**Departament d'Enginyeria Química**

**Escola d'Enginyeria**

**Universitat Autònoma de Barcelona**

# Study of relevant factors in the treatment of effluents by fungi for the degradation of emerging contaminants

**PhD Thesis**

**Marina Badia Fabregat**

**Bellaterra, Setembre 2013**

**Title:** Study of relevant factors in the treatment of effluents by fungi for the degradation of emerging contaminants

**Carried out by:** Marina Badia Fabregat

**Supervised by:** M. Teresa Vicent Huguet and Glòria Caminal Saperas

PhD program in Environmental Science and Technology

Departament d'Enginyeria Química

Escola d'Enginyeria

Universitat Autònoma de Barcelona

Bellaterra, 2014

This work has been funded by the Spanish Ministries of MICINN (projects CTQ2010-21776-C02, CTM2013-48545-C2 and AIB2010PT-00169) and MMAMRM (project 010/PC08/3-04.1). The author acknowledges the predoctoral fellowship from Universitat Autònoma de Barcelona and the research stay grant from the Generalitat de Catalunya (2012 BE1 00210).

Part of this work has been done in collaboration with the Institute of Environmental Assessment and Water Studies (IDAEA) from the Department of Environmental Chemistry, CSIC (Barcelona, Spain), the Catalan Institute for Water Research (ICRA) (Girona, Spain) and the Department of Crystallography, Mineralogy and Mineral Deposits in the University of Barcelona (Barcelona, Spain).

Part of the work presented has been performed in research stays at Centre of Biological Engineering (CEB) of University of Minho under the supervision of Dr. Alcina Pereira and Dr. Madalena Alves and at Finnish Forest Research Institute (METLA) under the supervision of Dr. Taina Pennanen and Dr. Hannu Fritze.

GLÒRIA CAMINAL SAPERAS, Científica Titula del CSIC a la Unitat de Biocatàlisis Aplicada associada al IIQAB, i M. TERESA VICENT I HUGUET, Professora Titular del Departament d'Enginyeria Química de la Universitat Autònoma de Barcelona,

CERTIFIQUEM:

Que la llicenciada en Biotecnologia Marina Badia Fabregat ha realitzat sota la nostra direcció, en els laboratoris del Departament d'Enginyeria Química, el treball que amb el títol "**Study of relevant factors in the treatment of effluents by fungi for the degradation of emerging contaminants**" es presenta en aquesta memòria, la qual constitueix la seva Tesi per a optar al Grau de Doctor per la Universitat Autònoma de Barcelona.

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

Bellaterra, Setembre 2014

## ACKNOWLEDGEMENTS

First of all, I would like to thank my thesis supervisors Teresa and Glòria for offering me the opportunity to work in their research group. I sincerely appreciate their advice and encouragement from the beginning until the end.

Thank you to all the “Toxics” people, with whom I shared some steps of this thesis: Edu, Marcel, Carles, Carlos, Belén, Rim, Guillem, Andrea and Cesc. Of course, I would also like to express my gratitude to all the “permanent staff” of the group, who in a way or another helped me at different points of my research: Lucia(s), Ernest, Paqui and Montse.

I also want to acknowledge the researchers of the chemical analytical groups with whom we have shared projects, without them this work with real effluents would not have been possible. Thus, I would like to specially thank Pablo Gago and Sílvia Díaz from IDAEA-CSIC and Daniel Lucas, Marta Llorca, Meritxell Gros and Sara Rodríguez from ICRA.

Thank you also to Dr. Benjamí Piña to provide us with the RYA strains and to his group, in particular to Alba Olivares, for her time, dedication and patience to show me the protocol for RYA assays.

Of course, I have a special mention to Ernest and Mònica for making me discover the world of stable isotopes. I would also like to acknowledge the advice from Dr. Petra Bombach about mathematical calculations behind isotope labelling and Dr. Magdalena Grifoll for her kindness on sharing her laboratory for some experimental settings.

I would also like to acknowlege the staff of the veterinary hospital of UAB to let us collect the water and thanks also to the maintenance team of the veterinary faculty for their help. Thank you to Jordi Moreno, Michele Stefani, Hèctor Monclús and Joaquim Comas from Lequia (UdG), as well, for providing us with the reverse osmosis concentrate.

I would like to aknowledge Dr. Madalena Alves and especially Dr. Alcina Pereira for their dedication at my work during the stay in Braga. Thank you also to all the labmates, especially Marta. Despite it was a short visit, they made me feel like I had been always there.

Thanks to Dr. Taina Pennanen and Dr. Hannu Fritze not only for their supervision in the lab but also for taking care of me in Finland. I would also like to thank Dr. Tero Tuomivirta. Of course, thank you to all Puimala people, who made those days a great experience. Finally, thanks to Lara to propose me to go to her former lab at METLA for my research stay, it was a good piece of advice.

I would also like to acknowledge the rest of the members of the Chemical Department of UAB: professors, technicians, secretaries and colleagues, for making these years more pleasant. Special thanks to everybody from “Dimecres de Pastís” for all the good moments inside and outside the Department.

Thank you too to my friends and Marcel’s family, with many of whom we have shared the same troubles and joys that theses entail.

And, last but not least, thank you to the three most important persons in my life and who gave me more support during all this time and that, inevitably, have also suffered the consequences. To my parents and Marcel, thank you for everything!

# LIST OF CONTENTS

## ABSTRACT

Emerging contaminants are a wide group of organic compounds detected in many environmental compartments. Even though their environmental concentration is usually in the range of ng $L^{-1}$ to low µg $L^{-1}$ (much lower than conventional organic pollutants), they still represent a threat to human health and the environment. Among emerging contaminants, pharmaceutically active compounds (PhACs) and endocrine disrupting compounds (EDCs) are of special concern. It is widely accepted that the main source to the environment are the effluents of wastewater treatment plants (WWTPs), where conventional activated sludge treatments are not able to degrade most of them. Therefore, alternative treatments should be found. One of those alternatives might be the use of ligninolytic fungi by taking advantage of their enzymatic system, that conferes them the ability to degrade a broad range of contaminants.

The present thesis assesses different factors related to the fungal degradation of emerging contaminants. The widely studied white-rot fungus *Trametes versicolor* has been chosen to carry out all the experiments of this thesis.

First of all, individual degradation of selected contaminants was studied. Taking into account that EDCs degradation has been less studied than PhACs, six EDCs belonging to the groups of UV filters (benzophenone-3 (BP3), benzophenone-1 (BP1) and 3-(4-methylbenzylidene) camphor (4-MBC)) and benzotriazoles (1H-benzotriazole (BTZ) and tolyltriazole, a mixture of 4-methylbenzotriazole (4-MBTZ) and 5-methylbenzotriazole (5-MBTZ)) were selected. Their degradation by *T. versicolor,* acute toxicity, estrogenic and dioxin-like activities were monitored, the fungal metabolites were identified and the first steps of the degradation pathway were suggested.

Moreover, the fate during fungal degradation of certain contaminants (BP3 and the analgesic and anti-inflammatory diclofenac) was assessed by means of compounds labelled with the stable isotope $^{13}C$. Combination of analyses of carbon isotopic composition of $CO_2$, bulk biomass and amino acids-stable

isotope probing (aa-SIP) allowed the distinction between simple transformation, oxidative mineralization or carbon incorporation into the biomass. Regarding the two studied compounds, both of them were mineralized, but only BP3 was found to be used as carbon source and incorporated in the fungal biomass.

On the other hand, two real effluents (veterinary hospital wastewater and a reverse osmosis concentrate from a pilot plant treating urban wastewater) were treated in fungal air-pulsed fluidized bioreactors under different operational conditions (sterile/non-sterile and batch/continuous) in view of a possible future implementation. With respect to that, the present thesis points out the importance of an external addition of nutrients and the control of aeration, which should be further optimized for an efficient removal of contaminants by the inoculated fungus.

The importance of conjugation and deconjugation processes is also highlighted in this thesis. They are a restriction in the assessment of emerging contaminants degradation in real effluents due to the absence of conjugates in the analytical methods and, at the same time, conjugates are important intermediate metabolites in the fungal degradation of the selected contaminants.

Molecular biology analyses (phospholipid fatty acids analysis (PLFA), real-time PCR (qPCR) and denaturing gradient gel electrophoresis (DGGE)) were performed as well with the aim of finding some correlation between the operation of the bioreactors and the performance of the inoculated fungus and the other microorganisms that could develop in the non-sterile bioreactors. Results suggest that the classical parameters monitored (i.e. laccase activity) might not be good indicators of inoculated fungus survival and predominance.

## RESUM

Els contaminants emergents són un ampli grup de compostos orgànics detectats en diversos compartiments ambientals. Tot i que la seva concentració normalment està compresa entre els ng/L fins a pocs µg/L (força inferior que els contaminants orgànics convencionals), poden representar una amenaça per a la salut humana i el medi ambient. D'entre tots els contaminants emergents, els principis actius dels fàrmacs (PhACs) i els compostos disruptors endocrins (EDCs) generen una especial preocupació. Per altra banda, està àmpliament acceptat que la seva principal font d'entrada al medi ambient són els efluents de les plantes depuradores, on els tractaments convencionals de llots actius no són capaços de degradar-ne la majoria. Per tant, s'han de buscar tractaments alternatius. Una d'aquestes alternatives podria ser l'ús de fongs ligninolítics, aprofitant el seu sistema enzimàtic que els hi confereix l'habilitat de degradar un rang molt ampli de contaminants.

Aquesta tesi avalua diferents aspectes relacionats amb la degradació de contaminants emergents per part de fongs. El fong de podridura blanca *Trametes versicolor*, àmpliament estudiat, és el que s'ha triat per a dur a terme els experiments d'aquesta tesi.

Primer de tot s'ha estudiat la degradació individual de determinats contaminants. Tenint en compte que la degradació dels EDCs ha estat menys estudiada que la dels PhACs, es van seleccionar sis EDCs pertanyents als grups dels filtres UV (benzofenona-3 (BP3), benzofenona-1 (BP1) i 3-(4-metilbenzilidè) càmfor (4-MBC)) i dels benzotriazols (1H-benzotriazol (BTZ) i toliltriazol, una mescla de 4-metilbenzotriazol (4-MBTZ) i 5-metilbenzotriazol (5-MBTZ)). S'ha fet, doncs, un seguiment de la seva degradació per part de *T. versicolor*, la toxicitat aguda i les activitats estrogènica i de tipus dioxina, s'han identificat els metabòlits generats pel fong i s'han suggerit els primers passos de la via de degradació.

A més a més, el destí de determinats contaminants (la BP3 i l'analgèsic i antiinflamatori diclofenac) durant la seva degradació per part del fong ha estat avaluada a través de compostos marcats amb l'isòtop estable $^{13}C$. La combinació d'anàlisis de la composició isotòpica del C del $CO_2$ i de la biomassa total i el sondeig d'isòtops estables en aminoàcids (aa-SIP) han permès la discriminació entre simple transformació, mineralització oxidativa o incorporació del carboni a la biomassa. Pel que fa als dos compostos estudiats, els dos s'han mineralitzat però s'ha vist que només la BP3 s'utilitza com a font de carboni i és incorporada a la biomassa del fong.

Per una altra banda, es van tractar dos efluents reals (l'aigua residual d'un hospital veterinari i el concentrat d'osmosi inversa d'una planta pilot de tractament d'aigües residuals urbanes) en un bioreactor de fongs fluïditzat per polsos d'aire i operat sota diferents condicions operacionals (estèril/no estèril i discontinu/continu) en vistes a una possible implementació futura. Amb aquesta intenció, aquesta tesi apunta a la importància de l'addició externa de nutrients i al control de l'aeració, els quals haurien de ser optimitzats per a obtenir una eliminació eficient de contaminants per part del fong inoculat.

En aquesta tesi també es remarca la importància dels processos de conjugació i desconjugació. Per una banda, són una una restricció en l'avaluació de la degradació en efluents reals a causa de la seva absència en els mètodes analítics i, per l'altra, els conjugats representen uns metabòlits intermedis importants durant la degradació per part del fong dels contaminants seleccionats .

També es van realitzar anàlisis de biologia molecular (anàlisi dels àcids grassos dels fosfolípids (PLFA), PCR quantitativa (qPCR) i gel d'electroforesis en gradient desnaturalitzant (DGGE)) amb l'objectiu de trobar alguna correlació entre l'operació dels bioreactors i el comportament del fong inoculat i els altres microorganismes que es desenvolupen en els bioreactors no estèrils. Els resultats suggereixen que els paràmetres de seguiment clàssics (com poden ser l'activitat lacasa) podrien no ser uns bons indicadors de la supervivència i predominança del fong inoculat.

# LIST OF ABBREVIATIONS

| | |
|---|---|
| 4DHB | 4,4’-dihydroxybenzophenone |
| 4HB | 4-hydroxy-benzophenone |
| 4-MBC | 3-(4’-methylbenzylidene)camphor |
| 4-MBTZ | 4-methyl-1H-benzotriazole |
| 5-MBTZ | 5-methyl-1H-benzotriazole |
| ABT | 1-aminobenzotriazole |
| ABTS | 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt |
| AhR | Aryl hydrocarbons receptor |
| AOP | Advanced oxidation processes |
| ARDRA | Amplified ribosomal DNA restriction analysis |
| BC | Blank control |
| BP1 | Benzophenone-1 or 2,4-dihydroxybenzophenone |
| BP3 | Benzophenone-3 or 2-hydroxy-4-methoxybenzophenone |
| BP4 | 2-hydroxy-4-methoxybenzophenone-5-sulfonic acid |
| BQL | Below quantification limit |
| BTZ | 1-H-benzotriazole |
| CAS | Conventional activated sludge |
| COD | Chemical oxygen demand |
| CRT | Cellular residence time |
| DCA | Detrended correspondence analysis |
| DCF | Diclofenac |
| DCW | Dry cell weight |
| DGGE | Denaturing gradient gel electrophoresis |
| DHMB | 2,2’-dihydroxy-4-methoxybenzophenone |
| DIC | Disolved inorganic carbon |
| DM | Defined medium |
| DMHAP | 3,5-dimethoxy-4-hydroxyacetophenol |
| DNA | Deoxyribonucleic acid |
| DOC | Disolved organic carbon |
| DW | Dry weight |
| E2 | Estradiol |
| EA-IRMS | Elemental analysis-isotope ratio mass spectrometre |
| EB | Experimental bottles |
| EBI | Experimental bottles with inhibitor |
| $EC_{50}$ | Half maximal effective concentration |
| EDC | Endocrine disrupting compounds |
| EEQ | 17β-estradiol equivalents |
| EMC | Ethylhexyl methoxycinnamate |

| | |
|---|---|
| ER | Estrogen receptor |
| FISH | Fluorescence *in situ* hybridization |
| GC-C-IRMS | Gas chromatography-combustion-isotope ratio mass spectrometry |
| HK | Heat killed controls |
| HOBT | 1-hydroxy-benzotriazole |
| HPLC | High performance liquid chromatograpy |
| HRT | Hydraulic residence time |
| I | Inoculated |
| IAMC | Isoamyl methoxycinnamate |
| ITS | Internal transcribed spacer |
| LOD | Limit of detection |
| LOEC | Lowest observed effect concentration |
| LOQ | Limit of quantification |
| MBR | Membrane bioreactor |
| MuGal | 4-methylumbelliferyl β-D-galactopyranoside |
| NF | Nanofiltration |
| NFQ | β-naphtoflavone equivalents |
| NI | Non-inoculated |
| NSAID | Non-steroidal anti-inflammatory drug |
| OC | Octocrylene |
| OD | Optical density |
| OD-PABA | Octyl dimethyl-p-aminobenzoate or 2-ethylhexyl-4-dimethylaminobenzoate |
| PAH | Polycyclic aromatic hydrocarbons |
| PBSA | 2-phenylbenzimidazole-5-sulfonic acid |
| PCA | Principal component analysis |
| PCR | Polymerase Chain Reaction |
| PhAC | Pharmaceutically active compounds |
| PLFA | Phospholipid fatty acids analysis |
| PNEC | Predicted no effect concentration |
| PPCP | Pharmaceuticals and personal care products |
| qPCR | Real time or quantitative PCR |
| rDNA | Ribosomal DNA |
| RO | Reverse osmosis |
| ROC | Reverse osmosis concentrate |
| RYA | Recombinant yeast assay |
| SAK | Sodium azide killed controls |
| SC | Selective medium |
| SIP | Stable isotope probing |
| SPE | Solid phase extraction |
| TCA | Trichloroacetic acid |

| | |
|---|---|
| TGGE | Temperature gradient gel electrophoresis |
| THB | 2,3,4-trihydroxybenzophenone |
| TOC | Total organic carbon |
| t-RFLP | Terminal restriction fragment lengh polymorphism |
| TSS | Total suspended solids |
| TTZ | Tolyltriazole |
| TU | Toxic units |
| UF | Ultrafiltration |
| UNI | Uninoculated controls |
| UV | Ultraviolet |
| VA | Violuric acid |
| VHW | Veterinary hospital wastewater |
| WRF | White-rot fungi |
| WWTP | Wastewater treatment plants |
| YPD | Yeast extract peptone dextrose |
| Y-PER | Yeast protein extraction reagent |

# Chapter 1

## General introduction

# 1.1. Emerging contaminants

### *1.1.1. From priority organic pollutants to emerging contaminants*

In the second half of 20$^{th}$ century, increasing concern about environmental pollution and the threat it represented for human health led to the implementation of laws regulating the use and disposal of those chemical compounds that presented highest toxicity and persistence. They included high acute toxic, genotoxic and carcinogenic compounds such as pesticides, polycyclic aromatic hydrocarbons (PAHs), chlorinated organic solvents and other industrial products. Attention to other kind of pollutants started with the estrogenic effects detected from wastewater effluents leading to hermaphrodite fish (Purdom et al., 1994). Then, improvements in the analytical methods allowed the detection of many other compounds, called emerging contaminants, in many aquatic environments at concentrations down to ng $L^{-1}$ (Daughton and Ternes, 1999). They are present at lower concentrations than the conventional priority pollutants but still they can represent a threat to human health and the environment. Most of them are not totally eliminated at conventional wastewater treatment plants (WWTPs). This issue becomes crucial when the WWTP effluent is reused for agriculture or other purposes. Therefore, the presence of emerging contaminants in the environment is an issue of concern among the scientific community due to the potential risks for human health and the environment itself. Among the emerging contaminants, pharmaceutically active compounds (PhACs) and endocrine disrupting compounds (EDCs) are of special concern.

Pharmaceuticals are consumed at high amounts for human diagnosis, prevention and treatment of diseases but also for veterinary purposes. PhACs are designed to be biologically active and to have low biodegradability to avoid their degradation before performing their effect. Those two properties make them the perfect candidates for having ecotoxicological effects if they are released in the environment. For example, diclofenac, a well known analgesic and non-steroidal anti-inflammatory drug (NSAID), commercialized in Spain under the brand name Voltaren, has high levels of consumption, with a calculated annual dose per capita of around 600 mg in the developed countries

(Zhang et al., 2008), which constitutes a consumption volume of around 30 tones only for Spain (Carballa et al., 2008). It was recently proposed to be included as priority substance in the Water Framework Directive 2000/60/EC (European Commission, 2012) due to its widespread occurrence and reported toxicity.

EDCs are micropollutants with biological activity that alter the hormonal system of non-target organisms. They are mainly hormones, such as 17α-ethinylestradiol from the anticonceptive pills (Ingerslev et al., 2003). However, other xenobiotics such as bisphenol A (vom Saal and Hughes, 2005), some ultraviolet (UV) filters (Schlumpf et al., 2004b) and benzotriazoles (Tangtian et al., 2012) have also been reported to have endocrine disrupting activity.

Increased consumer concern for protection against damaging UV radiation has led to the common use of UV filters. In particular, and due to the higher incidence of skin cancer in recent decades, several countries have promoted the use of sunscreen products worldwide. Therefore, as the use of UV filters has rised, so has their presence in the environment. Moreover, UV filters are a diverse group of chemical additives used as protection against solar UV radiation and they are not restricted to sunscreen products, in which amounts of up to 10% are added (Schreurs et al., 2002). UV filters are also found in everyday products such as other personal care products, food packaging, pharmaceuticals, domestic and industrial commodities or vehicle maintenance products (Ash and Ash, 2004) to prevent photodegradation of polymers and pigments (Lowe et al., 1997). UV filters can be classified into organic absorbers (chemical) and inorganic blockers (physical) on the basis of their mechanism of action. Organic UV filters absorb UV radiation and emmit the energy in a lower frequency. They include camphors, benzophenones, cinnamates and triazines, among others. Inorganic sunscreens, i.e. titanium dioxide and zinc oxide, protect the skin by reflecting and scattering UV radiation. Benzophenone-3 (BP3) is one of the most used UV filters in personal care products, found in more than 95% of samples in an extensive urine survey in US (Calafat et al., 2008). Regarding its toxicity, BP3 is under study by the European Commission (EU, 2007) as a substance with potential evidence of endocrine disrupting

effects and it is regulated by the EU 2002/72/EC Directive, relating to compounds in contact with food. 2,4-dihydroxybenzophenone or benzophenone-1 (BP1) is a human metabolite of BP3 but an UV filter itself as well. It presents higher estrogenic effects than BP3 (Takatori et al., 2003). Currently, 3-(4'-methylbenzylidene)camphor (4-MBC) is one of the most commonly employed UV filters as well. It has been reported in many environmental compartments and also in human samples, including human milk (Schlumpf et al., 2010).

Benzotriazoles are chemicals with a high production volume which are used in a wide variety of applications, like in dishwasher detergents (Janna et al., 2011), due to their high resistance to corrosion (Reemtsma et al., 2010). Nonetheless, the main applicability of those compounds is in the industry. For example, benzotriazoles are employed in cooling and hydraulic fluids, in antifreezing products, such as aircraft deicing fluids, as an ultraviolet light stabilizer in plastics or as an antifogging agent in photography and airports (Giger et al., 2006; Liu et al., 2011a). The use of benzotriazoles has been increasing during the last years and, according to Hart et al. (2004), they comprise 0.5–1% of antifreeze by weight, representing more than 1,000 tonnes per year generated in the United States alone and with a much greater global production considering the rest of the manufacturing countries (Hart et al., 2004; Liu et al., 2011a). Therefore, the high production of these compounds, as well as their high chemical stability, drives to an environmental concerning problem. 1-H-benzotriazole (BTZ) and tolyltriazole (TTZ), a mixture of 4-methyl-1H-benzotriazole (4-MBTZ) and 5-methyl-1H-benzotriazole (5-MBTZ), are the most commonly used benzotriazoles.

### *1.1.2. Sources of emerging contaminants*

As shown in Fig. 1.1, the main source of Pharmaceuticals and Personal Care Products (PPCPs) in the environment are the effluents of WWTPs (Kümmerer, 2009). After drug intake or personal care compounds absorption, the chemical compounds are metabolised by the human body and further excreted in urine and/or faeces as unchanged active compound or in form of metabolites (usually hydroxilated or conjugated compounds). Regarding topical drugs or personal

care products such as creams, lotions, shampoos and so on, release to the sewage system through the shower is also common. For example, for diclofenac, only 6% is not metabolised after oral application. However, many hydroxilated and conjugated metabolites can be also found in the urine and faeces. Furthermore, dermal application increases the amount of diclofenac released to the environment (Zhang et al., 2008). Therefore, municipal wastewaters are the main fate of PPCPs. Hospital effluents present high concentrations of PhACs (Santos et al., 2013), although their contribution to the total load of urban WWTPs is low because hospital wastewater is usually diluted by municipal wastewater by a factor of 100 (Kümmerer and Helmersb, 1997). Pharmaceutical industry can also contribute to the release of PhACs to the environment, although scarce data is available to this regard (Sim et al., 2011). When dealing with EDCs, industrial effluents can be the major source, as for example in the case of nonylphenol (Soares et al., 2008). Contribution of landfills leachate to the pollution of surface and groundwaters is unknown (Kümmerer, 2009).

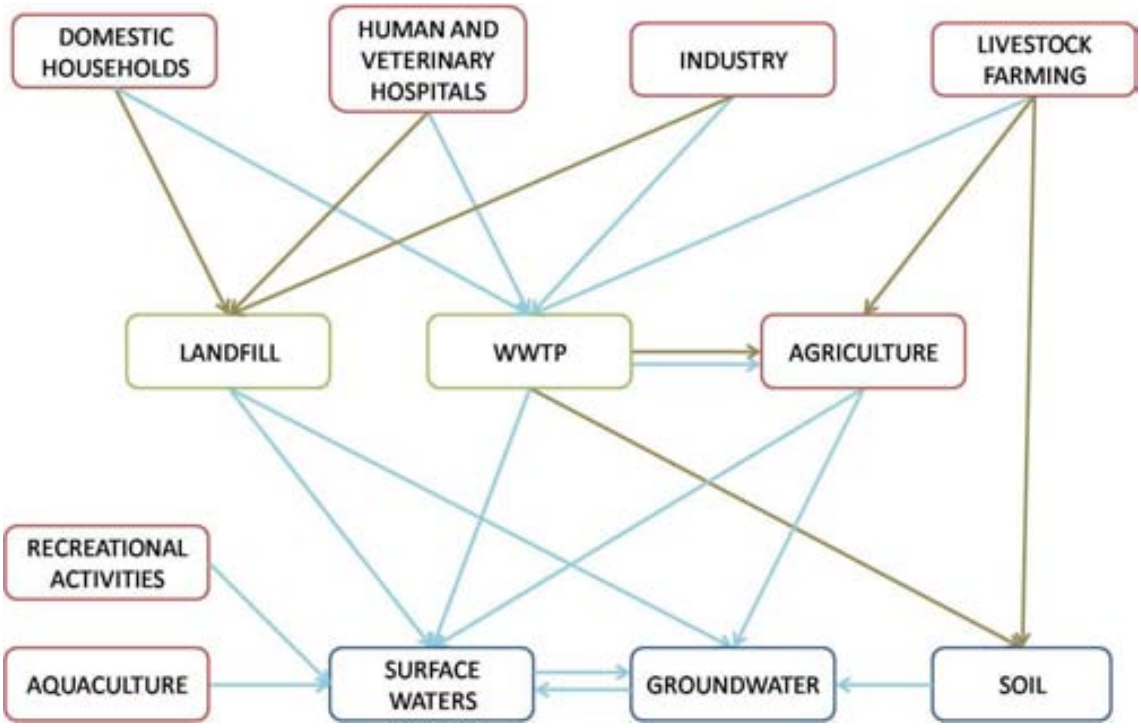



**Figure 1.1.** Scheme of the main sources of emerging contaminants (in red), their intermediate sources (green) and their fate in the evironment (blue). Lines in blue account for liquid effluents and in green for biosolids.

Regarding veterinary drugs, there are some specific compounds for veterinary purposes but the majority are the same used for humans but at different dosages. The entrance of those PhACs to the environment can be also through household or veterinary hospital effluents but in livestock farming and aquiculture, direct release might occur as well (Ellis, 2006). Wash-off during bathing activities represents the most direct input of UV filters to aquatic ecosystems. However, indirect input related to discharges from WWTPs is presently recognised to be the greatest source of these pollutants in the environment, as for the other pharmaceutical and personal care products (Buser et al., 2006). The use of benzotriazoles in aircraft deicers and their low biodegradability are the cause of finding concentrations in subsurface waters close to an airport of up to 126 mg $L^{-1}$ for BTZ and 198 mg $L^{-1}$ for TTZ (Cancilla et al., 1998). However, as benzotriazoles are widely used in dishwasher detergents, the presence of BTZ and TTZ in the environment is directly related to their discharge through the effluents of WWTPs as well (Giger et al., 2006).

Therefore, the vast majority of the emerging contaminants finally arrive to WWTPs. Most of the WWTPs operate with the biological process of conventional activated sludge (CAS). The problem arises when the CAS configurations have been demonstrated to not be successful in totally degrading many of those compounds (Joss et al., 2006; Verlicchi et al., 2012). Antibiotics and the psychiatric drug carbamazepine, for example, are usually degraded to a low extent (Verlicchi et al., 2010). In some cases, even negative removal percentages were recorded for compounds such as lipid regulators gemfibrozil, fenofibrate and atorvastatin, antibiotic clarythromycin, as well as for carbamazepine (Jelic et al., 2011). Analgesics and anti-inflammatories are the most studied compounds as they are detected at the highest concentrations in the WWTPs effluents. Among them, ibuprofen presents high average and maximal detected concentrations of 4.9 and 48 µg $L^{-1}$, respectively (Verlicchi et al., 2010). Removal percentages of diclofenac in WWTPs range from 0 to 80% but it is usually comprised between only 20 and 40% (Zhang et al., 2008). In fact, Joss et al. (2006) determined the biological degradation of diclofenac to be one of the lowest among several PhACs. Moreover, its removal by sorption into sludge is considered to be low, as its sorption coefficient is low too. Diclofenac

was found in the WWTPs effluents at concentrations ranging from 10 to $10^3$ ng $L^{-1}$ depending on the country (Zhang et al., 2008). Moreover, the removal of emerging contaminants in the WWTP, especially the more hydrophobic ones, does not necessarily indicate their biodegradation, as an important pathway for elimination can be the sorption to the sludge. Therefore, PPCPs can enter the environment not only through the liquid effluent but also through the reuse of treated sludge (i.e. for agriculture) and finally end up not only in the aquatic environment but also into the soil (Hörsing et al., 2011; Jelic et al., 2011). Jelic et al. (2011), for example, found that removal of hydrochlorotiazide and fenofibrates was totally due to sorption in the sludge. Removal and degradation of emerging contaminants depend on many factors (configuration of the plant, sludge retention time, pH, redox potential, chemical structure of the contaminant and the sorbent, concentration of the pollutant and so on). That is the reason for the variability on the removal and degradation percentages that can be found in the literature.

Several UV filters were found in treated urban wastewater (Li et al., 2007; Negreira et al., 2009; Rodil et al., 2008; Snyder et al., 2006) as removal percentages ranged from 18% until over 90% in full-scale plants (Balmer et al., 2005; Kupper et al., 2006). Concentrations found at raw wastewaters in Spain are 462 ng $L^{-1}$ for BP3 and 245 ng $L^{-1}$ for BP1 (Negreira et al., 2009). Maximum values reported in WWTP effluents in Switzerland were 2.7 µg $L^{-1}$ for 4-MBC and 0.7 µg $L^{-1}$ for BP3 (Balmer et al., 2005). However, for those most hydrophobic compounds, the major mode of elimination in treatment plants appears to be sorption/sedimentation onto sludge rather than degradation (Kupper et al., 2006). Therefore, higher concentrations of these UV filters are retained in the solid phase than in the aqueous effluent (Liu et al., 2011b) and, thus, high amounts have been reported in sewage sludge (Gago-Ferrero et al., 2011a; Nieto et al., 2009; Rodil et al., 2009b) since the first work of Plagellat et al. (2006) reporting concentrations of 4-MBC of 1.78 mg $kg^{-1}$ dry matter (DM).

On the other hand, the high water solubility in combination with the low biodegradability showed by benzotriazoles make these compounds moderately persistent in WWTPs effluent (Loos et al., 2013). Different authors have studied

the impact of discharges as well as the efficiency during removal treatments (Asimakopoulos et al., 2013; Gorga et al., 2013; Loos et al., 2013; Reemtsma et al., 2010; Stasinakis et al., 2013; Weiss et al., 2006). For example, Gorga et al. (2013) detected the presence of BTs in the 100% of samples coming from different WWTPs from Spain. Concentrations detected in the effluents were ranging from 0.4 to 3.0 µg $L^{-1}$ for BTZ and from 1.2 to 7.6 µg $L^{-1}$ for TTZs, similar to the concentrations found in the influent. These results denoted the low efficiency of WWTPs for the complete removal of these compounds. Another example was reported by Loos et al. (2013) during an EU-wide monitoring survey on emerging polar organic contaminants with even higher concentrations than those reported by Gorga et al. (2013) (reaching 221 µg $L^{-1}$ for BTZ and 24.3 µg $L^{-1}$ for TTZ). Similar pattern was also observed by Stasinakis et al. (2013). Regarding the degradation rates of TTZ, an additional problem has been detected for the two isomers, since they behave quite differently in the environment (Reemtsma et al., 2010). For example, a study carried out by Weiss et al. (2006) showed a removal rate of 11% for 5-MBTZ in front of the -6% for 4-MBTZ. Regarding their presence in sludge, Asimakopoulos et al. (2013) reported similar concentrations of around 100 ng $g^{-1}$ DW of TTZ and BTZ in the sludge despite TTZ's very low sorption due to its higher concentration in the influent. BTZ was calculated to remain up to 30% adsorbed in the sludge.

### *1.1.3. Occurrence and fate in the environment*

PhACs, EDCs and other emerging contaminants have been detected in many aquatic compartments, such as surface waters (rivers and lakes), groundwater, sea and even tap water around the world (Gros et al., 2012; Jonkers et al., 2005; Jurado et al., 2012). For example, diclofenac has a widespread presence in the environment (Duan et al., 2013; Fent et al., 2010b; Liu et al., 2011b; Morasch, 2013). Hughes et al. (2013) calculated the median of diclofenac occurence in USA, Europe and Asia freshwaters to be 136.5 ng $L^{-1}$, with a maximum detected concentration of 18.7 µg $L^{-1}$. In the same study, a complete review of worldwide occurrence of PhACs in freshwater ecosystems, authors reported that concentrations are ususally lower than those in the WWTP effluents; however in some cases they can reach several µg $L^{-1}$ or even mg $L^{-1}$ (e.g. ciprofloxacin). On the other hand, taking into account that many

compounds remain totally or partially adsorbed in the sludge, they were also found in soils amended with WWTP sludge, but also in soils irrigated with reclaimed water or amended with livestock manure (Li, 2014). High concentrations were found for the antibiotic doxycycline in Malaysia, that was detected up to 728 μg $kg^{-1}$ (Ho et al., 2012). PhACs and EDCs have been also detected in sediments. As an example, a comprehensive study of occurrence of PhACs in the Ebre river, detected many compounds in the sediments, being acetaminophen that found at higher concentration (222 ng $g^{-1}$) (Ferreira et al., 2011). Bioaccumulation in fish of those compounds more lipophilics is also reported (Huerta et al., 2013; Zenker et al., 2014). Diclofenac has been found in many fish tissues and calculated bioconcentration factor ranges from 0.3 to 2732 (Zenker et al., 2014). Huerta et al. (2013), for example, found it at a concentration of 8.8 ng $g^{-1}$ in an homogenate of *Barbus graellsii* collected in Mediterranean rivers. Finally, it should be highlighted the lack of knowledge about the occurrence, fate or activity of metabolites.

Although reports on the environmental concentrations of UV filters are limited, their occurrence has been demonstrated in various environmental compartments and biota, as reviewed in (Giokas et al., 2007). Residues of the most polar organic UV filters have been found in all kind of water sources: surface water (Balmer et al., 2005; Fent et al., 2010b; Rodil et al., 2008; Tarazona et al., 2010), groundwater (Jurado et al., 2014) and even tap water (Díaz-Cruz et al., 2012), at the ng $L^{-1}$ level. The organic UV filters most frequently found in water samples are BP1, BP3, 4-MBC, 2-hydroxy-4-methoxybenzophenone-5-sulphonic acid (BP4), 2-phenylbenzimidazole-5-sulfonic acid (PBSA), ethylhexyl methoxycinnamate (EMC), isoamyl methoxycinnamate (IAMC), octocrylene (OC) and octyl dimethyl-p-aminobenzoate (OD-PABA) (Kasprzyk-Hordern et al., 2008; Negreira et al., 2009; Rodil et al., 2008). Due to the high lipophilicity and poor biodegradability of many UV filters, they accumulate in soils, river sediments and industrial drainage (Gago-Ferrero et al., 2011b; Jeon et al., 2006; Rodil et al., 2008) and biota (Bachelot et al., 2012; Fent et al., 2010b; Pedrouzo et al., 2010; Rodil et al., 2008). For example, 4-MBC, BP3, EMC and OC have been detected in the lipidic tissues of fish in Swiss lakes (Balmer et al., 2005) in a range from 25 to

166 ng $g^{-1}$, and BP3, EMC, OD-PABA and OC were detected in Spanish river sediments (Gago-Ferrero et al., 2011b) in a range from 4.4 to 2,400 ng $g^{-1}$.

