and 134% more methane yield compared to untreated biomass, due to the improved biodegradability of the residue given by lignin and hemicellulose content reduction (Lalak *et al.*, 2015). Similarly, Zhao *et al.* studied the use of the WRF *Ceriporiopsis subvermispora* to treat lignocellulosic residues before AD-processes. The researchers proved that this fungus could degrade lignin and hemicellulose from yard trimmings, improving the AD of these residues: 154% increased in methane yield (Zhao *et al.*, 2014). Other biomasses have been also tested; Gonzalez-Fernandez *et al.* studied the AD of microalgae biomass as feedstock and concluded that not only was feasible, and the production of algae biomass and AD could be integrated, but also it was a promising technology for sustainable energy production (Gonzalez-Fernandez *et al.*, 2015).

5.1.3. Aim of the chapter

The aim was to evaluate the capacity of *T. versicolor* to remove PPCPs from selected MBR sludge, which was highly diluted and not previously treated. Initially, the ability of the fungus to degrade HZT in liquid cultures was assessed. Then, its degradation ability in different medium cultures was assessed in MBR bioslurry at Erlenmeyer scale with spiked HZT. The best operational conditions were chosen according to both fungal sludge colonization and HZT removal. Next, the best culture conditions were applied to treat non-spiked sludge under sterile and non-sterile conditions at Erlenmeyer scale, in order to compare the role of the fungus and the autochthonous microorganisms of the MBR sludge in the degradation of PPCPs. Finally, the valorisation of the fungal biomass, after the treatment of non-spiked MBR sludge under sterile and non-sterile conditions at reactor scale, to improve the formation of biogas in an anaerobic digester was evaluated.

5.2. Results and Discussion

5.2.1. Growth and removal capacities of *T. versicolor* in liquid cultures

HZT degradation experiments in spiked liquid medium cultures were carried out at optimal *T. versicolor* growth conditions in order to assess if the fungus was able to degrade this compound. The measured laccase activity for experimental, abiotic and killed cultures, and glucose consumption for experi–

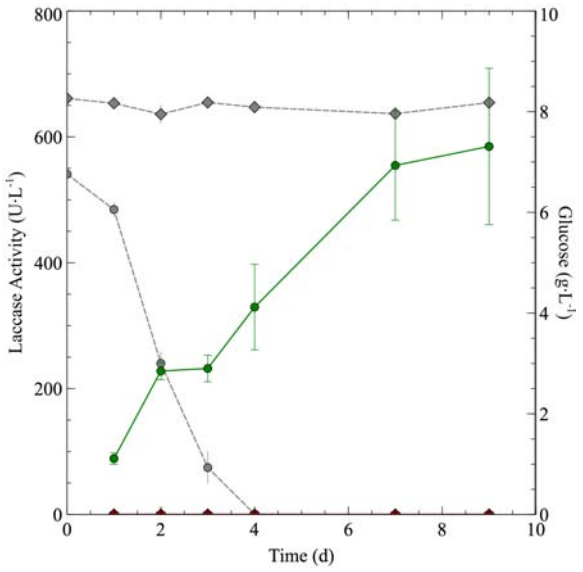


Figure 5.2. Laccase activity and glucose consumption of liquid medium cultures. Laccase activity: experimental cultures, abiotic controls and killed controls (graphs of both controls are overlapped because the activity is 0). Glucose consumption: experimental cultures and abiotic controls. Error bars represent standard error of triplicates. In this experiment killed controls were made with sodium azide.

–mental and abiotic cultures are shown in figure 5.2. Glucose analyses for killed controls are not shown since unexpected results with a bouncing trend were not in accordance with previous experiments; according to YSI 2700 owner's manual, the behaviour of killed control samples was caused by sodium azide interferences. In general, glucose concentration remained constant for abiotic controls ($8 g \cdot L^{-1}$) and fell rapidly for experimental cultures, reaching a minimum by day 4. In parallel,

experimental cultures showed an increasing laccase activity, reaching the highest activity the day 9 (ca. 600 $U \cdot L^{-1}$), and no laccase activity was detected for both abiotic and killed controls. This confirmed that *T. versicolor* had oxidative capacity under the selected conditions. Similar results have been reported for the same medium and fungus with other compounds. For instance, Jelic *et al.* (2012) obtained a comparable laccase activity in the degradation of CBZ, and Cruz-Morató *et al.* (2013b) obtained lower laccase production in the degradation of clofibric acid. However, previous reports had demonstrated that pollutants elimination can be achieved without laccase, suggesting that intracellular enzymes such as cytochrome P450 oxygenases would lead the degradation process (Marco-Urrea *et al.*, 2009c; Tran *et al.*, 2010).

After assessing the capacity of the fungus to grow in liquid media cultures, its capacity to remove HZT as target compound was assessed. HZT degradation results are presented in figure 5.3. It can be seen that experimental cultures had an increasing removal behaviour from day 0 to 7; with a levelled stage between days 2 and 4 followed by a rise until day 7. It can be considered that for killed and abiotic controls the removal rates were constant for the whole experiment, with removal percentages values below 10%. Nearly 45 % of measured HZT is eliminated from medium when experimental and abiotic cultures are equated. It can be considered that 10 % of the compound has been adsorbed onto fungal biomass, and 35% has been

degraded, as minimum, when killed and abiotic controls are compared. Although it has been previously reported that *T. versicolor* was able to degrade emerging pollutants from liquid media, e.g. Marco-Urrea *et al.* (2009c) was able to remove ibuprofen, clofibric acid and CBZ in liquid medium, and Badia-Fabregat *et al.* (2012) demonstrated that *T. versicolor* was able to remove 3-(40-methylbenzylidene) camphor; it is the first time that HZT removal by *T. versicolor* in liquid cultures is reported.

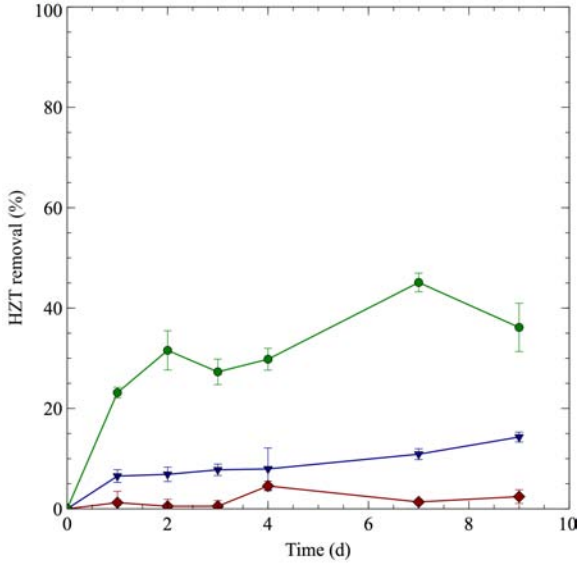


Figure 5.3. HZT degradation trends in liquid medium cultures: experimental cultures , abiotic controls and killed controls . Error bars represent standard error of triplicates.

5.2.2. Growth and removal capacities of *T. versicolor* in spiked bioslurry

Further experiments were designed to test both the ability of *T. versicolor* to grow on liquid MBR sludge, and its degradation capacity using HZT as target compound. Different culture media were used: (i) one with all the nutrients listed for liquid medium cultures (tables 3.3a and 3.3b), (ii) another with only glucose ($8 g \cdot L^{-1}$), and (iii) one without any additional nutrient; calling them from now as complete medium, glucose medium and no-nutrient medium, respectively. Also, sterile and non-sterile conditions were tested.

At first, fungus inactivation was performed with sodium azide instead of heat, because heat not only deactivates enzymes but also breaks the fungal membrane, increasing the pollutant adsorption. However, sodium azide did not completely inactivate cultures, and some HZT degradation was observed in the firsts killed controls (data not shown). Sodium azide inhibits the oxygen uptake of the fungus, but some enzymes can remain active, as reported for diclofenac degradation by Badia-Fabregat *et al.* (2014). Thus, sodium azide was substituted by heat in the fungus' deactivation in killed control cultures.

5.2.2.a. *Effect of the medium composition*

The effect of medium composition in the degradation of spiked HZT in sterile bioslurry systems was the first aspect tested. As it can be seen in figure 5.4, both complete and glucose medium experimental cultures consumed all the glucose

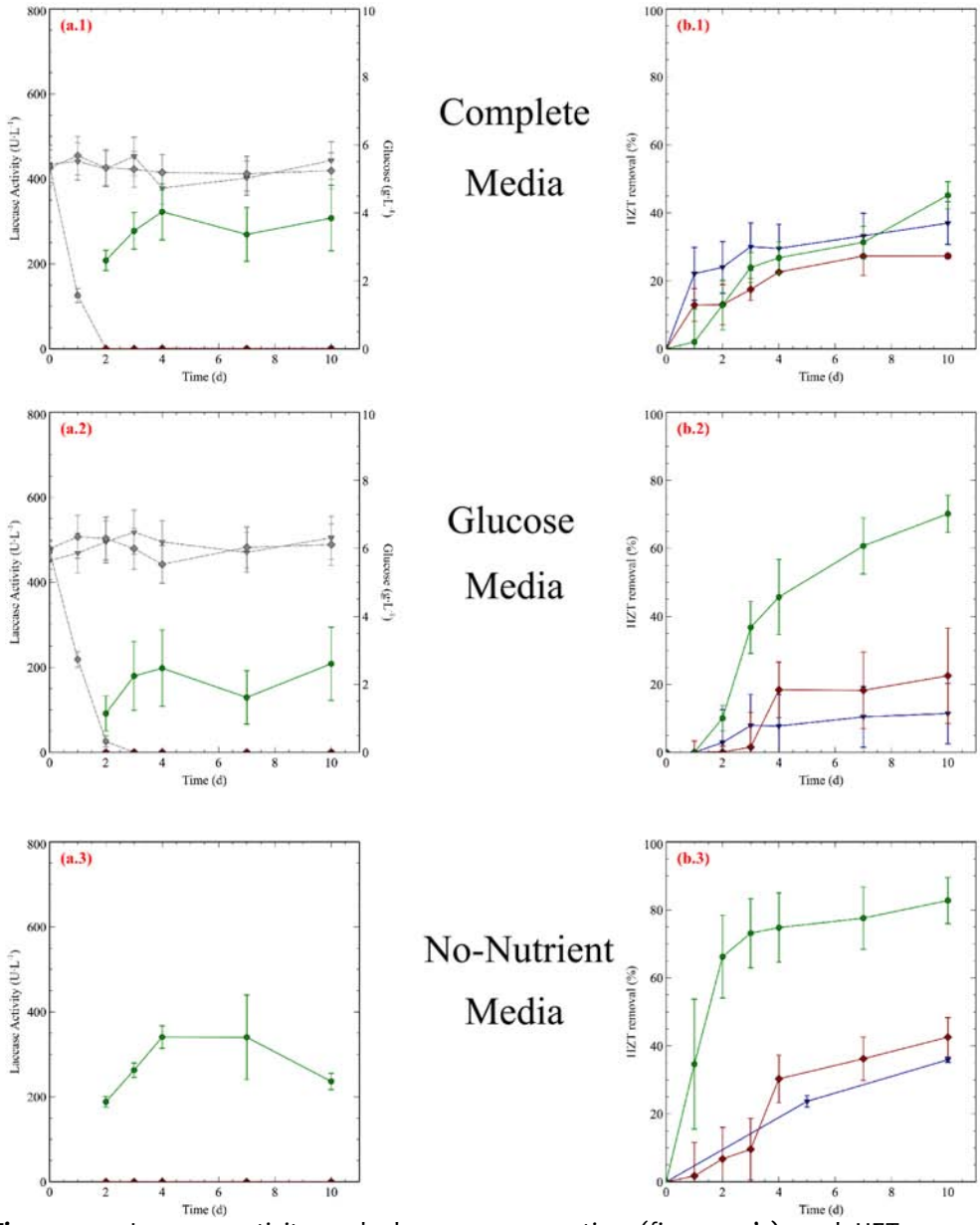


Figure 5.4. Laccase activity and glucose consumption (figures a's), and HZT removal (figures b's) in sterile Erlenmeyer bioslurry cultures with complete (1), glucose (2) and no-nutrient (3) media. Laccase activity and HZT degradation profiles: experimental cultures, abiotic controls and killed controls. Glucose concentration (only for complete and glucose media): experimental cultures and abiotic controls .and killed controls . Error bars represent standard error of triplicates.

by days 2 and 3 respectively, showing similar laccase activity behaviour and reaching a peak by day 4 (complete medium: 322 ± 66 $U \cdot L^{-1}$; glucose medium: 198 ± 90 $U \cdot L^{-1}$). No-nutrient medium also exhibited a gradual growth of laccase production from day 2 until 4 (331 ± 46 $U \cdot L^{-1}$), and then levelled out until day 7. Therefore, *T. versicolor* was able to grow and colonize MBR sludge with all the tested media, but better in the cases of complete and no-nutrient media were similar laccase activities were achieved.

T. versicolor's ability to remove spiked HZT from bioslurry systems with sterile MBR sludge was assessed, and the results showed that the fungus was able to degrade the target compound, as can be seen in figure 5.4. First, some HZT decrease in both killed and abiotic controls was observed for all tested media. On one hand, for complete medium experiments both controls followed a similar trend, reaching a final HZT concentration 37% lower than the initial. Differences between final HZT concentrations in abiotic (6.06$\mu g \cdot mL^{-1}$) and killed (5.25$\mu g \cdot mL^{-1}$) controls, indicates that part of the drug was adsorbed onto the fungal biomass. On the other hand, glucose and no-nutrient media controls showed no statistically differences between killed and abiotic controls, with removals at the end of the experiment ca. 11.4% in glucose medium and ca. 34.7% in no-nutrient medium.

Table 5.5. One-factor ANOVA on the result of HZT removal in fungal bioslurry for three different media under sterile conditions.

Source of variation	d.f.	S.S.	M.S	f ratios
Media culture	2	31.37	15.68	5.77^{s}
Error	18	48.89	2.72	-
Total	20	80.26	-	-

d.f.= degree of freedom; S.S.= sum of squares; M.S.= mean squares; s/ns= significant or not significant at $p < 0.10$

Second, experimental cultures with complete medium showed a final HZT degradation of 13.8%, with a 9.1% of the drug adsorbed onto the fungal biomass. In contrast, experimental cultures with glucose medium showed a higher degradation rate (71.4%); however, adsorption could not be measured due to controls' behaviour. Furthermore, experimental cultures in no-nutrient medium showed a similar HZT degradation (69.1%), with an adsorbed fraction of 4.3%. Even though, degradation in no-nutrient medium was faster, reaching a degradation rate of 66.7% by day 2. ANOVA analyses (table 5.5) showed significant statistical differences between tested groups. Consequently, it can be concluded that the medium affects the degradation of the selected drug. HZT was degraded in all experimental cultures, but the highest rate was obtained in systems without nutrient additions. Moreover, according to table 5.6 it can be assumed that MBR sludge has enough carbon and nitrogen to become the main source of nutrients for *T. versicolor*; however, not all the sewage sludge will be suitable for the fungus.

According to these results, and taking into account further applications of the technology, the no-nutrient medium was selected for subsequent experiments.

Table 5.6. MBR sludge characteristics.