BTZ and TTZ have been detected at relevant concentrations during the last years in surface river waters. For example concentrations for BTZ between 18 and 275 ng $L^{-1}$ and TTZs ranging from 29 to 568 ng $L^{-1}$ were found in the Ebre river (Gorga et al., 2013). However, concentrations up to 7 µg $L^{-1}$ for BTZ and 0.9 µg $L^{-1}$ for TTZ have been detected in other river waters samplings (Herrero et al., 2014). There is no data available about sorption to sediments or possible bioaccumulation of benzotriazoles.

### *1.1.4. Environmental toxic effects and risk assessment*

Regarding the presence of PPCPs in the environment, the key point is to elucidate if so low concentrations actually possess human or environmental risk or not. Even today little is known about this subject. There are many types of possible effects of xenobiotics to the aquatic biota. Despite acute toxicity and typical carcinogenic and mutagenic effects, ectopic activation of some receptor pathways can cause severe damage to organisms and their ecosystem by altering reproduction, hormonal and/or circulatory systems (Haarmann-Stemmann et al., 2009; Kerley-Hamilton et al., 2012; Sauzeau et al., 2011). Moreover, an especial case is that of the antibiotics, which not only can cause toxic effects to the biota but there is also a growing concern about generation and spread of resistances. In US, for example, the use of streptomycins in agriculture is being discussed as a possible reason for the high resistance of pathogenic bacteria against this antibiotic (Kümmerer, 2009).

Toxicity can be detected *in vivo* or at *in vitro* assays. Many *in vitro* assays have been developed. They are mainly recombinant yeasts expressing human or rainbow trout estrogen receptor (hER or rtER). But also human cell lines, e.g. MCF-7 cells (E-screen), are used. *In vitro* assays are faster and cheaper and allow a rapid screening of, for example, estrogenic compounds. Nevertheless, omission of some effects due to the specific target of the toxicity monitored in those assays might occur. *In vivo* assays are more tedious and time-consuming but their results are usually more reliable. Therefore, a combination of different

assays is recommended in order to have a wide spectrum of toxicity data that would allow performing a reliable risk assessment. Biological assays allow not only determining the toxicity of an especific compound but also to assess the possible decrease or increase in the toxicity after certain treatments. It allows taking into account the potential synergistic effects and the toxicity of the unknown metabolites generated.

Many toxicological studies reported lowest observed effect concentration (LOEC) of emerging contaminants at concentrations above those detected in the environment (Fent et al., 2006). For example, diclofenac has relatively limited acute toxicity, with an $EC_{50}$ in the Microtox analysis of 11.5 mg $L^{-1}$ (Ferrari et al., 2003). However, it is responsible for one of the most flagrant ecotoxical known effects of PhACs. Oaks et al. (2004) reported the link between the high mortality of vultures' population in India due to renal failure and their feeding with diclofenac-treated livestock. Thus, chronic toxicity is thought to be more feasible than acute toxicity due to the low concentrations but continuous presence in the environment of those compounds. In fact, some studies have reported that some compounds such as diclofenac, propranolol and fluoxetine show chronic effects for fish, zooplankton and benthic organisms at concentrations in the range of WWTPs effluents (Fent et al., 2006), i.e. reported $EC_{50}$ of diclofenac on zebra fish embryos was of 90 µg $L^{-1}$ (Dietrich and Prietz, 1999). Ferrari et al. (2003) calculated the predicted no effect concentration (PNEC) for diclofenac to be 116 µg $L^{-1}$.

Some reviews concerning the toxic effects of UV filters in the environment can be found in the literature (Brausch and Rand, 2011; Díaz-Cruz and Barceló, 2009; Schlumpf et al., 2008; Witorsch and Thomas, 2010). In general, risk for acute toxic effects posed by sunscreens is unlikely to aquatic organisms at usual environmental detected levels (Fent et al., 2010a), but chronic environmental toxic effects cannot be ruled out either (Fent et al., 2010b; Schmitt et al., 2008). Data of their effects in different aquatic organisms, such as the standard model *Daphnia magna* (Fent et al., 2010b), other invertebrates (Kaiser et al., 2012; Schmitt et al., 2008), fish (Christen et al., 2011; Kunz et al., 2006a, 2006b) and tadpoles (Kunz et al., 2004) among others, can be found.

Besides these toxic effects, most of the commonly used UV filters are known to cause endocrine disrupting effects in both aquatic and terrestrial organisms as well as in human skin cells. Recombinant yeast assays (RYA) indicates that a wide range of UV filters have potential to cause estrogenic effects. BP1 was found to be the most potent UV filter, with an $EC_{50}$ of 0.8 µM (171 µg $L^{-1}$). Other benzophenones, including BP3, present $EC_{50}$ in a range from 2.96 µM to 298 µM, and reaching in many cases 100% of estradiol (E2) effect (Kunz et al., 2006a). In studies with MCF-7 cells, $EC_{50}$ ranged from 0.68 to 3.73 µM (Schlumpf et al., 2004a, 2001). It can be seen that, in general, in vitro assays using MCF-7 cell lines are more sensitive, resulting in lower $EC_{50}$ than RYA assays. In vivo assays using fish indicate that numerous UV filters display estrogenic effects and adversely affect fecundity and reproduction as well (Coronado et al., 2008; Kunz et al., 2006b). Studies using rats have indicated that 4-MBC affects the hypothalamuspituitary–gonadal system in male rats thus altering gonad weight and steroid hormone production (Schlumpf et al., 2004b). Therefore, other alterations in the endocrine system than simply binding to the estrogen receptor can be generated by UV filters compounds. In fact, alterations in androgenic, progestogenic and thyroid system have already been pointed (Boas et al., 2011; Kunz and Fent, 2006; Schreurs et al., 2005). Moreover, effects of mixtures are another point not deeply studied, although the first works pointed to a synergetic effect (Heneweer et al., 2005; Kunz and Fent, 2009).

It is noteworthy the harmful interaction that benzotriazoles could have with the fauna. Cancilla et al. (2003), for example, detected that TTZs are lethal for feathead minnow (*Pimephales promelas*) at median aqueous concentrations of 22 mg $L^{-1}$. Concerning the characteristic effects of ECDs, Harris et al. (2007) showed the *in vitro* antiestrogenic activity of benzotriazoles while Tangtian et al. (2012) reported the hormonal disruption in fishes even at concentrations of 10 µg $L^{-1}$. Regarding the effect that these compounds could have on humans, Schriks et al., (2010) calculated the acceptable daily intake (ADI) for BTZ and TTZs (0.295 and 0.25 mg $Kg^{-1}$ bw $day^{-1}$, respectively). These ADI values are especially relevant since these compounds have been detected in drinking waters (Janna et al., 2011; Müller et al., 2012; Schriks et al., 2010) and it could

drives to a long-term exposure. Because of the levels detected in waters as well as the possible effects that they could have on biota and humans, the three BTs are included in a prioritizing list of trace pollutants and emerging contaminants to be studied in freshwater environments (Murray et al., 2010).

Almost all the studies revealed that the observed effects occur at higher concentrations than those found in rivers and lakes. Then, it should be taken into account that still remains the dilution of WWTP effluent into the surface water body where it is released. However, in many cases, especially in the highly populated dry areas, such as the Mediterranean ones, the main contribution of water to the rivers comes from the WWTP effluents, reaching in some cases over 90% (Carey and Migliaccio, 2009). Moreover, the risk increases when water for potable uses is collected downstream, which leads to *de facto* closed water circles. Nevertheless, the risk of adverse effects on humans due to the ingestion of PhACs present in drinking water seems to be negligible because the maximum possible intake within a life-span is far below the dosages used in therapy (Christensen, 1998). However, that assumption is based in the pharmacological studies for individual compounds and high dose in a short-term. Extrapolation of data to low dose and long-term ingestion of a mixture of compounds is still an unresolved issue in toxicology and ecotoxicology (Kümmerer, 2009).

Several attempts for risk assessment and prioritization of emerging contaminants have been performed (Hernando et al., 2006; Murray et al., 2010; Verlicchi et al., 2012). However, the scarcity of data on environmental concentrations, the few species used to identify toxic effects and the issue of the toxicity of mixtures, among others make that a reliable assessment of the potential risk of those compounds is not possible yet. Therefore, more environmental toxicity studies are needed in order to perform an appropriate risk assessment.

### *1.1.5. Treatments to degrade emerging contaminants*

Monitoring data of WWTPs indicate that current treatments based on conventional activated sludge are not effective at removing emerging

contaminants since several of them were found in untreated and treated wastewater and in sewage sludge in different countries (see section 1.1.2). Therefore, advanced approaches need to be developed and tested for a safer management of water and biosolids.

On the other hand, Joss et al. (2006) calculated that wastewater dilution, and hence dilution of PhACs concentration, is detrimental for their biological removal by activated sludge. Therefore, treatment at source point (i.e. hospital effluents, veterinary hospital effluents, pharmaceutical manufactures or livestock farms) could help to increase removal of PhACs, taking into account that those effluents present higher concentration of PhACs than WWTP influents after mixing with municipal wastewater (Santos et al., 2013; Sim et al., 2011). Some studies treating hospital wastewater at source point can be found in the literature (Kovalova et al., 2012; Verlicchi et al., 2010). However, to date, there is no work carried out with veterinary hospital wastewater; not even with wastewater characterisation purpose, despite similar PhACs concentration than in human hospitals are expected due to the use in animals of human pharmaceuticals apart from veterinary ones.

*Physico-chemical treatments*

In recent years the development and implementation of technologies such as advanced oxidation processes (AOPs), membrane filtration and activated carbon adsorption have been studied to improve removal efficiency of emerging contaminants.

Among oxidation technologies, ozonation is the most studied and the one with better perspectives for the removal of EDCs and PPCPs (Esplugas et al., 2007). Moreover, it has the advantage of providing simultaneous oxidation of xenobiotics and disinfection of wastewater. However, it is expensive because ozone is rapidly deactivated and it has to be continuously supplied. There is quite a high volume of information about removal of pharmaceuticals and pesticides via ozonation. For instance, many studies about degradation of diclofenac by ozonation have been performed. Huber et al. (2003) even tested it in real matrices instead of distilled water, with degradation percentages of

>97%. However, the data available for personal care products, and especially for UV filters, is rather limited and somewhat ambiguous. For instance, Rosal et al. (2009) did not observe any elimination of BP3 by ozone while other studies report removal efficiencies higher than 80% (Hernández Leal et al., 2010). Müller et al., (2011) studied the elimination of 4-MBTZ and 5-MBTZ from potable water thorough ozonation as a last step during water potabilization processes. Besides that, other oxidation technologies, mostly AOPs, have been studied for the degradation of EDCs and PhACs. Lee and von Gunten (2010), for example, compared the transformation of diverse oxidants (e.g. chlorine, ozone and hydroxyl radical). Results of the few studies examining UV filters response under photolysis and photocatalysis in aqueous samples indicate that the extent of degradation is quite variable (Rodil et al., 2009a; Sakkas et al., 2009; Vanquerp et al., 1999). Other studies were focused on electro-Fenton and photo-Fenton related reactions (Klamerth et al., 2010; Sirés et al., 2007). Nevertheless, all of them are expensive technologies, with high consumption of energy and chemicals. Moreover, generation of toxic transformation products is reported (Vogna et al., 2004) and hence sometimes combined AOPs followed by biological processes are suggested (Oller et al., 2011).

Adsorption to activated carbon has been reported to retain many compounds, although those more polar usually achieve lower removal percentages (Reungoat et al., 2010). Nevertheless, it has been also thoroughly studied, even at full scale with quite successful removal results (Reungoat et al., 2010). Its main drawback is the need for the regeneration of the activated carbon. However, nowadays, some studies are testing the use of agriculture residues for a more sustainable process (Baccar et al., 2012).

Filtration technologies, such as nanofiltration (NF), ultrafiltration (UF) and reverse osmosis (RO), are also highly studied (Urtiaga et al., 2013), with removal percentages of pollutants over 90% (Comerton et al., 2008; Kim et al., 2007) although not always. Radjenović et al. (2008), for example, found that gemfibrozil and mefenamic acid were less than 50% removed. The use of RO as tertiary treatments for the removal of organic micropollutants is already implemented in some WWTPs (del Pino and Durham, 1999). RO is very

effective in retaining micropollutants, and highly quality permeates can be obtained (Comerton et al., 2008; Dolar et al., 2012; Urtiaga et al., 2013). Comerton et al. (2008) reported total removal of BP3 with both NF and RO from a MBR effluent at initial concentration of 1 μg $L^{-1}$ and Loi et al. (2013) reported removal of BTZ around 70% and 85% of TTZs in a RO unit. However, as it is mainly a physical process, contaminants are not degraded but they remain in the so-called reverse osmosis concentrate (ROC) or brine: the liquid fraction with lower volume but higher concentration of pollutants. Technologies under study to treat ROC are mainly also AOPs, which imply high chemical dosages and energy consumption (Justo et al., 2013; Pérez-González et al., 2012). Nonetheless, alternative biological treatments might be applied as well.

*Alternative biological treatments to activated sludge.*

In order to avoid the release of xenobiotics, some biological alternative strategies to conventional WWTPs are under study too. They are ususally less expensive and present lower energy consumption than physico-chemical ones. The most popular is the use of membrane bioreactors (MBRs), which is already implemented in many WWTPs, although it was not initially designed for that purpose but for space-saving and reduction of sludge production. It was thouhgt that the higher cellular residence time (CRT) and the acclimatation of microorganisms could lead to higher removal percentages. However, although average removal of PPCPs in MBR is higher than in CAS, e. g. the later increases the average removal of diclofenac from 30 to 60% (Verlicchi et al., 2012), there is still a long way of improvement. Regarding benzotriazoles, Weiss et al. (2006) compared CAS *vs.* MBR treatment, showing that MBR was more efficient in terms of benzotriazoles removal. In addition, comparison between BTs biodegradation under aerobic and anaerobic conditions was performed by Liu et al. (2011a). The authors showed that the aerobic treatment was more efficient than anaerobic although half lives were still high (i.e. 315 days for BTZ). The authors also analyzed the transformation products (TPs) generated during the treatment, which have been found to be dependent on the predominant terminal electron-accepting condition because of the triazole ring (Liu et al., 2011a). When comparing both TTZ isomers in a membrane

bioreactor (MBR) treatment, differences in the removal rates were also obtained (61% for 5-MBTZ *vs.* the 14% for 4-MBTZ) (Weiss et al., 2006).

Artificial wetlands have been also proposed for the removal of emerging contaminants. However, they have some drawbacks, such as the high land-use and degradation percentages similar to those obtained at CAS (Li et al., 2014; Matamoros and Bayona, 2006; Verlicchi and Zambello, 2014). Diclofenac is among the better studied PhACs for their fate at constructed wetlands (Li et al., 2014). It presents low removal efficiencies (between 20 and 50%) with even negative removals in some studies, but presenting higher removal efficiencies when the constructed wetland acted as a tertiary treatment than as a primary treatment (Verlicchi and Zambello, 2014).

One of the few studies on UV filters biodegradation (Janzen et al., 2009) determined the microbial degradation of BP3 and 4-MBC in a vertical flow soil filter being only 12% of the total load in the first case and 75% in the latter. However, due to the high sorption affinity of the compounds the elimination percentages reached almost 100% in a low load experiment and 82-86% for BP3 and 91-96% for 4-MBC in a high load experiment. All tests were done at environmental relevant concentrations (3 µg $L^{-1}$). Hernández Leal et al. (2010) studied the removal of some UV filters (4-MBC, BP3, OC and avobenzone) in different biological systems under aerobic and anaerobic conditions. Based on their high log Kow values, they assume that their high removal under aerobic conditions and also the variable yields of removal under anaerobic conditions are driven by adsorption and not by biodegradation.

Finally, the use of ligninolytic fungi is another one of these alternatives and their application as decontaminating agent has been intensively studied during the last years. Promising results for the degradation of emerging contaminants have been reported. This treatment is explained in detail in the next section as it is the one studied in this thesis.

## 1.2. Biotechnological applications of white-rot fungi, and *Trametes versicolor* in particular

### *1.2.1. Why white-rot fungi?*

White-rot fungi (WRF) comprise the group of fungi most used in fungal bioremediation. WRF is an heterologous group of saprotrophic fungi, comprising basidiomycetes but also some ascomycetes that have the ability to degrade lignin of decaying wood to take profit of the cellulose and hemicellulose present inside (Kirk et al., 1976; Leisola et al., 2012). Lignocellulose comprises 95% of biomass in the earth but at the same time lignin is one of the most difficult compounds to degrade due to its composition and structure (highly branched phenylpropanoid polymer, see Fig. 1.2), being bacteria and many fungi unable to metabolise it.

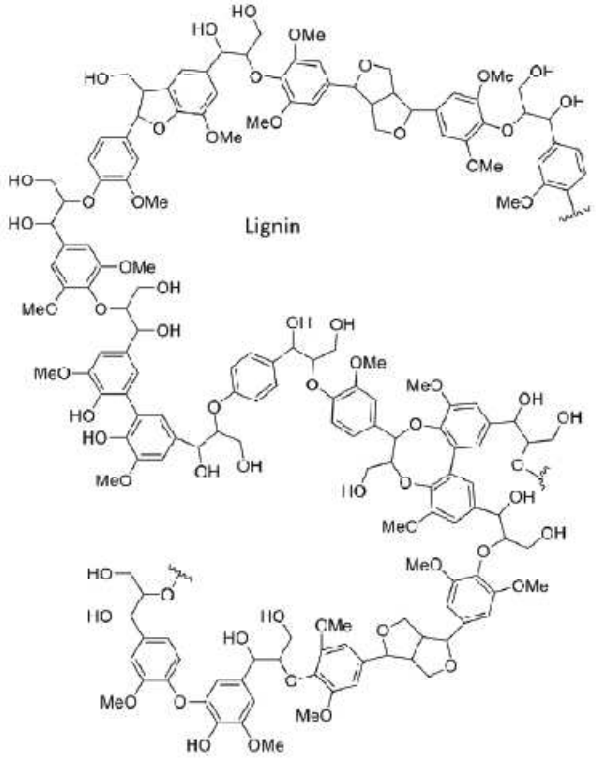



**Figure 1.2.** Example of the structure of lingin (Leisola et al., 2012).

Wood degrading fungi comprise brown-rot fungi, which are only able to degrade hemicellulose and cellulose, WRF, which can also degrade lignin, and soft-rot fungi, which have intermediate abilities, partly degrading lignin but at much slower rate (Moore et al., 2011). In order to acomplish such task, WRF have

developed a powerful enzymatic system, characterised by a high unspecificity given by the oxidation mechanism, partly based on the generation of free radicals and the presence of extracellular enzymes such as laccases and peroxidases. The presence of extracellular enzymes also conferes them a high tolerance to toxic compounds and the ability to transform poorly soluble compounds. Those two main characteristics, together with the fact that a pre-conditioning is not necessary gives WRF perfect features for their use for xenobiotics degradation (Barr and Aust, 1994).

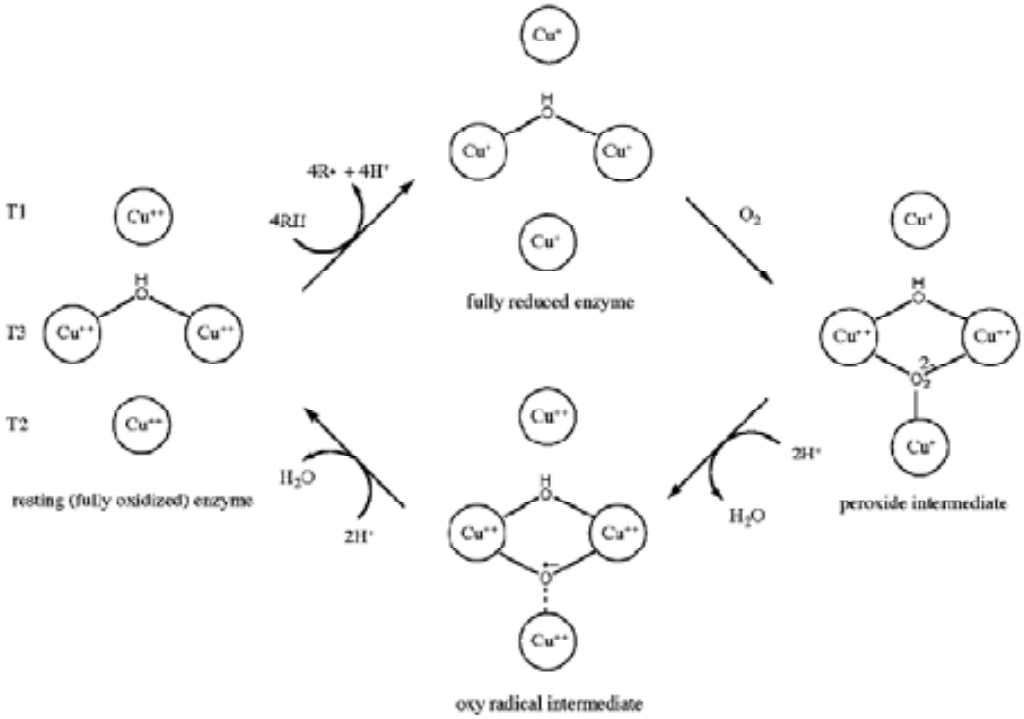



**Figure 1.3.** Catalytic cycle of laccase (Wong, 2009).

Laccases are oxygen oxidoreductases that belong to the group of multicopper blue phenoloxidases. They were already described in the 19$^{th}$ century in the lacquer tree *Rhus vernicifera* (Yoshida, 1883). They can catalize the oxidation of a wide variety of substrates, including diphenols, aminophenols, polypehnols, polyamines, methoxy phenols and aryl diamines and some inorganic ions with simultaneous reduction of $O_2$ to $H_2O$ (Morozova et al., 2007) (Fig. 1.3). They are highly unspecific enzymes, with broad functions, from the synthesis of lignin in plants to the synthesis of pigments, pathogenesis and degradation of lignin in fungi among others. However, they are considered not powerful enough to

oxydize the non-phenolic predominant part of the lignin theirselves alone. Then, high efforts were done in order to find the ligninolytic enzymes of WRF. At 1983, lignin peroxidase was the first fungal peroxidase described (Glenn et al., 1983; Tien and Kirk, 1983) and two years later manganese peroxidase (Glenn and Gold, 1985). Later, other enzymes such as versatile peroxidases and other groups of peroxidases were also described (Hofrichter et al., 2010; Mester and Field, 1998). Peroxidases are able to degrade compounds with high redox potential through reduction of $H_2O_2$ and trasference of two electrons. Nowadays, it is known that laccases can also degrade non-phenolic compounds by the action of small molecules called mediators (Bourbonnais and Paice, 1990). The production of HO• radicals by Fenton reactions can also contribute to lignin degradation (Henriksson et al., 2000). Finally, as fungi are eukariotic organisms, they also possess the intracellular monooxygenases cytochrome P450 enzymes. Cytochrome P450 enzymes are a wide family of enzymes with multiple functions. In humans, for example, they are involved in the transformation of xenobiotic compounds. Regarding lignin degradation, they could be involved in the hydroxylation and breakdown of lignin metabolites and detoxification of plant metabolites although no direct evidence of their role in lignin degradation has been reported (Subramanian and Yadav, 2008).

Therefore, taking into account their ecological role, first biotechnological applications of WRF were in the paper industry as biobleachings or biopulpings, pretreating the wood to reduce the price and the toxicity of the chemical treatments (Kirk and Yang, 1979; Sigoillot et al., 2005). Then, studies using WRF to detoxify paper, textile and agriculture industrial wastewater effluents were performed as well (Archibald et al., 1990; D'Annibale et al., 1998; Zhang et al., 1999). At the same time, considering that conventional contaminants, such as PAHs, dyes and pesticides, have a chemical structure that resembles the lignin one, many scientific groups started to investigate the possible use of WRF for decontamination of other polluted effluents and sites. This aspect is extensively review by Cerniglia (1997), Harms et al. (2011), Pinedo-Rivilla et al. (2009) and Pointing (2001) among others. Until then, bioremediation had been based on bacterial degradation. Bacteria grow faster and can use pollutants as source of carbon and energy. However, they use specific biochemical

pathways, which makes them successful degraders of structurally simple chemicals (Harms et al., 2011). However, when dealing with complex structures and/or mixtures of pollutants, fungi can be more effective due to their unespecificity above mentioned. Fungi can also help to mobilize bacteria and nutrients in soils to enhance bioremediation (Banitz et al., 2013).

*Phanerochaetes chrysosporium* is considered the model organism for WRF (Martinez et al., 2004), but other fungi are highly studied as well due to their high degrading capacities, such as *Pleurotus ostreatus, Bjerkandera adusta* and *Trametes versicolor.* In particular, *T. versicolor* strain ATCC#42530 has been extensively studied in our research group for the degradation of PAHs (Borràs et al., 2010), dyes (Blánquez et al., 2004), chlorinated compounds (Marco-Urrea et al., 2006) and resulted the best in a fungal screening for degrading PhACs as well (Marco-Urrea et al., 2009). Therefore it was also the fungus used in this thesis.

*T. versicolor* (Fig. 1.4) is a filamentous basidiomycete belonging to WRF. It is an aerobic saprotrophic fungus and it is present in many temperate forests of Europe, Asia and North America growing over trunks and branches of dead trees. It can express laccase, LiP and MnP but there is certain interstrain variability (Hofrichter et al., 2010; Tomsovský and Homolka, 2003). Many different names have been formerly used in the literature, including *Polyporus versicolor* and *Coriolus versicolor.*

**Figure 1.4.** *Trametes versicolor* growing in a decaying tree

### *1.2.2. Use of WRF for bioremediation*

Since the beggining of the studies on possible industrial applications of fungi, two lines of research have been stablished. Firstly, treatments with the entire fungal mycelia were performed (Archibald et al., 1990; Kirk and Yang, 1979). However, when ligninolytic enzymes were discovered, optimistic perspectives were glimpsed for enzymatic treatment. Promptly, it was shown that enzymatic treatments had also many disadvantages. Enzymatic treatment is not recommended when the enzymes are intracellular, the enzymes need cofactors or when it deals with multienzymatic processes (Carballeira et al., 2009). In the case that here concerns, the last two premises occur. On one hand, expensive and many times toxic mediators are needed for a good performace of ligninolytic enzymes. On the other hand, degradation of lignin is a cooperative process of multiple enzymes and, therefore, whole cell applications will always be higher efficient than single enzyme use. Moreover, fungal culture is still also need for the continuous production of enzymes due to their deactivation over time. Moreover, a decrease in the range of treatable contaminants occurs when using purified enzymes, even with the addition of mediators, in comparison with the use of the entire fungus. Therefore, although some improvements in the enzymatic treatments are taken place, such as the use of environmetally friendly mediators (Cañas and Camarero, 2010), whole fungal cells will always be more complete to perform total mineralisation of xenobiotics, either alone or when there is a complex mixture of compounds to oxidize. That is the reason of choosing the entire fungus *T. versicolor* for the experiments of this thesis. However, the use of those enzymes is still widely applied in industries such as pulp and paper mill where partial trasformation is sufficient (Widsten and Kandelbauer, 2008).

However, regarding the use of the whole fungi, there are also some points that have to be taken into account. First of all, xenobiotics degradation needs a suplementary source of energy (co-metabolism), as fungi cannot utilize them as sole source of carbon and energy such as happen with lignin (Cerniglia, 1997). Moreover, when working with WRF strongly dependend of ligninolytic such as *P.* chrysosporium, adequate growth conditions are needed because lignin-degradative system is usually activated after primary growth of the fungi, that is,

during secondary metabolism. It can be also induced by carbon or nitrogen starvation. However, in *T. versicolor*, laccase activity have been reported also in high nitrogen media (Collins and Dobson, 1997; Freitag and Morrell, 1992).

Fungal treatments can be performed for both solids, such as soils or sludge, or liquid effluents. Different types of reactors can be found for each case. To degrade pollutants in soils or sludge, the most common treatments are biopiles or bioslurry systems (Rodríguez-Rodríguez et al., 2012a, 2012b). To treat liquid effluents, many reactor conformations have been designed (Blánquez et al., 2004; Borchert and Libra, 2001; Hai et al., 2008; Rodarte-Morales et al., 2012a; Zhang and Geissen, 2012). The key design factors for achieving good performace are to provide the fungi with enough aeration, good contact between the fungus and the liquid, and to try to avoid the clogging of the reactor.

A great advantage of working in agitated submerged cultures is that at certain conditions, filamentous fungi can grow in form of pellets instead of dispersed mycelium. That method of growing represents an auto-immobilization of the fungus, with all the advantages of immobilization (lowering of the viscosity of the media, decrease of the clogging when working in bioreactors, and so on) but without the extra need of a support.

### *1.2.3. Fungal biodegradation of individual emerging contaminants*

Once demonstrated the ability of WRF to degrade hazardous pollutants and to detoxify some industrial effluents, since the beggining of the 21$^{st}$ century many studies moved on to the degradation of the newly detected emerging contaminants. Since then, the use of ligninolytic fungi has been already successfully applied for the degradation of many emerging ubiquitous pollutants such as estrogens (Blánquez and Guieysse, 2008; Cajthaml et al., 2009), but mainly pharmaceuticals such as antibiotics (García-Galán et al., 2011; Wetzstein et al., 1999) and psychiatric drug carbamazepine (Jelic et al., 2012; Zhang and Geissen, 2012) among many others in liquid media but also in solid-state media (Rodríguez-Rodríguez et al., 2011). There are also some works

dealing with the degradation of estrogens and PhACs by means of ligninolytic enzymes (Auriol et al., 2008; Lloret et al., 2010).

The study of the degradation pathways in fungi for pharmaceuticals and personal care products (PPCPs) is relatively recent and not fully developed. In general, the most studied xenobiotic degradation pathways in fungi are those related to PAHs (Cerniglia, 1997). As PPCPs present aromatic structures it is assumed that the enzymes implicated in their degradation might not differ considerably from the ones implicated in PAHs metabolism. Those are the extracellular enzymes laccase, manganese peroxidase and lignin peroxidase, and the intracellular cytochrome P450 system, performing Phase I transformations (e.g. oxidation, hydroxilation, demethylation and so on). However, fungi can perform Phase II reactions as well. Those are conjugations of the compound or its transformed metabolites (mainly hydroxylated) with certain molecules, being the most common glucuronides, sugar moieties and sulfate conjugates (Cerniglia et al., 1982; Hundt et al., 2000). Most of the studies related to PPCPs metabolism are performed in rats or humans, as toxicokinetics and degradative metabolism have been analysed to assess their associated risks.

Nevertheless, these degradation processes are only useful if they do not lead to the formation of new compounds with higher toxicity or bioaccumulation capacity. Therefore, it is necessary to identify and characterise the derivatives formed during the transformation processes and to assess the potential toxicity (or any potentially deleterious biological activity) not only of the parental compounds, but also of their degradation products, in order to draw a complete picture of the process.

Below are reviewed the studies published so far related to fungal degradation of those compounds which some aspects of their individual degradation have been studied in this thesis.

*UV filters*

Regarding UV filters, Garcia et al. (2010) applied the enzymatic treatment with purified laccase to the degradation of BP3 at low concentrations (10 µg $L^{-1}$). They achieved a complete removal of the compound in a primary effluent. However, the main inconvenient is that BP3, as many other xenobiotics, is not directly recognized by the enzyme laccase and it is necessary the addition of some mediator such as 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS**)**. Moreover, a higher mediator/contaminant molar ratio is needed as lower the contaminant concentration is. That raises the price of the treatment and adds extra compounds to be degraded in the effluent. To date, there are not articles studying the degradation of UV filters with the whole fungal cell.

*Benzotriazoles*

Partial degradation of BTZ and TTZ by the fungus *P. chrysosporium* was presented by Wu et al. (1998) in experiments performed at high concentrations of spiked contaminant (50-100 mg $L^{-1}$). However, possible generated metabolites were not studied. Enzymatic degradation of those compounds is not reported to date. Interestingly, the related compound 1-hydroxy-benzotriazole is considered as a laccase mediator (Rabinovich et al., 2004).

*Diclofenac*

More studied has been the fungal degradation of PhACs in general and the analgesic and anti-inflammatory diclofenac in particular. In previous works to this thesis, degradation percentages higher than 94% were achieved by *T. versicolor* after 1 hour of culture at initial spiked concentrations of 10 mg $L^{-1}$ and 45 µg $L^{-1}$ (Marco-Urrea et al., 2010b). Metabolites identified during fungal degradation were 4'- and 5-hydroxy-diclofenac, and both of them were thoroughly degraded after 24 hours of culture. As no other metabolites were found in the media, opening of the rings and complete mineralisation was suggested although not demonstrated. Cytochrome P450 was thought to be involved in the initial steps of degradation although laccase alone was also able to transform it. Lloret et al., (2010) and Margot et al. (2013) confirmed the degradation of diclofenac by laccase alone or with the addition of mediators

whereas Tran et al. (2010) also achieved 100% degradation with the use of the entire fungus. Other fungi, such as *P. chrysosporium* (Hata et al., 2010) and *B. adusta* (Rodarte-Morales et al., 2011), and enzymes such as versatile peroxidase from *B. adusta* (Eibes et al., 2011) and lignin peroxidase from *P. chrysosporium* (Zhang and Geissen, 2010) have been reported to degrade diclofenac as well.

### *1.2.4. Scale-up to bioreactors and treatment of real wastewater*

Despite the good results achieved in the above mentioned studies (section 1.2.3), most of the experiments were performed at Erlenmeyer scale under sterile conditions. Very few studies scale-up the treatment at bioreactor scale and fewer under non-sterile conditions and/or treating real effluents. Most of the studies at bioreactor scale were performed to treat industrial effluents, i.e. colored effluents, black liquor, paper bleaching wastewater and olive mill wastewater (Blánquez et al., 2008; D'Annibale et al., 2006; Font et al., 2003; Ortega-Clemente et al., 2009). In the last years, some studies reporting the ability of white-rot fungi to degrade PhACs at bioreactor scale have been also published. However, most of them were performed with sterile synthetic media (Jelic et al., 2012; Rodarte-Morales et al., 2012a). Other studies went a step further and worked with real wastewater spiked with a selected pollutant (Zhang and Geissen, 2012). Some of them have dealed with synthetic wastewater but under non-sterile conditions what led to a mixture culture of fungi and bacteria (Yang et al., 2013a). Recently, promising results were obtained treating urban and hospital wastewaters with *Trametes versicolor* (Cruz-Morató et al., 2014, 2013a).

However, biodegradation of PhACs by white-rot fungi is still a technology under study and, therefore, further efforts should be made to optimise the process. Although some authors have already reported carbon and nitrogen requirements for fungal survival and degradation (Casas et al., 2013; Zhang and Geissen, 2012), the amount of nutrients required by the fungus for treating real wastewater still needs to be optimised. Thinking of a further application, it would be optimal that the effluent would provide the fungus with all it needs.

The two main drawbacks reported when working under non-sterile conditions are the overtaking of the inoculated fungus by contaminating bacteria and the washing out of extracellular enzymes when working under continuous operation. However, it was previously reported that extracellular enzymes are not crucial for achieving good degradation percentages (Blánquez et al., 2004; Yang et al., 2013a).

Until now, most of the treatments of real wastewater under non-sterile conditions have been carried out for textile wastewater (Blánquez et al., 2008; Hai et al., 2008; Libra et al., 2003; Lu et al., 2009). To treat urban wastewater with fungi is a greater challenge due to its higher microbial titer that might lead to faster and higher contamination. Different strategies trying to avoid or minimise bacterial growth have been implemented in different studies. Some of them were successful but they are expensive, such as continuous ozonation of the media (Cheng et al., 2013) and others, such as only maintaining the pH acid did not supress bacterial gowth (Libra et al., 2003). Fungal reinoculation strategy was effective at previous studies (Blánquez et al., 2006; Dhouib et al., 2006). Additionally, some external nutrients have to be added to achieve good removal percentages because N and C present in wastewater are not enough (Cruz-Morató et al., 2013a). Use of low nitrogen media to avoid excessive fungal growth and to maintain them in secondary metabolism for extracellular enzymes productions has been extensively used for bioreactors working at sterile conditions (Casas et al., 2013; Moreira et al., 1998). Moreover, little is known about fungi and bacteria interactions in liquid media (Weber et al., 2007; Yang et al., 2011), as the vast majority of studies are in soils (Mikesková et al., 2012; Rousk and Bååth, 2007).

Finally, interpretation of degradation results from non-spiked real effluents is more complex than working with known spiked concentration. Deconjugation processes and other transformation reactions can mask degradation processes or vice versa, degradation could be overestimated by conjugation or simple transformation reactions. Some studies have already considered bacterial conjugations and deconjugations (Jewell et al., 2014; Khunjar et al., 2011; Kovalova et al., 2012; Lishman et al., 2006; Marvalin et al., 2012).

Suitable effluents to treat with WRF with the aim of degrading organic micropollutants before their disposal in the environment should be effluents with small volumes of concentrated effluents, rather than an entire WWTP effluent, where contaminants are usually more diluted. The reason is that, for a proper efficiency, fungal treatment requires hydraulic residence time (HRT) of 24-48 hours at least (Rodarte-Morales et al., 2012a; Yang et al., 2013a; Zhang and Geissen, 2012). Moreover, as smaller the volume of the bioreactor more economical will be the treatment taking into account that the main costs of fungal treatment are due to pellets production (Borràs et al., 2008; Gabarrell et al., 2012). Therefore, a cost-effective treatment might only be achieved treating concentrated wastewater of, for example, human and veterinary hospital effluents or pharmaceutical industry wastewater at source point or with other concentrated effluents such as reverse osmosis concentrate,.

## 1.3. Use of molecular tools for treatment assessment

Advances in genetics, biochemistry and molecular biological techniques have provided many research fields, among them environmental biotechnology, with a huge array of new tools to improve knowledge on biological processes and interactions among organisms (Fig. 1.5).

On one hand, deeper studies of individual organisms can be performed. For instance, easiness of access to sequencing have increased dramatically the number of genomes sequenced which will allow identification of novel biocatalysts and comparisons of degradation potential between organisms. *Phanerochaetes chrysosporium* was the first WRF basidiomycete to be fully sequenced (Martinez et al., 2004). Nevertheless, there are still much more bacteria sequenced than fungi. Information from sequenced organisms can lead to elucidation of new genes with unknown function or to better understand the metabolic network. Regardless genomic sequencing, transcriptome, proteome and metabolome analysis can also shed some light on their biological processes. However, all those analyses are beyond the scope of the present

work. The challenge arises when trying to link the molecular methods and process operation and thus the microbial structure and functions (Gilbride et al., 2006). Again, like in toxicity, as each technique has its strenghs and its scope, combinations of some of them allow a better understanding of the process.

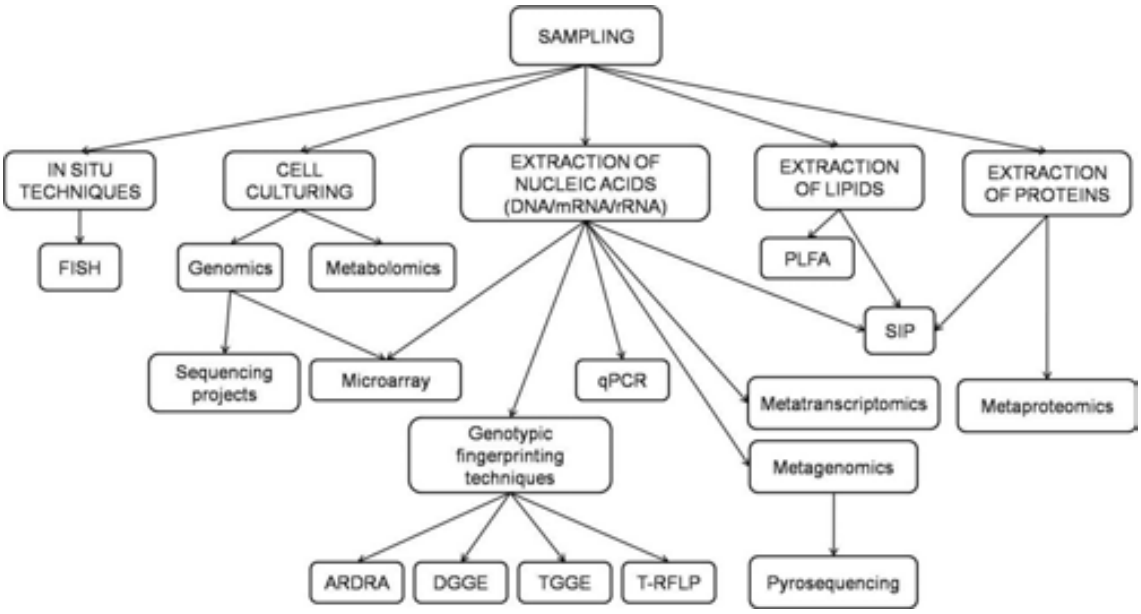



**Figure 1.5.** Scheme of some common molecular tools for analysis of microbial comunities in environmental biotecnology. Adapted from Desai et al. (2010).

On the other hand, molecular techniques allow better understanding and monitoring of *in situ* processes as well. Identification of active organisms, catabolic genes and interactions among microorganisms are some of the possibilities that are nowadays feasible.

### *1.3.1. Use of stable isotopes to assess fate of pollutants*

Many PPCPs, were previously shown to be biodegradable by white-rot fungi. However the mechanistic of PPCPs degradation is still not fully understood and has stated to proceed either cometabolically by means of extracellular enzymes such as laccases and peroxidases or via detoxification reactions such as cytochrom P450 and conjugations (Yang et al., 2013b). For bioremediation purposes, metabolic or growth-linked reactions are preferred over cometabolic or detoxification mechanisms since microorganisms can derive their carbon and energy directly from the pollutant. However, evidence of the use of xenobiotics

as a carbon source is limited in WRF. The experiments that could better approximate to that are those where degradation was observed without addition of extra nutrients (Prenafeta-Boldú et al., 2001). However, such studies do not allow assuring the incorporation of the contaminants in the fungal biomass.

To accomplish the aim of identifying biodegradation strategies that entail mineralisation, the use of contaminants labelled with carbon isotopes ($^{14}C$ and more recently the safer $^{13}C$) and further determination of carbon isotopic signatures of $CO_2$ has been widely applied. With the advent of stable-isotope probing (SIP) analyses, the range of applications increased including tracking the carbon flow through microbial communities (Bastida et al., 2010) and identifying unannotated pathways in certain microorganisms (Marco-Urrea et al., 2012), among others. The basis of this technique is labelling certain type of microbial biomarkers with stable isotopes (usually $^{13}C$) and then, using chromatography coupled to mass spectrometry (MS) or to isotope ratio mass spectrometry (IRMS) for higher sensibility, determine the increase in the $^{13}C$ atom percentage (at%) of the labelled biomarker pools. Thus, protein-SIP (Bastida et al., 2010), total lipid fatty acids (TLFA)-SIP (Bastida et al., 2011; Jakobs-Schönwandt et al., 2010), DNA-SIP (Lu and Chandran, 2010) or RNA-SIP (Bastida et al., 2011) analyses can be performed.

The application of isotope techniques to fungi can shed light on the role of these widespread organisms in decontamination processes and also predict contaminant fate in the environment (Harms et al., 2011). The use of SIP-techniques in fungi is scarce and limited to a recent study demonstrating the incorporation of the carbon-based nanomaterial $C_{60}$ fullerol into the lipid biomass of two white-rot fungi (*Trametes versicolor* and *Phlebia tremellosa*) (Schreiner et al., 2009).

### *1.3.2. Use of molecular techniques for fungal treatment assessment*

It is widely know the many limitations of the cultivable techniques for determining the diversity of the microorganisms. General purpose media select mainly fast growing heterotrophs (Gilbride et al., 2006). Therefore, all the genetic (or other biomarkers) based techniques have the advantage of collect

the diversity information regardless of the microorganisms are cultivable or not. Bacterial degradation is much more studied than fungal one and, therefore, the use of molecular biological tools for assessment of bacterial bioremediation is largely explored and developed. However, those techiques can be applied to both types of microorganisms. Nowadays, a broad range of techniques can be used. Below it can be find a short summary of them and a more extended description of those used in the present thesis (PLFA, DGGE and qPCR).

*Methods that allow monitor changes at the community level*

There are some chemical-based techniques that are culture-independent and allow to giving an insight into the microbial community. Analysis of the composition of PLFA is one of them. PLFA analyses are based on the fact that some PLFA are specifically produced or in much larger ammount by some groups of microorganisms. For example fungi have more long-chain polyunsaturated fatty acids (i.e. 18:2ω6) than bacteria. PLFA pattern mainly describes the living organisms as they are degraded quickly after cell death (White et al., 1979). PLFA use for the assessment of microbial community changes was firstly described in soil by Frostegård et al. (1993). Conversion factors to estimate total amount of bacterial, fungal and total microbial biomass carbon using PLFA of bacterial or fungal origin or total PLFA, respectively, have been calculated (Frostegård and Bååth, 1996; Frostegard et al., 1991; Klamer and Bååth, 2004).

*Methods that allow analysing the diversity of microorganisms*

Phylogenetic analysis through DNA libraries define the population of the microbial community if it is performed with ribosomal DNA (rDNA) fragments, while the metabolising capacity can be assessed by specific designed primers from catabolic operons (Kapley and Purohit, 2009). Analysis of those clone libraries or direct PCR products can be performed with different DNA fingerprinting methods: amplified ribosomal DNA restriction analysis (ARDRA), terminal restriction fragment lengh polymorphism (t-RFLP) and denaturing or temperature gradient gel electrophoresis (DGGE/TGGE). The last one has the advantage to also give direct access to sequence information. DGGE is based in the separation of rDNA fragments of a community, previously amplified by

PCR, in a polyacrylamide gel. The size of the fragments is similar and, thus, the separation is not based in the lenght of the fragment but in its GC content and resulting denaturalization in a urea gradient. Therefore, the diversity of bacterial or fungal communities of each sample can be analysed according to the patterns obtained. Moreover, individual bands can be excised, reamplified and sequenced or compared with the pattern of a clon further sequencied, to afterwards perform a phylogenetic analysis (Iwamoto and Nasu, 2001). Some studies have used DGGE analysis to monitor microbial communities *in situ* during bioremediation treatments (Iwamoto et al., 2000) or to link specific microbial populations with certain degradation profiles (Vila et al., 2010). On the other hand, new high-throughput sequencing techniques allow pyrosequencing of typically used rDNA but also targeting other specific genes (Vilchez-Vargas et al., 2010). However, for monitoring a treatment, where many samples are wanted to be analyzed, pyrosequencing is still an expensive technique.

*Methods that allow monitor changes in gene expression (at both organism and community level)*

Microarrays allow the simultaneous detection of multiple genes. They can be used for individual fungus study or for community analysis. For example, microarray analysis was performed for *T. versicolor* to determine differential gene expression during mycelial interactions with other fungi (Eyre et al., 2010) and for *P. chrysosporium* to study its transcriptome under different conditions (Wymelenberg et al., 2009). Microarrays for assessing bacterial degradation of contaminants are already developed too (He et al., 2007). However, they require time for careful design and are relatively costly.

*Methods targeting selected organisms or genes*

Fluorescence *in situ* hybridization (FISH) allows detection and monitoring of target microorganisms by hybridization of a fluorescent-labeled oligonucleotide probe to intracellular rRNA. Then, cells can be observed by a fluorescence microscopy (Iwamoto and Nasu, 2001). The drawbacks of this technique are its low sensitivity to cells with low metabolic activity and the low specificity of the available probes, as microorganisms (mainly bacteria) can only be classified inside large groups.

Real time PCR (RT-PCR) or also called quantitative PCR (qPCR) is based on the simultaneous amplification and detection of a certain fragment of DNA through specific labeled probes or non-specific fluorescent dyes that intercalates in the amplified DNA. qPCR has been used, for example, for the quantificatin of genes related to atrazine degradation in bacterial degradation microcosms (Sagarkar et al., 2013) but also to monitor certain microorganisms during field experiments of bioaugmentation (Smith et al., 2004).

# Chapter 2

## Objectives

The main aim of this thesis was to assess different factors of the fungal degradation of emerging contaminants, relevant for the treatment of real effluents. Those factors can be grouped in:

i) Factors related to the individual degradation of contaminants by the fungus *Trametes versicolor*

ii) Factors related to operational conditions in bioreactor treatments of real wastewater

iii) Factors related to the interactions of the fungus and the competing microorganisms growing in non-sterile conditions

In order to achieve that general goal, the following specific objectives were formulated:

- To determine the ability of *T. versicolor* to degrade individual emerging contaminants (selected UV filters and benzotriazoles) spiked in defined liquid medium under sterile conditions

- To identify the fungal metabolites of those emerging contaminants and to asses their acute toxicity and endocrine disrupting activity

- To study the mineralisation and the incorporation of micropollutants as carbon source by means of stable isotope labelled compounds

- To analyse the effect of nutrient addition in the activity of the fungus in real wastewater

- To identify some factors to take into account when quantifying emerging contaminants and evaluating their removal in real effluents

- To treat non-sterile reverse osmosis concentrate and veterinary hospital wastewater by *T. versicolor* in an air-pulsed fluidised bioreactor to

remove emerging contaminants at their pre-existent concentrations in continuous operation

- To study the microbial communities developed in the non-sterile bioreactors using PLFA, qPCR and PCR-DGGE analyses.

# Chapter 3



## List of articles and author’s contribution

This thesis is based on the following publications:

**I**, Journal Article: Gago-Ferrero, P., **Badia-Fabregat, M.**, Olivares, A., Piña, B., Blánquez, P., Vicent, T., Caminal, G., Díaz-Cruz, M.S., Barceló, D., 2012. Evaluation of fungal- and photo-degradation as potential treatments for the removal of sunscreens BP3 and BP1. Science of the Total Environment 427-428, 355-363.

*Author's contribution:* contribution to the experimental design, realization of the biodegradation experiments by *Trametes versicolor* and analysis of the samples by HPLC-UV, participation in the interpretation and discussion of the data and in the final manuscript elaboration.

Results included in this article are presented in the Chapter 5.

**II**, Journal Article: **Badia-Fabregat, M.**, Rodríguez-Rodríguez, C.E., Gago-Ferrero, P., Olivares, A., Piña, B., Díaz-Cruz, M.S., Vicent, T., Barceló, D., Caminal, G., 2012. Degradation of UV filters in sewage sludge and 4-MBC in liquid medium by the ligninolytic fungus *Trametes versicolor.* Journal of Environmental Management 104, 114-120.

*Author's contribution:* contribution to the experimental design, realization of the biodegradation experiments by *Trametes versicolor* in liquid media, enzymatic assays and analysis of the samples by HPLC-UV, participation in the interpretation and discussion of the data and in the final manuscript elaboration.

Results included in this article are presented in the Chapter 5.

**III**, Book Chapter: **Badia-Fabregat, M.**, Caminal, G., Vicent, T., Blánquez, P., Gago-Ferrero, P., Olivares, A., Piña, B., Díaz-Cruz, M.S., Barceló, D., 2013. UV Filters Biodegradation by Fungi, Metabolites Identification and Biological Activity Assessment *in* Emerging Organic Contaminants in Sludges. Analysis, Fate and

Biological Treatment. Vicent, T., Caminal, G., Eljarrat, E. and Barceló, D. Eds. Springer. The handbook of Environmental Chemistry 24, 215-240.

*Author's contribution:* participation in the elaboration of the manuscript

Results included in this article are presented in the Chapter 5.

**IV** Journal Article: **Badia-Fabregat, M.**, Rosell, M., Caminal, G., Vicent, T., Marco-Urrea, E., 2014. Use of stable isotope probing to assess the fate of emerging contaminants degraded by white-rot fungus. Chemosphere 103, 336-342.

*Author's contribution:* contribution to the experimental design, realization of all the experiments except for the analysis of the aa-SIP samples in the GC-C-IRMS, interpretation and discussion of the data and elaboration of the final manuscript.

Results included in this article are presented in the Chapter 6.

**V** Journal Article: **Badia-Fabregat, M.**, Lucas, D., Gros, M., Rodríguez-Mozaz, S., Barceló, D., Caminal, G., Vicent, T. Identification of some factors affecting pharmaceutical active compounds (PhACs) removal in real wastewater. Case study of fungal treatment of reverse osmosis concentrate. Journal of Hazardous Materials, submitted.

*Author's contribution:* contribution to the experimental design, realization of the biodegradation experiments by *Trametes versicolor*, interpretation and discussion of the data and elaboration of the final manuscript.

Results included in this article are presented in the Chapter 7.

**VI** Journal Article: Llorca, M., **Badia-Fabregat, M.**, Rodríguez-Mozaz, S., Caminal, G., Vicent, T., Barceló, D. Degradation of endocrine disruptors benzotriazole and tolyltriazole by *Trametes versicolor* in reverse osmosis concentrate and identification of degradation products. In preparation.

*Author's contribution:* contribution to the experimental design, realization of the biodegradation experiments by *Trametes versicolor* and toxicity analyses, participation in the interpretation and discussion of the data and in the elaboration of the final manuscript.

Results included in this article are presented in the Chapters 5 and 7.

**VII** Journal Article: **Badia-Fabregat, M.**, Lucas, D., Pereira, A., Alves, M., Rodríguez-Mozaz, S., Barceló, D., Vicent, T., Caminal, G. Fungal treatment of non-sterile veterinary hospital effluent: pharmaceuticals removal and microbial community assessment. In preparation.

*Author's contribution:* contribution to the experimental design, realization of the biodegradation experiments by *Trametes versicolor* and molecular biology analyses, interpretation and discussion of the data and elaboration of the final manuscript.

Results included in this article are presented in the Chapters 8 and 9.

**VIII** Journal Article: **Badia-Fabregat, M.**, Lucas, D., Tuomivirta, T., Fritze, H., Pennanen, T., Rodríguez-Mozaz, S., Barceló, D., Caminal, G., Vicent, T., in preparation. Bacterial and fungal communities assessment during *Trametes versicolor* continuous treatment of wastewater effluents.

*Author's contribution:* contribution to the experimental design, realization of the biodegradation experiments by *Trametes versicolor* and molecular biology

analyses, interpretation and discussion of the data and elaboration of the final manuscript.

Results included in this article are presented in the Chapters 7 and 9.

# Chapter 4



## Materials and methods

# 4.1. Microorganisms

### *4.1.1. Fungus*

For all the experiments of this thesis, the basidiomycete and white-rot fungus *Trametes versicolor* (ATCC#42530) was used. It was obtained from the American Type Culture Collection and was maintained by subculturing on petri dishes in malt extract (2%) and agar (1.5%) medium at 25ºC.

### *4.1.2. Recombinant yeasts*

For the recombinant yeast assays (RYA), two modified strains of the yeast *Saccharomyces cerevisiae* were used. They were kindly provided by the Environmental Toxicity Group of IDAEA-CSIC, led by Dr. Benjamí Piña. Strains were kept at -80ºC in 50% glycerin stocks.

The Estrogen Receptor assay (ER-RYA) was performed using the yeast strain BY4741 (MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0) from EUROSCARF (Frankfurt, Germany) transformed with plasmids pH5HE0 (containing the human estrogen receptor, hER) and pVitBX2 (with the estrogen response element of vitellogenin gene from *Xenopus laevis* controlling the gene reporter lacZ, ERE-LacZ) as described in Noguerol et al. (2006).

For the Aryl hidrocarbons Receptor assay (AhR-RYA), it was used the yeast strain described by Miller (1997). It is based on the strain YCM4 (derived from the strain W303a: MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100) harbouring a chromosomally integrated construct that co-expresses the human AhR and ARNT genes under the Gal1-10 promoter (induced by galactose) and the pDRE23-Z, containing the construct XRE5-CYC1-LacZ.

## 4.2. Chemical compounds and reagents

### *4.2.1. Selected contaminants*

The contaminants selected for their individual study of degradation by *T. versicolor* can be found at Table 4.1, with their physico-chemical characteristics, chemical structure and supplier.

[Phenyl-$^{13}C_6$]-oxybenzone ($^{13}$C-BP3) was obtained from Cambridge isotopes (Cambridge, UK) with a chemical purity > 99% and an isotope purity 99 at%. [Acetophenylring-$^{13}C_6$]-diclofenac ($^{13}$C-DCF) was obtained from Alsachim (Strasbourg, France) with a chemical purity > 99% and an isotope purity 99 at%.

### *4.2.2. Chemical products and reagents*

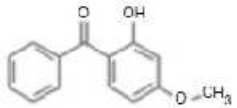
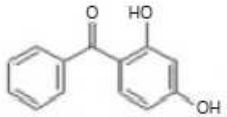
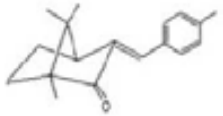
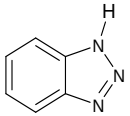
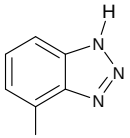
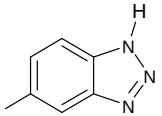
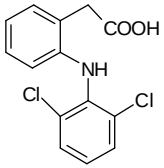
Glucose, ammonium tartrate dibasic, malt extract and other chemicals were purchased from Sigma-Aldrich (Barcelona, Spain).

The laccase mediators 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) and 3,5-dimethoxy-4-hydroxyacetophenol (DMHAP) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Violuric acid (VA) and hydrated 1-hydroxy-benzotriazole (HOBT) were purchased from Fluka (Barcelona, Spain). Enzyme was liofilized and stored at -20ºC.

The cytochrome P450 inhibitor 1-aminobenzotriazole (ABT) was purchased at Sigma-Aldrich (St. Louis, MO, USA).

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

**Table 4.1.** Physico-chemical characteristics, chemical structure and supplier for the selected contaminants studied in this thesis

| Compound | Abreviation | Chemical structure | CAS number | logKow | Supplier | Purity |
|---|---|---|---|---|---|---|
| Benzophenone-3 (2-hydroxy-4-methoxy-benzophenone) | BP3 |  | 131-57-7 | 3.79[a] | Merck | 99% |
| Benzophenone-1 (2,4-dihydroxy-benzophenone) | BP1 or DHB |  | 131-56-6 | 2.96[a] | Sigma-Aldrich | 99% |
| 3-(4′-methylbenzylidene) camphor | 4-MBC |  | 36861-47-9 | 4.95[b] | Alfa Aesar | 99% |
| 1H-benzotriazole | BTZ |  | 95-14-7 | 1.44[a] | Alfa Aesar | 99% |
| 4-methyl-1H-benzotriazole | 4-MBTZ |  | 29878-31-7 | 1.71[c] | Sigma-Aldrich | 99% |
| 5-methyl-1H-benzotriazole | 5-MBTZ |  | 136-85-6 | 1.71[c] | Sigma-Aldrich | 99% |
| Diclofenac | DCF |  | 15307-86-5 | 4.51[a] | Sigma-Aldrich | 99% |

[a] ChemID*plus* (http://chem.sis.nlm.nih.gov/chemidplus/); [b] Díaz-Cruz et al. (2008); [c] Matamoros et al. (2010)

# 4.3. Wastewater effluents

### *4.3.1. Reverse osmosis concentrate*

The reverse osmosis concentrate (ROC) effluent, subsequently treated by the fungus, was obtained from a pilot plant located in Castell-Platja d'Aro WWTP, in the north-east of Catalonia. The pilot plant is described in Dolar et al. (2012) and it consists first of a membrane bioreactor (MBR), which treats urban wastewater (96%), followed by a reverse osmosis unit (Fig. 4.1). The volume treated in the pilot plant is 200 L $h^{-1}$, with a recovery rate of 66%.

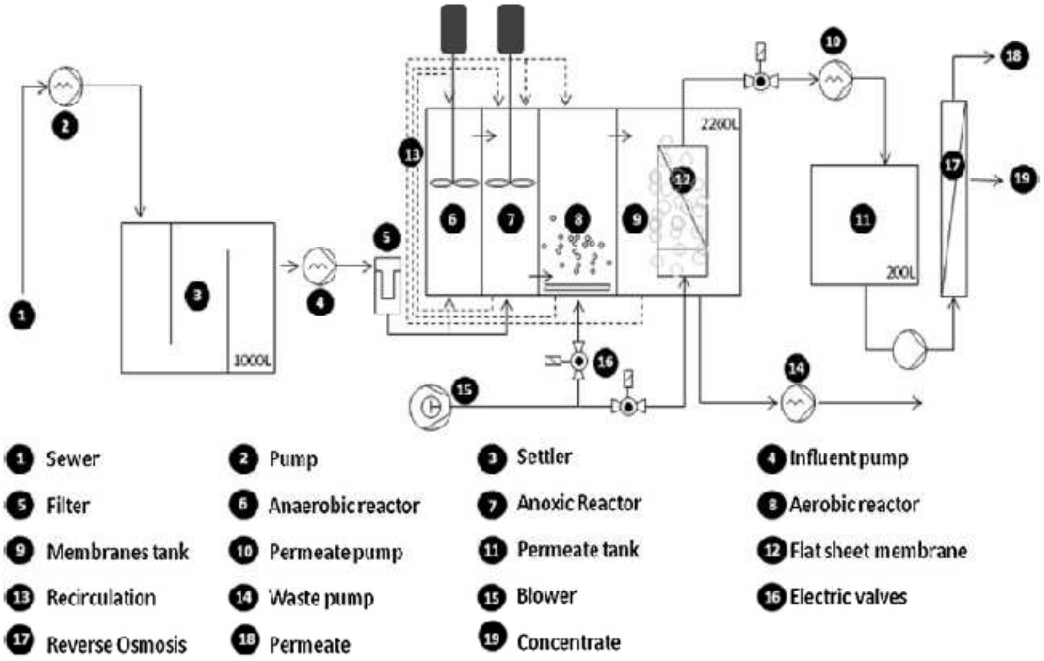



**Figure 4.1.** Pilot plant of Castell-Platja d'Aro, treating urban wastewater with an MBR followed by a RO unit from which ROC was sampled. Scheme taken from Dolar et al. (2012).

ROC samples for sterile batch treatments (Chapter 7.1.3) were sterilised the same day of sampling (March and September 2011) by autoclaving 30 min at 121ºC and then they were stored in the freezer at -20ºC until the experiments were set up. The day before starting the treatment, ROC was thawed at room temperature and autoclaved again. ROC for non-sterile continuous treatment (Chapter 7.2) was sampled on April 2013, after a change in the membrane from Ropur TR70-4021-HF (Toray Industries Inc., Japan) to Hydranautics ESPA2-LD-4040 (Hydranautics, USA), and stored at 4ºC until using it (one month).

Wastewater in the feed storage tank was replaced by fresh one stored at 4ºC every 3-5 days.

### *4.3.2. Veterinary hospital wastewater effluent*

Veterinary hospital wastewater (VHW) was obtained from a veterinary hospital located in the Universitat Autònoma de Barcelona campus (Bellaterra, Barcelona, Spain) the same day that bioreactor was set up. For continuous treatments (Chapter 8.3) it was sampled again once a week. Additionaly, veterinary hospital wastewater in the feed storage tank was replaced by fresh one stored at 4ºC every 2-3 days.

## 4.4. Media and culture conditions

### *4.4.1. Media for Trametes versicolor, culture conditions and pellets production*

Pellets production was done as previously described by Font et al. (2003). In the protocol, two steps can be differentiated: firstly the obtention of mycelium and afterwards the growth of fungal pellets. At every moment, sterility conditions were kept. For that purpose, all media and material were sterilised by autoclaving them 30 min at 121ºC.

For the obtention of mycelium, four 1 $cm^2$ cubes of fungal growing area of Petri plates were cut and inoculated in a 500 mL Erlenmeyer with 150 mL of growing media (malt extract 20 g $L^{-1}$ adjusted to pH 4.5). After 5-6 days at 25ºC and 135 rpm (r=25 mm) of orbital shaking, the mycelium was obtained. It was separated from the media by means of a strainer and triturated with a homogenizer Ystral GmgH X/10/20 in an 8 g $L^{-1}$ NaCl solution at a relation 1:1 v/v. The resultant suspension can be immediately used for the formation of pellets or kept at 4ºC.

For the formation of pellets, 1 mL of the triturated mycelia suspension was added to 1 L Erenmeyer with 250 mL of growing media. The culture was maintained during 5-6 days at orbital agitation (135 rpm) and 25ºC, to finally obtain the 2-3 mm pellets. Final pellets were separated from the media with a

strainer, washed with MilliQ water and resuspended in an 8 g $L^{-1}$ NaCl solution at a relation 1:1 v/v. They can be immediately used or kept at 4ºC for a maximum of one month.

For the degradation experiments, a defined media (Tables 4.3 and 4.4), modified from Kirk et al. (1978), with 8 g $L^{-1}$ of glucose, 1.9 g $L^{-1}$ of ammonium chloride, 1.168 g $L^{-1}$ of dimetyl succinic acid and macronutrients and micronutrients, adjusted at pH of 4.5 was used (Blánquez et al., 2004).

**Table 4.3.** Composition of defined medium

| Component | Concentration |
|---|---|
| Glucose (g $L^{-1}$) | 8 |
| Ammonium tartrate dibasic or ammonium chloride (g $L^{-1}$) | 3.3 or 1.9 |
| 2,2-dimethyl succinic acid (g $L^{-1}$) | 3.3 |
| Macronutrients (m $L^{-1}$) | 10 |
| Micronutrients (ml $L^{-1}$) | 1 |

**Table 4.4.** Composition of macronutrients and micronutrients of the defined medium

| Micronutrients | Concentration (g $L^{-1}$) | Macronutrients | Concentration (g $L^{-1}$) |
|---|---|---|---|
| Nitrile triacetic acid | 1,5 | $KH_2PO_4$ | 20 |
| $MgSO_4 \cdot 7H_2O$ | 3,0 | $MgSO_4 \cdot 7H_2O$ | 5 |
| $MnSO_4 \cdot H_2O$ | 0,5 | $CaCl_2$ | 1 |
| NaCl | 1,0 | | |
| $FeSO_4 \cdot 7H_2O$ | 0,1 | | |
| $CoSO_4 \cdot 7H_2O$ | 0,2 | | |
| $ZnSO_4 \cdot 7H_2O$ | 0,1 | | |
| $CuSO_4 \cdot 5H_2O$ | 0,01 | | |
| $AlK(SO_4)_2 \cdot 12H_2O$ | 0,01 | | |
| $H_3BO_3$ | 0,01 | | |
| $Na_2MoO_4$ | 0,01 | | |

For isotope stable experiments (Chapter 6), composition was changed to initial concentration of 0.5 g $L^{-1}$ of glucose and 2 g $L^{-1}$ of $KH_2PO_4$ instead of dimethyl

succinic acid to minimise other possible carbon sources than glucose and contaminant. The blended mycelia suspension used for the experiments was obtained by grinding 625 mg dry weight of pellets in 125 mL of 8 g $L^{-1}$ NaCl solution with a X10/20 homogenizer (Ystral GmbH, Dottingen, Germany).

For the bioreactor experiments, nutrients feed stock was prepared at 100 g $L^{-1}$ of glucose and 250 mg $L^{-1}$ of ammonia tartrate. In sterile batch bioreactors with defined media and spiked contaminant (Chapter 5.1.7), 3 drops $L^{-1}$ of antifoam (204, Sigma, USA) were also added to the media. Antifoam needs to be autoclaved separately and added later.

### *4.4.2. Media for RYA assays*

*Non-selective media (YPD)*

Non-selective media (YPD) was used for yeast growth from a Petri plate colony. It consists of 10 g of yeast extract (Scharlau) and 20 g of peptone (Oxoid) mixed with 50 mL of glucose 40% (20 g) mixed in a liter of MilliQ water after autoclaving them separately.

*Selective media (SC)*

The base of the selective media (SC) is a solution of 1.7 g $L^{-1}$ of Yeast Nitrogen Base without Amino acids or Ammonium Sulfate (YNB-AA/AS) (Sigma-Aldrich) and 5 g $L^{-1}$ of $(NH_4)_2SO_4$. Once autoclaved, the correct nutrients to complement auxotrophies depending on the strain were added.

For ER-RYA, the following solutions were added in 50 mL to prepare ER-SC media: 2.5 ml of glucose 40% (20 g in 50 ml), 50 µl of histidine 1% (500 mg in 50 ml) and 50 µl of methionine 1% (500 mg in 50 ml). All solutions were autoclaved 30 min at 121ºC and kept at room temperature except for histidine that was kept at 4ºC and protected from light.

For AhR-RYA, the following solutions were added in 50 mL to prepare YCM-SC media: 5 ml of galactose 20% (10 g in 50 ml), 100 µl of adenine 0.5% (250 mg in 50 ml), 400 µl of uracil 0.25% (125 mg in 50 ml) and 500 µl of CAS amino acids (CASaa) 10% (5 g in 50 ml). Solutions were sterilised by filtration (Millex-

GX 0.22 µm, Millipore) except for uracil that was autoclaved, and kept at room temperature except for CASaa that was kept at 4ºC and protected from light.

For Petri plates, 20 g L of bacteriologic agar was added to ER-SC and YCM-SC media.

*LacZ assay buffer (MuGAL)*

For fluorescent reading of activity the assay buffer called MuGAL was used, named after the compound responsible of fluorescence once activated by the enzyme β-galactosidase. The composition of the buffer is 21 mg of 4-methylumbelliferyl β-D-galactopyranoside (MuGAL) (Sigma-Adrich), 70 µl of β-mercaptoethanol, 1 ml of SDS 10% and 1 ml of Triton X-100 10% in 100 ml of Z Buffer.

Z Buffer contains 60 mM $Na_2HPO_4$, 40 mM $NaH_2PO_4$, 10 mM KCl and 1 mM $MgSO_4$, with the pH adjusted to 7.4.

### *4.4.3. Growth and maintainance of Saccharomyces cerevisiae strains*

*S. cerevisiae* strains were kept in SC media in sterile glycerol 1:1 (v/v) at -80ºC (glycerinate).

To grow yeast cells, a small portion of the glycerinate was streaked plated with an sterile Kolle loop in a Petri dish with the corresponding SC media to the strain. The plates, sealed with parafilm to avoid dehydratation, were incubated at 30ºC for aproximately 3 days, until colonies growth. Petri plates can be stored one month at 4ºC.

To create new glycerinates, a liquid culture in SC media was grown 20 hours, until stationary phase (aproximately $OD_{600}$ 1.5). Then 1 mL of culture is mixed with 1 mL of glycerol in a criotube and stored at -80ºC.