Parameter	Value
pH	5.16
TSS ($g \cdot L^{-1}$)	3.98 ± 0.04
VSS ($g \cdot L^{-1}$)	2.43 ± 0.03
TC ($mg \cdot L^{-1}$)	181.789 ± 4.72
TOC ($mg \cdot L^{-1}$)	74.348 ± 5.20
TAN ($mg \cdot L^{-1}$)	42.9 ± 0.04

The fungus' ability to survive and remove drugs in a bioslurry system was previously studied by Rodríguez-Rodríguez *et al.* (2010a). The authors tested the capability of *T. versicolor* to degrade naproxen and CBZ in bioslurry systems by adding water to dry WWTP sludge, obtaining a wide range of sludge concentrations: from 100 to 600 $g \cdot L^{-1}$. Whereas the present study tested the ability of *T. versicolor* to grow and eliminate drugs in bioslurry systems with naturally wet sludge, avoiding the prior drying treatment and the subsequent water addition in order to perform the bioslurry treatment. Despite the fact that the selected liquid-sludge was poor in solids (4 $g \cdot L^{-1}$), similar results were obtained. These authors found that degradation of naproxen and CBZ mainly occurred in the solid phase of the bioslurry, with adsorptions around 45%. Briefly, the fungus was able to degrade ca. 47% and 57% of naproxen and CBZ,

respectively, in bioslurry of dry sludge with water. In our study adsorptions percentages were lower and removal rates were higher, demonstrating that *T. versicolor* could not only colonize MBR sludge in bioslurry systems, but also it could efficiently degrade the target compound. In contrast to the mentioned work of Rodríguez-Rodríguez *et al.* (2010a), in the present experiments the PhACs were in both the sludge and the water at the same time; however, as drugs' concentration analysis were done with homogeneous samples of liquid/sludge, it was not possible to determine if the degradation occurred in the liquid, in the solid or in both.

5.2.2.b. *Effect of non-sterile conditions*

After assessing how the media affected the degradation of HZT, non-sterile conditions were tested only for no-nutrient cultures. The objective was to determine if *T. versicolor* could degrade spiked HZT in competition with autochthonous sludge microorganisms. First, the measured laccase activity of the fungus under non-sterile conditions was negligible, less than 0.5 $U \cdot L^{-1}$ (data not shown). This reduction could be caused as a result of the competition between *T. versicolor* and the autochthonous sludge microorganisms. Therefore, inhibitory effects should be considered to explain this reduction.

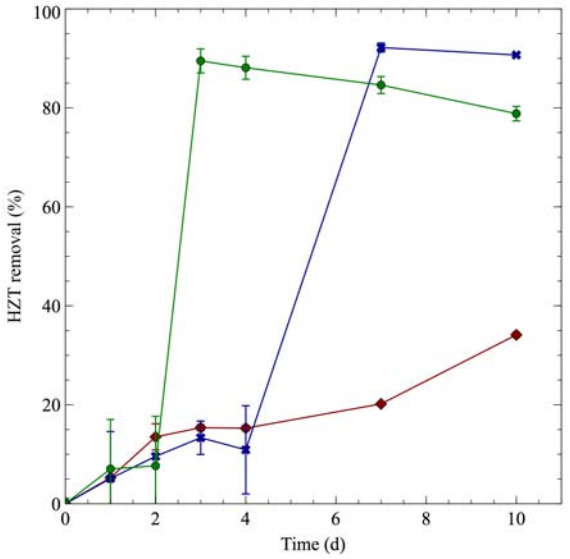


Figure 5.5. HZT removal in non-sterile flask-bioslurry cultures with no-nutrient medium: experimental cultures (non-sterile, inoculated), raw sludge controls (non-sterile, non-inoculated) and abiotic controls (sterile, non-inoculated). Error bars represent standard error of triplicates.

Second, degradation of the selected target compound in experimental culture was higher than under sterile conditions (figure 5.5); however, similar removal percentages for abiotic controls (ca. 34%) were achieved. When HZT degradation yields are compared, ANOVA analyses (table 5.7) did not indicate significant differences between inoculated and non-inoculated cultures with *T. versicolor*. However, HTZ degradation in experimental culture was faster than in raw sludge control, reaching the maximum degradation by days 3 and 7, respectively. In the case of the experimental culture under non-sterile conditions, it means a fungal degradation of 93.2%. Even

though it was possible to eliminate the spiked drug from MBR liquid-sludge with its autochthonous microorganisms, the bioslurry treatment with the fungus improved the elimination reducing the time needed. Although there were not significant differences between inoculated and non-inoculated cultures in terms of overall spiked HZT removal, it was unknown what happens with HZT and other drugs at real concentrations. So, further experiments without any spiked chemical and under non-sterile conditions were carried out, assessing both the total removal of PPCPs and the evolution of the microbial population.

Table 5.7. One-factor ANOVA on the result of HZT removal in fungal bioslurry for sterile and non-sterile conditions with no-nutrient media.

Source of variation	d.f.	S.S.	M.S	f ratios
Culture conditions	1	12.32	12.32	3.18^{ns}
Error	12	65.82	5.49	-
Total	13	78.14	-	-

d.f.= degree of freedom; S.S.= sum of squares; M.S.= mean squares; s/ns= significant or not significant at $p < 0.10$

Previously, WRF were already described to be able to growth and degrade PPCPs in bioreactor systems under non-sterile conditions. For instance, Li *et al.* (2015) studied the ability of *Phanerochaete chrysosporium* to degrade two target compounds, i.e. CBZ and naproxen, from non-sterile synthetic wastewater. These authors worked with the fungus immobilized into wood chips at reactor scale (3L of working volume),

obtaining high removal rates when both compounds where eliminated at the same time; however, the researchers needed a nutrient and carbon source similar to the one tested in the complete medium (tables 3.3a and 3.3b.). Similarly, Cruz-Morato *et al.* (2013a) demonstrated that *T. versicolor* was able to completely remove 7 out of 10 PPCPs from real wastewater under non-sterile conditions in a fluidized bed reactor (10L), but a source of minimal nutrients and carbon was supplied. Consequently, it has been described for first time that *T. versicolor* not only was able to growth and remove a selected drug in a bioslurry system made of non-sterile WWTP sludge, but also without any external source of neither nutrients nor carbon – which can vary depending if the TOC from the wastewater is easily assimilable for the fungus or not.

5.2.3. Pharmaceuticals degradation in non-spiked Erlenmeyer fungal bioslurry

Radjenović *et al.* (2009) showed that pharmaceutically active compounds were more easily eliminated in MBR systems than in conventional activated sludge reactors; however, some compounds, such as HZT, by-passed the reactor without further change in the concentration. In order to assess if *T. versicolor* could improve the PhACs removal from an MBR outlet, non-spiked fungal bioslurry experiments under non-sterile conditions were performed at Erlenmeyer scale. The aim was twofold: (i) to assess the efficiency of the fungus to eliminate PPCPs at real concentrations in bioslurry systems, and (ii) to evaluate how the

fungal inoculation would affect the autochthonous microbial population of the sludge. Two experimental groups were developed: one with sterile MBR sludge and another one with non-sterilized sludge, but both groups were made with sludge sampled the same day and inoculated with *T. versicolor*. Also, non-inoculated controls under non-sterile conditions were included.

5.2.3.a. *Total PPCPs removal*

Laccase activity trends were similar to previous experiments (data not shown). Non-sterile cultures showed negligible activity, while sterile cultures showed activities above 200 $U \cdot L^{-1}$. Table 5.8 shows the detected PhACs at initial MBR sludge and their removal yields after 15 days of bioslurry treatment, and table 5.9 presents a summary of PPCPs removal sorted by concentration range. In summary, out of the 50 PhACs analysed, 38 were detected in raw sludge's samples at the beginning of the experiment (11 of them below quantification limits), which represents a total amount of 10,152 ± 574 $ng \cdot L^{-1}$. The highest concentrations in MBR sludge were found for the antibiotics ciprofloxacin (3,727 ± 230 $ng \cdot L^{-1}$) and OFX (2,921 ± 174 $ng \cdot L^{-1}$). More than 66% of initial detected drugs were removed from both inoculated bioslurry, while non-inoculated removed approximately 54% of the initially detected drugs.

Table 5.8. Detected PhACs in MBR sludge and its removal yields after fungal bioslurry treatment.

Pharmaceutical	Initial concentration ± SD ($ng \cdot L^{-1}$)	Removal yields (%) [b]		
		Inoculated (sterile conditions)	*Inoculated (non-sterile conditions)*	*Non-inoculated (non-sterile conditions)*
Analgesics and anti-inflammatory drugs				
KTP	168.2 ± 9.0	39.2 ± 3.1	78.7 ± 3.1	[a]
Phenazone	88.0 ± 4.2	45.0 ± 1.1	[a]	[a]
Acetaminophen	77.4 ± 1.0	67.6 ± 7.5	67.6 ± 7.5	67.6 ± 7.5
Codeine	31.9 ± 1.3	39.6 ± 0.1	100.0 ± 0.1	91.2 ± 0.1
Propyphenazone	6.5 ± 0.3	72.9 ± 0.5	72.9 ± 0.5	72.9 ± 0.5
Piroxicam	5.1 ± 0.6	100.0 ± 0.3	100.0 ± 0.3	100.0 ± 0.3
Antihypertensive				
Valsartan	135.2 ± 1.9	36.7 ± 4.7	88.3 ± 4.7	88.3 ± 4.7
Anthelmintic				
Levamisol	10.7 ± 0.5	100.0 ± 0.5	4.1 ± 0.5	30.7 ± 0.5
Anti-H2				
Ranitidine	31.9 ± 3.9	100.0 ± 0.2	100.0 ± 0.2	92.4 ± 0.2
Calcium Channel Blockers				
Diltiazem	43.8 ± 0.7	100.0 ± 0.1	100.0 ± 0.1	100.0 ± 0.1
Norverapamil	18.7 ± 0.3	0.0 ± 1.6	71.3 ± 1.6	9.2 ± 1.6
Verapamil	16.4 ± 0.3	100.0 ± 0.5	100.0 ± 0.5	90.7 ± 0.5
Antibiotics				
Ciprofloxacin	3726.8 ± 229.9	89.2 ± 1.3	61.0 ± 1.3	46.5 ± 1.3
OFX	2921.4 ± 173.6	80.5 ± 0.8	64.5 ± 0.8	41.5 ± 0.8
Azithromycin	594.7 ± 43.0	98.6 ± 0.6	96.7 ± 0.6	92.8 ± 0.6
SMX	158.2 ± 2.7	95.0 ± 0.6	95.7 ± 0.6	92.7 ± 0.6
Clarithromycin	75.9 ± 1.6	95.9 ± 0.3	100.0 ± 0.3	42.4 ± 0.3
TRM	47.2 ± 8.5	91.1 ± 0.4	100.0 ± 0.4	78.7 ± 0.4

Pharmaceutical	Initial concentration ± SD (ng·L^{-1})	Removal yields (%) [b]		
		Inoculated (sterile conditions)	*Inoculated (non-sterile conditions)*	*Non-inoculated (non-sterile conditions)*
Antiplatelet drug				
Clopidrogel	11.1 ± 0.3	100.0 ± 0.1	100.0 ± 0.1	100.0 ± 0.1
Contrast medium				
Iopromide	490.5 ± 43.2	47.9 ± 5.5	100.0 ± 5.5	100.0 ± 5.5
Diuretics				
HZT	407.5 ± 9.3	99.1 ± 1.1	38.1 ± 1.1	[a]
Furosemide	356.0 ± 10.0	78.7 ± 7.5	24.4 ± 7.5	71.7 ± 7.5
Psychiatric drugs				
Citalopram	295.4 ± 13.3	91.8 ± 0.6	47.9 ± 0.6	42.1± 0.6
Venlafaxine	233.1 ± 7.7	66.8 ± 0.3	4.8 ± 0.3	[a]
Lorazepam	84.8 ± 2.0	100.0 ± 1.9	44.0 ± 1.9	38.4 ± 1.9
CBZ	66.9 ± 2.1	49.0 ± 0.4	[a]	[a]
Trazodone	34.1 ± 1.3	100.0 ± 0.4	100.0 ± 0.4	100.0 ± 0.4
Olanzapine	14.3 ± 1.6	100.0 ± 0.2	100.0 ± 0.2	79.6 ± 0.2
Total:	10,151.5 ± 574.1			

[a] Removal not assessed, final concentration was higher than the initial
[b] Removal errors expressed as the detection limit of each compound divided by 2

Generally, partial or total drug removal was observed, but 5 PhACs showed negative elimination rates: KTP, phenazone, CBZ, HZT, and venlafaxine. As described in chapter 4, this can be explained by the occurrence of conjugates in the bioslurry. Kovalova *et al.* (2012) also observed the occurrence of conjugation/deconjugation processes in the removal of PhACs in MBR systems. Some conjugates are formed by human metabolism to increase the solubility and excretion of the drug, which can be discomposed after a biological treatment back into

their original compound, leading to a higher concentration of the drugs in the effluent than in the influent. Otherwise, fungi can also conjugate PhACs during biodegradation treatments (Badia-Fabregat *et al.*, 2012). Since the evaluation of PPCPs' impact in water is still in their early stages, few data of their behaviour in sludge can be found. More attention has been paid to ECDs, due to male fish feminization and the possible alteration of human tissues' development (Auriol *et al.*, 2006), among others. Xu *et al.* (2014) found conjugated estrogens in the influents of some sewage treatment plants that could be deconjugated by intestinal bacteria in the sewer lines. These authors also obtained negatives removals that were attributed to sludge desorption, deconjugation of conjugates, and biotransformation between compounds. Therefore, negative PhACs' removal in fungal bioslurry systems could be related to one of the above mechanisms, but more research is needed in this field.

Table 5.9. Summary of pharmaceutical removal yield sorted by initial concentration range at non-spiked MBR fungal bioslurry treatments. Compounds that their concentration increased after treatment are not included.