Optimal conditions for S. cerevisiae growth are 30ºC and slow agitation (80 rpm).

# 4.5. Degradation experiments of selected contaminants

### *4.5.1. Degradation of selected contaminants in serum bottles*

All the experiments were performed on 125 mL amber serum bottles (Wheaton, Mealville, NJ), to avoid any type of photodegradation, and with cotton plugs to allow gas exchange, in a total reaction volume of 25 mL. The experiments were carried out at 25ºC and 130 rpm of orbital agitation. Pellets were added to a final concentration of aproximately 4 g dry cell weight (DCW) $L^{-1}$ and the selected contaminant at 10 mg $L^{-1}$. Stock solutions of selected contaminants (at concentrations around 1-5 g $L^{-1}$) were prepared dissolved in ethanol and stored at -20ºC protected from light with aluminium foil.

Treatments were done by triplicate (duplicates for BTZ and TTZ experiments) and bottles were sacrificed at each sample time point. Apart from the experimental bottles (EB), different controls were also included in the experiments. One of them are the uninoculated controls (UNI), without fungi but with contaminant to take into account potential natural photodegradation or other abiotic processes. Other controls are those with the same amount of fungi than EB but dead in order to determine the removal for adsorption to the biomass. That can be heat killed fungal control (HK), killed by the autoclave (30 min at 121ºC) or killed by the action of sodium azide (SAK) at a final concentration of 0.2 g $L^{-1}$. For BTZ and TTZ degradation experiments, EB+G treatment was also included. In those bottles, additional glucose was added every 3 d to recover the initial concentration of 8 mg $L^{-1}$. Finally, bottles with the fungus alive but without contaminant, blank controls (BC), were also included to detect changes in laccase activity and fungal growing due to toxicity of the compound, were also included.

At BP3, BP1 and 4-MBC experiments, at each sample time, 1 mL of liquid medium was filtered with a Millex-GV (Millipore) 0.22 µm syringe filter in order to determine the glucose concentration and the laccase enzymatic activity. Then, ethanol at a final concentration of 37.5% v/v was added to each bottle to achieve the total solubilisation of the UV filters. Finally, the bottle content was

filtered by vacuum with a Whitman GF/C glass fibber filter for determining fungal dry weight, and then analyzed by either HPLC-UV or HPLC-MS/MS. For BTZ and TTZ, at each sampling time, liquid medium was directly filtered by vacuum with Whitman GF/C for the fungal dry weight and 0.45 µm nylon filter (Millipore) for the other analyses.

Removal was determined by comparing contaminant concentration in the UNI controls with that in the EB flasks (Eq. 1). Degradation values were corrected for the sorption concentration values determined in HK or SAK control flasks (Eq. 2).

$$Removal\ (\%) = \frac{(UNI-EB)}{UNI} * 100 \qquad \text{Equation (1)}$$

$$Degradation\ (\%) = \frac{(HK-EB)}{UNI} * 100 \qquad \text{Equation (2)}$$

To quantify the amount of 4-MBC presumably taken up by the fungi, filtered pellets were resuspended in 5 mL of MilliQ water and sonicated under optimised conditions (10 min at 70% output capacity, as determined by observing fungal disruption in a phase contrast microscope) using aVibra Cell VC50 (Sonics & Materials, Inc.). Falcon tube was maintained in ice during all the disrupting time to avoid overheating of the samples. Disrupted fungus samples were thoroughly mixed with 5 mL of ethanol and centrifuged for 15 min at 6,240 × g. The supernatant was then filtered and analysed by HPLC-UV.

### *4.5.2. Degradation of selected contaminants in bioreactors*

A glass bioreactor (1.5 L) (Fig. 4.2) at experimental conditions described in Blánquez et al. (2004) was used for the degradation experiments in batch operational mode of spiked BP3 and BP1 in synthetic media. It consists on a glass bioreactor with air pulses in order to maintain a good agitation at the same time that assures the media aeration. Air pulses frequency is 3 sec closed and 1 sec opened; the oxigen supplied in that way is in excess of what fungus needs. pH was controled at 4.5 ± 0.5 with a probe (Mettler Toledo) and a controler (Mettler Toledo), adding HCl 1M or NaOH 1M. Temperature is mantained at

25ºC because the reactors were set up in a room with the temperature controlled at 25ºC. Pellets were added in an amount equivalent to a final concentration of 2 g DCW $L^{-1}$ and UV filters at 250 µg $L^{-1}$ from a concentrated stock standard solution in ethanol. Samples taken at scheduled times were vacuum filtered with Withman GF/C filters and concentrated by solid phase extraction (SPE).

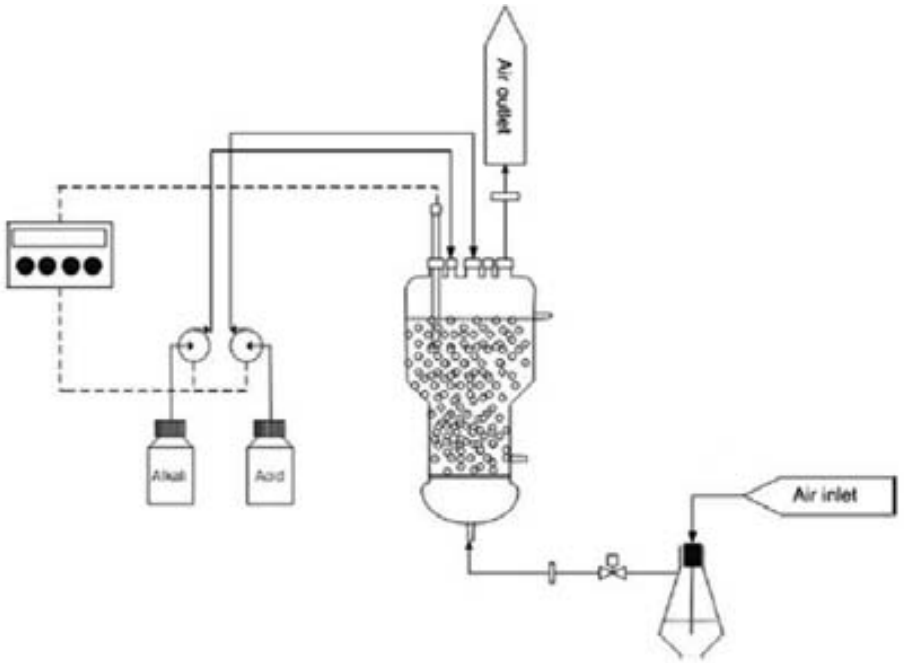



**Figure 4.2.** Scheme of the air-pulsed fluidised bioreactor. Modified from Blánquez et al. (2007).

### *4.5.3. Experiments with purified laccase*

To assess possible involvement of laccase in the first steps of contaminants degradation, *in vitro* experiments with comercial laccase of *T. versicolor* were performed. Laccase-mediated degradation experiments were done in amber bottles containing 10 mL of a purified laccase solution at a final activity of 300 U $L^{-1}$ (pH 4.5). The effect of the enzymatic mediators ABTS, HOBT, VA and DMHAP at a final concentration of 0.8 mM, except for HOBT at 1 mM, was also evaluated. 4-MBC was added at a final concentration of 10 mg $L^{-1}$, and the bottles were incubated under shaking conditions (130 rpm) at 25 ºC for 21 h.

### *4.5.4. Experiments with cytochrome P450 inhibitor*

To determine the involvement of cytochrome P450 in the degradation, 1-aminobenzotriazole (ABTS), a well known CYP450 inhibitor, was added to the

medium. A final concentration of 5 mM of ABTS was added from a stock solution in ethanol in a final volume of 10 mL while maintaining the fungal concentration at 5.0 g $L^{-1}$ dry weight, as described elsewhere (Marco-Urrea et al., 2006). These samples are referred to as experimental bottles with inhibitor (EBI). The same procedure, but without the addition of ABT or contaminant, was performed in inhibitor-free and UV filter-free controls, respectively. HK controls were also included.

## 4.6. Degradation experiments with stable isotope labeled compounds

### *4.6.1. Experimental design*

Experiments with stable isotope labeled compounds were also performed in 125 mL serum bottles (Wheaton, Mealville, NJ). Each experiment included, apart from the experimental bottles, uninoculated and sodium azide killed controls for abiotic degradation and biotic sorption determination respectively. DCF experiment also included heat killed controls. All experiments were conducted in duplicate. Cultures were incubated at 25ºC and 130 rpm orbital agitation.

$^{12}C$- or $^{13}C$-BP3/DCF were added from 100 mg $L^{-1}$ stock solution in acetonitrile (BP3) or ethanol (DCF) to a final concentration of 1 mg $L^{-1}$, in a total volume of 10 mL of medium. Acetonitrile and ethanol were totally evaporated with nitrogen before the addition of medium in order to avoid their possible use as carbon source by the fungus. Finally, one millilitre of blended mycelia was added to obtain a concentration of 0.5 g d.w. $L^{-1}$ in the bottles. In the sodium azide killed controls, 100 µL of sodium azide at 100 g $L^{-1}$ were additionally added the day before and left shacking overnight with the media and the fungus to ensure the total inactivation of the fungus prior to pollutant addition. Heat killed controls were previously autoclaved 30 min at 121ºC. The existing air inside the bottles was replaced by a higher oxygen content air by means of displacing the air with pure oxygen in order to avoid a potential oxygen limitation as *T. versicolor* is an aerobic organism (Marco-Urrea et al., 2008). Bottles were then closed with

Teflon-coated butyl-stoppers (Wheaton, Millville, NJ) and aluminium crimps (Baxter Scientific Products, McGaw Park, IL).

At each sampling point (initially, at 3, 6 and 9 days), the procedure was the same: for the non sacrificed bottles at that time, the air was replaced by blowing pure oxygen inside again and, for the sacrificed bottles, the procedure performed was as follows. $CO_2$ was sampled with a gas-tight syringe from the headspace of the bottle and directly injected to a gas chromatograph coupled to an isotope ratio mass spectrometer through a combustion interface (GC-C-IRMS). Then, for BP3 experiment, the bottles were opened and 1 mL was sampled and filtered through 0.22 µm PVDF syringe filter (Millipore, US) for glucose and laccase activity analyses. Afterwards, BP3 was solubilised by adding 6 mL of ethanol and the mixture was centrifuged for 10 min at 4ºC and 13000 *g*. In the case of DCF, ethanol addition was not needed due to its higher solubility in water. The supernatant was filtered by 0.45 µm nylon filter (Millipore, US) (BP3) or 0.22µm PVDF syringe filter (Millipore, US) (DCF) and analysed by HPLC-UV for contaminant quantification. The pellet was further processed for elemental analysis of biomass and amino acid-SIP (aa-SIP) analysis as described below.

### *4.6.2. Protein extraction, purification and amino acids derivatization*

Protocol used for protein extraction, purification and amino acids derivatization was adapted from Bastida et al. (2011). Fungal pellet was cleaned twice by resuspending it with 1 mL of Tris 20 mM pH 7.6 buffer and centrifuging 10 min at 4ºC and 5200 *g*. Part of the pellet was freeze-dried for elemental analysis and the rest was further processed for total protein extraction. Fungal pellet was resuspended with 1 mL of extraction buffer: 0.7 M sucrose, 0.5 M Tris, 50 mM EDTA, 0.1 M KCl and 40 mM dithiothreitol (Hernández-Macedo et al., 2002). Solution was lysed by probe sonication with 11 series of 11 seconds of sonication and 2 minutes of cooling in ice (Klimek-Ochab et al., 2011). Afterwards, lysate was centrifuged at 20.000 *g* and 4ºC during 15 min. The supernatant was transferred to two microtubes, adding one volume of 20% trichloroacetic acid (TCA) in each, and equally treated to perform the aa-SIP analysis. Mixture was incubated during 5 min at -20ºC and overnight at 4ºC.

Then, the solution was centrifuged 15 min at 20.000 *g* and 4ºC. The resulting pellet was dissolved in 200 µl of MilliQ-water and 1 mL of cold acetone was added. The solution was incubated at least 4-6 h at -20ºC. Finally, the solution was centrifuged 15 min at 20.000 *g* and 4ºC, the pellet was dried at 37ºC and resuspended with 50 µl of MilliQ-water.

For aa-SIP, protein pellet was firstly hydrolysed by adding 0.5 mL of HCl 6M and incubated at 110 ºC during 22 h. Then, the liquid was evaporated with nitrogen gas and afterwards the vial was cooled in ice 5 min. One millilitre of isopropanol and 250 µl of acetyl chloride were added and the mixture incubated overnight at 60ºC. Then, the solution was evaporated and the amino acids redisolved in 0.5 mL of dichloromethane and 0.5 mL of anhydrous trifluoroacetic acid and incubated 2h at 60ºC. Finally, derivatized amino acids were evaporated and dissolved again with 100 µL of dichloromethane and analysed by GC-C-IRMS.

## 4.7. Wastewater treatments

### *4.7.1. Fungal survival preliminar experiments*

Before performing the bioreactor experiments with real wastewater, a preliminar study about fungal survival growth in ROC was performed. Different treatments were thus performed in ambar serum bottles:

- ROC: reverse osmosis concentrate alone.
- ROC_pH: ROC adjusting the pH.
- ROC_N_pH: ROC supplemented with 3.3 g $L^{-1}$ of ammonia tartrate and adjustig the pH.
- ROC_CN: ROC supplemented with 8 g $L^{-1}$ of glucose, 3.3 g $L^{-1}$ of ammonia tartrate.
- ROC_CN_pH: ROC_CN adjustig the pH.
- DM_pH: defined medium (control)
- ROC_DM_pH: as DM_pH but using ROC instead of MilliQ water

In a second experiment, degradative capacity of fungus in ROC was assessed by means of the degradation of BP3 from an initial concentration of 10 mg $L^{-1}$ at ROC_pH, ROC_N_pH, ROC_CN_pH and DM_pH.

In all treatments, initial fungal biomass was 5 g DCW $L^{-1}$ and cultures were performed in a volume of 15 mL. In those treatments with the pH adjusted, it was set at 4.5.

### *4.7.2. Degradation experiment of UV filters present at ROC*

To determine the degradation of UV filters present in ROC, experiments with 200 mL of ROC in 500 mL bottles with a fungal concentration fo 2 g DCW $L^{-1}$ were performed. The treatments included in the experiment were abiotic controls without fungi (UNI), heat killed controls (HK) with the fungus autoclaved at 121ºC 30 min and some experimental bottles without the addition of extra nutrients and others where 8 g $L^{-1}$ of glucose and 3.3 g $L^{-1}$ of ammonia tartrate were initially added. Working with real wastewater samples, at pre-existent contaminant concentrations, implies higher contamination preventive methods, such as conscious cleaning of all material and rising with destilled water, ethanol and acetone. At each sampling point, duplicate samples were sacrificed. Samples were vaccuum filtered through a glass fiber Whatman GF/C filter and a 0.45 µm nylon filter (Millipore).

### *4.7.3. Bioreactors and operating conditions*

For PhACs and EDCs degradation studies at bioreactor scale, different experiments were performed under different conditions: sterile and non-sterile conditions, batch and continuous operation, and so on. At Table 4.4, the nomenclature and characteristics of the experiments is provided.

Bioreactors employed were fungal biomass air-pulsed fluidised bed glass bioreactors with an useful volume of 1.5 L (Fig 4.3A). For VHW2 experiment, a 10 L bioreactor was also used (Fig 4.3B). For ROC2, two 1.5 L bioreactors were set up in parallel under the same theoretical conditions (ROC2A and ROC2B). For ROC3, VHW1, VHW3 and VHW4, two 1.5 L bioreactors were set up in

parallel as well; in those cases one inoculated with *T. versicolor* (I) and the other non-inoculated as a control (NI).

**Table 4.4.** Characteristics of experiments

| Experiment | Wastewater | Sterile/non-sterile | Operational mode | Bioreactors | Other | Analyses |
|---|---|---|---|---|---|---|
| ROC1 | ROC | Sterile | Batch | 1.5 L | No extra nutrients | PhACs |
| ROC2 | ROC | Sterile | Batch | 2 x 1.5 L (A and B replicates) | - | PhACs, EDCs |
| ROC3 | ROC | Non-sterile | Continuous | 2 x 1.5 L (I and NI) | - | MB, PhACs, EDCs |
| VHW1 | VHW | Non-sterile | Batch | 2 x 1.5 L (I and NI) | - | MB, PhACs |
| VHW2 | VHW | Non-sterile | Batch | 10 L | - | MB, PhACs, EDCs |
| VHW3 | VHW | Non-sterile | Continuous | 2 x 1.5 L (I and NI) | - | MB |
| VHW4 | VHW | Non-sterile | Continuous | 2 x 1.5 L (I and NI) | - | MB, PhACs |

ROC: reverse osmosis concentrate; VHW: veterinary hospital wastewater; I: fungal inoculated bioreactor; NI: non-inoculated control bioreactor; MB: molecular biology studies of microbial community; PhACs: analysis of pharmaceutically active compounds; EDCs: analysis of endocrine disrupting compounds.

Temperature was set up at 25ºC and pH was controlled to be constant at 4.5 by HCl 1 M or NaOH1 M addition. Pellets of *T. versicolor* were added at 2-4 g dry cell weight (DCW) $L^{-1}$ (Table 4.5). Fungal pellets were kept inside the bioreactor by means of a mesh. At continuous treatments, 1/3 of the biomass was replaced by fresh one every 2-5 days, as determined by Blánquez et al. (2006), corresponding to a cellular retention time between 6 and 15 days (Table 4.5). Glucose and ammonia tartrate were added from a concentrated stock at pulses of 0.6 min $h^{-1}$. Initial addition rates are shown in Table 4.5 and they were changed during the experiment in some treatments in order to avoid glucose accumulation in the media or fungal lysis.

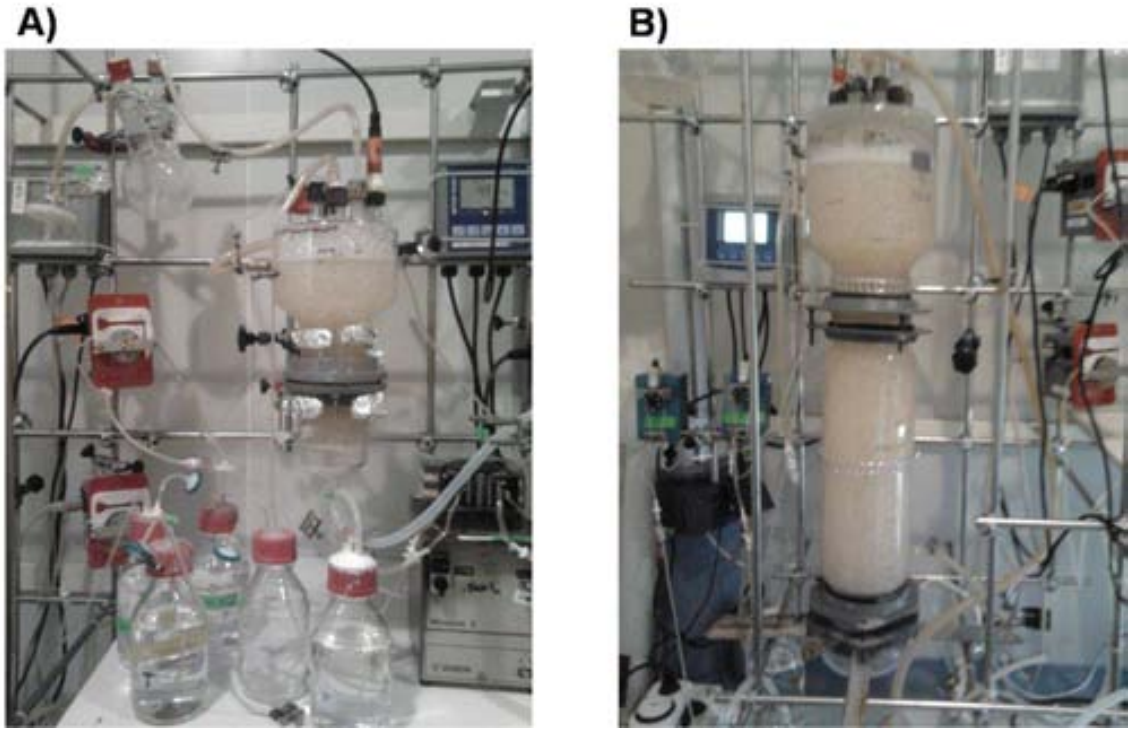



**Figure 4.3. A)** 1.5 L and **B)** 10L air-pulsed bioreactors employed in this thesis.

**Table 4.5.** Operational parameters of the different experiments

| Experiment | Glucose initial feed rate (mg $g^{-1}$ $d^{-1}$) | Ammonium tartrate initial feed rate (mg $g^{-1}$ $d^{-1}$) | HRT | CRT | Experiment lenght (d) | Initial fungal biomass (g DCW $L^{-1}$) |
|---|---|---|---|---|---|---|
| ROC1 | 0 | 0 | n.a. | 7 | 7 | 1.59 |
| ROC2 | 552-696[a] | 1.24-1.57 [a] | n.a. | 6 | 6 | 2 |
| ROC3 | 192 | 0.43 | 3 and 2 | 15 | 24 | 3.6 |
| VHW1 | 277 | 0.62 | n.a. | 15 | 15 | 2 |
| VHW2 | 300 | 0.67 | n.a. | 14 | 14 | 2 |
| VHW3 | 200 | 0.45 | 3 and 2 | 15 | 30 | 4.4 |
| VHW4 | 343 | 0.77 | 3.3 | Variable | 26 | 3.7 |

HRT: hydraulic residence time; CRT: cellular residence time; DCW: dry cell weight; n.a.: not aplicable

[a] data for ROC2A and ROC2B, respectively.

In the continuous treatments, wastewater in the feed storage tank was replaced every 2-3 days by fresh one (from the most recent sampling) stored at 4ºC. Wastewater already stored in the feed tank for 2-3 days is called “old wastewater”.

Samples at batch experiments were obtained by making negative pressure through a silicone tube that ends at a sterilised glass vial (hereafter refered to as the sampling system). In continuous experiments, samples were collected from the outlet effluent storage bottle, replaced by a new one every 24 hours.

Samples for microbial community analyses were kept frozen at -20ºC in sterile 2 mL eppendorf tubes and 50 mL falcon tubes without any processing. Samples for contaminants quantification and other routine analyses were vacuum filtered with 1.2 µm Wathman GF/C filters followed by 0.45 µm nylon filter (Millipore). Sample concentration and dilution caused by acid/base addition were taken into account for pharmaceuticals removal calculations.

## 4.8. Analytical methods

### *4.8.1. HPLC-UV analysis*

Quantification of contaminants to determine their degradation in spiked experiments was perfomed in a Dionex 3000 Ultimate HPLC equipped with UV detector and autosampler Dionex. Samples of biodegradation experiments were placed in amber HPLC vials to avoid natural photodegradation during the analysis. The chromatographic separation was achieved on a LiChrosphere RP-18 (125 mm x 4 mm, 5 µm) LC column from Merck.

The method used for UV filters was based on that of Salvador and Chisvert (2005), with modifications for the detection of each compound (Table 4.6). Flow rate and injection volume were set up to 0.5 mL $min^{-1}$ and 20 µL, respectively. The mobile phase consisted of ethanol (A) and acetic acid 1% in MilliQ water (B). The detection of the analytes was performed at 313 nm

The method for DCF separation was modified from Marco-Urrea et al. (2010b), changing the isocratic mobile phase for a gradient elution. Flow rate and injection volume were set up to 1 mL $min^{-1}$ and 20 µL, respectively. The mobile

phase consisted of acetonitrile (A) and acetic acid 6.9 mM in MilliQ water adjusted to pH 4 (by NaOH) (B). The detection of the analytes was performed at 275 nm

For BTZs, a chromatographic gradient adapted from Müller et al. (2011) was used for compounds separation. 20 µL of sample was injected at flow rate of 0.6 mL $min^{-1}$. The mobile phase consisted of methanol (A) and MiliQ water (B). The detection of the analytes was performed at 275 nm as described elsewhere (Hart et al., 2004).

Calibration curves were done from multiple dilutions of stock solutions in ethanol. Concentration ranges were from 0.01 mg $L^{-1}$ to 25 mg $L^{-1}$. R values obtained were all over 0.99.

**Table 4.6.** Chromatographic methods for the different studied contaminants

| Compounds to analyse | | % of eluent at each time | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| BP3, BP1, 4-MBC, OC, EMC | Time (min) | 0 | 5 | 20 | 35 | 35.5 | 40 | 40.5 | 50 |
| | A (Ethanol) | 20 | 20 | 70 | 70 | 100 | 100 | 20 | 20 |
| | B (1% acetic acid) | 80 | 80 | 30 | 30 | 0 | 0 | 80 | 80 |
| BP3, BP1 | Time (min) | 0 | 5 | 10 | 15 | 15.5 | 20 | 20.5 | 30 |
| | A (Ethanol) | 20 | 20 | 50 | 70 | 100 | 100 | 20 | 20 |
| | B (1% acetic acid) | 80 | 80 | 50 | 30 | 0 | 0 | 80 | 80 |
| 4-MBC | Time (min) | 0 | 5 | 20 | 25 | 25.5 | 30 | 30.5 | 40 |
| | A (Ethanol) | 20 | 20 | 70 | 70 | 100 | 100 | 20 | 20 |
| | B (1% acetic acid) | 80 | 80 | 30 | 30 | 0 | 0 | 80 | 80 |
| DCF | Time (min) | 0 | | 20 | 25 | 30 | | 30.5 | 35 |
| | A (Acetonitrile) | 35 | | 55 | 100 | 100 | | 35 | 35 |
| | B (Acetic acid 6.9 mM) | 65 | | 45 | 0 | 0 | | 65 | 65 |
| BTZ, 4-MBTZ and 5-MBTZ | Time (min) | 0 | | 1 | 13 | 18 | | 18.5 | 24 |
| | A (Methanol) | 5 | | 5 | 95 | 95 | | 5 | 5 |
| | B (MilliQ water) | 95 | | 95 | 5 | 5 | | 95 | 95 |

### *4.8.2. SPE extraction*

SPE for BP3 and BP1 bioreactor samples were performed with 3cc and 60 mg Oasis HLB cartridges (Waters, Mildford, USA) as described in Radjenovic et al. (2007). A Baker connected to a vacuum pump was used. Firstly, cartridge was conditioned with 2 x 2.5 mL methanol and 2 x 2.5 mL MilliQ water adjusted to pH 4.5, eluted by gravity. 20 mL of samples were passed through the cartridge

and compounds eluted with 2 x 2 mL of methanol. The extracts were reconstituted with 0.5 ml 40:60 v/v ethanol:water 1% acetic acid. The percentage of recovery was 78.2 ± 7.5 for BP3 and 96.3 ± 1.3 for BP1. For ER-RYA assay, samples were evaporated and reconstituted with 0.5 ml of methanol (MeOH).

SPE for UV filters extraction from ROC were performed with 3cc and 200 mg Isolute C18 cartridges (Biotage). Firstly, cartridges were conditioned by 5 mL of ethyl acetate, 5 mL of methanol and 5 mL of MilliQ water. 200 mL of sample were passed through the cartridge in a constant and moderate speed and dried under vaccuum. Finally UV filters were eluted in two tubes, the first with 6 mL of ethyl acetate and the second with 8 mL of methanol. Samples were partially evaporated under a nitrogen stream and the content of the tubes was mixed in a chromatography vial. Tubes were cleaned with methanol/ethyl acetate 1:1 (v/v). Finally, the vial was totally evaporated and redisolved in 0.5 ml 40:60 v/v ethanol:water 1% acetic acid.

### *4.8.3. Stable isotope analysis*

Analysis of carbon isotopic composition in the different samples were performed in the Scientific and Technological Centers service of Universitat de Barcelona (UB) in a collaboration with Dr. Monica Rosell from the department of crystallography, mineralogy and mineral deposits in UB.

*$CO_2$ analysis by GC-C-IRMS*

$^{13}C/^{12}C$ ratios of headspace $CO_2$ were determined by a GC–C–IRMS system consisted of an Agilent 6890 gas chromatograph (Palo Alto, CA, USA) equipped with a split/splitless injector, coupled to a Delta Plus isotope ratio mass spectrometer through a GC-Combustion III interface (ThermoFinnigan, Bremen, Germany). The injector was set at 220ºC in split mode (1:10) and the amount of headspace gas sample (from 3 to 600 µL) was selected in each injection depending on $CO_2$ concentration in order to get good amplitudes and reproducibility in duplicate analysis. The GC was equipped with two different capillary columns: a GS-GasPro column (30 m x 0.32 mm, Agilent Technologies, Palo Alto, CA, USA) set at isotherm 30ºC for the BP3 experiment

and a HP-PLOT/Q (30 m x 0.32 mm x 20 µm film, Agilent Technologies, Palo Alto, CA, USA) set at 50ºC. The gas chromatograph and the mass spectrometry system were controlled using the Thermo Isodat NT 2.0 (Bremen, Germany) software, which also allowed the acquisition of data and the calculation of the $\delta^{13}C$ values against our $CO_2$ gas reference. $CO_2$ calibration curve was obtained by injection of the content of previously evacuated Vacutainers™ filled with argon and known volumes of $CO_2$.

*Bulk biomass analysis by EA-IRMS*

The carbon isotopic composition of the bulk freeze dried biomass was determined using a Flash EA1112 elemental analyser (EA) coupled to a Delta C isotope ratio mass spectrometer through a Conflo III interface (ThermoFinnigan, Bremen, Germany).

*Amino acids SIP analysis by GC-C-IRMS*

Carbon isotopic compositions of individual amino acids were determined with the same GC–C–IRMS system described for $CO_2$. A HP-5MS UI column (30 m x 0.32 mm x 1 µm film, Agilent, Palo Alto, CA, USA) was used for chromatographic separation with helium as a carrier gas at a flow rate of1.8 mL $min^{-1}$ and the following temperature program: the initial 40ºC were held for 2 min, heated to 160ºC at 10ºC $min^{-1}$ and immediately heated to 190ºC at 4ºC $min^{-1}$ and finally heated to 300ºC at 10ºC $min^{-1}$ and held for 10 min. The injector was set at 300ºC and the samples (1 µL from dichloromethane extracts) were injected using either the splitless mode or with a split ratio of 1:5 (only the standard). The individual amino acids were identified by comparison of the retention times and mass spectra (by conventional GC-MS) with those of the standard mixture (Sigma-Aldrich, Saint Louis, USA).

### *4.8.4. Monitoring analysis of the treatments*

*Glucose analysis*

Glucose concentration was measured with a biochemical analyser YSI 2700 SELECT (Yellow Spring Instruments) in the concentration range 0-20 ± 0.04 g $L^{-1}$. Analysis is based on the enzymatic reaction of glucose oxidation to oxygen peroxide throught the enzyme gluocose oxidase immobilized in a membrane

and the subsequent reduction of oxygen peroxide in a platinium anode, that converts the signal in electric intensity.

*Laccase activity*

Laccase activity was measured through the oxidation of 2,6-dimetoxyphenol (DMP) by the enzyme laccase in a modified version of the method for the determination of manganese peroxidase of Kaal et al. (1993). The analysis process is based on the measure of the absorbance variance at 468 nm at 30ºC during 2 min in a Varian Cary 3 UV/Vis spectrophotometer. The reaction was done with 600 µL of sample, 200 µL of sodium malonate 250 mM at pH 4.5 and 50 µL of DMP 20 mM. Activity units per litre (U $L^{-1}$) are defined as the amount of DMP in micromoles per litre which are oxidized per minute (µmol DMP $L^{-1}$ $min^{-1}$). The molar extinction coefficient of DMP was considered as 24.8 $mM^{-1}$ $cm^{-1}$ (Wariishi et al., 1992).

*Fungal dry weight*

Biomass pellets dry weight was determined after vacuum-filtering the cultures through pre-weighted glass-fiber filters (Whatman, Spain). The fungal amount was determined as the constant weight at 100 ºC.

*pH and conductivity*

Conductivity was determined by a CRISON MicroCM 2100 conductimetre and pH by a pHmetre CRISON MicropH 2001.

*Organic matter and ions*

Dissolved organic carbon (DOC) and total suspended solids (TSS) were analysed according to APHA (APHA-AWWA-WEF, 1995). The $N\text{-}NH_4^+$ concentration and chemical oxygen demand (COD) were analysed by using commercial kits LCH303 and LCK114 or LCK314 respectively (Hach Lange, Germany). Clorur, nitrate, nitrite and sulphate anions were quantified by a Dionex ICS-2000 ionic chromatograph. Phosphate was analysed by a phosphate analyzer (Hach Lange 115 VAC Phosphax sc).

### *4.8.5. Analysis for metabolites identification*

Determination of fungal metabolites of the selected contaminants was performed by the chemical analytical groups of Institut de Diagnosi Ambiental i Estudis de l'Aigua (IDAEA-CSIC) and Institut Català de Recerca de l'Aigua (ICRA), led by Dr. Damià Barceló.

Analysis of BP3, BP1 and 4-MBC were performed at IDAEA-CSIC by Pablo Gago, supervised by Dra. Sílvia Díaz as described at Badia-Fabregat et al. (2012) and Gago-Ferrero et al. (2012). Briefly, target analysis of BP3 and its known metabolites, namely BP1, 4HB, 4DHB, DHMB and THB were performed by HPLC using a hybrid triple quadrupole-linear ion trap mass spectrometer (HPLC–QqLIT-MS/MS) from Applied Biosystems-Sciex (Foster City, California, USA). Non-target analyses for the identification of unknown metabolites were performed by ultra high performance liquid chromatography coupled to quadrupole-time of flight-tandem mass spectrometry (UPLC-QqTOF-MS/MS) using a Waters Acquity UPLC$^{TM}$ system attached to a Waters/Micromass QqToF-Micro$^{TM}$ (Waters/Micromass, Manchester, UK).

Analysis for BTZ, 4-MBTZ and 5-MBTZ were performed at ICRA by Dr. Marta Llorca, supervised by Dr. Sara Rodríguez. The equipment employed was an on-line turbulent flow chromatography system coupled to a hybrid linear ion trap – high resolution mass spectrometer LTQ Orbitrap (TFC-LTQ Orbitrap).

### *4.8.6. Analysis of PhACs and EDCs*

Quantification of PhACs and EDCs was performed by the chemical analytical group of Institut Català de Recerca de l'Aigua (ICRA), led by Dra. Sara Rodríguez and Dr. Damià Barceló.

Quantification of 76 PhACs at ROC1 was performed by Dr. Meritxell Gros and in all the other experiments by Daniel Lucas. The analytical procedure performed is based on Gros et al. (2012) and briefly it consists on an SPE (Solid Phase Extraction) using Oasis HLB (3cc, 60 mg) cartridges (Waters Corp. Mildford, MA, USA), followed by a Ultra-Performance liquid chromatography (Waters Corp. Mildford, MA, USA) coupled to 5500 QqLit, triple

quadrupole–linear ion trap mass spectrometer (5500 QTRAP, Applied Biosystems, Foster City, CA, USA).

Quantificatin of 27 EDCs and related compounds was performed by Dra. Marta Llorca. EDCs were analyzed by on-line turbulent flow chromatography coupled to a liquid chromatography tandem mass spectrometry (LC-MS/MS) system through the methodology previously developed by Gorga et al. (2013).

## 4.9. Toxicity assays

### *4.9.1. Microtox*

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

### *4.9.2. Recombinant yeast assay (RYA)*

Yeast-based bioassays, in which yeast harbours the human oestrogen receptor (hER) or the human aryl hydrocarbon receptor (hAhR), can be used to monitor and quantify interactions between ER or AhR, respectively, and the compounds present in the medium by activation of the LacZ gene. The protocol carried out is described at Noguerol et al. (2006).