Concentration Range ($ng \cdot L^{-1}$)	**Initial Concentration ± SD ($ng \cdot L^{-1}$)**	**Removal Yields (%)**		
		Inoculated (Sterile)	*Inoculated (Non-Sterile)*	*Non-inoculated (Non-Sterile)*
C ≥ 200	8384.6 ± 1517.5	84.1	65.0	52.1
200 > C ≥ 50	531.4 ± 37.9	77.1	82.1	72.1
50 > C	271.8 ± 14.5	83.8	93.6	83.1
Total	9187.8 ± 944.6	83.6	66.9	54.2

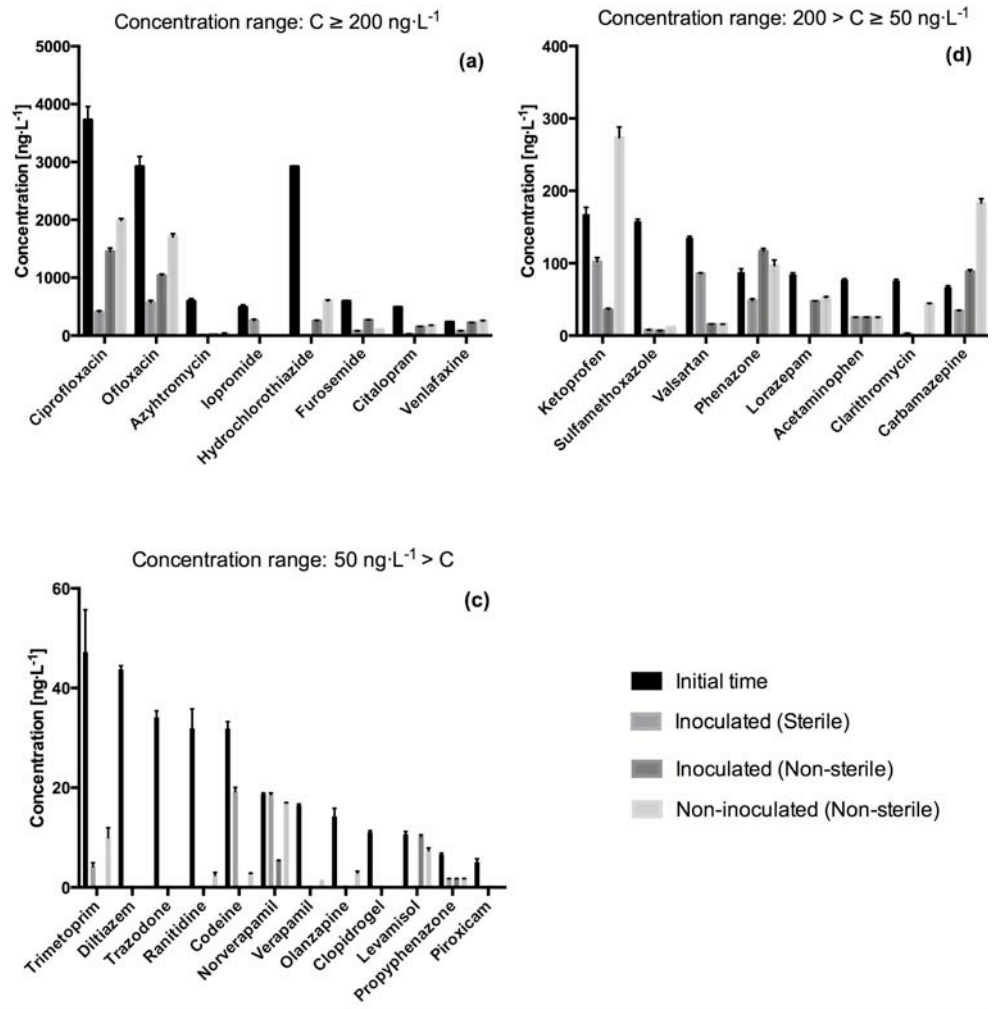


Figure 5.6. Concentration of the PhACs in the initial MBR sludge and after 15 days of treatment in the 3 bioslurry systems: inoculated under sterile conditions, inoculated under non-sterile conditions and raw sludge controls. The PhACs had been grouped into 3 groups according to the initial concentration in the sludge: (a) more than 200 $ng \cdot L^{-1}$, (b) between 200 and 50 $ng \cdot L^{-1}$ and (c) below 50 $ng \cdot L^{-1}$.

Figure 5.6 compares the initial and final concentrations of the detected PhACs. For an easier understanding of the data, drugs had been grouped by initial concentration into three ranges: 0-50 ng·L^{-1} (plot ***c***), 50-200 ng·L^{-1} (plot ***b***) and more than 200 ng·L^{-1} (plot ***a***). ANOVA analysis had not been carried out because of the complexity of the matrix, the elevated number of PhACs in the MBR sludge, and the differences in drugs concentration – up to 3 orders of magnitude. When inoculated and non-inoculated cultures under non-sterile conditions are compared, three main behaviours can be observed: (1) inoculated cultures led to lower drug concentration, (2) inoculated and non-inoculated cultures got the same final drug concentration, and (3) non-inoculated cultures led to lower drug concentration. The predominant behaviour was (1) and it can be noticed for 13 PhACs (6 fully degraded): ciprofloxacin, OFX, azithromycin, citalopram, SMX, lorazepam, clarithromycin, TRM, ranitidine, codeine, norverapamil, verapamil and olanzapine. In contrast, only 2 drugs, i.e. furosemide and levamisol, were better removed in (3) non-inoculated cultures; and none of them were totally degraded. Finally, the behaviour (2), where both mentioned cultures got equal final concentration, was given for 8 compounds: iopromide, valsartan, acetaminophen, diltiazem, trazodone, clopidrogel, propyphenazone and piroxicam of which 5 were completely degraded. Other two observable behaviours were: (i) deconjugation of drugs, which appear with a higher concentration after the treatment, and (ii) higher final drug

concentration in inoculated cultures under sterile conditions, i.e. iopromide, KTP, valsartan, codeine and norverapamil. In general, cultures inoculated with *T. versicolor* under non-sterile conditions obtained better results in the removal of PPCPs than non-inoculated cultures for any concentration range.

Should be noted that due to the nature of the experiment conducted in Erlenmeyer flasks, the monitoring and adjustment of pH was not feasible. pH has been proved as an important parameter for the fungus role in the degradation of pollutants (Folch *et al.*, 2013), as well as the fungus growth (Borràs *et al.*, 2008). Moreover, during the development of the experiment we noticed that fungus pellets were not visible in cultures under non-sterile conditions after 5-7 days of treatment. This, together with the negligible laccase activity, resulted in a microbial analysis of the cultures in order to find out if *T. versicolor* was able to survive in these conditions, and how the initial microbial diversity was affected by the inoculation of the fungus.

5.2.3.b. *Microbial analysis*

DGGE profiles of fungal and bacterial PCR products at initial time before inoculation and at the end of the fungal inoculated non-sterile experiment are shown in figure 5.7. Their phylogenetic affiliations are presented in tables 5.10 and 5.11.

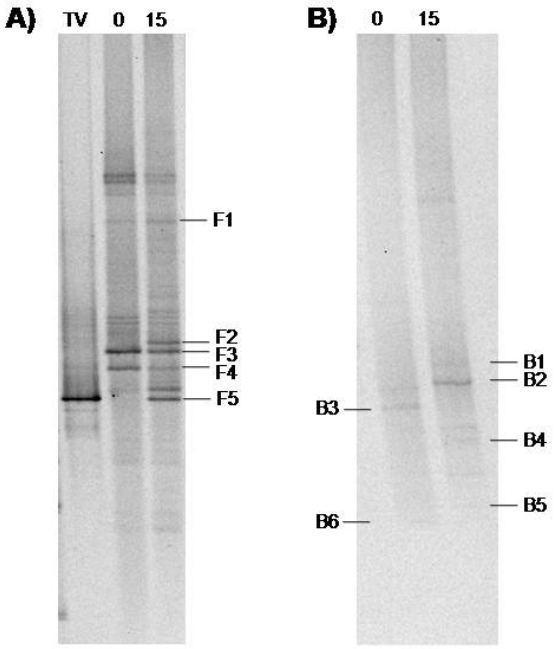


Figure 5.7. DGGE profiles of **A)** fungal PCR-amplified ITS fragments of 18S rDNA and **B)** bacterial PCR-amplified 16S fragments rDNA from fungal bioslurry experiments at initial time (0) and after 15 days (15) of fungal inoculated non-sterile treatment. TV corresponds to the amplified product of a *T. versicolor* pure culture.

Initially, in the MBR sludge, there were two main fungal species (F3 and F4); however, after 15 days of experiment, the microbial diversity of the mixture increased. In the bacterial profile, more bands were obtained in the 15d samples than initially as well. All fungal bands at exception of that belonging to *T. versicolor* (F5) correspond to unknown fungi, as the best alignments gave identities of only 79 and 77% to different uncultured fungi for F1/F2 and F3/F4 respectively. The identity between F1 and F2 is 81% while between F3 and F4 is 97%.

Bacteria found in the bioslurry treatment belonged to diverse classes, from *Alphaproteobacteria* (B1) to *Chloroflexi* (B6), going through *Gammaproteobacteria* (B3), *Holophagae* (B2), *Bacteroidetes* (B4) and some unclassified bacteria (B5). All sequences retrieved a match with 95% of identity or higher with sequences already deposited in the GenBank; however, most of them were from uncultured bacteria. Sequences identified in the initial sludge were related with treatment of wastewater or degradation of selected pollutants: the closest organism for B3 was found in a MBR treating volatile organic compounds (VOCs) at Valladolid, Spain (Lebrero *et al.*, 2013) and the closest for B6 was found in a biotrickling filter also in Valladolid (Lebrero *et al.*, 2012). Sequences found after 15 days of treatment were more related with soil bacteria such as B1, B2 (Turlapati *et al.*, 2013) and B4 (Toyota and Kuninaga, 2006).

Table 5.10. Phylogenetic affiliation of the retrieved fungal ITS sequences of the selected DGGE bands.

DGGE Band	Seq. length	Closest relative[a]	Covery	Identity (%)	Accession number
F1	183	Uncultured fungus clone J2102	106/134	79	JX974768
F2	194	Uncultured fungus clone J2102	102/129	79	JX974768
F3	170	Uncultured Glomeromycota clone 4Bart180S	132/172	77	HQ021976
F4	164	Uncultured Glomeromycota clone 4Bart180S	128/166	77	HQ021976
F5	224	Trametes versicolor culture-collection ICMP:19973	224/224	100	KF727428

[a] Closest organism at GenBank

Table 5.11. Phylogenetic affiliation of the retrieved bacterial 16S sequences of the selected DGGE bands.

DGGE Band	Seq. length	Closest relative[a]	Covery	Identity (%)	Accession number	Class or Order[b]
B1	241	Uncultured Asticcacaulis sp. clone AS85P1	228/241	95	KC172633	Unclassified α-proteobacteria
		Asticcacaulis excentricus strain CB 48	226/241	94	NR_074137	
B2	265	Uncultured bacterium clone H-HN-E4-Min_444417	262/266	98	JQ054706	Holophagae/ Holophagales
		Acidobacterium sp. ORAC	258/265	97	FN689719	
B3	280	Uncultured bacterium isolate DGGE gel band 18	272/278	98	JX627832	γ-proteobacteria/ Xanthomonadales
		Dokdonella soli strain KIS28-6	271/278	97	NR_044554	
B4	266	Uncultured bacterium	256/266	96	AB087366	Unclassified Bacteroidetes
		Bacteroidetes bacterium N2	249/266	94	AB540001	
B5	254	Uncultured bacterium clone ncd265f05c1	248/252	98	HM270445	Unclassified Bacteria
B6	246	Uncultured bacterium isolate DGGE gel band 14	246/246	100	JQ038792	Chloroflexi/ Chloroflexales
		Kouleothrix aurantiaca strain:MYSI-A	223/246	91	AB079639	

[a] Closest organism at GenBank and, when possible, cultured closest match
[b] Classified using the Ribosomal Database Project (RDP)

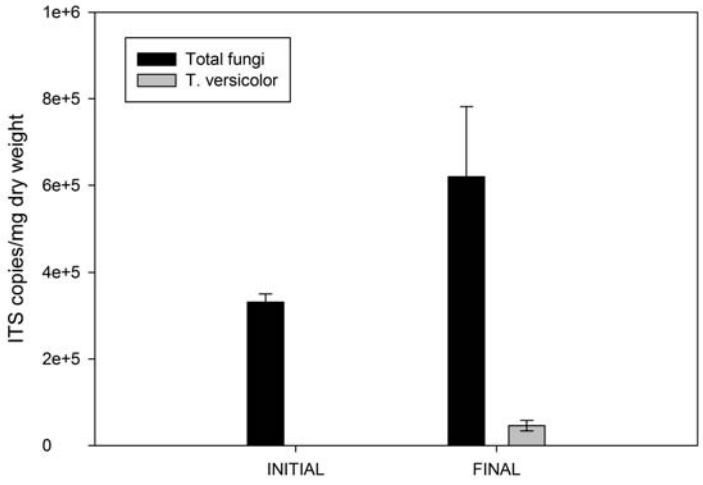


Figure 5.8 Amount of total fungi and *T. versicolor* (related to ITS copies/mg dry weight) at initial time and after 15 days of inoculated non-sterile bioslurry treatment.

The main conclusion one can deduce from the results is that after 15 days of treatment there was still some *T. versicolor* in the bioslurry mixture. With DGGE analysis it is only possible to determine presence and absence of the species. Therefore, in order to quantify *T. versicolor* in relation to total fungus, quantitative PCR was performed. At 15 days, total fungi increased 87% with respect to the initial time, what means almost doubling the amount. However, *T. versicolor* only accounts for the 7.4% of the total fungi at 15 days (figure 5.8). Those low values agree with the visual disappearance of *T. versicolor* at that experimental time. These results are in concordance with the findings of Rodríguez-Rodríguez *et al.*

(2012b) working with *T. versicolor* in biopiles. They found that after 21 days of treatment other fungi replaced *T. versicolor*. Therefore, although matrices are different, similar re-inoculation strategy (Rodríguez-Rodríguez *et al.*, 2014) could be also applied at bioslurry treatment to achieve higher removal percentages.

5.2.4. Bioslurry at reactor scale

Once it was assessed that *T. versicolor* under non-sterile conditions was not only able to survive in bioslurry systems in competition with the autochthonous sludge population, but also it was able to remove PPCPs at real concentrations, we investigated if it was possible to increase the scale of the process: from a 0.5L Erlenmeyer flask to a 8L stirred bioreactor. Additionally, we hypothesised that it was possible to use the biomass of the bioslurry as a co-substrate in a sewage sludge AD process, so the production of biogas and the methane yield would be improved. Firstly, fungal bioslurry treatments with non-spiked and non-sterile MBR sludge were performed, evaluating the laccase activity in order to estimate the growth and colonization level of *T. versicolor* in those systems. Secondly, anaerobic batch tests with three different inoculums from diverse WWTPs were performed so as to determine how would affect the use of biomass from the fungal bioslurry as a co-substrate, monitoring the production of biogas and methane yield as indicators. Finally, the total removal of PhACs at real concentrations was assessed.

5.2.4.a. *Bioreactor performance*

Previously, Rodríguez-Rodríguez *et al.* (2012a) studied the growth and removal capacities of *T. versicolor* at reactor scale; however, the researchers worked with sludge from the outlet of an anaerobic digester located at the Prat de Llobregat's WWTP. In the present research, the aim was to treat the sludge from a MBR before its anaerobic digestion, and to use the resulting biomass as a co-substrate in the AD process, improving both the gas production and the removal of emerging pollutants. Additionally, a lower HRT of 5 days for the fungal bioreactor was selected, since under non-sterile conditions without external sources of nutrients the microbial competition would affect the fungus degradation capacities, and because it has been proved that *T. versicolor* could degrade emerging pollutants within a period of 5 days (Marco-Urrea *et al.*, 2009c).

In order to evaluate the state of *T. versicolor* in the bioreactor, and given the unfeasibility of direct biomass quantifications, laccase activity was monitored. As it has been mentioned before, the activity of this enzyme indicates the oxidative potential of the fungus, which is partially involved in the degradation of some pollutants. In figure 5.9 the laccase activity in the fungal bioslurry with non-sterile MBR sludge is showed. It can be seen that after the inoculation of the bioreactor with the fungus (day 0) null laccase activity was detected, something normal since the fungus had not enough

time to grow. After inoculation, the laccase activity tended to increase, reaching a maximum by day 2 (14.5 ± 0.1 $U \cdot L^{-1}$); however, this maximum was followed by a decrease (9.6 ± 0.1 $U \cdot L^{-1}$ at day 3 and 1.7 ± 0.1 $U \cdot L^{-1}$ at day 4) until no laccase activity was measured the last day of experiment (day 5).

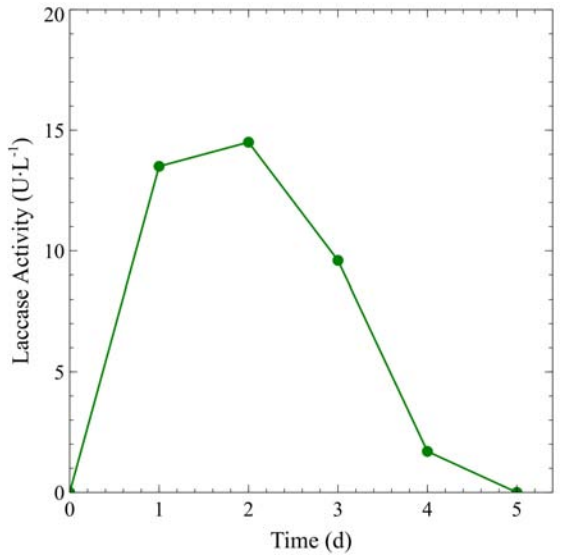


Figure 5.9 Laccase activity in the fungal bioslurry at reactor scale with MBR sludge under non-sterile conditions and no further source of nutrients or carbon. Standard error bars are not appreciable.

These results confirmed that no longer HRT was needed, as *T. versicolor* did not have further oxidative potential in the bioreactor after day 5. In contrast, Rodríguez-Rodríguez *et al.* (2012a) observed no laccase production at the beginning of their experiment; only between days 14 and 18 laccase activity was detected, with a 28.5 $U \cdot L^{-1}$ peak at day 18. This could indicate that

the fungus needed more time to adapt to AD sludge than MBR sludge, although neither nutrient nor carbon sources were provided.

5.2.4.b. *Anaerobic batch assays*

During the anaerobic digestion of simple or complex materials, methanogenic or hydrolytic stages can be the limiting steps of the process. Traditional parameters such as COD, TOC and TSS could provide an idea about the nature of the residue; however, no information about neither the limiting step nor the microbial activity for a singular substrate during an AD process is given (Soto *et al.*, 1993). Instead, the BMP test can be useful in order to establish not only the potential of a certain sludge to produce methane, but also to determine if the sludge can be stabilized with an AD process. Given that, an initial screening of the three selected AD inoculums was performed according to their methanogenic activity (ACT), which allowed to select the most suitable AD sludge in terms of CH_4 production; choosing the best-adapted inoculum to the operational BMP tested conditions (Angelidaki *et al.*, 2007). Next, net methane production of fungal bioslurry's biomass as substrate was monitored using the inoculums that showed higher ACTs.

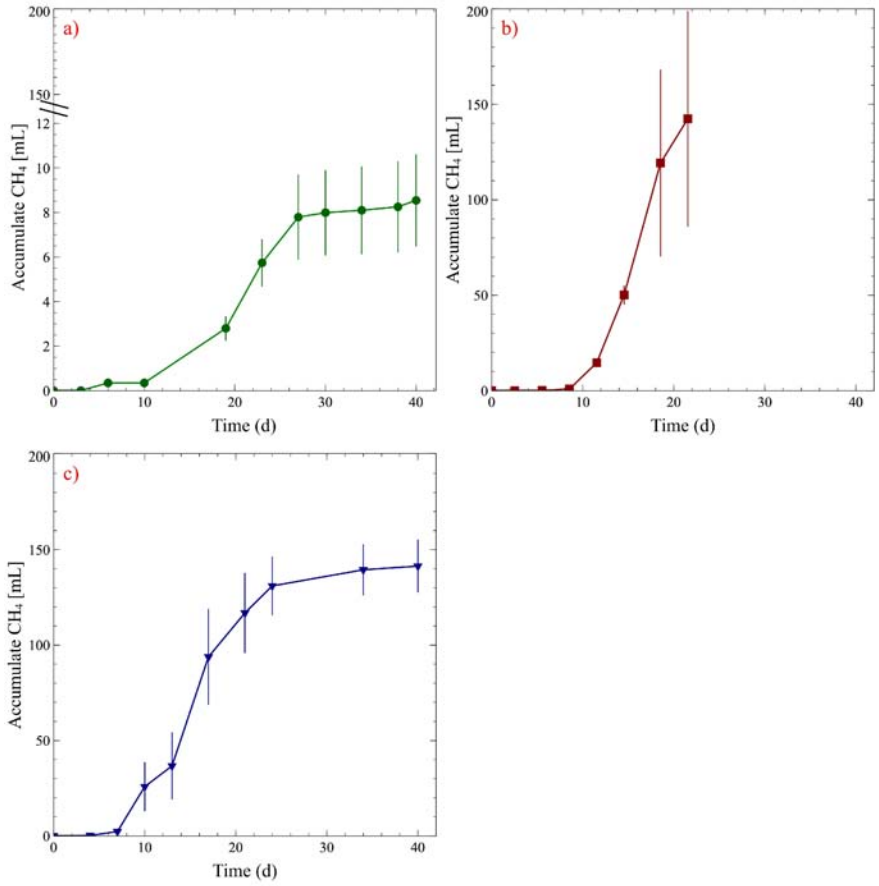


Figure 5.10 Evolution of the net methane production of Terrassa (a), Sabadell (b), and Blanes (c) inoculums in batch reactors. Notice that for plot a, Y's axis has been re-escalated in order to display the results. Error bars represent the standard error.

(i) Initial Screening

The evolution of the methane production during the methanogenic activity tests for the three inoculums is shown in figure 5.10 and the ACTs values are summarized in table 5.12. As it can be seen, all the cultures present a similar behaviour. First, a phase between days 0 and 8/10 where no methane production –

or less than 1mL – was detected. This lag phase was given by the acclimation of inoculums' organisms to the new operational conditions. Second, a growth phase between days 8/10 and 20/25, where methane production experienced an exponential increase, was observed. During this stage the inoculums' population expanded rapidly, as enough food remained in the culture. Finally, a levelled or stationary stage from day 20/25 was seen, detecting little or no further methane production. In this phase, although some methane could still be produced, the feed reached its limit, and the death rate for inoculums' organisms equated their reproduction rate.

Table 5.12. Accumulated net methane production at the end of the screening and the methanogenic activity for each AD inoculum.

Inoculum	Net accumulated methane (mL CH_4)[a]	Methanogenic Activity	
		[$gCOD_{CH4}$·$gSSV^{-1}$·d^{-1}]	[L_{CH4}·$gSSV^{-1}$·d^{-1}]
Terrassa	8.54 ± 2.07	0.007	0.003
Sabadell	n.a.	0.127	0.050
Blanes	141.38 ± 13.85	0.078	0.031

[a]Concentration values ± standard error

The AD inoculum from Terrassa's WWTP (plot ***a*** in figure 5.10) showed the lowest methanogenic production, with a final net production of 8.54 ± 2.07 mL CH_4 by day 40, and an ACT of 0.007 $gCOD_{CH4}$·$gSSV^{-1}$·d^{-1}. According to the operators of this plant, the main purpose of the AD process was the reduction of sludge

volume and not the methane production, which was burned without further use. In contrast, Sabadell and Blanes WWTPs employs the biogas in order to generate heat for the plant. As it can be observed (plot ***b*** in figure 5.10), the ACT for Sabadell's AD inoculum was the highest (0.127 $gCOD_{CH4}{\cdot}gSSV^{-1}{\cdot}d^{-1}$); however, and due to technical problems with the temperature control after day 22, the experiment was finished earlier, which did not affect the ACT's determination – as it is based in the maximum slope of the graph – but it was not possible to determine the net accumulated methane. Finally, Blanes' AD inoculum (plot ***c*** in figure 5.10), which displayed a gradual and extensive growth from day 7 to 21, showed a lower ACT (0.078 $gCOD_{CH4}{\cdot}gSSV^{-1}{\cdot}d^{-1}$). According to these results, the AD inoculums from Sabadell and Blanes where chosen for the following BMP test of the fungal bioslurry, discarding Terrassa's inoculums due to its low ACT.

Comparable biogas production behaviours have been previously observed with other types of sludge. For instance, Martín-González *et al.* (2010) while studying the co-digestion of the organic fraction of municipal solid waste with FOG (i.e. fat, oil and grease waste), reported alike trends during anaerobic batch tests: initial lag phase of 10–12 days followed by a growth period of 10 days, and a final stationary stage. Also, similar behaviour was also observed during the anaerobic co-digestion of pig-manure and dewatered sewage sludge in batch reactors (Zhang *et al.*, 2014).

(ii) BMP Test

Once the best inoculums were chosen, the biogas production of the fungal bioslurry was evaluated with them. Additionally, two controls were made in order to assess how a 5 days treatment of MBR sludge with *T. versicolor* would affect the AD process: (i) cultures with inoculum and fungal biomass and (fungal control) (ii) cultures with inoculum, fungal biomass and untreated raw MBR sludge (sludge control). The results on the biogas production are displayed in figure 5.11, where inoculum production has been withdrawn. Fungal controls showed the higher biogas productions, while sludge controls and fungal bioslurry cultures had similar net biogas production until ca. day 10, when fungal bioslurry cultures reached the stationary phase whereas sludge controls increased slightly for one more point-time.

The highest net biogas production value was reached with Sabadell's WWTP inoculum (plot ***a*** from figure 5.11), achieving a net accumulated biogas production of 289.51 ± 19.68 mL for fungal control; followed by the Blanes' fungal control with 126.30 ± 0.00 mL of accumulated biogas (plot ***b*** from figure 5.11). Although it is known that fungal biomass can be anaerobically digested (Rouches *et al.*, 2016a, 2016b), these results were not in accordance with the previous experience of the research group, where bare fungal biomass was digested in a few days with low biogas production if compared with experimental cultures. In

the present experiment, the high biogas production of these controls could be due to an inadequate wash up of the fungal biomass prior its addition to the AD bottles; resulting in extra nutrients – from the growth media of *T. versicolor* – for the BMP tests of the fungal controls.

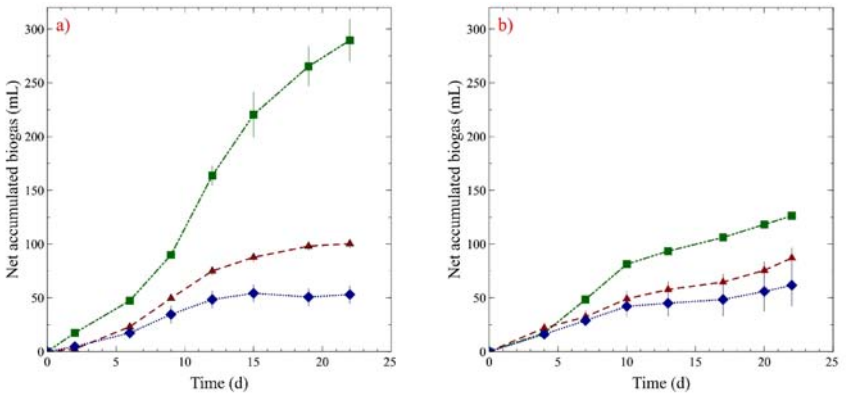


Figure 5.11. Accumulated biogas production of fungal bioslurry cultures (◆), fungal controls (■) and sludge controls (▲) with both Sabadell (a) and Blanes (b) inoculums. Inoculum production has been withdrawn. Error bars stand for standard error.

The next cultures with higher net accumulated biogas production were the sludge controls for both inoculums, with 100.35 ± 4.16 mL and 87.08 ± 9.44 mL for Sabadell and Blanes respectively; indicating that MBR sludge was degraded during the BMP tests with the selected inoculums. Finally, the lowest values of accumulated net biogas production were for the fungal bioslurry with 53.06 ± 8.32 mL and 61.71 ± 19.71 mL for Sabadell and Blanes inoculums respectively. So, the treatment of MBR

sludge with *T. versicolor* affected in some way the AD process. The fungus alone (fungal controls) produced high amounts of biogas, but when MBR sludge was added (sludge controls) the production decreased. This can be explained by the increasing complexity of the matrix: while the fungal biomass of the control represented a homogeneous matrix with high amounts of nutrients – due to the solution where the fungus was produced and kept –, the MBR sludge (both the treated and the untreated) represented a complex biomass with different organic and inorganic compounds and a rich microbial population. In any case, it has been proved that the fungal bioslurry can be anaerobically digested.

In general, the BMP tests with the inoculum from Sabadell obtained better net biogas production for both controls than with Blanes inoculum, and similar for fungal bioslurry cultures. However, when referring to the percentage of CH_4 production in the fungal bioslurry, this was higher with Blanes inoculum (19.14% of methane) than with Sabadell inoculum (13.81% of methane). Also, this happened with net accumulated methane production values, where fungal bioslurry cultures digested with Blanes inoculum showed higher values: 11.82 ± 0.93 mL net CH_4 in front of 7.33 ± 0.45 mL net CH_4 of fungal bioslurry cultures with Sabadell inoculum. These values contrast with those obtained during the initial screening, where observed methane productions were one order of magnitude greater. A rapid check

of the literature indicates that the methane production was low (Kim *et al.*, 2003; Martín-González *et al.*, 2010), but, as mentioned before, the fungal bioslurry can be anaerobically digested since neither the fungal biomass nor the MBR sludge were not toxic for any of the tested AD inoculums.

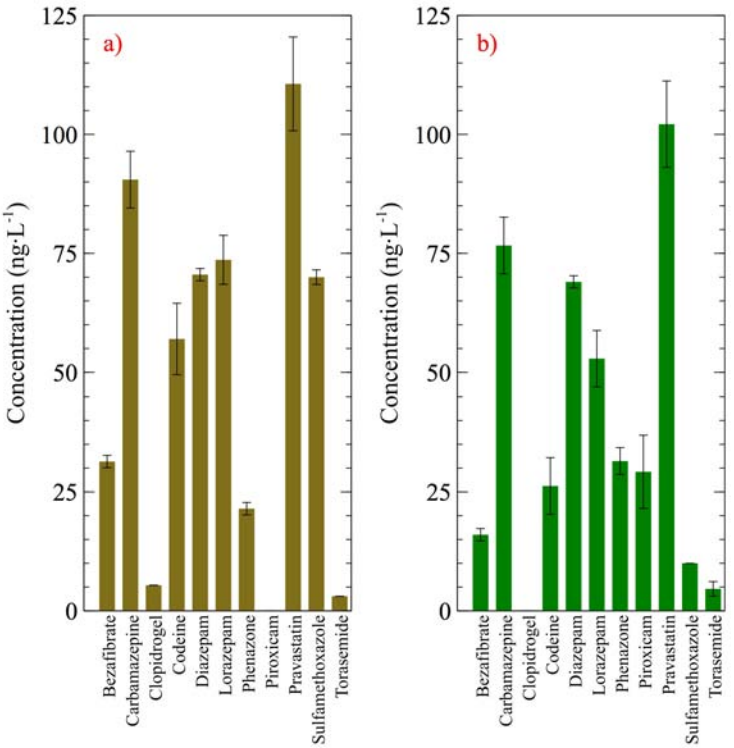


Figure 5.12. EPs concentration in the (a) raw MBR sludge and (b) after its treatment with *T. versicolor* for 5 days in bioslurry at reactor scale. Error bars stand for standard deviation.

As a result and given the methane production values, only the cultures with fungal bioslurry and inoculum from Blanes' WWTP were analysed in order to determine the total removal of

PhACs in each stage of the process: before and after the fungal bioslurry treatment, and after the AD process.