Firstly, a colony of a Petri dish was grown overnight in assay tubes containing 2 mL of YPD media. It was incubated at 30ºC ans gently agitation of 80 rpm. Then, a selection of plasmid containing clones was performed with an SC media overnight culture: 10 µL of YPD culture were inoculated in 10 mL of SC media in a 50 mL erlenmeyer. Incubation was also at 30ºC and 80 rpm.

For RYA assay, SC culture needs to be in the exponential phase, meaning a $DO_{600}$ of 0.1-0.15. If DO is higher it can be diluted with new SC media, however, always taking into account that if dilution is higher than ¼, one hour more of incubation is needed. Assay was performed in polypropilene, a non-estrogenic plastic, microtiter plates (NUNC, Thermo Scientific). Once working in the plates, esterility conditions were more flexible. 50 µL of SC culture were added to each well, except for the first one of each dilution set, where 100 µL were added. 10 µL of sample were added to this first well, obtaining a 1/10 dilution. After mixing it well, ½ successive dilutions were performed for the rest of the wells, leaving 50 µL of mixture at each one for the assay. Every plate must contain also a line of negative control and another of positive control. Negative control consists on the SC culture with the yeast but without the addition of sample, to determine the basal fluorescence of the yeast. For the positive control, 17β-estradiol (5 µl of 10 µM solution in methanol, in 5 mL of SC culture) or β-naphtoflavone (50 µl of 100 µM solution in 5 ml of SC culture) was added at ER-RYA or YCM-RYA, respectively. The last well of each sample raw was reserved for possible toxicity assay. It was performed mixing 50 µL of positive control with 50 µL of the first sample dilution (form the first well of the row). The aim of this control is to determine if a negative response of the samples is actually due to the no interaction with the receptor or a possible toxicity of the sample with the subsequent die or inhibition of the yeast. For possible anti-estrogenic activity assays, samples were diluted with medium containing 17β-estradiol at 10 nM instead of medium alone and serial dilutions were also performed. For the positive control, tamoxifen at a final concentration of 673 nM was used.

The plates were incubated 6 hours at 30ºC and 80 rpm, covered with aluminium foil. After that period, 50 µL of the detergent yeast protein extraction reagent (Y-PER®, Thermo Scientific) were added at each well in order to lyse the cells and release the intracellular enzymes. In this point, right after Y-PER® addition, plates can be frozen at -20ºC for further analysis. Then, plates were incubated half an hour more. Finally, after incubation, 50 µL of MuGal media were added. Quickly, plate was centrifuge 1 min at 1000 rpm and read in a fluorimetre (Victor III, Perkin Elmer). Wave lenghts of absorbtion and emission were 365 and 445

nm, respectively. Values of each well were taken every 63 sec during 20 min (20 complete readings of the plate).

Some considerations for the assay are that samples must be dissolved in water or methanol (or mixtures of both); however, final concentration in the assay cannot be higher than 10% to avoid toxicity for yeast cells. For the addition of the samples and compounds, glass capillaries were used to avoid the adsoption of compounds to the plastic of pipete tips or the release of estrogenic compound from them. Analysed sampes from fungal degradation experiments of real effluents were those processed for PhACs or EDCs quantification analysis because they need to be concentrated by SPE. Samples for the $EC_{50}$ determination of selected compunds were directly analysed from stock solutions in methanol. The results are expressed as estradiol equivalents (EEQ, ER-RYA) or ß-naphthoflavone equivalents (NFQ, AhR-RYA), as described (Noguerol et al., 2006).

## 4.10. Microbial community analysis

### *4.10.1. Phospholipid fatty acids analysis (PLFA)*

Phospholipid fatty acids extraction and analysis were done according to Frostegård et al. (1993) with some modifications to improve extraction in liquid samples. Briefly, 40 mL of sample were centrifuged 20 min at 10000*g* and supernatant was filtered through a 0.7 µm glass fiber filter (Millipore). Cell pellet and filter were extracted together overnight with 1.5 mL citrate buffer (0.15M, pH 4.0), 1.9 mL of chloroform, 3.75 mL of methanol and 2 mL of Blight&Dyer solution (chloroform:methanol:citrate buffer 1:2:0.8 v/v/v). Mixture was centrifuged 10 min at 2500 rpm and supernatant was transferred in a clean tube. Pellet was cleaned with 2.5 mL of Blight&Dyer solution and supernatants collected toghether. Then, 3.1 mL of chloroform and 3.1 mL of citrate buffer were added and left overnight for separation of phases. Lower phase was transferred to a clean tube and evaporated under nitrogen stream. Polar lipids were separated through a silicic acid column previously activated at 120ºC.

Sample was dissolved in 100 µL of chloroform and added to the column. Neutral lipids were discarded with 5 mL of chloroform and glycolipids with 20 mL of acetone. Finally, polar lipids were eluted with 5 mL of methanol and evaporated under nitrogen stream. At that point, internal standards (19:0 and 13:0) were added. 1 mL of methanol:toluene (1:1 v/v), 2 mL of hexane:chloroform (4:1 v/v), 0.3 mL of acetic acid 1 M and 2 mL of water were added and centrifuged 3 min at 2500 rpm after vortexing. Upper phase was transferred to a clean tube and the original tube was cleaned with 2 mL of hexane:chloroform (4:1 v/v). Collected upper phases were evaporated under nitrogen stream and redisolved in 50-100 µL of hexane. Samples were analysed with a gas chromatograph (Hewlett Packard Series II 5890) with flame ionization detector through a 50 m HP-5 capilar column.

To determine bacterial biomass, the following fatty acids were taken into account: i15:0, a15:0, 15:0, i16:0, 16:1ω9, i17:0, a17:0, 17:0, cy17:0, 18:1ω7 and cy19:0. To determine fungal biomass, only 18:2ω6 was taken into account. To calculate the aproximate amount of C that can be assigned to bacteria and fungus, conversion factors obtained from the literature were applied: 363.6 nmol bacterial PLFA $mg^{-1}$ C for bacteria (Frostegård and Bååth, 1996) and 11.8 nmol fungal PLFA $mg^{-1}$ C for fungi (Klamer and Bååth, 2004). Principal component analyses (PCA) of the results, taking into account the areas of those quantified peaks, were performed with the program PC-ORD 5.0.

### *4.10.2. Nucleic acid extraction, PCR-DGGE, sequencing and phylogenetic analyses*

DGGE and subsequent phylogenetic analyses were performed at Departamento de Engenharia Biologica of Universidade do Minho for VHW1 and VHW2 experiments and at Finnish Forest Research Institute (METLA) for VHW3, VHW4 and ROC3 experiments. Therefore, two methodologies are described corresponding to each research center.

For VHW1 and VHW2 experiments, homogenized samples were taken from the bioreactors at different times and stored at -20 ºC. DNA extraction, PCR amplification, DGGE analysis, cloning and sequencing was performed as

previously described (Rodríguez-Rodríguez et al., 2012b). Briefly, total genomic DNA was extracted from approximately 4 mL for each sample using the Fast DNA® Spin kit for soil (MP Biomedicals, USA). Bacterial 16S rRNA genes and internal transcribed spacer (ITS) regions of fungal rRNA genes were amplified from total genomic DNA. Complete bacterial 16S rRNA genes were amplified for cloning and sequencing by using the forward primer Bact27-f and the universal reverse primer Uni1492-r (Weisburg et al., 1991). The thermocycling program was: 95 ºC for 2 min; 95 ºC for 30 s, 52 ºC for 40 s and 72 ºC for 90 s (30 cycles), and 72 ºC for 5 min. In the case of fungal ITS regions the forward primer ITS1F (Gardes and Bruns, 1993) and the reverse primer ITS4 (White et al., 1990) were employed. The thermocycling program was as described above but with an annealing temperature of 55ºC and duration of 30 s. For DGGE analysis, PCR products were generated using bacterial 16S rRNA gene primers U968-f and L1401-r (Nübel et al., 1996) and the fungal rRNA primers ITS1F and ITS2 (White et al., 1990). A 40-base GC clamp was attached to the primers U968-f and ITS1F at the 5' end. The thermocycling programs were as described above but with 35 cycles, an annealing time of 40 s and elongation time of 1 min and annealing temperature of 56ºC for bacterial primers. The size and amount of PCR products was estimated by electrophoresis in 1% agarose gels (w/v) using a DNA marker and SYBR®Safe staining. DGGE analysis of the PCR products was performed with a DCode system (Bio-Rad, Hercules, CA, USA). Gels containing 8% (w/v) polyacrilamide (37.5:1 acrylamide/bis-acrylamide) with a denaturing gradient of 30-60% for bacteria and 20-50% for fungi were employed. The 100% denaturant corresponded to 7 M urea and 40% (v/v) formamide. Electrophoresis was performed for 16 h at 85 V in 0.5X TAE buffer at 60ºC. Gels were then stained with silver nitrate and scanned in an Epson Perfection V750 PRO (Epson, USA). Fragments previously amplified by PCR using the primers Bact27-f/Uni1492-r and ITS1F/ITS4 were purified by means of the PCR cleanup kit NucleoSpin Extract II (Macherey-Nagel, Germany). The fragments were then incorporated into a pGEM-T vector using the pGEM Easy Vector Systems kit (Promega, Madison, WI, USA). The vector was employed in the transformation of *Escherichia coli* competent cells JM109 Competent Cells (Nzytech). Positive transformants were selected after growth in LB medium supplemented with ampicillin, IPTG and X-Gal. After PCR

amplification with U968GC-f/L1401-r or ITS1FGC/ITS2, clones were screened in DGGE by means of comparing with the corresponding band-patterns of the samples. Those clones matching different bands in the total community profile were selected, and their inserts were amplified by PCR using the pGEM- T vector-targeted sequencing primers Sp6 and T7, purified (NucleoSpin Extract II kit) and subjected to DNA sequence analysis. Inserts were bidirectionally sequenced with the primers Sp6/T7 at Eurofins MWG Operon (Ebersberg, Germany). For phylogenetic analysis, partial sequences were assembled using the CAP application included in the BioEdit v7.0.9 software package (Hall, 1999). Chimeras were checked with Mallard 1.02 and Pintail 1.1 program. Similarity searches for the assembled sequences were performed using the NCBI Blast search program within the GenBank database. All sequencies have been deposited to GenBank database under Accession Numbers from KM 392022 to KM392055.

For VHW3, VHW4 and ROC3 experiments, total DNA was extracted from 4 mL samples with FastDNA SPIN Kit for Soil (MP Biomedicals) following the procedure described by the company. Fragments of bacterial 16S and fungal Internal Transcribed Spacer (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 U968 forward (5’ ACC GCG AAG AAC CTT AC 3’) and R1401 reverse (5’ CGC TGT GTA CAA GAC CC 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 U968 at the 5’ end. The PCR program for fungi was 5 min at 95ºC followed by 40 cycles of 30 sec at 95ºC, 40 sec at 55ºC and 1 min at 72ºC, ending with a final elongation step of 5 min at 72ºC. Bacterial program was the same except for the hibridation temperature which was 56ºC. The length and ammount of PCR products were estimated in 1% agarose gel with DNA ladder and labeled with ethidium bromide. Denaturing Gradient Gel Electroforesis (DGGE) were performed in an INGENYphorU (Ingeny, The Netherlands) machine. Urea gradients were adjusted in order to optimise 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. Electroforesis were performed during 16 hours at 75 V in 1x TAE buffer at 60ºC. 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 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 R1401 primers. Partial fungal and bacterial DGGE-derived sequences were aligned with sequences retrieved from databases of GenBank/EMBL/DDBJ with Blastn algorithm. Bacterial and fungal sequence data have been deposited to GenBank database under Accession Numbers KM355623-KM355667 and KM361323-KM361352, respectively. Those sequences that could not be deposited because their length was less than 200 bp can be found in the Tables 4.7 and 4.8). DGGE images were analysed with GelCompar II (ver. 5.1.; Applied Maths BVBA, Belgium), generating a binary matrix (presence/absence of band) that it was furhter analysed by detrended correspondence analyses (DCA) with the program PC-ORD 5.0.

**Table 4.7**. Sequence of those fungal sequences that could not be submitted at GenBank.

| DGGE Band | Sequence (plus strain, 5'→3') |
|---|---|
| VHW3-F1 | ATTACTGAATGATAATTTCAACACTTGTGAATTATAAATTAATTTGTTTGGGTGTTTTTCGAAAGAGAAATGCCCAAAGATTTTTTAAAAAACACTTATTTTAAACCAATATCTGAGAAAATTTTAATAAATAAATTAAAACTTTCAACAACGGATCTCTTGGTTCTC |
| VHW3-F6 | CATTACCGAGTTTACAACTCCCAAACCCCTGTGAACATACCACTTGTTGCCTCGGCGGATCAGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGAAGACCCCTAAACTCTGTTTCTATATGTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTG |
| VHW3-F9 | GTCATTAACAAATGCTTTATCTGTCCTTGATTGTGGACAGACATTCGTGCCTCCAAGCACAAAATGTGTACCAGTGTTACAGTAGTCTATATGCGAAAAATATAAAAACTTTTAACAATGGATCTCTTGGCTCTT |
| VHW4-F1 | TGCGGAGGATCATTACTGAATGATAATTTCAACACTTGTGAATTATAAATTAATTTGTTTGGGTGTTTTTCGAAAGAGAAATGCCCAAAGATTTTTTAAAAAACACTTATTTTAAACCAATATCTGAGAAAATTTTAATAAATAAATTAAAACTTTCAACAACGGATCTCTTGGTTCTC |
| VHW4-F4 | TCATTAGTGAATATAGGACGTCCAACTTAACTTGGAGTCCGAACTCTCACTTTC |

| | |
|---|---|
| | TAACCCTGTGCACTTGTTTGGGATAGTAACTCTCGCAAGAGAGCGAACTCCTA TTCACTTATAAACACAAAGTCTATGAATGTATTAAATTTTATAACAAAATAAAACT TTCAACAACGGATCTCTTGGCTCTC |
| VHW4-F5 | AACTCCCAAACCATATGTTTATCTACCTGTTTCGTTGCTTCGGCAGGCGGTTCC TCTGGAGCCTTCGCCCTACGGGTGCCTGCCGGAGTATCTAAAACTCTTGTTAA TTACTGTTCTGTCTGAGTAAACTTTTAATAAGTTAAAACTTTCAACAACGGATCT CTTGGTTCTG |
| VHW4-F6 | AACTCCCAAACCATATGTTTATCTACCTGTTTCGTTGCTTCGGCAGGCGGTTCC TCTGGAGCCTTCGCCCTACGGGTGCCTGCCGGAGTATCTAAAACTCTTGTTAA TTACTGTTCTGTCTGAGTAAACTTTTAATAAGTTAAAACTTTCAACAACGGATCT CTTGGTTCTG |
| VHW4-F8 | GGATCATTACCGAGTTATACAACTCATCAACCCTGTGAACATACCTAAACGTTG CTTCGGCGGGAACAGACGGCCCCGTAACACGGGCCGCCCCCGCCAGAGGAC CCCCTAACTCTGTTTTTATAATGTTTTTTCTGAGTAAACAAGCAAATAAATTAAA ACTTTCAACAACGGATCTCTTGGCTCTG |
| VHW4-F9 | GGATCATTACCGAGTTATACAACTCATCAACCCTGTGAACATACCTAAACGTTG CTTCGGCGGGAACAGACGGCCCCGTAACACGGGCCGCCCCCGCCAGAGGAC CCCCTAACTCTGTTTTTATAATGTTTTTTCTGAGTAAACAAGCAAATAAATTAAA ACTTTCAACAACGGATCTCTTGGCTCTG |
| VHW4-F10 | GGATCATTACCGAGTTATACAACTCATCAACCCTGTGAACATACCTAAAACGTT GCTTCGGCGGGAACAGACGGCCCTGTAACAACGGGCCGCCCCCGCCAGAGG ACCCCTAACTCTGTTTTTATAATGTTTTTCTGAGTAAACAAGCAAATAAATTAAA ACTTTCAACAACGGATCTCTTGGCTCTG |
| VHW4-F21 | GCGATCCCCCCAATCTCCCGCCCCCCGCCGGGGGCGGCGGGCCCTCTGAAC CTTTTTTTAAACCACTGAAACCGTCTGAGCAAACCAAAAACAAAATTATCAAAA CTTTCAACAACGGATCTCTTGGCTCTG |
| ROC3-F5 | TCATTAGTGAACATAGGACGTCCAACTTAACTTGGAGTCCGAACTCTCACTTTC TAACCCTGTGCATTTGTTTGGGATAGTAACTCTCGCAAGAGAGCGAACTCCTA TTCACTTATAAACACAAAGTCTATGAATGTATTTAATTTTATAACAAAATAAAACT TTCAACAACGGATCTCTTGGCTCTC |
| ROC3-F6 | ATATAGGACGTCCAACTTAACTTGGAGTCCGACCTCTCACTTTCTAACCCTGTG CATTTGTTTGGGATAGTAACTCTCGCAAGAGGGCGAACTCCTATTCACTTATAA AACACAAAGTCTATGAATGTATTAAATTTTATAACAAAATAAAACTTTCAACAAC GGATCTCTTGGCTCTC |
| ROC3-F7 | ATATAGGACGTCCAACTTAACTTGGAGTCCGAACTCTCACTTTCTAACCCTGTG CACTTGTTTGGGATAGTAACTCTCGCAAGAGAGCGAACTCCTATTCACTTATAA ACACAAAGTCTATGAATGTATTAAATTTTATAACAAAATAAAACTTTCAACAACG GATCTCTTGGCTCTC |
| ROC3-F8 | GATCATTAGTGATTGCCTTAATTGGCTTAAAACTATATCCATCTACACCTGTGA ACTGTTTGATTGAATCTTCGGATTCGATTTTTATACAAACATTGTGTAATGAACG |

| | |
|---|---|
| | TCATTAGATCATAACAAAAAAAAACTTTCAACAACGGATCTCTTGGCTCTC |
| ROC3-F9 | TCATTACCGAGTTTACAACTCCCAAACCCCTGTGAACATACCACTTGTTGCCTC GGCGGATCAGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCT AAACTCTGTTTCTATATGTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTT CAACAACGGATCTCTTGGTTCTG |
| ROC3-F10 | GGATCATTACCGAGTTTACAACTCCCAAACCCCTGTGAACATACCACTTGTTG CCTCGGCGGATCAGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGAGGAC CCCTAAACTCTGTTTCTATATGTAACTTCTGAGTAAAACCATAAATAAATCAAAA CTTTCAACAACGGATCTCTTGGTTCTG |
| ROC3-F12 | TCATTATCGAGTTACCGAGGGGGCATTAGATGCTGGCCGCAAGGCACTGTGC TCGGCCCCTTTGTTCAACTAGTTCCAACCACCGTGCATCACTTCGAGGGGTTC GCCCCTTGATATCACACAAACCACTGTATTATGAAGTCTAATAGAATGCAAATT TGATTAAATACAACTTTCAACAACGGATCTCTTGGCTCTC |
| ROC3-F13 | TCATTATCGAGTTACCGAGGGGGCATTAGATGCTGGCCGCAAGGCACTGTGC TCGGCCCCTTTGTTCAACTAGTTCCAACCACCGTGCATCACTTCGAGGGGTTC GCCCCTTGATATCACACAAACCACTGTATTATGAAGTCTAATAGAATGCAAATT TGATTAAATACAACTTTCAACAACGGATCTCTTGGCTCTC |
| ROC3-F14 | AAATTTCGAGTGCTGAGCAGCGGCTCGCCAGGTGCCAGCAATGGCGCTGGGT GAGTTTGCTGCCGACACCACTTTACGCTTGTTGTGTTTGACAGAGTTATTGTAC CTTTAAAAATAGACAACTTTTAACAATGGATCTCTTGGCTCTT |
| ROC3-F22 | GGATCATTACTGAGTTACCAACTCCAAACCCTCCCGTGGACCCCTAGAACGTT GCCTCGGCGGGCCCGCCCGCCGGTGGCCCCAACAAACAAAAACTCTCCAAC GTACTTCTGAGTGGCGCAGACTAAAAATAAGTCAAAACTTTCAGCAACGGATC TCTTGGCTCTG |

**Table 4.8**. Sequence for those bacterial sequences that could not be deposited.

| DGGE Band | Sequence (plus strain, 5'→3') |
|---|---|
| VHW3-B8 | GTCGAGTTGCAGACTACAATCCGAACTGAGACGTTATTTTTGAGATTTGCTC CGCCTCACTCCTTCGCTTCCCTTTGTTTACGCCATTGTAGCACGTGTGTAGC CCAAATCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCAGGT TATCCCTGGCAGTCTCCCCAGAGTGCCC |
| VHW3-B22 | GTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCA GCACCTGTGTATCGGTTCTCTTTCGAGCACTTCCACCTCTCAGCGGAATTCC GACCATGTCAAGGGTAG |
| ROC3-B5b | GAGCCGAGCCTGCTGGCTTCTATCTTCATCTCCTGGTCCCCGATGCTGCTTC TCTTCGGCGTCTGGATCTTCTTTATGCGCCAGATGCAGGGCGGCGGGGGCA AAGGTGCCATGTCGTTCGGTAAGA |
| ROC3-B6 | TTCTGACTAAGAACGTCAAGGTGGTTGGTGAGCCGCCAGAAGAGCCGAGCC |

| | |
|---|---|
| | TGCTGGCTTCTATCTTCATCTCCTGGTCCCCGATGCTGCTTCTCTTCGGCGT CTGGATCTTCTTTATGCGCCAGATGCAGGGCGGCGGGGGCAAAGGTGCCAT GTCGTTCGGTAAGA |

### *4.10.3. Quantitative PCR (qPCR)*

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 20 µL of the reaction mixture contained 10 µL of Maxima SYBR Green qPCR Master Mix (Fermentas), 0.375 µM of each primer and 1 µL 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. (2005) for *T. versicolor* and the program described at Rajala et al. (2013) for total fungi. Standard curves were performed with known ammounts 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.

## 4.11. Calculations and statistical analysis

### *4.11.1. Calculations and data analysis of RYA*

The RYA system is based on the different transciptional rate of a reporter gene, lacZ, which expression depends on the union of the receptor to a ligand, either natural or xenobiotc. In the present assay, to quantify gene reporter (β-galactosidase) expression at each well, MuGAL was added in excess in fluorescence generation is measured along the time. Standard linear regression methods were applied to calculate the slope, which is expressed in terms of fluorescent units per second (FU $s^{-1}$). From those data, the so-called dose-response curve was obtained, where β-galactosidase activity was represented versus ligand concentration or sample dilution.

When working with known ligand concentrations, $EC_{50}$ of the compound can be determined from the dose-response curve. $EC_{50}$ is the concentration of

compound that causes half of the maximum response, and it can be calculated from the fitting of the data to the Hill equation. This equation determines the fraction of receptor bound to the hormone in a certain moment assuming that hormone concentration is much higher than receptor one, and that only one agonist molecule binds to each receptor. Then, Hill equation is formulated as below:

$$\Phi_{\mathrm{I}} = \frac{[h\,R]}{R_{\mathrm{t}}} = \frac{1}{1 + (K_{\mathrm{d}}/[h])}$$

Equation (3)

where *Ø* is the fraction of receptor bound to the hormone, *hR* is the concentration of receptor bound to the hormone, $R_t$ the total concentration of receptor, $K_d$ corresponds to $EC_{50}$ and *h* to the concentration of hormone (ligand). The Hill equation only describes the equilibrium for receptor-hormone interaction. However, it is assumed that this is the limiting step for gene expression. That equation, reformulated, is what expresses the phisiological response (in this case the increase of fluorescence per unit of time) at a certain concentration of ligand:

$$U_{\mathrm{L}} = \frac{A}{1 + \frac{K}{[L]}} + B$$

Equation (4)

In this case, $U_L$ is the increase in the response, *A* is the maximum increase of the response (positive control minus negative control), *B* is the basal level of activity (without ligand, negative control), *K* is the aparent $K_d$ (equivalent to $EC_{50}$) and *L* is the ligand concentration.

In order to be able to calculate and compare the estrogenic or dioxin-like activity, results are given in 17β-estradiol (EEQ) and β-naftoflavone (NFQ) equivalents, respectively. The equivalents represent the concentration of reference ligand needed to obtain the same response than the analysed sample. Those EEQ or NFQ are calculated taking into account the $EC_{50}$ of the

reference ligand and the dilution where $EC_{50}$ of the sample ($f_{dil}$) is obtained, according to the formula below (example for the EEQ):

$$EEQ = EC_{50\ Estradiol} \times f_{dil} \qquad \text{Equation (5)}$$

### *4.11.2. Calculations and statistical analysis for stable isotope experiments*

Isotope ratios were reported in δ-notation (‰) relative to the Vienna Pee Dee Belemnite standard (V-PDB, IAEA-Vienna). The $\delta^{13}C$ value is defined as:

$$\delta[(‰)] = \left(\frac{Rs}{Rr} - 1\right) * 1000 \qquad \text{Equation (6)}$$

where Rs and Rr are the $^{13}C/^{12}C$ ratios in the sample and V-PDB standard, respectively. To convert $\delta^{13}C$ to atom% $^{13}C$, the following equation was used:

$$atom\%^{13}C = \frac{100}{\frac{1}{\left(\frac{\delta}{1000} + 1\right) R_{PDB}} + 1} \qquad \text{Equation (7)}$$

where δ is the measured $\delta^{13}C$ (‰) of the sample and $R_{PDB}$ is the isotope ratio of V-PDB ($R_{PDB}$=0.0112372).

Delta values ($\delta^{13}C$) of the samples for bulk analysis were corrected using the linear regression derived from three international reference materials (USGS 24, IAEA-CH-6 and IAEA-CH-7) and with respect to the Vienna Pee Dee Belemnite (VPDB) standard according to Coplen et al. (2006).

*$CO_2$ calculation*

In order to correctly quantify the $^{13}CO_2$, some corrections were done according to Morasch et al. (2007). Depending on the experimental conditions, two main corrections have to be performed. The first one would be taking into account the equilibrium between the $CO_2$ in the gas phase and the $CO_2$ dissolved in the liquid, that is, the Henry constant. The other one would be to consider the dissociation constants (pK) of the different species of carbonate in the liquid

media. In the experimental conditions above described, the last is not needed because the acidic pH of the media (4.5) entails having all the dissolved inorganic carbon in form of $CO_2$ instead of $HCO_3$. Regarding the equilibrium between gas and liquid phases, the value of $K_H = [CO_2]_g/[CO_2]_{aq}$ at 25ºC is 0.83 (Sander, 1999) and, taking into account that in the experimental bottles, the gas phase is 15 times higher than the volume of the liquid media, the amount of $CO_2$ dissolved in the liquid media compared to that in the head-space accounts for 5.5%. Even being low it has been taken into account. Finally, as $\delta^{13}C$ values are not compared to initial time (absence of $CO_2$) but to $\delta^{13}C$ values of experimental bottles spiked with $^{12}$C-DCF/$^{12}$C-BP3, the threshold of 5‰ in the $\delta^{13}C$ variation that Morasch et al. (2007) also proposed can be neglected.

The percentage of $^{13}CO_2$ released from the labelled phenyl moieties of $^{13}$C-DCF and $^{13}$C-BP3 was calculated taking into account the amount of $CO_2$ produced and the $\delta^{13}C$ values at each time.

*Amino acids calculation*

Regarding amino acids $^{13}$C incorporation quantification, necessary adjustments were done in order to correct for the carbon introduced during derivatization process. Silfer et al. (1991) also described that even during derivatization process some fractionation occurs and it should be taken into account to give the exact atom percentage of each amino acid. However, if the given values are compared to the experimental bottles spiked with $^{12}$C-DCF/$^{12}$C-BP3 value, that fractionation is already corrected. Thus, only differences in at% values and number of carbons in the amino acid related to those incorporated during derivatization between experimental bottles spiked with the labelled compound compared to those spiked with the unlabelled compound were taken into account.

To determine pairwise differences at SIP experiments, the data was subjected to two-way ANOVA. The Holm-Sidak method was applied at the 95% confidence level. The software used for the statistical analysis was Sigmaplot 11.0.

### *4.11.3. Calculations and statistical analysis for PhACs and EDCs removal*

For pharmaceuticals removal calculations, those compounds detected below quantification limit (BQL) were considered to have a concentration ½ of the limit of quantification (LOQ) (EPA, 2000). One-factor analysis of variance (ANOVA) for experimental data was done with Sigmaplot 11.0.

### *4.11.4. PCA and DCA analysis*

Principal component analyses (PCA) were performed for PLFA results, taking into account the areas of those quantified peaks: 16:0, i14, 14:0, i15, a15, C15:1, 15:0, i16:1, C16:0, i16:0, 16:1ω9, 16:1ω7c, 16:1ω7t, 16:1ω5, br17, 17:1, 10Me16, C17:0, i17, a17, 17:1ω8, cy17, C17:1, 17:0, br18, 10Me17, 18:2a, 18:2ω6, 18:1ω9, 18:1ω7, 18:1, 18:0, 19:1a, 18-OH, 10Me18, 18:2c, br19, delta18, cy19, 20:5, 20:4, 20:2 and 20:0. DGGE gel images were analysed with GelCompar II (ver. 5.1.; Applied Maths BVBA, Belgium), generating a binary matrix (presence/absence of band) that it was furhter analysed by detrended correspondence analyses (DCA). Binary matrix for bacteria and fungi were combined for all the samples to perform the analysis in order to have a similar approach to that of PLFA, where all the community is represented. PCA and DCA analyses of total samples (all the samples from the three experiments), total VHW samples (VHW1 and VHW2 experiments) and only ROC samples were performed with the program PC-ORD 5.0. 13 bioreactor variables such as time, glucose concentration and COD were tested for their possible axis distribution explanation.

# Chapter 5

## Individual degradation study in spiked synthetic media of some endocrine disruptors belonging to UV filters and benzotriazoles by *Trametes versicolor*

## INTRODUCTION

Fungal degradation of PhACs has been extensively studied during the last years as reviewed by Cruz-Morató et al. (2013). However, the ability of white-rot fungi to degrade several EDCs is still fairly unknown. Among them, UV filters and benzotriazoles are compounds detected at high concentrations in the environment but knowledge about their potential degradation by WRF is rather scarce. Therefore, in this chapter, the potential ability of using the entire fungus, in particular *Trametes versicolor,* to degrade selected UV filters (benzophenone-3 (BP3), benzophenone-1 (BP1) and 3-(4'-methylbenzylidene)camphor (4-MBC)) and benzotriazoles (1H-benzotriazole (BTZ), 4-methyl-1H-benzotriazole (4-MBTZ) and 5-methyl-1H-benzotriazole (5-MBTZ)) was studied.

Experiments were performed in liquid media because it allowed a better analysis and monitoring of many parameters, including the contaminant concentration and the fungal metabolic state such as glucose consumption and enzyme production. In those studies the degradation treatment was performed with the fungus in form of pellets. Several controls were included apart from the experimental bottles (EB) in order to determine if removal is due to abiotic processes, adsorption to the fungus or fungal degradation. Therefore, uninoculated bottles (UNI) allow the rejection of abiotic degradation processes, such as spontaneous conjugation with glucose molecules present in the medium (Winklert and Sandermann, 1992). Heat killed (HK) and sodium azide killed (SAK) dead fungi controls were also performed in order to ensure that the compound elimination was not only due to its adsorption onto the biomass. The two killed controls were initially included because fungus can be inactivated in different ways; the drawback is that all of them act differently and thus the adsorption can become different. The most used ones are the thermal inactivation (HK) and the killing by sodium azide (SAK). These differences come from the distinct action of high temperatures and sodium azide to the fungus. The first one causes total desactivation of the enzymes and physicochemical changes in the surface morphology of the fungus, resulting in variations in the biosorptive capacity of the heat-killed cells respect to living cells (Bayramoğlu

and Yakup Arica, 2007), and the latter is a metabolic inhibitor with a yet unclear action mode. Some authors propose that it is a cytochrome c oxidation inhibitor (Verdin et al., 2005), while others consider it as an ATPase inhibitor (Vasilyeva and Forgac, 1998). Anyhow, sodium azide stops the metabolism, thus inhibiting the active transport through the membrane but not avoiding the first step of binding to the surface, which is a rapid and energy-independent phenomenon, some passive membrane transport, which is also energy-independent (Jarosz-Wilkolazka et al., 2002; Verdin et al., 2005) or the temporary activity of some other enzymes. Finally, blank controls (BC), which consist of the fungus alive without the addition of the contaminant, were also included in some experiments to determine if the contaminant induces any change in the fungus such as an increase in the laccase activity. Another important issue to take into account is the low solubility of many EDCs, such as some of the UV filters, which makes it totally necessary to work with unit samples due to the indispensable solubilisation step before the analytical determination by high performance liquid chromatography (HPLC).

If there is only interest in determining the elimination or degradation percentages, experiments can be performed at low contaminant concentrations, similar to those found in the environment. However, degradation processes are only useful if they do not lead to the formation of new compounds with higher toxicity or bioaccumulation capacity. Therefore, it is necessary to identify and characterise the derivatives formed during the transformation processes and to assess the potential toxicity not only of parent compounds but also of the degradation products formed, in order to draw a complete picture of the process. Therefore, if analyses for identification of metabolites are wanted to be performed, experiments have to be done at higher concentrations (i.e. 10 mg $L^{-1}$). This is because degradation products are usually at much lower concentrations than the initial parent compound. The identification of the metabolites also allows their subsequent inclusion in the analytical methods for an improved monitoring of the fungal treatments of effluents. Identifications of the fungal metabolites obtained during the degradation of the different pollutants were performed by the environmental chemical analyitical groups of Institut de Diagnosi Ambiental i Estudis de l'Aigua (IDAEA-CSIC) and Institut Català de

Recerca de l'Aigua (ICRA). Concretely, UV filters were analysed by Pablo Gago supervised by Dr. Silvia Díaz of IDAEA-CSIC and benzotriazoles by Dr. Marta Llorca supervised by Dr. Sara Rodríguez of ICRA, both groups led by Dr. Damià Barceló.

Some enzymatic studies were also performed in order to be able to determine, together with the identification of the generated metabolites, the degradation pathway of the studied contaminants. The most common pathways in the degradation of xenobiotics by *T. versicolor* are the oxidation by extracellular ligninolytic enzymes (mainly laccase) or by the intracellular cytochrome P450. Thus, laccase enzymatic assays and *in vivo* studies, consisting of the addition of a P450 inhibitor in a fungal culture with a contaminant that enables to know if that enzymatic system participates or not in the first steps of the contaminant's transformation, were performed.

Then, degradation studies of the less hydrophobic UV filters BP3 and BP1 at concentrations near the environmental ones (250 µg $L^{-1}$) at bioreactor scale were carried out and optimisation of SPE protocol is presented. Due to the high hydrophobicity of UV filters, adsorption experiments in both batch and continuous abiotic processes were performed.

Finally, acute toxicity (Microtox anaysis) and estrogenic, anti-estrogenic and dioxin-like activities of the studied compounds were assessed. $EC_{50}$ values were calculated for each contaminant and evolution of toxicity and endocrine disrupting activity was monitored during the experiments. Estrogenic and dioxin-like assays were performed through a recombinant yeast assay (RYA) developed by the environmental toxicity group of IDAEA-CSIC led by Dr. Benjamí Piña. In fact, the analyses regarding UV filters were performed by Alba Olivares, a researcher from his group, and they kindly gave the strains to us and trained us to perform the analyses.