5.2.4.c. *Total PPCPs removal*

Although the observed low methane production values, the removal of PhACs at real concentration was monitored for those cultures with the inoculum from the WWTP located in Blanes. The aim was to determine if the AD process would improve the removal of emerging contaminants. Hence, both fungal bioslurry and untreated MBR sludge were digested separately. The initial concentration of PPCPs was determined in fresh raw MBR sludge and compared with the measured concentrations of drugs in the fungal bioslurry after 5 days of treatment. Figure 5.12 shows the emerging contaminants found in both the raw MBR sludge (plot ***a*** of figure 5.12) and the bioslurry (plot ***b*** of figure 5.12). It can be seen that only 11 drugs were detected, less than the 28 compounds found in the first PPCPs determinations, (table 5.8); however, the concentrations in both experiments were similar (differences below one order of magnitude). Three drugs showed a lower concentration at the beginning of the experiment due to conjugation/deconjugation processes: the non-steroidal anti-inflammatory drugs (NSAIDs) phenazone (13.26 ± 0.00 $ng \cdot L^{-1}$) and piroxicam (0 ± 0.00 $ng \cdot L^{-1}$), and the diuretic toresamide (8.84 ± 0.00 $ng \cdot L^{-1}$). Deconjugation of PPCPs was previously observed in prior fungal bioslurries at Erlenmeyer scale; which has also been reported by researchers who worked

with wastewaters that overcame a biological process (Celiz *et al.*, 2009; Petrovic *et al.*, 2009).

On one hand, the compound with the higher concentration at the raw MBR sludge (plot ***a*** of figure 5.12) was SMX (201.11 ± 4.42 $ng{\cdot}L^{-1}$), followed by lorazepam (196.69 ± 11.05 $ng{\cdot}L^{-1}$), codeine (141.44 ± 6.63 $ng{\cdot}L^{-1}$) and CBZ (137.02 ± 2.21 $ng{\cdot}L^{-1}$); and all the others PhACs had a concentration below $50ng{\cdot}L^{-1}$, except for the fibrate drug bezafibrate (64.09 ± 0.00 $ng{\cdot}L^{-1}$). Consequently, a total PPCPs concentration of 828.75 ± 26.52 $ng{\cdot}L^{-1}$ was detected in the bioreactor inlet. On the other hand, after the treatment of MBR sludge with *T. versicolor* in bioreactor (plot ***b*** of figure 5.12), the total amount of pollutants was reduced, reaching a final PPCPs concentration of 497.25 ± 48.62 $ng{\cdot}L^{-1}$. Only one compound showed a concentration above $100ng{\cdot}L^{-1}$ (lorezapam with 137.02 ± 13.26 $ng{\cdot}L^{-1}$); three drugs had a concentration between 100 – 50 $ng{\cdot}L^{-1}$ (CBZ: 97.24 ± 2.21 $ng{\cdot}L^{-1}$; piroxicam: 83.98 ± 22.10 $ng{\cdot}L^{-1}$; and codeine: 53.04 ± 2.21 $ng{\cdot}L^{-1}$); and the other 7 PhACs had concentrations below $50ng{\cdot}L^{-1}$. It was not the first time that these drugs were detected in sewage sludge (table 5.8) or in anaerobic digesters. For instance, Martín *et al.* (2015) were able to detect 22 PPCPs in diverse sludges from different treatment plants, and bezafibrate, CBZ and SMX were found in anaerobically digested sludges from primary and secondary treatments. Subedi *et al.* (2015) and Subedi and Kannan (2015) also found clopidrogel, codeine, diazepam, and lorazepam in several WWTP of India and USA with a wide range

of concentrations Wassenaar *et al.* (2015) detected piroxicam and pravastatin in sludge applied as soil amendment; phenazone was previously reported by Rodríguez-Rodríguez *et al.* (2014) in thermally dried sludge; and finally, torasemide has only been found in water bodies such as reservoirs, sea and waste waters (Gros *et al.*, 2012; Jelic *et al.*, 2015; Moreno-González *et al.*, 2014).

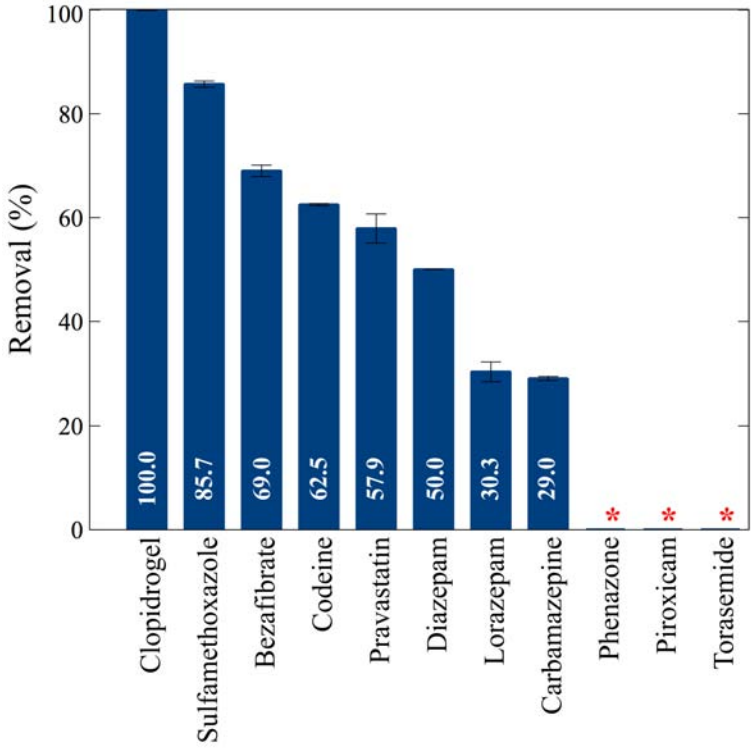


Figure 5.13. Removal percentages for each emerging contaminant after MBR sludge was treated with *T. versicolor* for 5 days in bioslurry at reactor scale. Removals for phenazone, piroxicam and torasemide were not assesse due to final concentration was higher than initial. Error bars represent the individual detection limit for each compound divided by 2.

The total removal after the fungal bioslurry treatment with the fungus was 40.0 ± 0.9 %; however, high individual removals were achieved. In figure 5.13 the removal rates for each compound after the fungal bioreactor treatment can be observed. Clopidrogel was totally removed from the MBR sludge, followed by SMX (85.7 ± 0.6%). Four compounds were removed with rates above 50%: bezafibrate (69.0 ± 1.1 %), codeine (62.5 ± 0.2 %), pravastatin (57.9 ± 2.8 %), and diazepam (50.0 ± 0.0 %). Comparing fungal bioslurry treatment at reactor scale with the previous fungal bioslurry at Erlenmeyer scale (inoculated with *T. versicolor* under non-sterile conditions), it can be seen that: clopidrogel was the only compound totally removed in both experiments; codeine and piroxicam were completely removed at Erlenmeyer scale, but only partially eliminated within bioreactor (i.e. codeine) or deconjugated (i.e. piroxicam); CBZ was partial removed in the current bioreactor (29.0 ± 0.4 %), but deconjugated in the Erlenmeyer bioslurry; phenazone was deconjugated in both experiments; SMX and lorezapam were better removed in Erlenmeyer experiments (95.7 ± 0.6 % and 84.8 ± 2.0 %, respectively) than in bioreactors (85.7 ± 0.6 % and 30.3 ± 1.9 %, respectively); and bezafibrate, diazepam, pravastatin, and torasemide were only detected and eliminated in the present experiment.

Table 5.13. Removals of the detected PPCPs before the anaerobic digestion of both untreated and treated MBR sludge with *T. versicolor.*

Compound	Initial Concentration ($ng \cdot L^{-1}$)[b]	Removal (%)[c]	
		MBR sludge + AD	*Bioslurry + AD*
Bezafibrate	31.38 ± 2.21	22.5 ± 1.1	36.6 ± 1.1
CBZ	90.49 ± 8.84	[a]	[a]
Clopidrogel	5.38 ± 0.00	[a]	100.0 ± 0.1
Codeine	57.00 ± 8.84	38.0 ± 0.2	65.1 ± 0.2
Diazepam	70.53 ± 2.21	[a]	[a]
Lorazepam	73.64 ± 2.21	49.0 ± 1.9	64.0 ± 1.9
Phenazone	21.48 ± 2.21	[a]	[a]
Piroxicam	0	[a]	[a]
Pravastatin	110.61 ± 2.21	[a]	12.1 ± 2.8
SMX	69.99 ± 4.42	59.0 ± 0.6	100.0 ± 0.6
Torasemide	3.08 ± 0.00	100.0 ± 0.0	100.0 ± 0.0
	Total Removal:	***46.4 ± 0.8***	***52.2 ± 1.0***

[a] Removal not assessed, final concentration was higher than the initial
[b] Concentration values ± standard deviations
[c] Errors expressed as the detection limit of each compound divided by 2

The AD of the fungal bioslurry improved the global elimination of PPCPs, reaching a combined total removal of 52.2 ± 1.0 %. In table 5.13 can be seen that SMX and torasemide were finally removed from the sludge after the AD treatment of the bioslurry. Additionally, the final removals of two compounds were improved: codeine (65.1 ± 0.2 %) and lorazepam (64.0 ± 1.9 %). However, other three drugs showed increased concentrations after the AD process, decreasing their general removal yields: bezafibrate, CBZ, diazepam and pravastatina. On

the contrary, the MBR sludge that was not previously treated with the fungus achieved not only low individual removal rates, but also a lessen global removal yield (46.4 ± 0.8 %); with torasemide as the only drug that was completely removed. So, all the compounds reached higher removal percentages in the AD treatment of fungal bioslurry than in the solely AD treatment. Other researchers have found similar removal rates during AD, such as Narumiya *et al.* (2013) that, while studying the phase distribution of PPCPs in real scale anaerobic digesters, observed that SMX was almost eliminated (~99 %), bezafibrate was partially removed (<50%), and CBZ was unaffected; or Reyes-Contreras *et al.* (2011) that were able to degrade, among other PPCPs, CBZ in a pilot plant, although the removal rate (ca. 7 – 10 %) was lower than the obtained with the bioslurry treatment.

In general, the removal of PPCPs during anaerobic digestion of sewage sludge and wastewater has been deeply studied by other researchers. As mentioned before, Carballa *et al.* (2007b) found that the presence of PPCPs did not affect the methane yield of the AD, and drugs could be anaerobically degraded under mesophilic and thermophilic ranges of temperatures without significant differences. Furthermore, Alvarino *et al.* (2014), while working with a lab-scale Up-flow Anaerobic Sludge Blanket (UASB) reactor of 4.5L, observed not only that PPCPs biodegradation was correlated with methane production and drugs sorption was significant for lipophilic compounds, but also that heterocyclic compounds – e.g. pyridines, uracils or furans –

resisted the degradation under anaerobic conditions; however, the presence of a substituted group increased the bio-availability of the compound – such as pyrimidine. Nevertheless, it has been the first time that an anaerobic digestion process was used together with a bioslurry system with *T. versicolor* in order to improve the general removal of non-spiked emerging pollutants in real WWTP sludge under non-sterile conditions. Although the removal of PPCPs at real concentrations was high, further research should be done in order to (i) study the possible inhibitory effects of some compounds and their conjugates, (ii) evaluate the changes and interactions of the microbial AD populations, and (iii) increase the scale of the anaerobic digesters and work in continuous process.

5.3. Conclusions

It has been proved that MBR sludge can be treated with *T. versicolor* at laboratory scale. The fungus has grown under non-sterile conditions without any extra nutrients, and it was able to remove a widely range of emerging pollutants. The activity of the MBR sludge's autochthonous microorganisms at Erlenmeyer bioslurry scale was enough to eliminate emerging pollutants, but when it was treated with *T. versicolor* the time to eliminate the detected PPCPs was reduced, resulting in an improved removal of these pollutants. The fungus was also able to remove PPCPs

at reactor scale under the same conditions, resulting in middle-high removals.

The fungal treatment of MBR sludge has been proved as an adequate pre-treatment of the MBR bioslurry prior its stabilisation via anaerobic digestion. As no toxic TPs, which could have affected the AD process, has been produced, and the global elimination of PhACs has been increased in the linked treatment – fungal treatment plus AD process.

MBR sludge has been successfully treated, but the low solids content of the sludge makes difficult to assess whether the solid or just the liquid was treated, maybe the two phases should be analysed separately. Despite these drawbacks, the removal treatment of emerging contaminants, which at real scale would be carried out under non-sterile conditions, presents better results with the inoculation of *T. versicolor*.

CHAPTER 6

Post-Treatment of a WWTP effluent

Summary

The efficient removal of pharmaceuticals was assessed in soil amended with biochar – charred eucalyptus leafs – and NUA from the mining industry. The selected pharmaceuticals were three antibiotics (OFX, TRM and SMX), two analgesics and anti-inflammatories (ibuprofen and ketorpofen), one psychiatric drug (CBZ), and one ß-blocker (propranolol). First, the capacity of bare soil to adsorb the pharmaceuticals was assessed in 24h batch experiments in order to quantify the distribution coefficient (K_d) of the selected drugs. The most adsorbed compound was OFX and the lowest KTP. Second, the adsorption of three drugs with similar $logK_{ow}$ at different soil:amendment ratios was assessed and carried out in 24h batch experiments, in order to determine which ratio would be the most suitable. A ratio of 1% was selected, according not only to the adsorption rates, but also to pH change, economic costs and theoretical adverse effects of the treatment. Finally, the removal percentage of each pharmaceutical compound was measured in soil amended with biochar and NUA during 21 days. The results showed high removal percentages for all the tested pharmaceuticals, proving the efficiency of biochar and NUA to remove emerging contaminants from water by amending the soil.

6.1. Introduction

6.1.1. Groundwater recharge

The global population increase leads to a growing demand and consumption of natural resources, and water is one of the most vulnerable capitals. Freshwater is expected to be one of the first natural resources that will run low in the near future (Wagner *et al.*, 2002). Modern society needs water for a wide range of tasks, being the irrigation of crops the main consumption activity (65% of the total water use). As freshwater use implies a modification of its properties and a reduction of its availability, but not a total depletion, water can be reused for other activities after being treated (Levine and Asano, 2004). Linking treated wastewater discharge with downstream water uptake is the key to water reuse.

Groundwater is an important natural resource that can be used to supply water for municipal, agricultural, and industrial purposes. While natural recharge of underground basins is a slow process, artificial recharge methods are faster and can be linked with other water management systems. Also, artificial recharge of aquifers can (i) reduce or stop the drop of groundwater level, (ii) protect underground freshwater resources from saltwater intrusion, and (iii) be a method to store an excess of surface water (i.e. floods and reclaimed wastewater) (Asano and Cotruvo, 2004). Nowadays, both

surface spreading and direct injection are the main artificial recharge systems in use.

Direct aquifer injection systems are used in order to deposit water directly into the underground water basins. These systems are applied when there is an unsaturated zone between the surface and the aquifer (Oaksford, 1985). However, the water introduced must be treated before its injection, especially if it is reclaimed wastewater, as no natural attenuation or elimination of pollutants will occur, since the water will not percolate through the soil (Asano and Cotruvo, 2004).

Groundwater recharge by surface spreading of reclaimed wastewater is a widely used system to replenish aquifers, especially in arid zones with plenty of land. The reclaimed wastewater stream is intermittently introduced into spreading or recharge basins, allowing sufficient time for its infiltration. The percolation of the water across the ground, and its movement throughout the aquifer will result in a quality improvement thanks to the experimented physical, chemical, and biological natural processes. Both the percolation of the water and the resulting treatment processes, are the so-called soil-aquifer treatment (SAT) (Fox, 2001; Miotlinski *et al.*, 2010). However, it is difficult to get rid of certain trace contaminants in the reclaimed wastewater (i.e. industrial and pharmaceutical chemicals, home and personal cleaning products, salts, and heavy metals) by natural means. Trace pollutants must be eliminated from the reclaimed wastewater, as long-term health

risks can be associated with chronic exposure to such type of pollutants (Levine and Asano, 2004).

6.1.1.a. *Alice Springs SAT*

The selected SAT for this study is located in Alice Springs at the Arid Zone Research Institute (Northern Territory, Australia), and it was originally planned to recharge the aquifer with 600 $ML \cdot year^{-1}$. SAT's inlet stream is a treated wastewater from Alice Springs' WWTP. From June 2008, when the SAT commenced its activity, to December 2009, 317.4 ML of reclaimed water were discharged into four basins (total area: 7,640m^2), giving a final infiltration rate of 240$mm \cdot d^{-1}$ (evaporation loss and rainfall gains had been taken into account). In December 2009 a fifth basin was incorporated to the project, giving a final recharge area of 10,269 m^2. The aquifer is an unconfined Quaternary aquifer with a water table occurring at a depth of 15 – 20 m, with natural water level fluctuations of 1–2m (Miotlinski *et al.*, 2010).

6.1.2. Low-cost sorbents

There are a great variety of technologies to control and minimize the water pollution by organic contaminants, e.g. advanced oxidation, aerobic degradation, filtration, flocculation, and precipitation among others; however, the complex operating procedures of these technologies implies high operational and maintaining costs. In contrast, adsorption processes for removing pollutants at low concentrations are more economical and efficient, making them a better alternative

(Bhatnagar *et al.*, 2015; Tran *et al.*, 2015). Adsorption can be defined as the mass transfer of a substance from a fluid to a solid's surface, becoming bounded by physical and/or chemical interactions (Kurniawan and Babel, 2003).

Activated carbon is considered as the universal adsorbent for water treatment, but its high cost reduces their use in wastewater treatment. Local materials available in large quantities, like natural products from agricultural activities or waste products and by-products from industrial operations, can be utilized as inexpensive sorbents. Furthermore, the valorisation of these materials into low-cost sorbents will help local industries to reduce its outlay by decreasing the waste disposal costs. A material can be qualified as a low-cost sorbent when requires a few or no processing before its utilisation, and it is abundant in nature or as industrial waste. Some of the most used low-cost sorbents are: bark, lignin, chitin, dead biomass, algae, xanthate, zeolite, clay, fly ash, moss, wool, and cotton (Bailey *et al.*, 1999; Kurniawan *et al.*, 2006).

6.1.2.a. *Biochar*

Biochar is a recent term utilized to describe the partial carbonized organic matter applied in soil management (Lehmann *et al.*, 2006). According to the International Biochar Initiative (IBI), biochar can be defined as a solid material obtained from the pyrolysis or gasification of biomass, which can be added to soil to improve its functions and to reduce the greenhouse emissions from biomass (International Biochar

Initiative [IBI], 2015). Biochar properties depend on production factors such as: the chemical and physical properties of feedstock, the heat treatment, the pressure, and the pre- and/or post-treatment. However, biochar can be classified into three general categories depending on the feedstock ash composition (Joseph and Taylor, 2014):

- Ash composition between 3% and 5%: wood, bamboo, nut shells and some seeds produce a hard biochar with high porosity, surface area and water holding capacity.
- Ash composition ranged from 3 – 5 % to 10 – 13 %: agricultural residues, bark and green-waste make biochar with high cation exchange.
- Ash composition higher than 13%: sludge, manures, rice husk, municipal waste and paper waste produce biochars that are very variable, but some general trends can be observed: low adsorption surface area, high liming ability (when the feedstock are manures and sludge), high EC and pH, and high heavy metal concentration if compared with other feedstock.

The utilization of biochar in environmental management practices responds to four different, but highly linked, applications: as a soil amendment, as a way to manage agricultural waste, as an alternative to produce energy, and as an instrument to mitigate the climate change (Lehmann and Stephen, 2009; Sohi *et al.*, 2010).

In the recent years, it has been proved that biochar is more efficient at improving soil quality than other organic amendments, since it is more stable and has a higher capacity to hold nutrients. Additionally, modern agricultural methods are being criticized for unsustainable practices that lead to the consumption of high external inputs, the releasing of green house gasses (GHGs) from soils and emissions from agricultural machinery, and the erosion and degradation of soils. Biochar could provide the opportunity to switch into more sustainable practices, as it can improve the fertility, the holding capacity and the structure of the soil by using local and renewable materials as feedstock (Lehmann and Stephen, 2009; Reddy, 2014).

According to Ackerman, an appropriate waste management can reduce the climate change by: (i) decreasing methane emissions from landfills, (ii) reducing industrial usage of energy, (iii) recovering energy from waste, (iv) increasing the carbon sequestration in forests – due to decrease demand for virgin paper –, and (v) decreasing the energy used in long-distance transport of waste (Ackerman, 2000). Since biochar can be produced from the partial combustion of organic waste – i.e. animal and crops waste –, it can be considered as a suitable waste management technique, because it reduces waste disposal costs, prevents the pollution of ground and surface water, and provides cost-effective renewable energy. The pyrolysis of agricultural waste not only decreases the volume and weight of those materials, but also generates biochar and

renewable energy in the form of electricity, biofuels and heat (Gaunt and Lehmann, 2008; McHenry, 2009).

Energy demands of modern society are being intensified due to rapid increase of world's population and developing technologies. A great part of this demand is satisfied by fossil fuels, which reserves are decreasing. In contrast, renewable energy is produced from sources that can be maintained indefinitely, such as solar, wind, hydro, geothermal, wave, and biomass. Despite the other sources, biomass could be a suitable alternative to traditional fossil fuels, because it is the precursor of biosyngas, bio-oil and biochar fuels, and modern biomass energy systems can be set up in any location where animals and/or plants can live. Energy recovery from biomass has been focused on thermochemical processes such as combustion, gasification and pyrolysis; however, among these three, pyrolysis is the most popular because the conditions can be optimised to maximize the production of gases, oils or chars, depending on the demand. Using pyrolysis to produce renewable energy and biochar for land application is a joint strategy to decrease climate change (Lehmann, 2007; Özçimen and Ersoy-Meriçboyu, 2010; Özçimen and Karaosmanoğlu, 2004; Sánchez *et al.*, 2009).

The climate change is one of the most important challenges that modern society has to overcome. Anthropogenic GHGs are released not only when fossil fuels or biomass are burned, but

also with decomposition of organic matter. Carbon sequestration in the environment has aroused as a suitable way to reduce GHGs emissions. In terrestrial ecosystems, although soils show a low potential to accumulate C, the C sequestration can be achieved by increasing soil C stocks. A way to perform that is to apply biochar to soil, offering a large and long-term C sinks (Lehmann *et al.*, 2006). However, two conditions have to be met: (i) biochar has to be produced at the same rate that plants grow, as they will transform atmospheric CO_2 into organic C by photosynthesis; and (ii) the resulting biochar must be more stable than the precursory biomass (Lehmann and Stephen, 2009).

As it has been shown, not only biochar has been deeply studied and utilized in environmental management in four different ways, but it also has an important role in the elimination of pollutants from water streams due to its reported effectiveness to sorb organic compounds (natural and man-made chemicals). Adsorption is becoming an interesting way to remove pollutants that are difficult to eliminate with other methods, and hence biochar has the change to become a cheap treatment method. Biochar's high sorption is given by high specific surface area, aromaticity, and microporosity, but not only the sorption ability is important. Desorption behaviour of biochar must be considered when it is used as a soil amendment and as a low-cost sorbent, since bioaccessibility, bioavailability, efficacy, and toxicological impacts of certain pollutants depend

on this. That is, a compound will not affect its target organism if it is not released into the environment again (Kookana *et al.*, 2011). Biochar has been applied in water remediation to remove dyes, phenolic compounds, PAHs, pesticides, organic solvents, and heavy metals. Although biochar presented a wide range of adsorption capacities depending on the pollutant, the biochar type, and the chemical and physical properties of the matrix (Mohan *et al.*, 2014), its low cost – compared with activated carbon – and the possibility to link its production with energy generation make biochar as an attractive alternative to traditional water treatments.

6.1.2.b. *NUA*

Despite the low cost of biochar produced with agricultural residues, there are still a few expenses associated with its manufacturing process. An alternative to this is the utilization of industrial by-products that do not need a pre-treatment before their use. For instance, a by-product from the heavy mine industry, known as NUA, has shown a high capacity to sorb P and N when applied to soils. This residue is generated in important amounts during the production of synthetic rutile (TiO_2) from ilmenite ($FeTiO_3$) as follows: sulphuric acid is used to purify ilmenite, and the consumed acid is neutralised with quicklime (CaO), resulting in the generation of NUA. The chemical reaction involved in NUA formation is (Douglas *et al.*, 2010):

$$2Fe^{3+} + 3SO_4^{2-} + 3CaO + 3H_2O \rightarrow 2FeOOH + 3CaSO_4 \cdot 2H_2O$$

Douglas *et al.* (2010) were the first to observe that the amendment of a soil – from a turf farm – with NUA (at a ratio of 5 – 10 % by mass) change the physical and chemical soil properties. First, the content of nutrients into leachates were reduced: phosphorus (PO_4-P) leaching was reduced by 97%, nitrogen (both NH_3-N and NO_3-N) was better retained in soil (82% and 40%, respectively), and the concentration of other trace elements (i.e. La, As, Cd, Cr, Th, Fe, etc.) was substantially reduced in leachates. Second, the leachate pH of the amended soil increased by 1 – 1.5 units, but its volume decreased ca. 1.8 times. Third, amended soils showed a higher environmental radioactivity (measured as absorbed dose rates) than unamended, but lower than soils where fertilizers or other amendments were applied. Hence NUA had potential to be used not only as a soil amendment for unretentive soils, but also to reduce and retain nutrients in a wide range of soils.

Wendling *et al.* (2012) used NUA to remove nutrients and to dissolve organic carbon (DOC) from water bodies, preventing the deficient oxygen conditions caused by algal blooms. In this study, the authors used the mining by-product as a filtration media in steel columns filled with both NUA and sand, obtaining high sorption values: 7.5 gDOC·$KgNUA^{-1}$, 20.88 mg total P·$KgNUA^{-1}$ and 357 mg total N·$KgNUA^{-1}$. Also mixes of NUA with

other mining and metallurgical by-products were tested, achieving high removal efficiencies: > 95% of total P was removed, 25 – 51 % greater DON sorption, and 18% greater DOC elimination. The high capacity of NUA to retain P was driven by two mechanisms: P was mainly retained on the surface of iron oxides impurities, and dissolved Ca led to precipitation and co-sorption of both Ca and P; DOC attenuation occurred due to several sorption and precipitation reactions; and dissolved organic nitrogen (DON) decline can be explained not only by the Ca precipitation, but also by its sorption and/or ion exchange onto solid surfaces. Consequently, NUA demonstrated effective properties to remove nutrients and DOC from water streams, mitigating the eutrophication of freshwater systems.

Once it was assessed that NUA could attenuate DOC in water streams, Oliver *et al.* (2013) investigated its effectiveness to remove organic compounds from water. In this study, the capacity of NUA to remove commercial pesticides (atrazine, diuron, 2,4-D and chlorpyrifos) by sorption was evaluated in 6 hours' experiments. First, the removal of each compound was assessed individually, obtaining good sorption rates and demonstrating that the sorption of each compound was related with its water solubility: 69% sorption of atrazine, 89% diuron, 61% 2,4-D, and 83% chlorpyrifos. Second, it was assessed the removal of 3 pesticides in mixtures, showing that diuron removal was not affected, atrazine was less sorbed (11% less) but it was

not significant, and 2,4-D was notably less sorbed. The removal diminution of 2,4-D was explained because pesticides with higher affinity are less affected in multiple systems with competitive sorption. Finally, the sorption of these pesticides onto NUA was evaluated in the presence of nutrients (NO_3^- and PO_4-P) at different concentrations. Results showed that diuron was not affected (>90% removal), atrazine removal was only affected by the presence of 0.2 mg·L^{-1} NO_3^- (10% less), and 2,4-D was significantly affected (more than 15% less), which can be explained by the competition of the ionic species. In conclusion, this study confirmed that NUA could be applied as low cost sorbent to treat waterways containing organic compounds such as pesticides.

6.1.3. Aim of the chapter

The aim of this chapter was to determine if it was possible to treat a WWTP effluent with low-cost sorbents, improving its final quality and avoiding expensive and highly technified post-treatments technologies. First, the adsorption of a recharge basin soil from an aquifer treatment was evaluated for a selected group of PhACs. Next, an amendment ratio was selected according to its adsorption values for three drugs at different ratios. Finally, the removal of six compounds was measured in soil amended with biochar and NUA.

6.2. Results and discussion

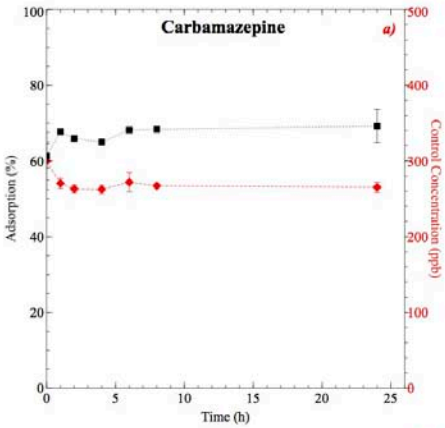
6.2.1. Soil capacity to adsorb pharmaceuticals compounds

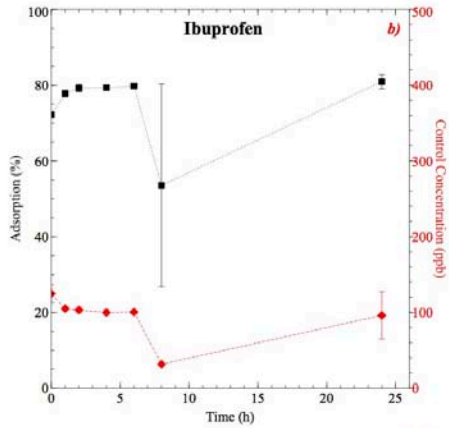
The adsorption percentages of each compound onto the soil are shown in figure 6.1. As can be seen, three main patterns can be used to describe PhACs behaviour in soil: gradual increment (plots ***d***, ***e***, and ***f*** in figure 6.1), rapid initial increase (plots ***a***, ***b***, and ***c*** in figure 6.1), and level behaviour (***g*** in figure 6.1). Additionally, Table 6.1 summarises the calculated K_d values obtained at equilibrium for each compound.

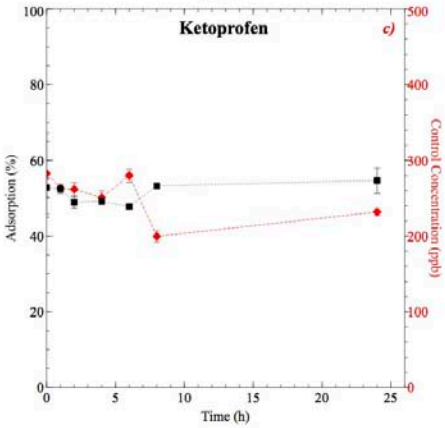
Table 6.1 K_d values for each compound in soil at equilibrium.