# 5.1. Degradation of UV filters by *Trametes versicolor*

### *5.1.1. Degradation study of BP3 and BP1*

First of all, taking into account the high log$K_{ow}$ of the UV filters targeted for their individual study (i.e. 3.79 for BP3 and 5.13 for 4-MBC, see Table 4.1), a step of extraction or solubilization was needed for their correct quantification such as for other hydrophobic compounds (Blánquez and Guieysse, 2008). Therefore, experiments in serum bottles were performed in order to optimise this solubilization step prior to the degradation study. Ethanol was chosen as extractant because of the high solubility of the studied compounds in ethanol (Salvador and Chisvert, 2005). Both time of contact and amount of ethanol were optimised. At Fig. 5.1A, it can be seen how equilibrium was immediately reached either at 16.7% or 50% of ethanol. Regarding the optimal concentration of ethanol, the maximum percentage of recovery was already achieved with ethanol at a final volume percentage of 37.5% (Fig. 5.1B). Therefore, 5 min of contact, manual agitation and 37.5% of the final volume of ethanol were considered as enough for a correct quantification of the compounds. That was the protocol followed except when working at low concentrations and a SPE step was necessary.

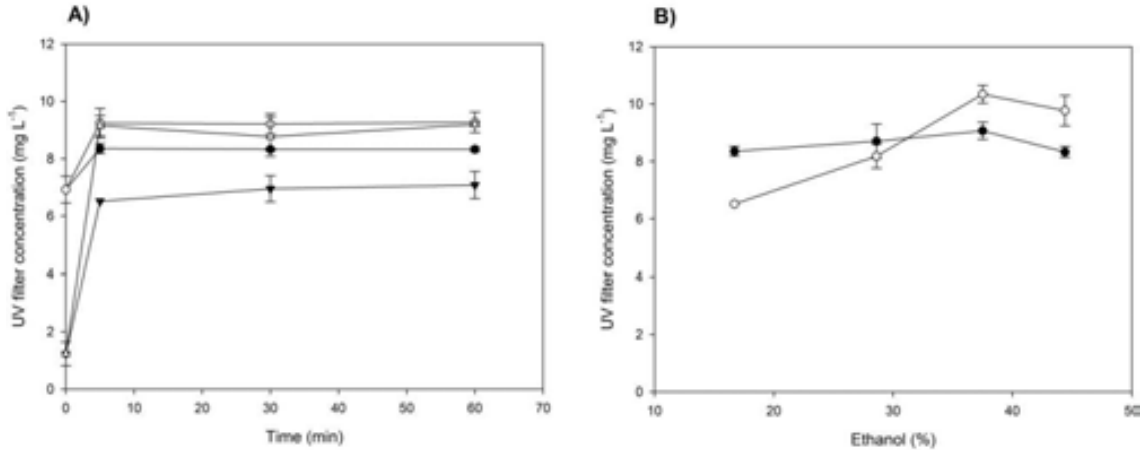



**Figura 5.1. A)** Recovered BP3 at (●) 16.7% or (○) 50% etanol final concentration and recovered 4-MBC also at (▼) 16.7% or (△) 50% etanol final concentration, at different times of solubilization. **B)** Concentration of (●) BP3 and (○) 4-MBC at different final concentrations of ethanol after 5 min of solubilization.

Once optimised the solubilization step, experiments in serum bottles were carried out in order to assess the capacity of the fungus to degrade BP3 and BP1 and to characterise the possible biodegradation products (see detailed methodology at Section 4.5.1). To facilitate the identification of possible metabolites, higher concentrations of UV filters (10 mg $L^{-1}$) than those reported in the environment or in wastewater effluents were used. Experiments for BP3 and BP1 are presented together because BP1, despite being used as UV filter itself, has also been described to be a BP3 metabolite in rats and humans.

The first experiment lasted 24h to assess the possible degradation of BP3. The results obtained are shown in Fig 5.2. Abiotic degradation processes, were discarded, since no decrease of the BP3 concentration in the UNI controls was observed. Dead fungi controls (HK and SAK) were also performed in order to ensure that the sunscreen agent's elimination was not only due to its adsorption onto the biomass. However, in the case of BP3, no differences were found between HK and SAK regarding adsorption, being in both cases around 20%. Therefore, in the following experiments, SAK controls were not included.

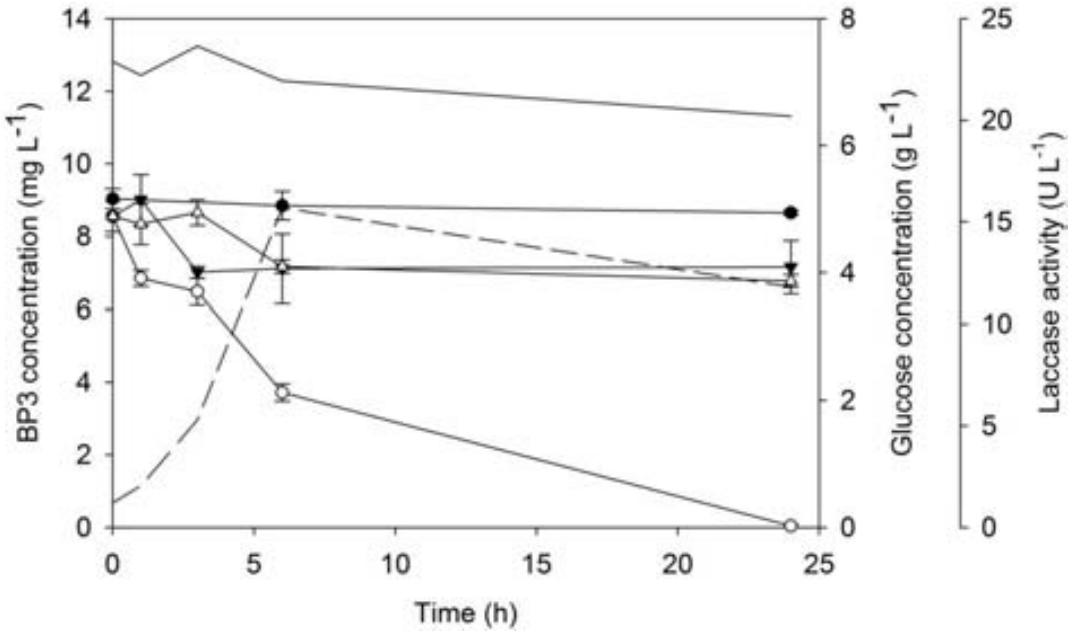



**Figure 5.2.** BP3 concentration profile at 24 h degradation experiment. Treatments: (●) UNI, (○) EB, (▼) HK and (△) SAK. Glucose concentration and laccase activity in EB are also plotted in a solid line and long dashes, respectively. Values plotted are means ± standard error for triplicates.

In the experimental bottles, BP3 exhibited a high degree of elimination, reaching >99% between 6 and 24 hours (Fig. 5.2). Therefore, taking into account the amount of BP3 adsorbed on the biomass, at least 80.4% of the initial concentration could be assigned to degradation processes at 24 hours. During the biodegradation monitoring by HPLC-UV, two new peaks were detected in the chromatograms at retention time ($t_R$) of 13.4 min (M1) and $t_R$=12.7 min (M2) (data not shown). None appeared at HK or BC controls. Their lower $t_R$ than BP3 one ($t_R$=18.5 min) indicates that they are compounds more polar.

Therefore, a longer experiment of 20 days was performed in order to determine if those metabolites generated during the fungal treatment were further degraded as well or not. At Fig 5.3A is shown how elimination of BP3 was maintained near 100% without abiotic elimination or higher adsorption in HK controls during the 20 days. Regarding the metabolites produced, the maximum concentration of both was observed at 24 hours of culture, M1 with a relative area of 40% compared to the initial concentration of BP3, while M2 reached only a relative area of 5% (Fig. 5.3B). Thus, the sum of all degradation products areas did not achieve the initial concentration of BP3. As between 10 and 15 days all peaks disappeared, it can be assumed that, finally, the aromatic ring was broken. It must be remarked that this biotransformation occurred approximately 10 days after glucose depletion in the media. Thus, fungus was still alive even after 15 days of culture. Low laccase activity was detected during all the treatment, with a maximum of 23 U $L^{-1}$ at 20 days (Fig. 5.3A). That increase at the end of the treatment could mean that fungus was still active or, by contrary, cellular lysis was taking place with the subsequent release of intracellular laccase.

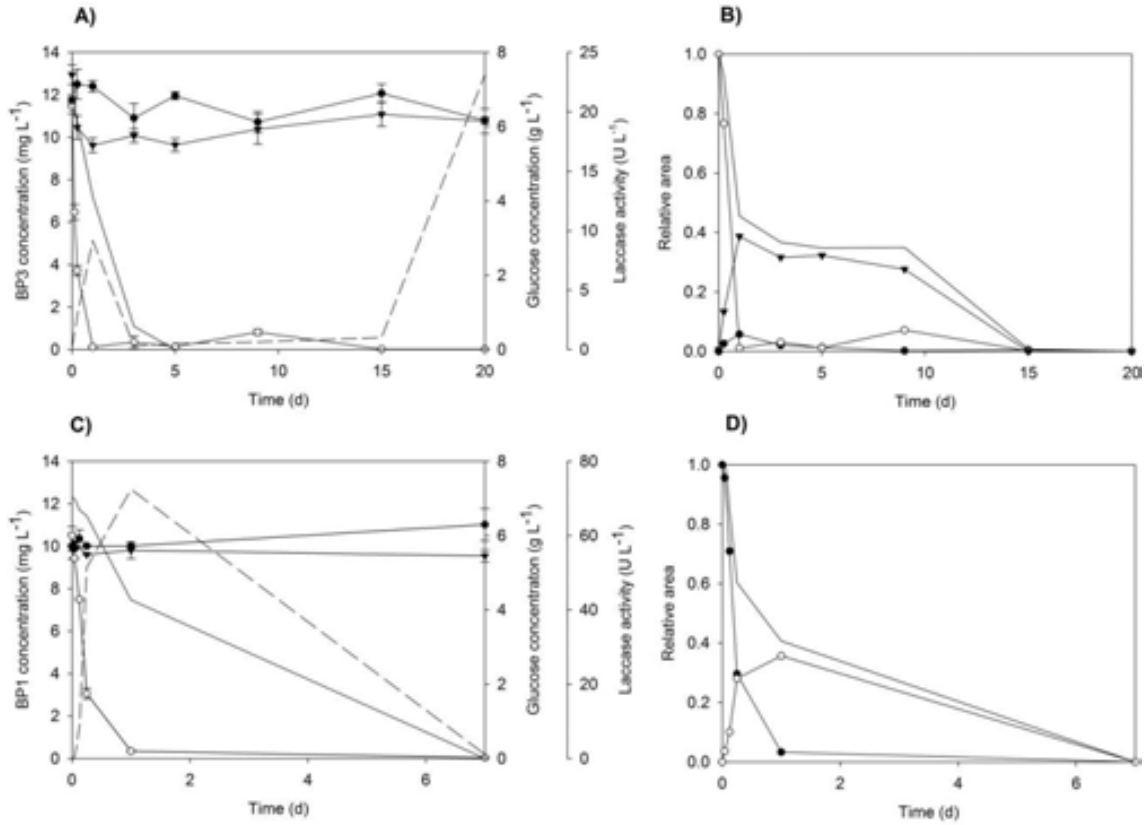



**Figure 5.3. A)** BP3 concentration profile degradation experiment by *T. versicolor* at Erlenmeyer scale at 10 mg $L^{-1}$ BP3 initial concentration and **B)** evolution of peak areas in HPLC chromatograms. Legend: (○) BP3 peak, at $t_R$ 18.5 min; (▼) M1 peak, at $t_R$ 13.4 min; (●) M2 peak, at $t_R$ 12.7 min. **C)** BP1 concentration profile in degradation experiment by *T. versicolor* at Erlenmeyer scale and at 10 mg $L^{-1}$ BP1 initial concentration and **D)** evolution of peak areas in HPLC chromatograms. Legend: (○) BP1 peak, at $t_R$ 15.5 min; (●) N1 peak, at $t_R$ 13.7 min. In **A)** and **C)**: Treatments: (●) UNI, (○) EB and (▼) HK. Glucose concentration and laccase activity in EB are also plotted in a solid line and long dashes, respectively. Values plotted are means ± standard error for triplicates. In **B)** and **D)**: Sum of all areas of the main peaks is plotted in a solid line.

Similarly, 7 days-long fungal degradation experiments were performed for BP1. Results showed a similar but faster degradation profile to that of BP3 (see Fig, 5.3C and D). No adsorption was observed at HK controls and, therefore, all removal is considered degradation. In the chromatograms obtained with the HPLC-UV, a new peak also apeared at EB at $t_R$=13.7 min (N1), its area reaching 36% of the initial area of BP1 at 24 h, but it totally disapeared after 7 days. Its retention time in the analysis was lower than BP1 ($t_R$=15.6 min) and, therefore, it is postulated to be a less hydrophobic compound as well. Laccase activity was higher than at BP3 experiments, reaching 72 U $L^{-1}$ at 24 h.

### *5.1.2. Enzymatic studies of laccase and cytochrome P450 involvement in BP3 degradation*

In order to characterise the initial steps of BP3 degradation, *in vitro* assays with commercial laccase were performed. The degradation capacity was determined for laccase alone and also with the addition of mediators, as in some cases laccase alone is not capable of oxidizing some compounds, but the fungi have the ability to produce endogenous mediators to make the reaction possible (Morozova et al., 2007). This could be the case of BP3, for example. Commercial laccase was not able to transform BP3 by itself, although it can be degraded if external mediators increasing the oxidising range of the enzyme are added. In the presence of ABTS, HOBT and VA, it was totally degraded after 21 hours (data not shown). Regarding the mediator DMHAP, only 19.9% of degradation was achieved. Taking into account that fungi have their own mediators, the oxidation of BP3 by the endogenous laccase-mediator system is in principle feasible. These results are in accordance with those published by Garcia et al. (2010). They also observed that low concentrations of BP3 (1 ng $L^{-1}$) require higher mediator/BP3 molar ratios than high BP3 concentration (1 mg $L^{-1}$) to maintain the same degradation rate at a given enzyme concentration (1U $mL^{-1}$). This laccase enzymatic treatment was found to generate oxidative coupling reactions, with dimmers, trimmers and similar products.

The involvement of cytochrome P450 intracellular enzymatic system was tested by adding the inhibitor ABT to *T. versicolor* cultures with BP3. As shown in Fig. 5.4, its participation in the first steps of BP3 degradation is uncertain, as some delay in the percentage of degradation was observed but degradation slope was the same for the cultures with (EBI) and without (EB) inhibitor.

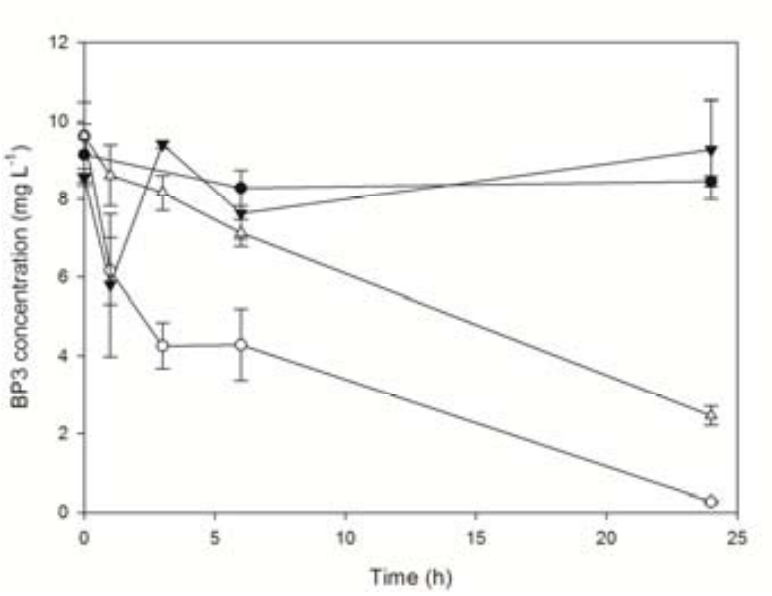



**Figure 5.4.** Concentration profile of BP3 during cytochrome P450 inhibition experiment. Treatments: (●) UNI, (▼) HK, (○) EB and (△) EBI.

### *5.1.3. Identification of fungal BP3 and BP1 metabolites and elucidation of degradation pathways*

Once demonstrated the degradation of BP3 and BP1 by the fungus *T.* versicolor, the formation of metabolites during biodegradation was investigated. Samples analysed were those of the 20 days-long experiment for BP3 degradation and the 7 days-long experiment for the study of BP1. Firstly, target compounds of BP3, namely BP1, 4-hydroxy-benzophenone (4HB), 4,4'-dihydroxy-benzophenone (4DHB), 2,2'-dihydroxy-4-methoxybenzophenone (DHMB) and 2,3,4-trihydroxy-benzophenone (THB), which have been previously identified as metabolites of BP3 in rats and humans, were considered (Felix et al., 1998; Jeon et al., 2008). BP1, 4DHB and 4HB were identified as metabolites produced during the degradation experiments of BP3 with the fungus. As shown in Fig. 5.5, BP1 was detected at 6 h of treatment at 3.6 µg $L^{-1}$ maximum concentration. 4DHB and 4HB were identified after 3 days of treatment. 4DHB was detected at a maximum concentration of 50.7 µg $L^{-1}$ at day 5, while 4HB was always below limit of quantification (BQL). THB and DHMB were apparently not produced by the treatment with the fungus, since they were not detected in any sample.

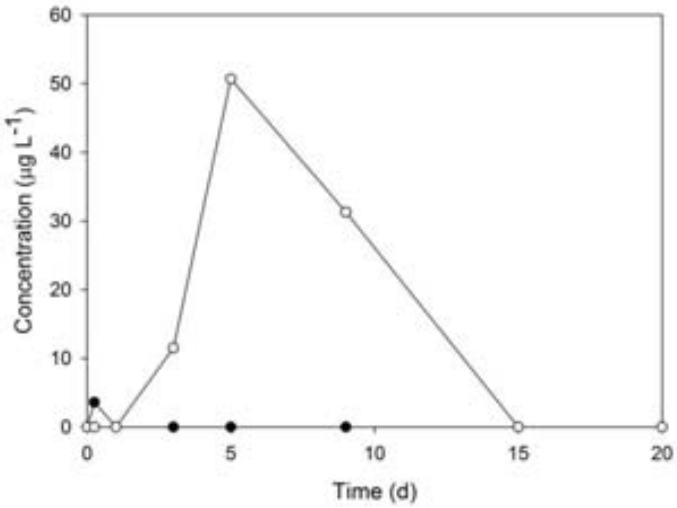



**Figure 5.5.** Concentration profile of BP3 metabolites during a degradation experiment with *T. versicolor.* Legend: (●) BP1 and (○) 4DHB.

Fungal degradation of BP1 resulted in the formation of 4HB and 4DHB. Their concentrations were similar to those reported for BP3 degradation experiments. Likewise in BP3 degradation tests, THB and DHMB were not detected in the analysed samples.

Since the relative concentrations of these metabolites were quite low suggesting that other major metabolites might be formed, further studies were carried out in order to identify them. Data suggested that the M1 metabolite detected with the HPLC-UV might be the BP3 conjugated with a molecule of 132 Da. Several studies claim that the formation of conjugated metabolites with pentoses (mainly xylose and ribose) and hexoses (mainly glucose) is a predominant pathway in the degradation of chemicals by WRF, especially in presence of phenolic hydroxyl groups (Gesell et al., 2004; Kondo et al., 1993). In this case, the addition of a pentose via glycosidic bond to the BP3 with the consequent loss of one molecule of water corresponds to an increase in the molecular mass of 132 Da. Thus, M1 metabolite may be produced by the addition of one pentose molecule to BP3. Following the rationale discussed above, it appears that the other metabolite (M2) might be the result of the addition of one hexose molecule to BP3, likely glucose, via glycosidic bond. The increase in the polarity of the identified metabolites caused by the addition of

sugars is in agreement with the lower $t_R$ associated to them in comparison to those of the respective parental compounds.

The results obtained for BP1 were pretty similar. N1 metabolite MS/MS spectra were very similar to those obtained for BP3 and, as it was reported for BP3, the difference in 132 Da in the molecular mass suggests the addition of one pentose molecule to BP1.

Therefore, non-target identified metabolites constitute conjugated forms of BP3 and BP1, and thus, further enzymatic activity of the fungus might revert them to the parent compound as later observed in experiments with oxygen limitation (see Chapter 6). Nevertheless, the fact that all identified metabolites, together with BP3 and BP1, disappeared along the treatment confirms further fungal degradation with potential cleavage of the aromatic rings.

Fig. 5.6 summarises the first steps of BP3 and BP1 degradation pathways. They are a combination of phase I and phase II reactions. Phase I reactions consist on enzymatic transformations, usually oxidations, whereas phase II reactions generate conjugated metabolites. These processes generally result in a decrease of the hydrophobicity, although in some cases the higher availability of the resulting metabolite may also increase its toxicity (Marco-Urrea et al., 2009; Mcgrath and Singleton, 2000). In the case of BP3 and BP1, they are rapidly transformed in their glycoconjugated forms. Conjugation processes constitute one of the defensive mechanisms that fungi have against toxic hydroxylated compounds (Hundt et al., 2000). Conjugation increases the polarity of the compounds, their bioavailability and their subsequent degradation. The active enzymes appear to be UDP-xylosiltransferase when conjugation occurs with a xylose (Kondo et al., 1993) or UDP-glucosyltransferase if the added molecule is a glucose, as it has been previously reported for other xenobiotics upon the action of *T. versicolor* (Hundt et al., 2000). Conjugations with ribose, also described for other fungi (Gesell et al., 2004), constitute a possibility that cannot be ruled out since the molecular weight of this conjugate fits with the results obtained in the HPLC-MS/MS analyses. Sugar residues would bind to the molecule through an O-glycosidic

bond to the unique free hydroxyl of BP3 and to one of the two free hydroxyl groups present in BP1. Fragmentation of the conjugates yield BP3 and BP1 molecules, but any other breackdown product. Additionally, the problem of detecting conjugated metabolites is that they can be easily reverted back to the parent compound. Thus, it must be confirmed that at the end of the treatment both the parental compound and the conjugated forms have been degraded. In the case of BP3 it occurred between 9 and 15 days of culture.

Later, O-glycosidic bond would break down and other fungal enzymes of phase I would act. Likely, the monooxygenase cytochrome P450 would oxidize BP3 and BP1 by adding hydroxyl groups or eventually demethylation as happen for other compounds as well (Campoy et al., 2009; Hammer et al., 2001). This would lead to the formation of BP1, 4HB and 4DHB, metabolites also reported for mammals (Jeon et al., 2008) since fungi are eukaryotic cells too. In fact, the experiment of cytochrome P450 inhibiton pointed to the possible involvement of this intracellular enzymatic system in the first steps of BP3 degradation. Then, further ring cleavage might occur. Laccase could oxidize BP3 as well, although only with the help of mediators.

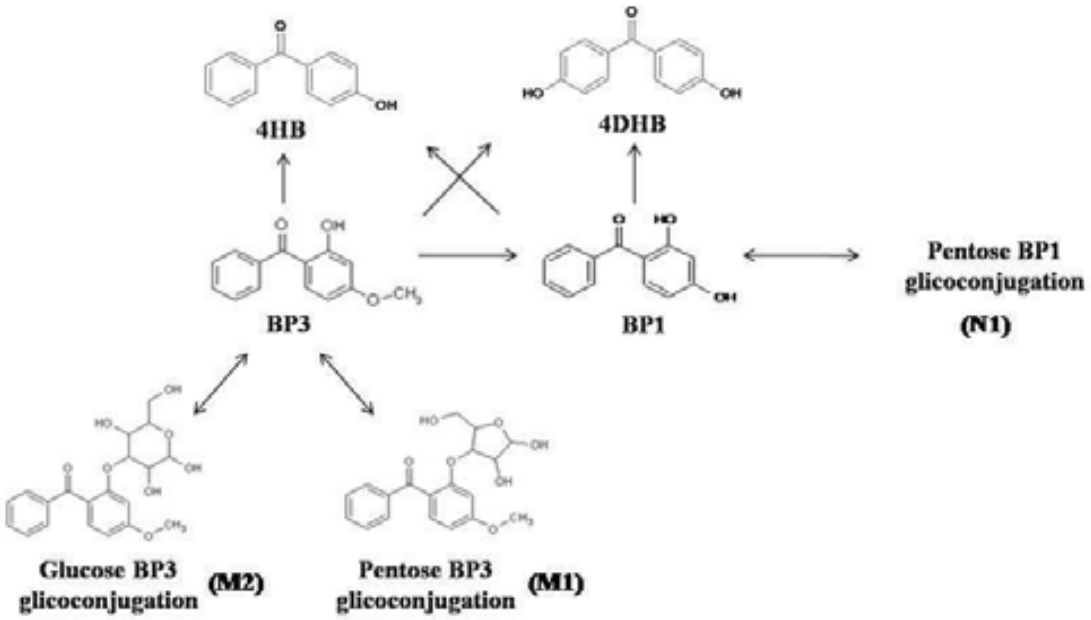



**Figure 5.6.** First steps of BP3 and BP1 transformation by *T. versicolor.*

Thus, altough some enzymes involved are similar in fungi and mammals, different metabolites are generated. Toxicokinetic studies in rats revealed that the main metabolic pathway of BP3 is the dealkylation of the methoxy side chain, which lead to the metabolite BP1. Secondary pathways would be aromatic hydroxylations carried out by the cytochrome P450 leading to the hydroxylated metabolites THB and DHMB (Jeon et al., 2008). Nevertheless, the predominant metabolites in human urine and semen samples during the first 24 h after topic application are the glucuronide conjugates of BP3 and BP1 (León et al., 2010). None of those compounds, except BP1, were found in the fungal culture.

### *5.1.4. Degradation study of 4-MBC*

4-MBC, another common UV filter was also studied for its degradation by *T. versicolor.* Fig. 5.7A presents the results of a 24 h degradation experiment in liquid medium at an initial 4-MBC concentration of 10 mg $L^{-1}$. Abiotic degradation processes were not observed, as no concentration reductions were observed in the uninoculated controls (UNI). Dead fungus controls were also used to ensure that contaminant elimination from the medium was not occurring merely because of its physical sorption onto the biomass. HK and SAK controls were included because for some xenobiotics, differences in elimination levels have been reported between them (Marco-Urrea et al., 2010a). The fact that every unit sample was extracted with ethanol before quantification led us to consider that the observed sorption would be minimal, as reported in the previous experiments with BP3 and BP1. Nevertheless, after 6 h of incubation, when the values stabilised, the sorption percentages for the SAK and HK controls were 36% and 52%, respectively. These results demonstrated that the solvent did not totally counteract the binding of 4-MBC to the fungal wall or its entrance into the cell in either the SAK or HK controls. The EB treatments exhibited a high degree of 4-MBC elimination, >95%, between 6 and 24 h. Taking into account the amount that had possibly been adsorbed onto the biomass (also at 24 h) in the worst-case scenario, the elimination of at least 48% of the initial concentration can be ascribed to fungal degradation processes (Fig. 5.7A).

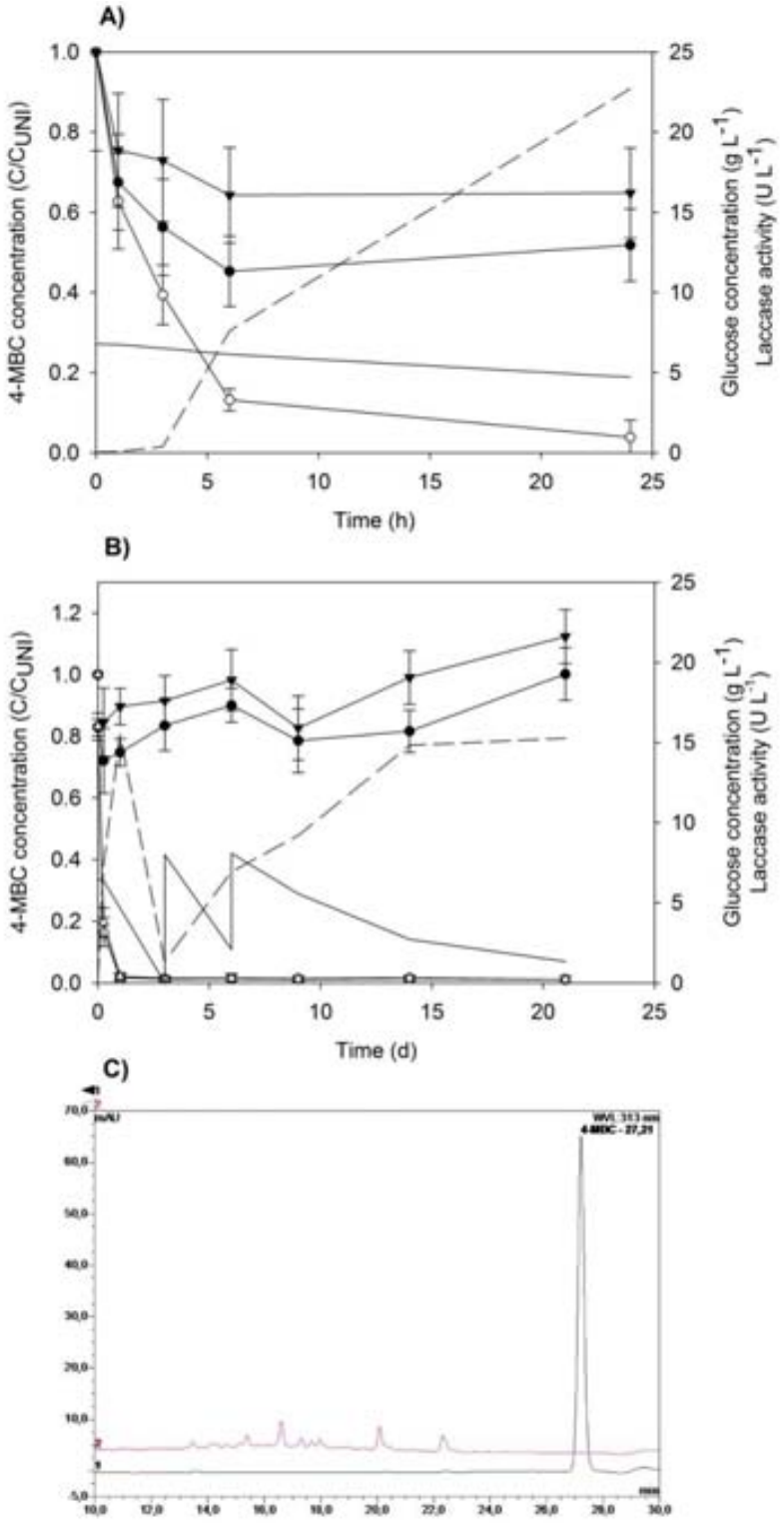



**Figure 5.7.** Concentration profiles of 4-MBC at initial concentration of 10 mg $L^{-1}$ after **A**) 24 h and **B**) 21 d of degradation experiments with *T. versicolor* in liquid medium. Values are expressed as mean ± standard deviation (n=3).Treatments in **A**): (○) EB, (●) HK and (▼) SAK. In graph **B**), (○) EB, (Δ) EB with the amount of 4-MBC extracted by sonication added, (●) HK and (▼) HK with the amount of 4-MBC extracted by sonication added. Glucose concentration is plotted as a solid line and laccase activity as a dashed line. **C)** Superposed HPLC chromatograms of the initial EB sample (below) and the EB sample at 21 d (above).

Therefore, as solubilization with ethanol was not enough to recover all the contaminant in the killed controls, disruption of the pellets was included in a long-term experiment to quantify the amount of 4-MBC that had been taken up by the fungus in both the HK control and the experimental bottles (EB) (Fig. 5.7B). The SAK controls were not included in subsequent experiments, as the HK were considered to be the best model of the two for determining the maximal sorption capacity of the fungus. Fig. 5.7B shows that after quantification of the 4-MBC extracted from the disrupted pellets, the total normalised recovery from the HK was near unity, whereas in the EB, it was negligible. Thus, as it was possible to recover the entire initial amount of 4-MBC in the inactivated controls but not in the experimental samples, it appears that 4-MBC removal in the EB was due not only to fungal uptake but mainly to a degradation process, raising the degradation percentage from 48% to almost 100%. However, it has to be taken into account that in this experiment, adsorption values were lower than in previous ones, leading in some samples to non significative differences between the ammount recovered with solubilization and sonication steps or only with solubilization with ethanol.

Throughout the process, some small peaks that were not detected in any of the controls appeared in the EB chromatograms (Fig 5.7C). Thus, they were assumed to be metabolites of the parent compound. At 24 h, although 4-MBC was completely removed, some of these peaks were still detected. Moreover, longer incubation times did not yield an improvement in the metabolisation process. In fact, at 21 d, the EB chromatogram profile was very similar to that at 24 h. The lack of a carbon source was eliminated as a possible cause for the absence of further degradation, as glucose was added at 3, 6 and 9 d to avoid depletion (Fig 5.7B).

In summary, *T. versicolor* was able to totally degrade 4-MBC from an initial concentration of 10 mg $L^{-1}$ in less than 24 h of treatment. However, total metabolisation of its degradation products was not achieved even after 21 d of culture. Therefore, these compounds were identified, and toxicity assays were

performed to assess the potential hazards of the process, as explained in the next sections.

### *5.1.5. Enzymatic studies of laccase and cytochrome P450 involvement in 4-MBC degradation*

Additional experiments were performed to characterise those initial steps of 4-MBC degradation by *T. versicolor* leading to the above mentioned intermediate products. Laccase enzymatic assays and fungal degradation in presence of a cytochrome P450 inhibitor were performed as they are the most common pathways in the degradation of xenobiotics by *T. versicolor*.

Results showed that laccase would not be involved, at least in the first steps, in the degradation of 4-MBC, because no 4-MBC transformation was achieved *in vitro* with commercial laccase, not even with the addition of mediators.

On the other hand, cytochrome P450 was postulated to be responsible for the first hydroxylation of the contaminant. As shown in Fig 5.8, no 4-MBC degradation was obtained when ABT was added to the media. That means that fungus might follow a similar detoxification pathway to those described in mammals (Völkel et al., 2006).

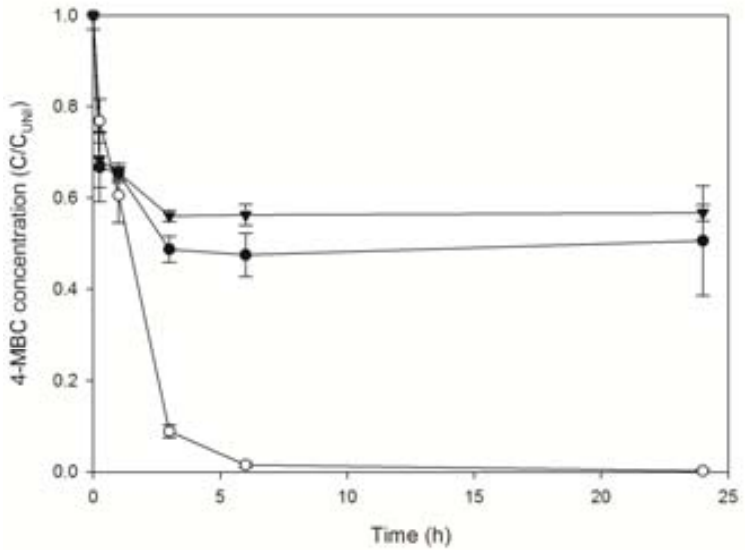



**Figure 5.8.** Concentration profile of 4-MBC during cytochrome P450 inhibition experiment at 10 mg $L^{-1}$ initial concentration. Treatments: (●) EB, (○) HK and (▼) EBI.