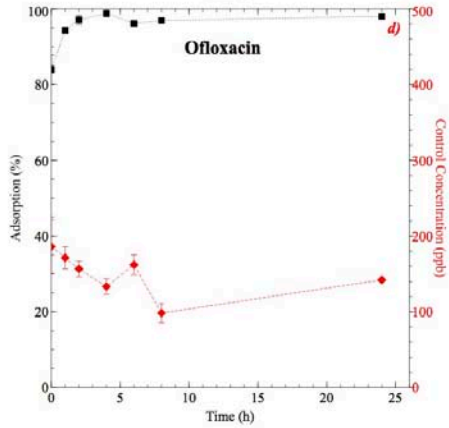
Compound	K_d (mL·g^{-1}) ± SD
KTP	1.21 ± 0.03
Ibuprofen	4.24 ± 0.02
CBZ	2.25 ± 0.05
SMX	4.38 ± 0.03
Propranolol	22.88 ± 0.01
TRM	14.46 ± 0.02
OFX	2487.9 ± 0.4

Compounds that were gradually adsorbed to soil (OFX, PRN, and SMX – figure 6.1 plots ***d***, ***e***, and ***f*** respectively) exhibited a sorption fluctuation between times 4h and 8h – this variation


Carbamazepine
a)
Adsorption (%)
Control Concentration (ppb)
Time (h)


Ibuprofen
b)
Adsorption (%)
Control Concentration (ppb)
Time (h)


Ketoprofen
c)
Adsorption (%)
Control Concentration (ppb)
Time (h)


Ofloxacin
d)
Adsorption (%)
Control Concentration (ppb)
Time (h)

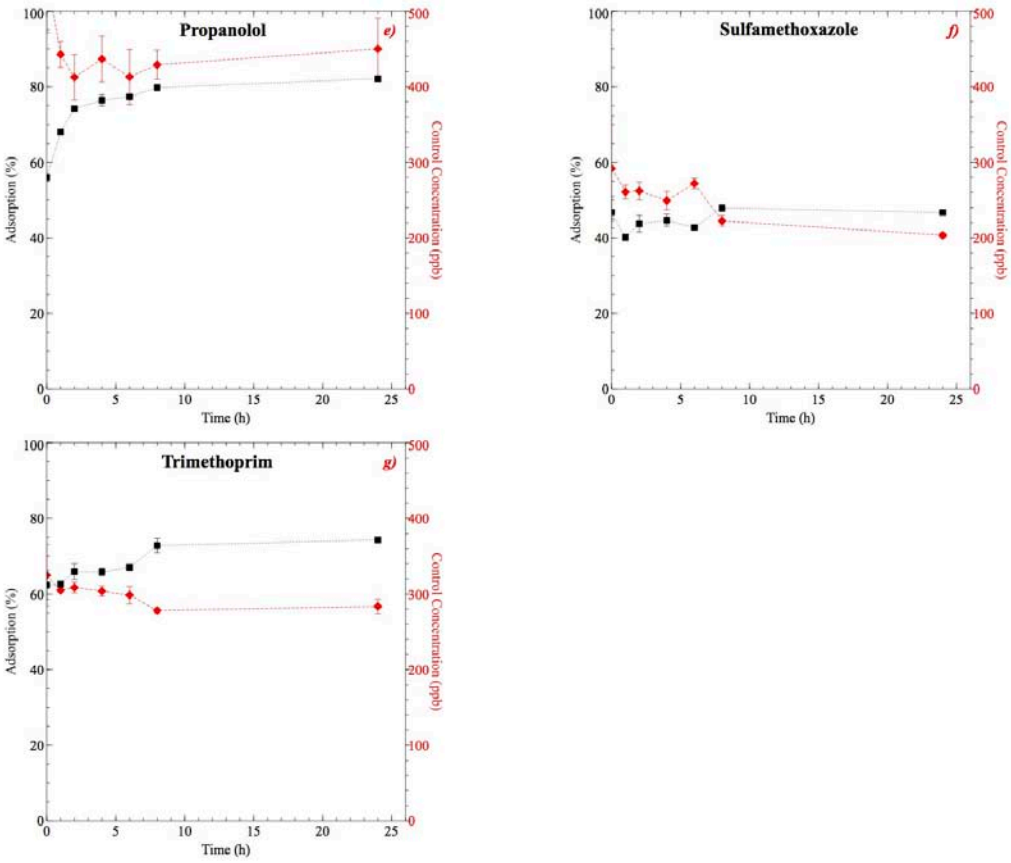


Figure 6.1 Adsorption percentages for each compound onto SAT recharge basin soil. Left axis represents adsorption percentage onto soil ( ), and right axis represents PPCPs concentrations on chemical controls ( ). Error bars stand for the standard error.

was sharper for SMX – followed by a faint increase. The maximum sorption was achieved at time 24h for PRN (82.07%) and SMX (46.72%), while OFX reached the maximum at time 4h (98.78%). In contrast, carbamezapine, IBU, and KTP (***a***, ***b***, and ***c***) had a rapid adsorption increase during the first hour followed by a decrease, especially acute in the case of IBU. After those falls, the adsorption percentage tended to grow up, reaching previous values. For those compounds, the maximum adsorption capacity was achieved at time 24h: 69.19% carbamezapine, 80.91% IBU, and 54.68% KTP. On the contrary, TRM showed a level behaviour with three stages. The first stage, between times 0h and 1h, had an adsorption percentage around 62.5 ± 0.2 %. The second stage comprised times from 2h to 6h and ranged 66.3 ± 0.7 % of adsorption. The third and last stage, between times 8h and 24h, reached an adsorption percentage around 73 ± 1 %.

K_d is a very important parameter to estimate the adsorption potential of a dissolved contaminant in contact with soil or sediments. K_d values are a simplistic but useful model of sorption onto solid matrices because it represents the ratio of an element on the solid phase, implies a linear relationship between sorbed and non-sorbed spices, and attenuation mechanisms and environmental factors are included in empirical K_d values (Leo *et al.*, 1971; Sheppard *et al.*, 2011). As shown in table 6.1, OFX is the compound with the highest K_d value (2487.9 ± 0.4 $mL \cdot g^{-1}$) for the

Alice Springs SAT soil and hence the most adsorbed. While KTP, with a K_d value of 1.21 ± 0.03 mL·g^{-1}, is the weakest adsorbed compound. K_d values for TRM, sulfanethoxazole and CBZ are similar to the results from Kodešová *et al.* (2015), where the researchers tested the adsorption of seven drugs onto 13 different agricultural soils, and IBU's K_d value (4.24 ± 0.02 mL·g^{-1}) was similar to the reported value (3.71 ± 0.46 mL·g^{-1}) for a Palouse silt loam agricultural soil in the USA (Xu *et al.*, 2009). According to these results, the soil from basin A of Alice Springs SAT can adsorb the tested PhACs, but with a wide range of adsorption capacities depending on the compound. Improving soil capacity to adsorb more mass of drugs with low-cost sorbents could lead to a better SAT performance with a low economical impact.

6.2.2. Selection of the amendment ratio

After assessing the capacity of the recharge basin soil to adsorb drugs at a concentration of 500ppb, different ratios of soil:amendment were tested in order to assess which one would be the most suitable for a further treatment according to the K_d values for three PhACs (Table 6.2). First, TRM showed the higher adsorption onto the soil:amendment matrix with NUA at 1% (13.42 ± 0.23 mL·g^{-1}) and the lowest with biochar at 0.5% (1.31 ± 0.58 mL·g^{-1}); however, the higher result was slightly lower than the K_d value obtained in bare soil (14.46 ± 0.02 mL·g^{-1}). Second,

Table 6.2 K_d values for tested compounds at equilibration point [$mL \cdot g^{-1}$] (± standard error).

Trimethoprim				
Ratio (%)	***NUA + Sand***	***NUA + Soil***	***Biochar + Sand***	***Biochar + Soil***
0.1	2.92 ± 0.44	13.42 ± 0.35	0	3.12 ± 0.17
0.5	*	10.37 ± 0.33	*	1.31 ± 0.58
1	0.37 ± 0.30	13.42 ± 0.23	0	2.09 ± 0.11
2	0.88 ± 0.39	9.45 ± 0.09	0.43 ± 0.32	1.46 ± 0.49
5	0.48 ± 0.21	11.60 ± 0.03	*	1.83 ± 0.10
Propanolol				
Ratio (%)	***NUA + Sand***	***NUA + Soil***	***Biochar + Sand***	***Biochar + Soil***
0.1	2.69 ± 0.04	18.44 ± 0.12	8.74 ± 0.30	14.06 ± 0.27
0.5	5.23 ± 0.04	18.38 ± 0.03	11.48 ± 1.02	24.52 ± 0.27
1	3.76 ± 0.29	17.53 ± 0.04	17.11 ± 0.04	15.81 ± 0.07
2	5.47 ± 0.13	17.68 ± 0.10	39.39 ± 0.13	28.54 ± 0.14
5	9.59 ± 0.29	40.17 ± 0.04	39.81 ± 0.31	33.42 ± 0.07
Sulfamethoxazole				
Ratio (%)	***NUA + Sand***	***NUA + Soil***	***Biochar + Sand***	***Biochar + Soil***
0.1	*	4.83 ± 0.09	34.70 ± 0.08	29.62 ± 0.02
0.5	*	3.76 ± 0.07	36.97 ± 0.00	35.80 ± 0.01
1	*	4.53 ± 0.00	38.85 ± 0.04	44.88 ± 0.03
2	*	4.63 ± 0.04	56.30 ± 0.04	68.35 ± 0.04
5	0	4.55 ± 0.04	181.74 ± 0.14	181.04 ± 0.14

*K_d value not assessed, measured concentration was higher than the initial

PRN was better adsorbed in soil and NUA, reaching a maximum K_d value of 40.17 ± 0.04 $mL \cdot g^{-1}$ with a NUA ratio of 5%. PRN's adsorption for soil (22.88 ± 0.01 $mL \cdot g^{-1}$) was improved with NUA at 5% – although for other ratios the K_d value was high – and with biochar at 0.5, 2 and 5%. Third, SMX adsorption was enhanced for the greatest part of the tested treatments – except for NUA at 0.5% –, and it was especially high using biochar as amendment:

from 29.62 ± 0.02 $mL \cdot g^{-1}$ at 0.1% to 181.04 ± 0.14 $mL \cdot g^{-1}$ at 5%. When the tested ratios were compared in each group using ANOVA, only PRN in soil amended with NUA (Table 6.3) and TRM in soil with biochar (Table 6.4) did not show significant differences between ratios. Briefly, PRN and SMX were better adsorbed with biochar, while TRM was more adsorbed in NUA.

Table 6.3 One-factor ANOVA on the result of TRM adsorption in soil amended with biochar.

Source of variation	d.f.	S.S.	M.S	f ratios
Soil/Amendment	4	102.8101	25.7025	0.7315^{ns}
Error	30	1054.1188	35.1373	-
Total	34	1156.9289	-	-

d.f.= degree of freedom; S.S.= sum of squares; M.S.= mean squares; s/ns= significant or not significant at $p < 0.5$

Comparing drugs' K_d values for NUA and biochar (Table 6.2) with the ones obtained for only soil (Table 6.1), it can be seen that TRM was better adsorbed in unamended soil, but with similar values to a NUA addition of 0.1% and 1%; PRN adsorption was improved using NUA at 5% and biochar at 0.5, 2 and 5%; and SMX was better adsorb in all the cases – except NUA at 0.5% –, but especially high when biochar was used. pH evolution for soil amended with NUA or biochar at 1%, and for unamended soil is showed in figure 6.2. While pH solution of bare soil was stable with few oscillations (ranged ca. 7.5 – 7.75 units), amended soils exhibited higher initial pH values (8.6 for biochar and 8.3 for

NUA), followed by a decrease until reaching a final pH of 7.8 and 7.5 for biochar and NUA treatments, respectively.

Table 6.4 One-factor ANOVA on the result of propranolol adsorption in soil amended with NUA.

Source of variation	d.f.	S.S.	M.S	f ratios
Soil/Amendment	4	181.3721	45.3430	0.1911^{ns}
Error	30	7119.2122	237.3071	-
Total	34	7300.5844	-	-

d.f.= degree of freedom; S.S.= sum of squares; M.S.= mean squares; s/ns= significant or not significant at $p < 0.5$

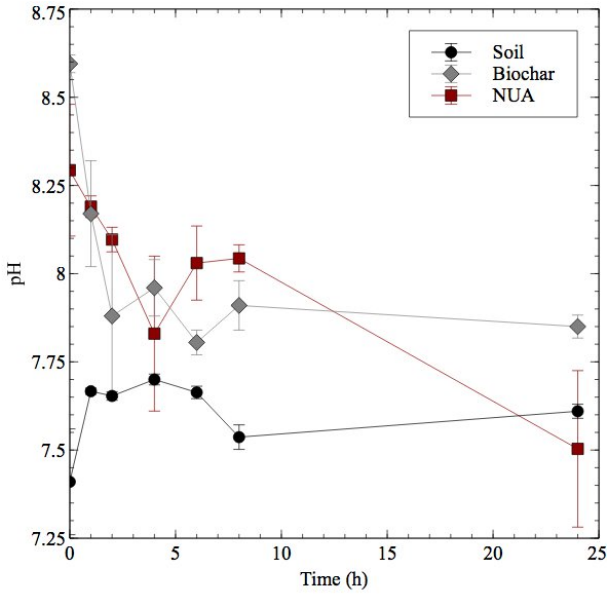


Figure 6.2 pH evolutions for bare soil and for soil amended with NUA and biochar (1% ratio). The lines represent the pH of the $CaCl_2$ solution, with a soil:solution ratio of 1:5 g:mL. Error bars stand for the standard error.

Borgman and Chefetz (2013) found that not only the loading of biosolids (i.e. organic matter from sewage used in agriculture) would affect the transport of pollutants trough soil, but also pH was an important factor in the mobility and retention of PhACs in saturated soils. These researchers observed that some pharmaceutical compounds were poorly retained in soil columns with biosolids, even when the amounts of biosolids were increased. Furthermore, they observed that pH was the predominant factor affecting pollutants mobility in saturated soil columns; especially for weak acids, where pH values 3-4 units above the pK_a of the compound would lead to significant mobility changes. Also specific interactions – such as hydrophobic effect and van der Waals interactions – could explain the improvement or diminishment of compounds retention on solid matrices. Essandoh *et al.* (2015) observed similar pH effects in the removal of salicylic acid and IBU from aqueous solutions with biochar. These authors found that the highest removal percentage was achieved under acid conditions, and adsorption decreased with pH increase. Biochar's charge depends on pH: rising pH values lead to negatively charged biochars, and hence increases electrostatic repulsion and decreases adsorption capacity; however, these researchers also found an intermediate pH region – 2 – 3 units above the compound's pK_a –, where adsorption experienced a brief increase followed by a fall. At this pH range, carboxylate anion

forms of the compounds predominated on biochar's surface, which momentarily adsorbed more mass of the same compound.

The amount of amendment to be used was another key factor for further experiments and for real application. Even though a better retention of the compounds was reached with higher amounts of NUA and biochar, these could lead no only to higher operational costs, but also to modifications in soil microcosm. In general, microbial populations influence soil fertility, plant growth, and degradation of pollutants. Previous studies showed that a soil amended with biochar could provide a suitable habitat and both nutrient and carbon sources (Steinbeiss *et al.*, 2009); however, high levels of immobilized drugs in the amendment:soil matrix could generate adverse effects on the autochthonous microbial population. Yao *et al.* (2012) observed that soils amended with biochars that adsorbed high levels of SMX showed an antibiotic effect on *Escherichia coli* populations; thus, high amendment ratios, which led to high adsorption rates, should not be the best choice to treat water streams via soil amendment.