### *5.1.6. Identification of fungal 4-MBC metabolites and elucidation of the degradation pathway*

In order to increase the knowledge about fungal degradation of 4-MBC, metabolites generated during biodegradation by *T. versicolor* were determined and monitored during the 21 days-long experiment. To account for the possible formation of other compounds apart from 4-MBC fungal degradation products, the HK and BC chromatograms were recorded. At the initial time of the experiment, only the signal corresponding to 4-MBC was observed, which almost disappeared upon 24 h of fungal treatment as already observed by HPLC-UV. During all the treatment, a total of 4 compounds were identified as the major metabolites. They differ from those detected in the HPLC-UV chromatograms, which were probably compounds generated in a lower amount whereas the major metabolites could not be detected by HPLC-UV because the method was only optimised for the parent compound. Thus, two products with m/z=271 (Pr271) and m/z=287 (Pr287) were observed from the early hours. Pr271 reached the maximum concentration at 24 h to finally disappear at 3 d of culture. From its molecular weight and structure it is assumed to be an hydroxylation in the aromatic ring or the methyl group next to the aromatic ring; and Pr287, in lower amounts, a double hydroxylation.

Also in the first hours of treatment a peak with the same m/z ratio and MS/MS fragmentation pattern to those of 4-MBC could be observed. This evidenced the transformation of the commercially available 4-MBC(E) into its isomer, 4-MBC(Z). This isomerization process has been found to occur upon the action of living organisms (Buser et al., 2005).

However, the main metabolite detected was Pr425 (m/z=425). It corresponds to the conjugation of Pr271, the hydroxylated metabolite, with a molecule of pentose by a glycosidic bond. Conjugated compound of the Pr287 metabolite (Pr441) was also found but at lower concentration. Both conjugates were also finally totally degraded at day 12 after reaching their maximum concentration at day 3 of treatment.

In previous 4-MBC assays, the metabolites identified in rats and humans were 3-(4-carboxybenzylidene)-6-hydroxycamphor (the major metabolite) and 3-(4-carboxybenzylidene) camphor and their respective glucuronide conjugates (Völkel et al., 2006). None of them were detected in the fungal cultures. As shown by experiments inhibiting the cytochrome P450, the first step of 4-MBC degradation by *T. versicolor* is the hydroxylation of the aromatic ring, forming Pr271 and PR287 metabolites. Subsequently, the addition of a pentose via a glycosidic bond to the hydroxilated or dihydroxilated 4-MBC occurs. These results are in agreement with previous works reporting that the formation of conjugated metabolites with pentoses (mainly xylose and ribose) and hexoses (mainly glucose) is a predominant pathway in the degradation of chemicals by white rot fungi, especially those with phenolic hydroxyl groups (Gesell et al., 2004; Kondo et al., 1993). Conjugation processes constitute one of the defensive mechanisms that fungi activate against toxic hydroxylated compounds (Hundt et al., 2000). The fact that all those metabolites were also finally transformed implies that other enzymes attack these conjugated intermediates, breaking them into smaller, unidentified fragments. Fig. 5.9 summarises the proposed initial steps of the 4-MBC degradation pathway.

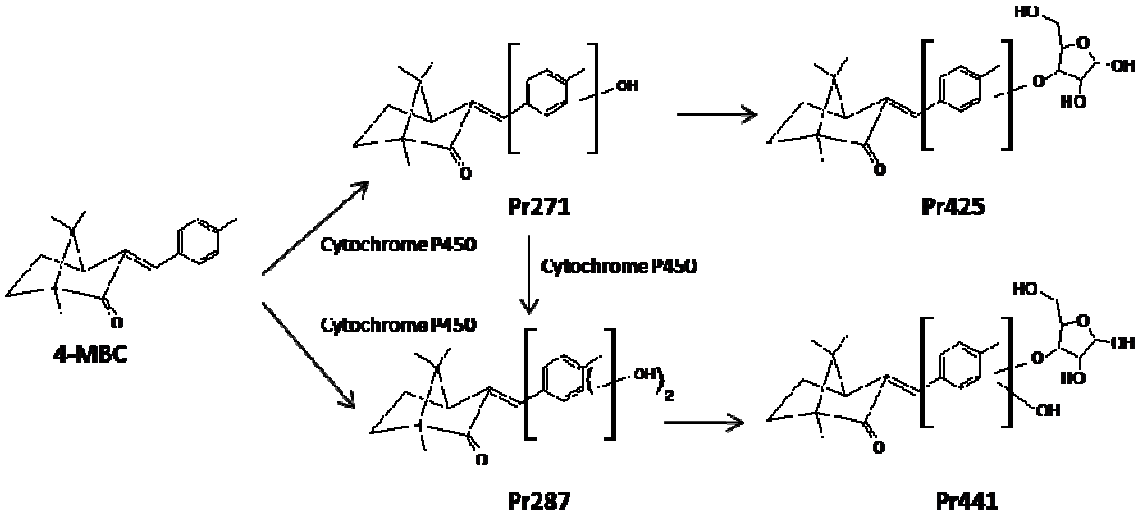



**Figure 5.9.** First steps of 4-MBC transformation by *T. versicolor.*

For all the studied UV filters, the main metabolites identified were the conjugated forms. From them, the predominant product in BP3, BP1 and 4-MBC degradation was the pentose conjugate. There are many fungal

conjugated forms reported in the literature. The more common are glucuronide, glutathione, sulphate and glycoside conjugations (Cerniglia et al., 1982; Zhang et al., 1996). However, the conjugated metabolites of 4-MBC, BP3 and BP1 by *T. versicolor* are only formed by the addition of one pentose molecule to the parent compound or also of one glucose in the case of BP3. Ribose and xylose are the most common conjugated pentoses (Gesell et al., 2004). Sugar residues attach to the molecule through an O-glycosidic bond with the hydroxyl groups present in the aromatic ring (Gesell et al., 2004; Kondo et al., 1993). In BP3, the conjugation should occur through the unique free hydroxyl of the molecule, whereas BP1 presents two possible alternatives. In 4-MBC, conjugation would occur only after the mono- or di-hydroxylation of either the aromatic ring or the adjacent methyl group.

These results show the importance of phase II reactions besides oxidations in the degradation pathway of some toxic xenobiotics. Glycoconjugations decrease their toxicity and increase bioavailability, allowing further transformations. However, this further transformation must occur during the fungal treatment to efficiently remove the original pollutants. Otherwise, conjugation reversion could occur.

Finally, fungi are eucariotic organisms and, thus, they have been sometimes considered as model for degradation processes. As in mammals, phase I and phase II enzymes appear to participate in the degradation of BP3, BP1 and 4-MBC by *T. versicolor*. However, UV filters are an example where the degradation metabolism differs in mammals and in fungi (Asha and Vidyavathi, 2009), despite the enzymes involved are of the same family (cytochrome P450 monooxygenases and transferases).

### *5.1.7. Biodegradation of BP3 and BP1 in bioreactor*

Afterwards, the fungal degradation of some UV filters (BP1, BP3, 4-MBC, octocrylene (OC) and ethylhexyl methoxycinnamate (EMC)) was wanted to be studied at concentrations near those found in the environment (e.g. some µg $L^{-1}$) and at reactor scale in a 1.5 L bioreactor operated in batch mode. Therefore, as experiments in bioreactors were planned to be performed at lower

concentrations, solid-phase extraction (SPE) procedure was firstly optimised for all UV filters that we wanted to study. Optimisation of SPE was also needed for the experiments described in the Section 7.1.

SPE optimisation started based on the multicontaminant concentration method described by Radjenovic et al. (2007). Originally, cartridge used was Oasis HLB conditioned with 2 x 2.5 ml of methanol and 2 x 2.5 ml of MilliQ water at pH 4.5. Table 5.1 shows the recovery percentage of triplicate samples spiked with 50 µg $L^{-1}$ for each UV filter at every elution conditions. Elution with methanol results in very low recovery values for the most hydrophobic compounds, even when cleaning the glass tube of the sample with the methanol used for elution. Then, an apolar solvent such as ethyl acetate was tested but results did not improve.

**Table 5.1.** Recovery values for SPE using Oasis HLB cartridges

| Sample volume | Elution | BP1 | BP3 | 4MBC | OC | EMC |
|---|---|---|---|---|---|---|
| 20 ml | 2 x 2 ml methanol[a] | 96.3±1.3 | 78.2±7.5 | 31.7±0.7 | 27.3±3.3 | 20.3±3.7 |
| 200 ml | 3 x 10 ml methanol + cleaning | 90.8±1.8 | 69.6±9.5 | 58.4±7.7 | 51±11 | 47.1±8.5 |
| 20 ml | 2 x 2 ml ethyl acetate | 75±16 | 47±18 | 22±10 | 21±16 | 26±21 |

[a] (Radjenovic et al., 2007)

Then, changing the cartridges to Isolute C18, more specific for hydrophobic compounds, was tested. In this case, the changes in the protocol were based on that of Díaz-Cruz et al. (2012). Conditioning of the cartridge was always done with 2 x 2.5 mL ethyl acetate:dichlormethane (EtAc:DCM) 1:1, 2 x 2.5 mL methanol and 2 x 2.5 mL of MilliQ water at pH 4.5. Table 5.2 shows the recovery percentage of triplicate samples spiked with 125 µg $L^{-1}$ for each UV filter at every elution conditions. Optimised procedures for vial (20 mL) and bottle (200 mL) samples that will be used hereafter are highlighted in bold. From the obtained results, cleaning of the recipient containg the samples was found to be crutial to increase the percentages of recovery of the compounds, especially those more hydrophobic. It was also pointed out that two elutions was enougth as increasing the number and volume of elutions did not improved the figures or even decreased them due to losses.

**Table 5.2.** Recovery values for SPE using Isolute C18 cartridges

| Sample volume | Elution | BP1 | BP3 | 4MBC | OC | EMC |
|---|---|---|---|---|---|---|
| 200 ml | 4 x 2 ml EtAc:DCM 1:1 [a] | 85.7±2.0 | 78.9±2.5 | 68.3±0.1 | 34.7±1.4 | 28.6±2.3 |
| 20 ml | 4 x 2 ml EtAc:DCM 1:1 | 86.9±3.2 | 55.5±4.0 | 46.7±3.8 | 19.7±4.5 | 21.8±4.6 |
| 20 ml | 4 x 2 ml EtAc:DCM 1:1 + cleaning | 85.4±3.6 | 62.7±7.0 | 61.6±5.5 | 70.0±2.5 | 77.8±2.3 |
| **20 ml** | **2 x 2 ml EtAc:DCM 1:1 + cleaning** | **97.0±1.3** | **86.2±2.8** | **79.7±1.4** | **74±12** | **94.3±9.6** |
| **200 ml** | **6 ml EtAc:DCM 1:1 + cleaning** | **91.3±1.2** | **81.7±2.3** | **69.4±1.1** | **75.5±1.4** | **66.7±5.1** |

[a] (Díaz-Cruz et al., 2012)

On the other hand, as stated before, UV filters are compounds with logKow values quite high. Therefore, prior to the fungal treatment in bioreactor, a 3 days long abiotic (without fungus) experiment was performed to assess their adsorption in the bioreactor walls. Resuls are shown in Fig. 5.10 and Table 5.3. After emptying the bioreactor, 50 mL of methanol were added to solubilize the compounds that could be adsorbed in the walls; however, low recoveries were found except for BP1 (79.7%).

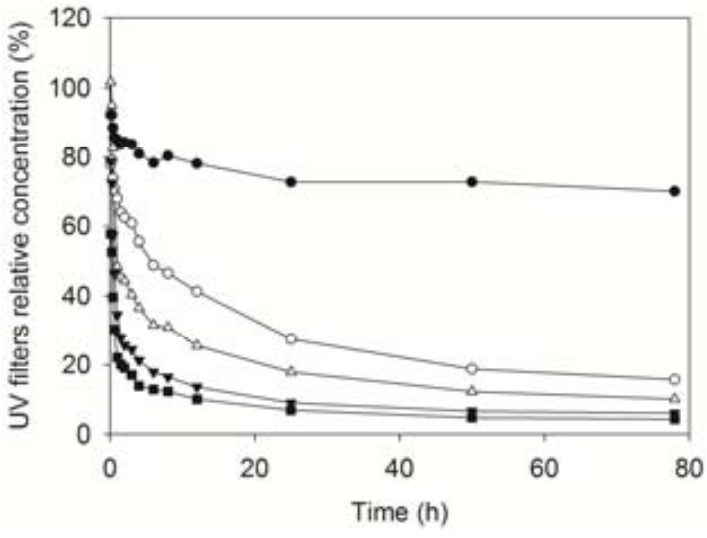



**Figure 5.10.** Concentration evolution of UV filters in an abiotic batch reactor. Legend: (●) BP1, (○) BP3, (△) OC, (▼) 4-MBC and (■) EMC.

**Table 5.3.** Results of the amount of UV filters in an abiotic batch bioreactor experiment

| | BP1 | BP3 | 4-MBC | OC | EMC |
|---|---|---|---|---|---|
| Initial theoretical (µg) | 378 | 381 | 375 | 492 | 375 |
| Initial[a] (%) | 92.3 | 77.9 | 78.7 | 101.7 | 57.7 |
| Final[a] (%) | 70.0 | 15.7 | 6.0 | 10.0 | 4.2 |
| Recovered by solubilization (µg) | 29.4 | 20.8 | 80.0 | 187.5 | 105.8 |
| Total final[a] (%) | **79.7** | **38.9** | **37.5** | **62.1** | **40.1** |

[a] All calculations are based on the theoretical initial concentration

Then, a continuous abiotic bioreactor was set up in order to determine if an equilibrium of adsorption was reached. The problem was that UV filters were then not only adsorbed on the bioreactor but also to the silicone tubes and the storage tanks of the feed water influent and of the effluent. Fig. 5.11 and Table 5.4 show the results.

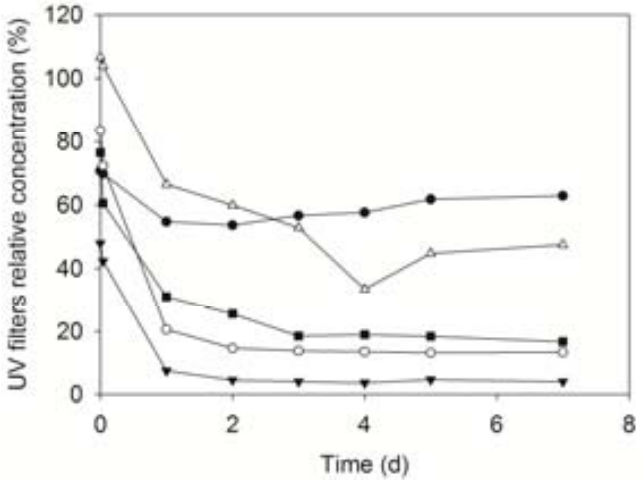



**Figure 5.11.** Concentration evolution of UV filters in an abiotic continuous reactor. Legend: (●) BP1, (○) BP3, (△) OC, (▼) 4-MBC and (■) EMC.

**Table 5.4.** Results of the amount of UV filters in an abiotic continuous bioreactor experiment

| | BP1 | BP3 | 4-MBC | OC | EMC |
|---|---|---|---|---|---|
| Initial theoretical (µg) | 474 | 372 | 375 | 380 | 515 |
| Feed tank final (%) | 75.7 | 78.0 | 29.1 | 74.1 | 42.5 |
| Bioreactor final (%) | 61.0 | 13.7 | 4.1 | 44.6 | 18.1 |
| Outlet storage tank final (%) | 51.1 | 5.1 | 3.2 | 22.0 | 8.8 |

[a] All calculations are based on the theoretical initial concentration

Taking into account the results of these two abiotic reactors, 4-MBC and EMC were discarded for their study in a fungal bioreactor due to their very high

adsorption values and OC for its lower recovery in SPE and higher deviation. Moreover, for BP3 and BP1, we could follow the degradation process by the formation and disapearance of their main metabolites (the BP3 conjugates M1 and M2 and the BP1 conjugate N1). Thus, two batch bioreactors were carried out, one for BP3 degradation and the other one for BP1 degradation. Higher removal rates were observed by *T. versicolor* during 24 h batch operation. Initial levels of BP3 dropped to non-detectable levels in 8 h (Fig 5.12A). In the case of BP1, about 95% of the initial concentration was removed after 2 h of treatment and completely eliminated at 24 h (Fig 5.12B). When comparing with the removal curves obtained in abiotic reactors, at least 80% of BP1 and 40% of BP3 degradation can be assured. However, taking into account the results obtained previously in the serum bottles, 100% degradation was probably achieved in both cases.

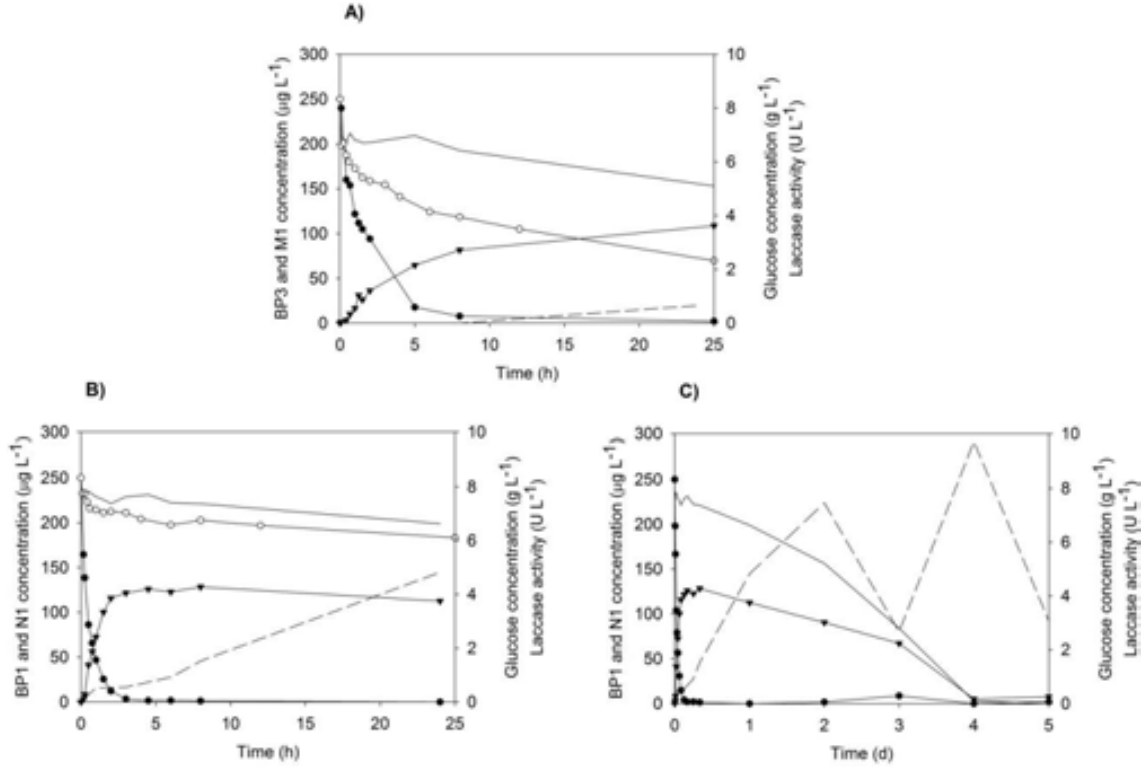



**Figure 5.12.** Concentration profiles of **A)** BP3 and **B)** and **C)** BP1 during fungal degradation in the bioreactor at initial concentration of 250 µg $L^{-1}$. Legend: (●) BP3 or BP1 concentration at fungal batch, (○) BP3 or BP1 concentration at abiotic batch, (▼) M1 or N1 metabolite concentration at fungal batch. Glucose concentration and laccase activity in fungal bioreactors are plotted in dotted line and long dashes, respectively.

During the biodegradation treatments, the peaks assigned to M1 and N1 (conjugates with pentose of BP3 and BP1, respectively (see Fig. 5.6)) were observed in the HPLC-UV chromatograms indicating that these contaminants were indeed degraded by the fungus and that their elimination from the medium was not only driven by their adsorption. M1 reached a 40% of the initial area of BP3 upon 24 h of treatment. Similarly, for BP1 N1 reached the maximum (50% of initial BP1 signal) after 8 h, to further decrease and finally be totally transformed after 4 days (Fig 5.12C). Laccase activity was also higher at BP1 than at BP3 in bioreactor treatments as well as in the serum bottles experiments.

### *5.1.8. Determination of acute toxicity and estrogenic and dioxin-like activities*

Microtox is a simple and standardized test, useful to have a first impression of the acute toxicity of a sample. $EC_{50}$ value for BP3 and 4-MBC was determined to be 2.60 ± 0.34 mg $L^{-1}$ and 9.15 ± 7.91 mg $L^{-1}$, respectively, and in the same order of magnitude than other PPCPs (Farré et al., 2001). Toxic units (TU) were calculated for experimental samples at initial time and at the end of the treatment in the 24 h experiments. For BP3 experiment, toxicity decreased from 10 TU to 0.5 TU due to the activity of the fungus and for the 4-MBC experiment, acute toxicity was reduced from 12.5 TU at initial time to no acute toxictiy after 24 h.

Estrogen Receptor and Aryl hydrocarbon Receptor Recombinant Yeast Assay (ER-RYA and AhR-RYA) were performed to determine the $EC_{50}$ of each compound and to evaluate the treatments. Estrogenic activity of BP3 and BP1 presented $EC_{50}$ values of 12.5 mg $L^{-1}$ and 0.058 mg $L^{-1}$, respectively, and a lowest observed effect concentration (LOEC) of 1.6 mg $L^{-1}$ and 0.015 mg $L^{-1}$. These results indicate that BP1 is three orders of magnitude less estrogenic than the model compound 17β-estradiol and 200-fold more estrogenic than its parent compound (BP3). Metabolites 4HB and DHMB also show estrogenic activity with $EC_{50}$ values of 0.92 mg $L^{-1}$ and 7.9 mg $L^{-1}$, respectively, whereas 4DHB and THB were considered non estrogenic in this assay. Similar values

have been described by Kunz and Fent (2006). The estrogenic potency of 4-MBC was half of BP3, with and $EC_{50}$ value of 28.5 mg $L^{-1}$.

Analyses by ER-RYA during the 21 days-long experiment of 4-MBC biodegradation by *T. versicolor* showed no estrogenic activity, indicating that any potential estrogenic metabolite were not present at enough concentration to elicit biological response. In order to evaluate the evolution of the endocrine disruption during the degradation processes at bioreactor-scale, the estrogenic activity was monitored at the same scheduled times than chemical analyses. During BP3 bioreactor treatment, estrogenic activity was below the detection limit in all check points indicating that putative estrogenic metabolites formed by T. *versicolor* degradation such as BP1 and 4HB were readily metabolised and, thus, they were not present at sufficiently high concentration to elicit biological response. This is in agreement with the low concentrations found for that metabolite during fungal degradation. In the case of BP1, the estrogenic activity decreased at the same rate than it was degraded. Fig. 5.13 shows how T. *versicolor* upon 4h of treatment eliminated almost completely the estrogenic activity. These outcomes suggest that bidegradation by *T. versicolor* of BP3 and BP1 did not produce significant amounts of estrogenic metabolites.

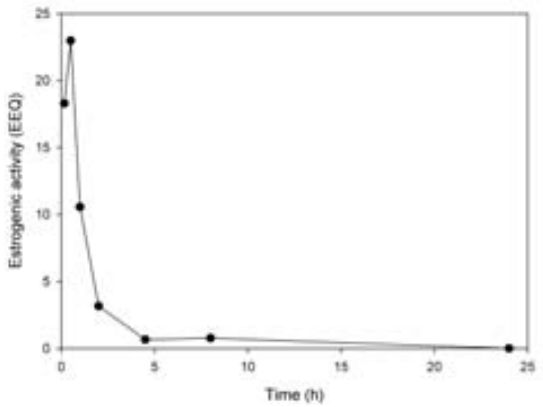



**Figure 5.13.** Estrogenic activity profile of BP1 fungal bioreactor treatment.

Dioxin-like activity was also assessed by means of RYA assay. BP1, with an $EC_{50}$ value of 0.61 mg $L^{-1}$ showed 10 fold more dioxin-like activity than BP3, with an $EC_{50}$ value of 6.8 mg $L^{-1}$. However, two of their metabolites, 4HB and

DHMB, presented four and eleven times higher activities, with $EC_{50}$ values of 0.16 mg $L^{-1}$ and 0.59 mg $L^{-1}$, respectively. No dioxin-like activity was observed for either 4DHB or THB. Dioxine-like activity during biodegradation processes of BP3, BP1 and 4-MBC by *T. versicolor* was below detection limits for the three compounds. These data indicate that metabolites produced by *T. versicolor* where in small amounts or rapidly metabolised in non dioxin-like compounds.

In order to incorporate methodology RYA assays in the research group, $EC_{50}$ of the reference compounds (17β-estradiol and β-naftoflavone) and the UV filters BP3, BP1 and 4-MBC were calculated again in our laboratory to compare the results with the previously obtained to validate the protocol. Table 5.5 shows the results compared with those of IDAEA group. $EC_{50}$ for β-naftoflavone gives the same result in both laboratories. However, $EC_{50}$ for ER-RYA strain results in values of one order of magnitude higher for all compounds which means less sensitivity of the assay despite it was performed following the same protocol. We were unable to find the reason behind that behaviour; however, as we basically used it to compare the initial and the final effluents of the fungal treatments, results were likewise reliable.

**Table 5.5.** $EC_{50}$ values for reference compounds and UV filters

| | ER-RYA | | AhR-RYA | |
|---|---|---|---|---|
| | Literature | Validation | Literature | Validation |
| 17β-estradiol (ng $L^{-1}$) | 29 ± 4[a] | 350 ± 64 | b.d.l.[a] | n.a. |
| β-naftoflavone (mg $L^{-1}$) | b.d.l.[a] | n.a. | 0.14 ± 0.02[a] | 0.14 ± 0.05 |
| BP3 (mg $L^{-1}$) | 12.5[b] | 90 ± 25 | 6.8[d] | n.a. |
| BP1 (mg $L^{-1}$) | 0.058[b] | 0.46 ± 0.08 | 0.61[d] | n.a. |
| 4-MBC (mg $L^{-1}$) | 28.5[c] | 106 ± 113 | b.d.l.[d] | n.a. |

b.d.l.: below detection limit; n.a.: not analysed
[a] Noguerol et al. (2006), [b] Gago-Ferrero et al., (2012), [c] Badia-Fabregat et al. (2012), [d] Badia-Fabregat et al. (2013)

## 5.2. Degradation of benzotriazoles by *Trametes versicolor*

### *5.2.1. Degradation of BTZ, 4-MBTZ and 5-MBTZ in synthetic media*

Benzotriazole (BTZ) and the two isomers of tolyltriazole (4-MBTZ and 5-MBTZ) were chosen for their individual study because they were the EDCs detected at higher concentration in one of the effluents treated by *T. versicolor* in this thesis, a reverse osmosis concentrate (ROC) (see Chapter 7), and there were not studies about their degradation by this fungus. Thus, experiments at high concentration (10 mg $L^{-1}$) in synthetic media were performed to identify the fungal metabolites in order to incorporate them in the analytical methods and try to detect them in the fungal treatment of the ROC (Chapter 7). Two experiments were performed: EB, lasting 9 days with an initial glucose concentration of 8 g $L^{-1}$, and EB+G, lasting 14 days with extra glucose addition at days 3, 7 and 11 until recover the initial concentration to avoid nutrients depletion.

Degradation results for each experiment are shown in Figure 5.14. The major process was attributed to degradation since the sorption observed in HK experiments (difference between removal and degradation in Table 5.6) was almost negligible for the three compounds. The highest degradation percentage corresponded to 5-MBTZ, arriving to 85% after 14 d of exposure. In contrast, the structural isomer 4-MBTZ showed higher stability (55% of removal) as it was previously observed by Weiss et al. (2006) in a MBR. However, the most stable compound was BTZ, with a degradation yield of 37% after 14 days of exposure. Similar results were observed by Liu et al. (2011) by bacteria under aerobic conditions.

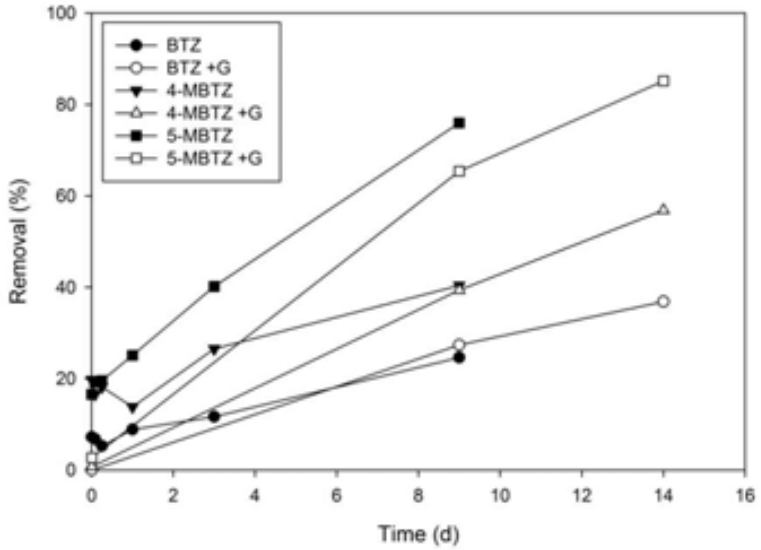



**Figure 5.14.** Removal of BTZ, 4-MBTZ and 5-MBTZ in the experimental bottles and the experimental bottles with addition of glucose every 3 days (+G treatments). Results are the mean of duplicates.

**Table 5.6.** Minimum degradation and removal percentages after 14 days of experiment (EB+G treatment)

| | **Removal (%)** | **Degradation (%)** |
|---|---|---|
| **BTZ** | 37 | 37 |
| **4-MBTZ** | 55 | 50 |
| **5-MBTZ** | 85 | 77 |

Focusing in the fungal dry weight, this one increased 20% between the 1$^{st}$ and the 3$^{rd}$ day in EB treatment. After this period, the weight decreased to achieve similar levels that the initial weight (Figure 5.15). In contrast, when glucose was added periodically to avoid starvation of *T. versicolor*, the fungal dry weight increased between 50 and 70% after 14 days in the EB+G and reached 88% in the blank control with extra addition of glucose (BC+G). However, no remarkable differences between degradation percentages were observed after the 9 days between EB and EB+G experiments (Figure 5.14) leading to think that transformation reactions were independent of *T. versicolor* metabolic state. Laccase activity was very low in all degradation experiments (less than 5 U $L^{-1}$) and similar to the BC (data not shown).

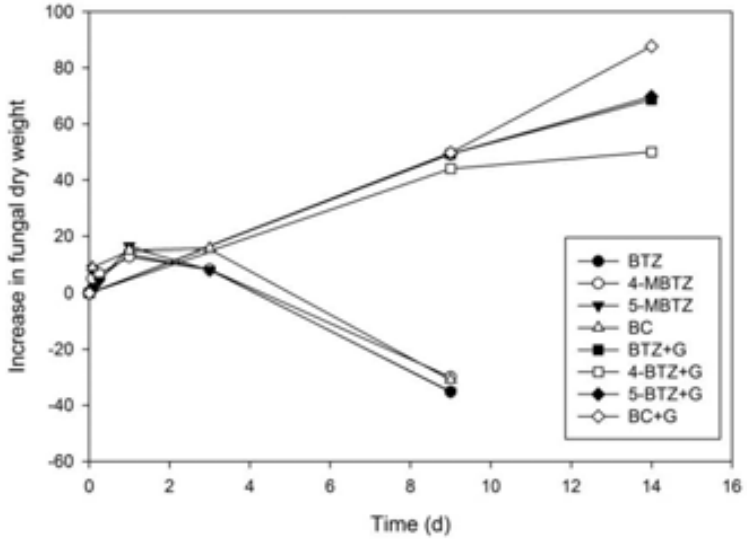
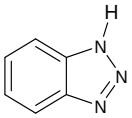
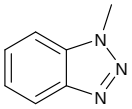
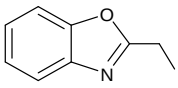


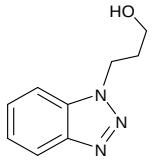
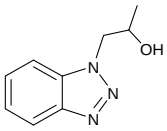
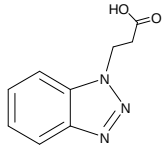
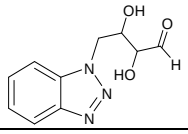
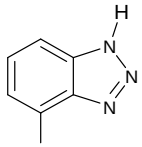
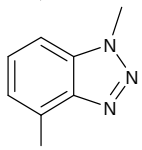
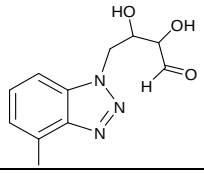
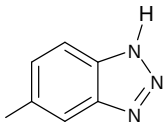
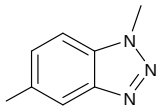
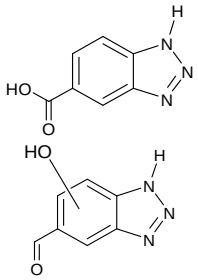
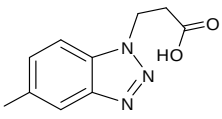
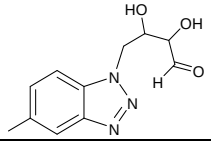

**Figure 5.15.** Evolution of fungal dry weight along the different degradation experiments in synthetic media. Legend: +G, experiments with extra addition of glucose; BC, blank control without contaminant.

### 5.2.2. Identification of transformation products

Samples of both EB and EB+G experiments were analyzed in order to identify any possible metabolite generated during biological treatment with *T. versicolor*. Table 5.7 shows the results of proposed elemental compositions for the detected metabolites. All the reported metabolites were identified in both EB and EB+G experiments, which leads to think that metabolic state of the fungus is not important for their degradation in this case.

**Table 5.7.** List of proposed metabolites identified during analysis by TFC-LTQ-Orbitrap.

| Compound | tr (min) | m/z | Proposed Structure | Percentage of initial parent compound at day 14 |
|---|---|---|---|---|
| **BTZ** | 4.5 | 120.0556 |  | 63% |
| **TP134** | 5.2 | 134.0713 |  | 0.25% |
| **TP148A** | 5.11 | 148.0756 |  | 3% |

| | | | | |
|---|---|---|---|---|
| **TP178A** | 4.7 | 178.0975 |  | 2% |
| **TP178B** | 5.0 | 178.0975 |  | 9% |
| **TP192** | 5.06 | 192.0767 |  | 2% |
| **TP222** | 4.62 | 222.0873 |  | 27% |
| **4-MBTZ** | 4.9 | 134.0715 |  | 43% |
| **TP148B** | 5.57 | 148.0868 |  | 0.1% |
| **TP236** | 4.93 | 236.1031 |  | 0.1% |
| **5-MBTZ** | 5.0 | 134.0715 |  | 15% |
| **TP148C** | 5.61 | 148.0868 |  | 4% |
| **TP164** | 4.4 | 164.0454 |  | 0% |
| **TP206** | 5.12 | 206.0923 |  | 1% |
| **TP236** | 4.95 | 236.1031 |  | 12% |

As regards to BTZ, six possible metabolites were identified: TP134, TP148A, TP178A, TP178B, TP192 and TP222. In order to make an approximation of the ratio formation of each metabolite, the peak area of the corresponding compound was normalized with the peak area of BTZ at initial time. By this approximation, the major ratio formation was detected for TP222 (11% after 9 days and 27% after 14 days) followed by TP178B (3 and 9% after 9 and 14 days, respectively). TP222 corresponds to a tetrose sugar attached through a N-glycoside bond. Other two minor compounds were identified: TP192 (ratio formation of 1 and 2% after 9 and 14 days, respectively) and TP148A (ratio of 1 and 3% after 9 and 14 days, respectively). A similar degradation product to TP148A has been postulated by Liu et al. (2011) during aerobic degradation of 5-MBTZ but not during the degradation of BTZ. Finally, TP134 was detected as a minor TP (0.23 and 0.25% after 9 and 12 days, respectively). The same structure has been proposed by Liu et al. (2011) and Huntscha et al. (2014) for the degradation of BTZ under aerobic conditions. Huntscha et al. (2014) also reported similar conjugated metabolites with tetroses attached to the aromatic ring. Therefore, as removal at 14 d was 37% (Table 5.5), BTZ was only transformed to these metabolites but not further metabolised.