According to these, for further experiments a soil:amendment ratio of 1% was selected because at this ratio the tested compounds were well retained, pH was similar to unamended soil, and a higher percentage of amendment would turn into higher operational costs.

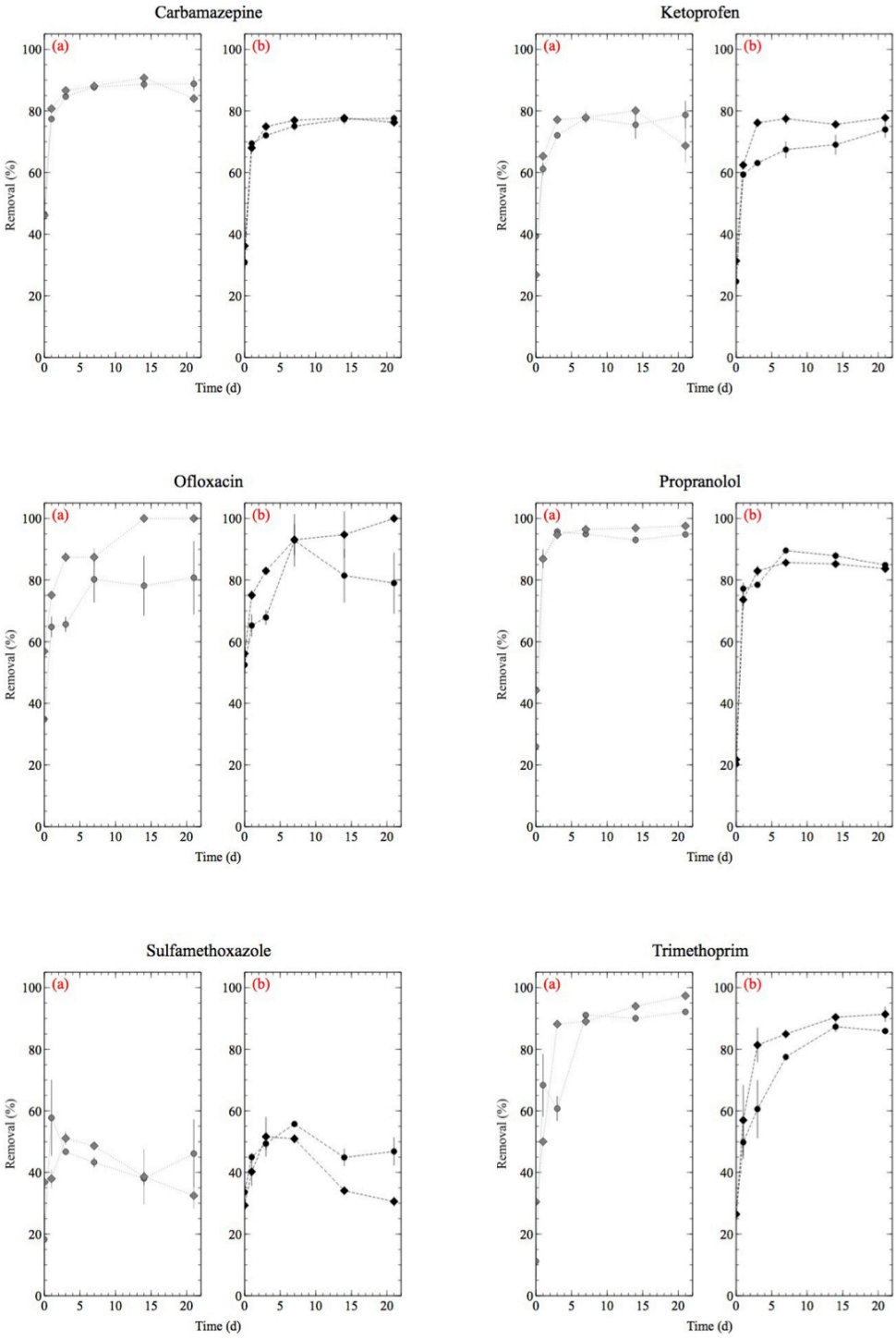


Figure 6.3 Removal percentage of the selected drugs. For each compound, biochar (plots a) in soil (◆) and sand (●), and NUA (plots b) in soil (◆) and sand (●) were applied at a ratio of 1%. Error bars stand for the standard error.

6.2.3. Removal of selected pharmaceuticals from amended soil

As can be seen in figure 6.3, the main pattern for CBZ, KTP, OFX, PRN and TRM was a constant and gradual increase of the removal percentage. In contrast, SMX showed a maximum removal during the firsts 3 – 7 days, followed by a regular decrease. On the contrary, IBU (data not shown) exhibited a different evolution: constant fluctuations – from 0% to 77% – with high errors – more than 15% of the value. Furthermore, IBU's chemical control showed a low recuperation ratio. For these reasons, IBU removal was not assessed and it was not taken for further deliberations.

The highest removal in soil was obtained for OFX, reaching a 100% removal by day 14 with biochar and day 21 with NUA. TRM was the second better-removed pharmaceutical: 97.4 ± 0.4 % in biochar and 91.4 ± 2.5 % in NUA, both at day 21. Likewise TRM, PRN obtained a high removal with biochar (97.6 ± 0.3 %) at the end of the experiment (21d); however, this percentage was lower with NUA (85.6 ± 0.3 %), and it was obtained the day 7 of treatment. CBZ followed a similar trend: the higher removal was obtained with biochar (90.7 ± 0.7 % at day 14), while the removal with NUA was significantly lower (77.7 ± 1.1 % at day 14). In contrast, KTP showed similar patterns for both biochar and NUA, reaching similar removal rates: 80.1 ± 0.4 % at day 14 and 77.8 ± 0.6 % at day 21 for biochar and NUA, respectively. On the contrary, SMX was the lowest-removed compound with values

below 60%, and it followed a different trend: highest removals were achieved at the beginning (51.1 ± 2.1 % in biochar and 51.7 ± 6.4 % in NUA, both at day 3) and the lowest values were obtained the last day of experiment (32.5 ± 4.2 % and 30.6 ± 1.6 % for biochar and NUA, respectively). Despite the fact that the highest removal percentages were obtained with soil amended with biochar, there are no statistical differences between amendments according to ANOVA analyses (data not shown).

Biochar has been used as soil amendment for a long time in order to improve its physical and chemical properties, and in recent years it has been applied as low-cost sorbent in wastewater treatments, replacing high cost treatments such as activated carbon. NUA started to be a novel amendment a few years ago, initially as a soil amendment and then as a low-cost sorbent to remove nutrients, DOC and pesticides. Indeed, only a few articles studied these materials as a way to treat polluted water bodies or streams with emerging contaminants by amending soils with them, in particular in soil aquifer treatment of reclaimed water. However, Jung *et al.* (2015) studied the adsorption of three NSAIDs on biochar. The authors tested the ability of two biochars to adsorb three NSAIDs (i.e. diclofenac, naproxen, and IBU) in single and multiple solutions, with adsorptions above 200 $mg \cdot g^{-1}$. Authors suggested that not only biochar structure played an important role in drugs adsorption, but also the chemical and physical properties of the compounds.

As biochars had limited adsorption places, drugs in decreasing order of size, bond energy and polarity occupied these places. Consequently, a shield or cloud of adsorbed PhACs around the biochar was formed, avoiding the adsorption of other compounds. In our study, compounds removal was assessed in a multiple solution and followed the order of OFX > TRM = PRN > CBZ > KTP > SMX for biochar, and OFX > TRM > PRN > CBZ = KTP > SMX for NUA. These removal orders were similar to the decreasing order of polarizability (OFX > PRN > TRM > KTP > CBZ > SMX) with minor changes: the shift of TRM for PRN – which had similar polarizability values – and the shift of CBZ for KTP.

Yao *et al.* (2012) applied biochar to soil in order to treat reclaimed water used for irrigation, as this type of water was pointed as a potential pollution source of soils and ground water. The authors tested two biochars in soil columns and measured the amount of SMX in leachates. Although all leachates had SMX, unamended soil columns had 6 – 65 times more SMX in the leachates than amended columns. In addition, after flushing the columns with an SMX-free solution, the researchers found that unamended soil columns still showed high SMX concentration in the leachate; stating that SMX had a greatest mobility in these soils. Therefore, amending soils with low-cost sorbent could be novel technology to treat reclaimed water and prevent pollution of ground water.

Advanced treatments or post-treatments for WWTP effluents have became interesting technologies to remove those

pollutants that cannot be removed in traditional secondary treatments. Among others, filtration and ozonation techniques have been studied due to their capacities to eliminate PhACs from reclaimed water streams. Nakada *et al.* (2007) studied both techniques in a joint treatment to eliminate drugs in a real secondary effluent of a WWTP. The researchers observed that the lowest removals were achieved during sand filtration (the first stage of the advanced treatment): removal percentages below 50% for low hydrophobic compounds (such as SMX and CBZ) and above 80 % for more hydrophobic compounds (such as IBU); however, these removal percentages were increased with the ozonation of the stream, reaching an average removal percentage greater than 80%. Compared to soil amendment with biochar or NUA as a treatment for reclaimed water, it can be seen that, although our experiments were performed at higher drugs concentration (500ppb in front of the 0.29 – 0.01 ppb range concentration), a similar removal rate was reached for TRM (97.4% with soil:biochar and 96.0% with sand filtration and ozonation); KTP and SMX were better removed with sand filtration followed by ozonation than with biochar (93.2% and 80.1% for KTP, and 87.4% and 51.1% for SMX; percentages for sand filtration/ozonation and biochar treatments, respectively); and CBZ was better removed in amended soil treatments (90.7% biochar and 77.7% NUA) than in filtration/ozonation treatment (8.25%). However, the amendment of soil with NUA and biochar

would imply lower costs than the ozonation treatment. According to Filiberto and Gaunt (2013), producing biochar would cost between $50 and $500 (United States dollars) per ton – depending on manufacturing factors –, and application into soils would cost $60-70/ha (cost for 25 ton per hectare). Given a total soil mass for the Alice Springs SAT's recharge basins of 315,000 Ton (surface: 10,269 m^2; depth: 17.5 m; and bulk density: 1.8 Ton·m^{-3}), 3,150 Ton of biochar – or NUA – would be necessary. Consequently, the cost of amending the soil in order to treat 317.4 ML·$year^{-1}$ of reclaimed water would range from $170,000 to $1,700,000. In contrast, the ozonation of the same amount of reclaimed water would have an implementation cost of ca. $300,000 and operational and maintenance costs per year around $20,000 (U.S. EPA., 1999).

6.3. Conclusions

Biochar and NUA land application in order to improve soil properties, enhance microbial communities and sequester carbon has been deeply studied in the past. In recent years, these by-products have been demonstrated as suitable low-cost sorbents to retain not only nutrients in soils, but also to remove contaminants such as pesticides. Nowadays, these sorbents are being used to adsorb emerging contaminants from water in filters with or without soil or sand.

The adsorption of 7 PhACs onto unamended soil from a recharge basin of a soil aquifer treatment was evaluated. The highest adsorption was measured for OFX followed by propranolol and the lowest for KTP. Additionally, the best amendment ratio was assessed according to the adsorption of three drugs at different ratios of the two tested amendments. A ratio of 1% was selected, as the compounds were well retained and the pH was similar to unamended soil. Finally, the removal of 6 compounds was measured in soil amended with biochar and NUA at 1% during 21 days. The results showed high removal percentages for all the tested PhACs, achieving a 100% removal of OFX in both biochar and NUA amendments, followed by TRM with removal percentages above 95% in both amendments.

It has been demonstrated the efficiency of biochar and NUA to remove emerging contaminants from water by amending soil with them. Although biochar showed higher and faster removal rates, there was no statistically difference with NUA. Further studies should assess which amendment is more suitable after testing real reclaimed water from Alice Springs SAT, and how the autochthonous microbes are affected by the treatment.

CHAPTER 7

Concluding Remarks

The removal of micro-pollutants from different streams of wastewater treatments with fungal mediated systems and adsorption to low-cost sorbents has been demonstrated. First, the utilisation of fungal biopiles with a forestry by-product as substrate and bulking material has been proved as a viable method to treat thermal dried sewage sludge, obtaining elevated removal yields. Furthermore, the microbial analysis of the biopiles demonstrated that not only *T. versicolor* survived more than 22d under non-sterile conditions, but also it accelerated the switch to fungal and bacterial communities more adapted to degrade organic pollutants in solid-phase systems. Second, the treatment of MBR sludge in a bioslurry system with WRF has been described for the first time, achieving high removal rates for the total PPCPs content. Additionally, a revalorisation method for the effluent of the fungal bioslurry has been proposed. Third, the post-treatment of WWTP effluent in order to remove the EPs from reclaimed water used for artificial aquifer recharge has been studied, reaching considerable adsorptions with the studied materials.

High removal yields were reached with the fungal bioslurry when treating MBR sludge. However, in pilot (or higher) scale this waste stream would not be treated as sludge, instead bioreactors would be used or liquid and solid phase would be separated; treating each one with different methods. In contrast, biopiles with thermal dried sludge achieved middle-

high removal yields, but the process was feasible and it could be scalable. Additionally, a low-cost measure helped to reduce the concentration of drugs in the final effluent of a WWTP, improving the overall system. Globally, it has been proved that the different streams of a WWTP can be treated in order to remove the PhACs and reduce their global impact in the environment. Hence, the cycle of the PPCPs could be closed in the WWTPs improving the plants with remediation techniques.

Although extensively research has been performed, further research in this field should be carried out and focused to:

- Increase the scale of the biopiles: since it has been proved that the fungus was able to colonize and degrade EPs using pine bark as lignocellulosic substrate and bulking material, a pilot scale biopile (500- 2,000 kg) to treat sewage sludge should be performed. Other researchers have demonstrated that it was possible to treat soils polluted with recalcitrant organic pollutants in biopiles at pilot scale, but there is a lack of information regarding the elimination of PPCPs from sewage sludges in biopiles systems at this scale.
- Improve the treatment of MBR sludge: the concentration of solids in this type of sludge was low, so other ways to treat it should be studied. The solid and liquid phase of the selected MBR sludge could be separated by continuous centrifugation, making it

possible to treat each stream separately: the liquid flux in bioreactor and the solid flux in biopiles.

- Study the PPCPs adsorption in continuous mode and with real reclaimed water: the adsorption dynamics should be studied in continuous mode so as to obtain a better understanding of the adsorption/desadsorption processes in SAT systems improved with biochar or NUA. Also, real reclaimed water should be used, in order to assess and evaluate the behaviour of both low-cost sorbents under real conditions.
- Search new ways to valorise the resulting biomass from bioremediation processes: the anaerobic digestion of the slurry biomass was studied, but the low solids content of the MBR sludge, and the poor methanogenic activity of the selected AD inoculums lead to unsatisfactory re-use of the biomass. Further experiments should search not only new AD inoculums, but also search new ways to revalorise the resulting solid from biopiles systems, such as amendment for forestry or agricultural soils or to restore landfills and quarries.
- Establish the environmental and economical feasibility of the treatments: Life Cycle Assessment and Risk Assessment tools should be used in order to ascertain if

the proposed biological and physical treatments are adequate from an environmental point of view.

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