Regarding 4-MBTZ, their degradation led to two metabolites: TP236 and TP148B, with ratio formations between 0.1–0.3% in front of a disappearance of 55% of 4-MBTZ. Thus, in this case, further metabolism could occur, probably with the opening of the rings and maybe even mineralisation. TP236 could be again a conjugation with a tetrose sugar.

5-MBTZ was the more efficiently degraded. Four potential metabolites were identified: TP148C, TP164, TP206 and TP236. The major ratio formation was for TP236 (12.44% after 14 days) followed by TP148C (4% after 14 days). Then, at the end of the experiment, the major percentage of the initial 5-MBTZ was further transformed to other compounds or even mineralised as it could occur with 4-MBTZ. The major metabolite (TP236) was also the conjugated with a tetrose sugar. TP164 was previously detected during the ozonation of 5-

MBTZ (Müller et al., 2012) and the degradation by activated sludge (Huntscha et al., 2014).

In the case of BTZ and TTZ, no studies of laccase and cytochrome involvement were performed because the objective of the study was to identify the transformation products and try to detect them in real wastewater degradation experiments, rather than elucidate the exact degradation pathway.

### 5.2.3. Evaluation of the toxicity

BTZ has been described as anti-estrogenic compound in *in vitro* assays, whereas no *in vivo* activity has been detected (Harris et al., 2007). In this work, estrogenic and anti-estrogenic activities for BTZ, 4-MBTZ and 5-MBTZ were assessed by recombinant yeast assay (RYA) as well as the acute toxicity by the bioluminescence assay with *V. fischeri*.

Regarding the acute toxicity measured by bioluminescence assay (Microtox® assay), the $EC_{50}$ after 15 min were determined for the three compounds being 25, 26 and 4 mg $L^{-1}$ for BTZ, 4-MBTZ and 5-MBTZ, respectively. Those results are in accordance to the previous work carried out by Pillard et al. (2001). The authors also found a structural isomer toxicity for TTZ where 5-MBTZ was more toxic than 4-MBTZ. Additionally, no estrogenic activity was detected for concentrations up to 5.3 g $L^{-1}$ for BTZ and 2.4 g $L^{-1}$ for 4-MBTZ and 5-MBTZ. However, possible anti-estrogenic activity was found for the three compounds at concentrations above 0.34 g $L^{-1}$ for BTZ and above 0.15 g $L^{-1}$ for both TTZs (Figure 5.16). Taking into account that our sensitivity in the ER-RYA assay was 10 fold lower than values reported by the literature, the obtained anti-estrogenic response of BTZ was the same than the one obtained by Harris et al. (2007) in a previous work.

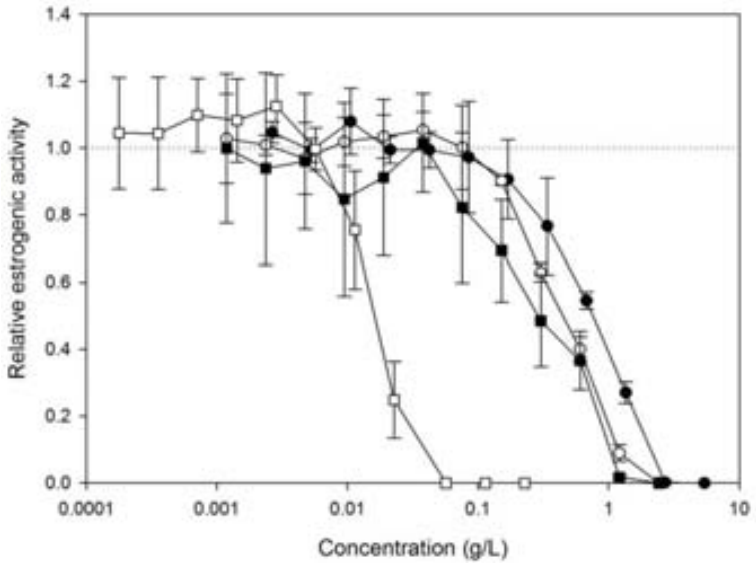



**Figure 5.16.** Anti-estrogenic activity measured as a decrease in the relative estrogenic activity in a medium with 17β-estradiol at 10 nM and (□) tamoxifen, (●) BTZ, (○) 4-MBTZ and (■) 5-MBTZ in serial diluted concentrations.

Anti-estrogenicity and acute toxicity by bioluminescence assay were also monitored during the fungal degradation of the pollutants in synthetic media during the EB+G experiment. Initial TU for acute toxicity were below 4 and not statistically significant different than the blank control. This values were maintained during all the treatments with the exception of 4-MBTZ degradation experiments after 14 days (TU=11±3). This fact could point out the possible production of some metabolites more toxic than the parent compound. In terms of anti-estrogenic activity, it was not detected for any sample. The hypothesis is that concentrations along experiments (<10 mg $L^{-1}$) were below effective anti-estrogenic concentrations.

## CONCLUSIONS

Based on fungal degradation experiments in synthetic media, all the selected compounds can be removed by the action of *T. versicolor.* For the three UV filters studied (BP3, 4-MBC and BP1) the elimination in the experimental bottles can be completely assigned to fungal degradation. On one hand, in BP1 and BP3 experiments, because including the solubilisation step allows a complete recovery of the contaminant in the inactivated controls, and on the other hand, in the case of 4-MBC, where the adsorption values reached almost 50%, all the initial contaminant is recovered after the fungal pellets disruption. In this way, with the inclusion of a sonication step, it is possible to recover all the contaminant that the dead fungus had taken from the medium, probably by means of some passive mechanism. Elimination of studied UV filters and also both TTZ isomers is not only due to a single transformation but a further degradation, and maybe even mineralisation, because quantification analyses of the main metabolites produced revealed that they also disappeared along the treatment period. However, BTZ has been the most recalcitrant compound with degradation below 50% after 14 days. Moreover, its removal was only due to transformation, mainly to a conjugated compound, and not further degraded. Therefore, there are some compounds, such as BTZ, that are very recalcitrant because they cannot be degraded even with the broad and unspecific enzymatic machinery system of white-rot fungi under the experimental conditions applied. Therefore, other experimental conditions should be tested trying to improve the degradation of BTZ.

For all the compounds included in the present study, the main metabolites identified were conjugated forms that were further degraded. From them, the predominant product in BP3, BP1 and 4-MBC degradation was the pentose conjugate and for BTZ, 4-MBTZ and 5-MBTZ the glycoconjugates with a tetrose. The presence of the cytochrome P450 inhibitor 1-aminobenzotriazole (ABT) affected the degradation rates of both BP3 and 4-MBC, but in different ways. For 4-MBC, any conjugation reaction requires a previous step of hydroxylation (or dihydroxylation) by cytochrome P450. This is the reason accounting for the complete inhibition of 4-MBC degradation in the presence of

ABT. On the other hand, glycoconjugation would be the first step in the BP3 and BP1 (included in the degradation pathway of BP3) metabolism and, thus, ABT reduces, but does not inhibit, BP3 transformation because BP3, unlike 4-MBC, presents hydroxylated groups that allow conjugation in the absence of cytochrome P450 hydroxylase activity. In all cases, conjugation would revert gradually and oxidation of compounds and subsequent ring cleavage would take place, leading to smaller unidentified fragments. However, unless similar degradation pathways (oxidations by cytochrome P450 and conjugations) are followed by fungi and mammals, obtained metabolites are not always the same. In addition, the importance of isomers must be pointed out. Firstly, regarding differences observed in degradation yields between the isomers 4-MBTZ (55% degradation) and 5-MBTZ (85% degradation), which is in accordance with previous published works. Also, concerning the production of isomers of the parent compound during degradation, as happened with 4-MBC.

Degradation products must be identified but also characterised in any treatment in order to assess the suitability of the method with respect to toxicity hazard. Estrogenic activity was detected for the UV filters and possible anti-estrogenic activity for benzotriazoles. As studied compounds present some hormonal activity, specific assays for monitoring endocrine disrupting activities, such as estrogenic and dioxin-like (ER-RYA and AhR-RYA, respectively) assays, are needed during the degradation treatments. Degradation by *T. versicolor* decreases the toxicity (also acute toxicity measured by Microtox) to undetectable levels, what means that degradation did not result in a formation of estrogenic metabolites or at least at high enough concentrations to overcome the activity of the parental compounds. RYA assay was established in our laboratory.

Finally, studies at lower concentrations, near the environmental ones (250 µg $L^{-1}$), in a first scale-up to an air pulsed reactor of 1.5 L, showed that the removal of both studied benzophenones is also very high at lower concentrations. In the case of 4-MBC, as well as other highly hydrophobic compounds, those kind of studies in lab-bioreactor in the given conditions are not suitable to perform because a complete solubilisation of the compound is totally necessary for a

reliable quantification. Therefore, liquid samples can not be taken at different time points because they would not be representative. That fact, at different scale, would be what happens with those compounds in the WWTP (and also in the environment): the more soluble compounds, like benzophenones, are those found at a higher proportion in the liquid effluent, while the UV filters with a higher log Kow (such as 4-MBC and OC) are mainly found adsorbed onto the sludge.

# Chapter 6

## Use of stable isotope probing to assess the fate of emerging contaminants degraded by white-rot fungus

## INTRODUCTION

In Chapter 5, typical studies of fungal degradation of selected contaminants have been presented. In those experiments, identification of metabolites was performed and their disappearance after some days of experiment allowed us to guess that further transformations were taking place. However, no direct evidences of mineralisation could be obtained. Moreover, despite there are some articles reporting the degradation of some compounds as the sole carbon source (Prenafeta-Boldú et al., 2001), there is not direct evidence of the use of xenobiotics as carbon source by fungi either. In fact, the dominant theory is that fungi degrade xenobiotics co-metabolically, even mineralising them in some cases, but without taking profit of them as energy or carbon source. Indeed, it is thought that bacteria can outcompete fungus in contaminated sites because the latter cannot obtain energy for growth from contaminants (Harms et al., 2011).

The use of compounds labelled with stable isotopes (mainly $^{13}C$) allows the safe determination of the mineralisation of certain compounds. Moreover, the use of stable isotope probing (SIP) analyses increased the range of applications of these labelled compounds. SIP is based in the monitoring of the changes in the $^{13}C$ atom percentage (or the related $\delta^{13}C$ (‰)) of certain biomarkers such as DNA (DNA-SIP), proteins (protein-SIP) or total lipid fatty acids (TLFA-SIP) (Bastida et al., 2011, 2010; Jakobs-Schönwandt et al., 2010; Lu and Chandran, 2010). Therefore, with the use of SIP methodology, the incorporation of carbon from micropollutants to the fungal biomass can be demonstrated.

In this chapter, we take advantage of the increasing disponibility of commercially available compounds labelled with stable isotopes with the aim of fulfilling some of the above mentioned lacks of knowledge. Therefore, from those commercially available labelled emerging contaminants, we chose the UV filter benzophenone-3 (BP3) and the anti-inflammatory diclofenac (DCF) because they were candidates to be mineralised by *T. versicolor* (Chapter 5; Marco-Urrea et al., 2010).

Therefore, in the present study, methodology to assess contaminants degradation pathway by fungi by means of stable isotopes was established. We combined the analysis of carbon isotopic composition of $CO_2$, bulk biomass and individual amino acids (by aa-SIP) during the degradation of $^{13}$C-DCF and $^{13}$C-BP3 by the white-rot fungus *T. versicolor* to track the $^{13}$C fate of these emerging contaminants and the degradation mechanism used by the fungus.

Firstly, $^{13}CO_2$ production was quantified in order to determine if the contaminants were mineralised or not by *T. versicolor.* Then, stable isotope labelling in the total fungal biomass was analysed to know if the contaminants are incorporated into the fungal biomass and/or if parent compound or its metabolites are uptaken by the fungus. Finally, aa-SIP was implemented as an unambiguous evidence for the fungal assimilation of the contaminant as a carbon source. Taking into account the results of all the above mentioned experiments, the fate of the contaminants during fungal degradation can be elucidated.

The analyses of the experiments presented in this chapter have been performed in collaboration with Dr. Monica Rosell from the Department of crystallography, mineralogy and mineral deposits in the Universitat de Barcelona (UB).

# 6.1. Benzophenone-3

### *6.1.1. Protocol optimisation*

Before performing the experiments with the labelled compounds, protocol of the methodology had to be optimised. Therefore, different aspects were studied such as the effect of the lack of oxygen (due to the need of replace the cotton plugs for Tefon stoppers in order to retain the gas phase to quantify the $CO_2$ produced) and the changes in fungal, contaminant and glucose concentrations in the degradation (due to the low amount of available labeled BP3). Moreover, protein extraction had to be adapted and optimised for fungi as well.

Effect of oxygen and concentrations of BP3, fungus and glucose in the fungal degradation

As stated before, in order to be able to quantify the $CO_2$ produced, experimental bottles have to be completely sealed. This could be an important drawback as fungi are aerobic organisms, that need oxygen for their survival and degradation of contaminants. Therefore, some experiments were performed with Teflon stoppers instead of cotton plugs for sealing the serum bottles. Fig. 6.1A shows how this limitation in the exchange of gases decreased the degradation yield to only 79% removal at 24 h and after day 3, even an increase of BP3 concentration was detected reaching 70% of initial concentration. Regarding the metabolites generated, the same conjugates M1 and M2 were observed (see Chapter 5), with higher concentration of M1 as well (Fig. 6.1B). At day 8, both of them have disapeared but in this case it would not be due to further transformation but a reversion to BP3 due to the lack of oxygen.

Therefore, the gas phase had to be replaced more frequently in order to avoid oxygen limitation. Then, another experiment with only partial oxygen limitation due to the periodical opening of the bottles (every 3 days) was performed. Under those conditions, stable 80% degradation of BP3 was achieved during 15 days (Fig. 6.2A). However, no further transformation of M1 and M2 metabolites was observed (Fig. 6.2B). M1 concentration accounted for a 20%.

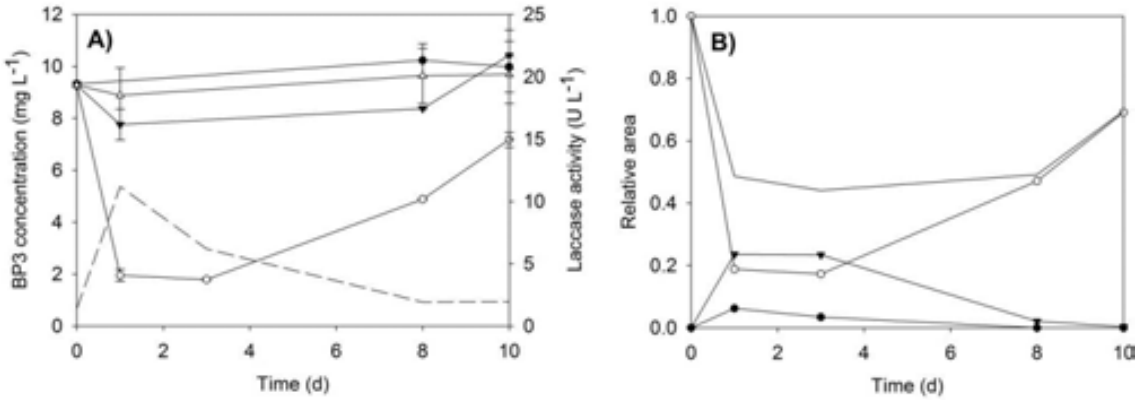



**Figura 6.1.** BP3 and its metabolites concentration evolution in oxygen limited experiments. **A)** BP3 concentration at (○) EB, (●) UNI, (▼) HK and (△) SAK. Laccase activity in EB is also plotted in long dashes. Values plotted are means ± standard error for triplicates. **B)** Evolution of peak areas in HPLC chromatograms. Legend: (○) BP3, (▼) M1 and (●) M2. Sum of all areas of the main peaks is plotted in a solid line.

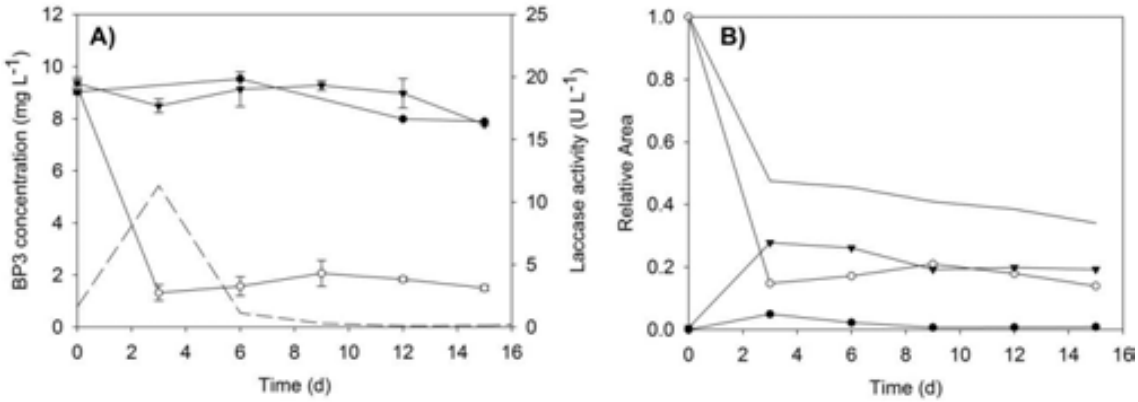



**Figura 6.2.** BP3 and its metabolites concentration evolution in partially oxygen limited experiments. **A)** BP3 concentration at (○) EB, (●) UNI, (▼) HK and (△) SAK. Laccase activity in EB is also plotted in long dashes. Values plotted are means ± standard error for triplicates. **B)** Evolution of peak areas in HPLC chromatograms. Legend: (○) BP3, (▼) M1 and (●) M2. Sum of all areas of the main peaks is plotted in a solid line.

Therefore, taking into account the results presented in this section, aeration is very important to avoid reversion of BP3 first steps transformation and finally achieve further degradation. Thus, as only opening the bottles every 3 days was not enough to totally degrade BP3, in the next experiments the gas phase in the serum bottles was enriched with oxygen (not only air) at the beginning of the experiment, as already performed Marco-Urrea et al. (2008), and every three days. Moreover, taking into account that labeled compounds are expensive products, the amount of BP3 was reduced to 1 mg $L^{-1}$. Therefore, in order to

increase the possibilities to detect incorporation in the amino acids and minimise carbon dilution, the amount of fungus and glucose were also reduced, maintaining the ratios of the previous degradation experiments. Fungal biomass was fixed at 0.5 g DCW $L^{-1}$ and exploratory experiments with non-labeled BP3 at 0, 0.5 and 1 g $L^{-1}$ of glucose were performed. Control bottles to compare the degradation profiles between cotton plugs and Teflon stoppers at the different conditions were also included. The maximum laccase activity (around 20 U $L^{-1}$) was detected at cultures with 0.5 g $L^{-1}$ of glucose although differences between treatments are low (Fig. 6.3). As shown at Fig. 6.4, degradation of BP3 was dependent on glucose concentration, with only 40% removal and very low generation of pentose conjugated metabolite (M1) when no glucose was added at the medium. Degradation percentage with the 0.5 g $L^{-1}$ of glucose medium was acceptable (60%) and, thus, used for the stable isotope experiments to minimise the dilution of labeled carbons. Time of oxygen replacement was fixed at 3 days, in a compromise between the lenght of the experiment, the amount of samples to be taken and the degradation percentage achieved, that was only around 20% lower than using cotton plugs (see Fig 6.4A).

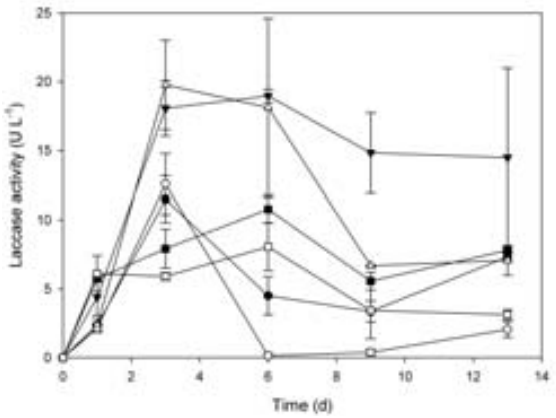



**Figure 6.3.** Laccase activity. Symbols: experiments at 1 g $L^{-1}$ glucose with (●) cotton plugs or (○) teflon stoppers, experiments at 0.5 g $L^{-1}$ glucose with (▼) cotton plugs or (△) teflon stoppers and experiments at 0 g $L^{-1}$ glucose with (■) cotton plugs or (□) teflon stoppers.

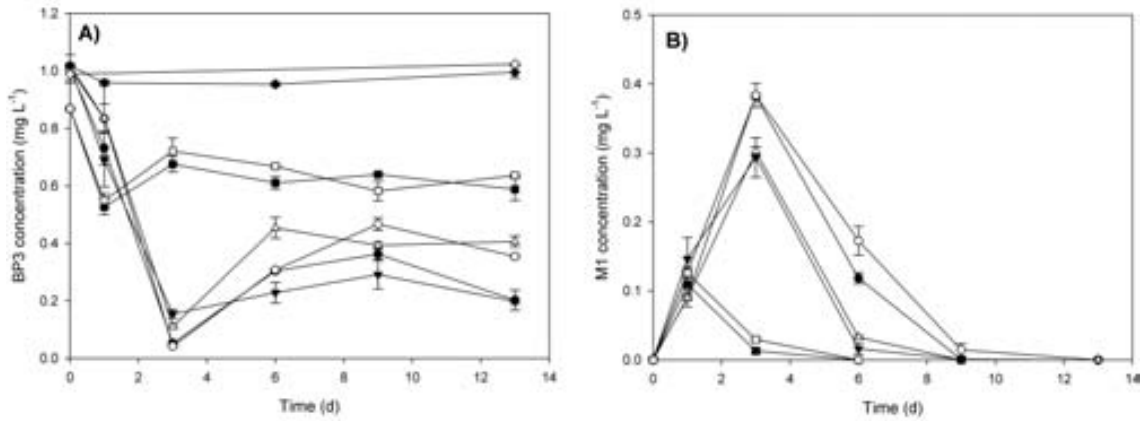



**Figure 6.4. A)** BP3 and **B)** M1 concentration. Symbols: experiments at 1 g $L^{-1}$ glucose with (●) cotton plugs or (○) teflon stoppers, experiments at 0.5 g $L^{-1}$ glucose with (▼) cotton plugs or (△) teflon stoppers, experiments at 0 g $L^{-1}$ glucose with (■) cotton plugs or (□) teflon stoppers, (♦) inactivated control with sodium azide and (◊) uninoculated control.

Protein extraction protocol

Protein extraction and amino acid derivatization protocol was adapted for fungi from those of Bastida et al. (2011) and Jechalke et al. (2013) for larvae and bacteria. First of all, fungi cell disruption was optimised. Based on Blánquez et al. (2004) and Klimek-Ochab et al. (2011), lysis with Constant Cell Disruption System (Constant Systems, LTD) and probe sonication (Vibra Cell, S&M) were compared at high fungal amount (160 mg DCW, 7.6 g DCW $L^{-1}$). At lower fungal concentration (8.8 mg DCW, 8.8 g DCW $L^{-1}$) probe sonication and extraction with silica beads disruption from a kit (Rneasy Plant mini kit, Qiagen) were compared as well. As shown in Fig. 6.5, the highest protein concentrations, or at least the same than with the cell disruptor, were achieved with probe sonication. Therefore, probe sonication was performed from here on as it was considered to be more adequate for low volume samples than cell disrupter. However, when working at lower amount of fungi, extracion efficiency was found to be lower (Fig. 6.5).

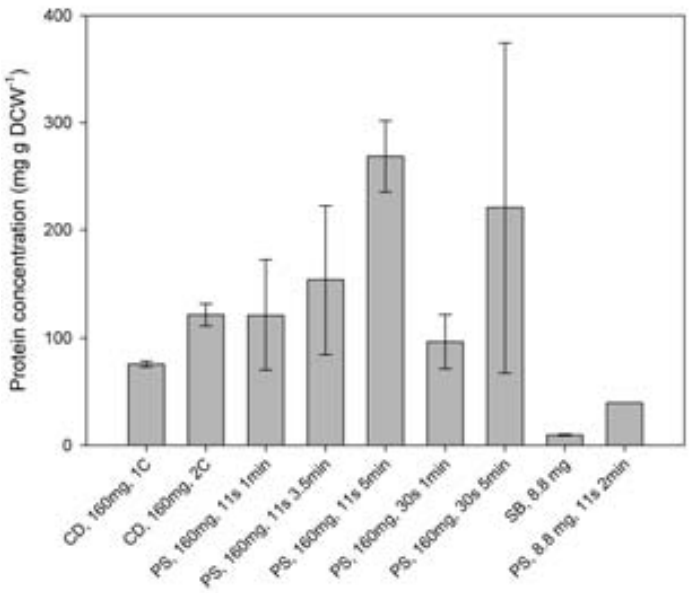



**Figure 6.5.** Protein concentration achieved after cell disruption with cell disruptor (CD), probe sonication (PB) or silica beads (SB). Cell disruptor was set at 2.86 atm 1 shot (1C) or 2 (2C). Probe sonication was performed at cycles of 11 or 30 s, which summed 1, 2, 3.5 or 5 min of sonication. Proteins were quantified by Bradford assay of the supernatant of centrifugation after cell lysis.

Afterwards, losses of protein during the extraction protocol were assessed, as shown in Fig. 6.6, by means of taking samples at different steps of the protocol (from the culture medium, after sonication, after proteins precipitation with TCA, after cleaning the proteins with ammonium acetate and, finally, after a second cleaning with acetone). Losses after protein precipitation and cleaning were considerable. Therefore, protocol was reduced to only precipitation with TCA and one cleaning step with acetone after checking by GC-MS that it was enough for a proper quantification of amino acids (data not shown). Moreover, homogenization of the mycelia prior to cell disruption was found to be unnecessary (Fig. 6.7).

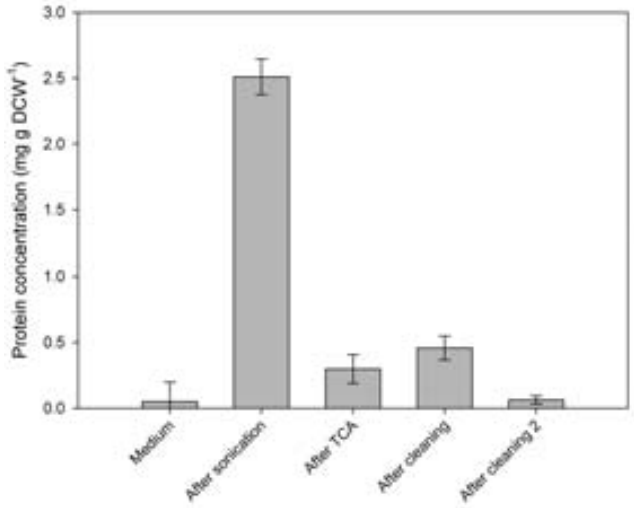



**Figure 6.6.** Protein concentration at different steps of protein extraction protocol. Proteins were quantified by Bradford assay. The first cleaning was performed with 1 mL of ammonium acetate incubated overnight at -20ºC. The second cleaning was performed with 1 mL of acetone incubated overnight at -20ºC.

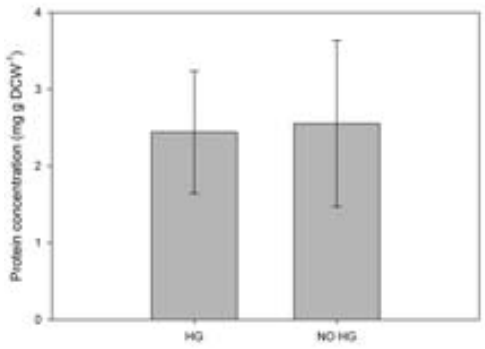



**Figure 6.7.** Protein concentration with and without homogeneization (HG) step prior to sonication. Proteins were quantified by Bradford assay.

Thus, simplification of the protocol was performed, remaining as described in Materials and Methods (Section 4.6.2).

### *6.1.2. Mineralisation of benzophenone-3 (BP3)*

Once protocol was optimised, the experiment with the labeled BP3 ($^{13}$C-BP3) was performed, with a length of 9 days and replacing the air with oxygen. $CO_2$ was measured every 3 days and again the air was replaced with oxygen and the bottles sealed. At the same sampling times that $CO_2$ was measured, samples for BP3 quantification by HPLC-UV, elemental analysis by isotope ratio

mass spectrometre (EA-IRMS) and protein extraction for further performing aa-SIP were taken as well. As Figure 6.8 shows, in the experiments spiked with $^{13}$C-BP3, BP3 concentration decreased quite fast from the liquid the first 3 d, but afterwards a plateau was reached achieving a final removal of 81.2±5.6%. Negligible removal was observed in the inactivated controls, indicating that removal was not due to sorption as already determined in Chapter 5. Glucose consumption rate and laccase activity showed the previously observed profiles. Glucose was totally consumed during the first 3 days and laccase maximum activity was around 20-25 U $L^{-1}$, concluding that the activity of the fungus was the usual.

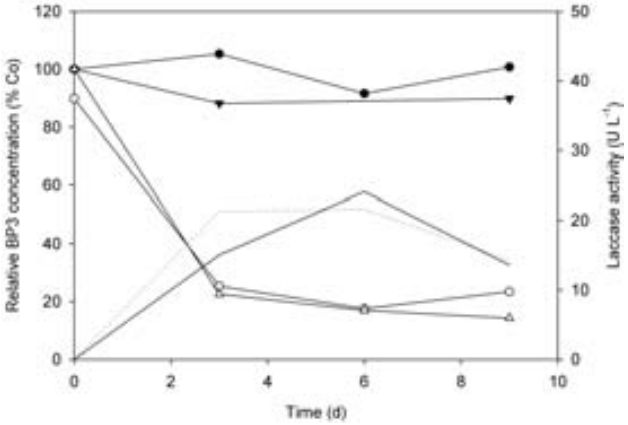



**Figure 6.8.** Time course degradation of BP3 and laccase activity. Symbols: (●) uninoculated controls with $^{12}$C-BP3, (▼) controls containing sodium azide with $^{13}$C-BP3, (○) experimental cultures with $^{12}$C-BP3, (△) experimental cultures with $^{13}$C-BP3, (—) laccase activity at experimental cultures with $^{13}$C-BP3 and (···) laccase activity at experimental cultures with $^{12}$C-BP3.

As shown in Fig. 6.9, $CO_2$ production rate in the experimental bottles reached a peak within the first 3 d of incubation, corresponding with the period of glucose consumption. No significant differences in total $CO_2$ production were detected between samples containing labelled and unlabelled compounds. As shown in Fig. 6.9, 30% of $^{13}$C from BP3 was already detected as $CO_2$ on day 3 and 45% on day 9. At 6 and 9 d the increase in the labelled ring mineralisation was still considerable because $CO_2$ was much more enriched in $^{13}$C ($\delta^{13}$C was higher) although the production was lower. The $\delta^{13}$C values of controls containing sodium azide plus $^{13}$C-BP3 were identical to those of controls containing sodium azide with $^{12}$C-BP3 indicating that $^{13}CO_2$ production was biotic.

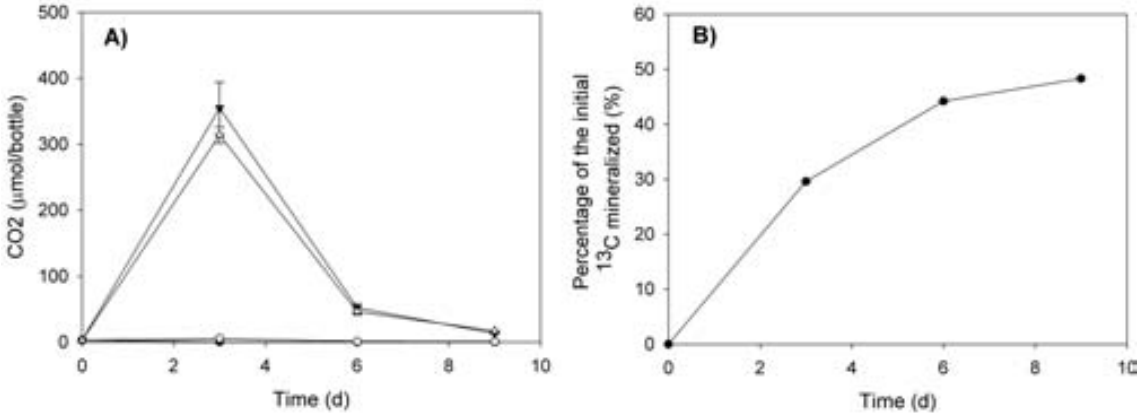



**Figure 6.9. A)** Carbon dioxide produced every three days in cultures of *T. versicolor* spiked with BP3. Symbols: (●) non-inoculated controls with $^{12}$C-BP3, (○) controls containing sodium azide with $^{13}$C-BP3 and experimental cultures with (▼) $^{12}$C-BP3 and (△) $^{13}$C-BP3. **B)** Cumulative $^{13}CO_2$ production in experimental cultures spiked with $^{13}$C-DCF.

This is the first study reporting biological mineralisation of BP3. Mineralisation of other xenobiotics had already been reported for *T. versicolor*: [$^{13}C_2$]-trichloroethylene (Marco-Urrea et al., 2008), [U-$^{14}$C]-pentachlorophenol (Tuomela et al., 1999) and 2,4',5-[U-$^{14}$C]-trichlorobiphenyl (Beaudette et al., 1998). However, mineralisation percentages in those cases were lower (11% on day 22 for TCB, 29% in 42 d for PCP). On the other hand, other labelled PPCP compounds such as [$^{13}C_6$-phenyl]-sulfamethazine and [$^{13}C_3$-propionic]-ibuprofen, did not show $^{13}CO_2$ production after being degraded by white-rot fungi (García-Galán et al., 2011; Marco-Urrea et al., 2009).

### *6.1.3. $^{13}$C incorporation into fungal biomass by EA-IRMS and amino acid-SIP analyses*

The bulk analysis of the $\delta^{13}$C values of fungal biomass by EA-IRMS showed $\delta^{13}$C enrichment in cultures spiked with $^{13}$C-BP3 in comparison with the $\delta^{13}$C values observed in the unlabeled controls which remained constant over time (Fig. 6.10). A marked shift in the $\delta^{13}$C enrichment was observed the first 3 d of incubation in cultures spiked with $^{13}$C-BP3 (from -21.9 ± 0.3 to -6.7 ± 2.5) and afterwards remained constant. The carbon isotopic signature in heat killed and sodium azide killed controls spiked with either labelled or unlabelled BP3 resulted in $\delta^{13}$C values statistically identical, indicating that $^{13}$C-incorporation in

live cultures was not due to passive uptake associated to physico chemical processes such as sorption. The time-course of $^{13}C$ enrichment into biomass is depicted in Fig. 6.10 as percentage of $^{13}C$ atom (at%).

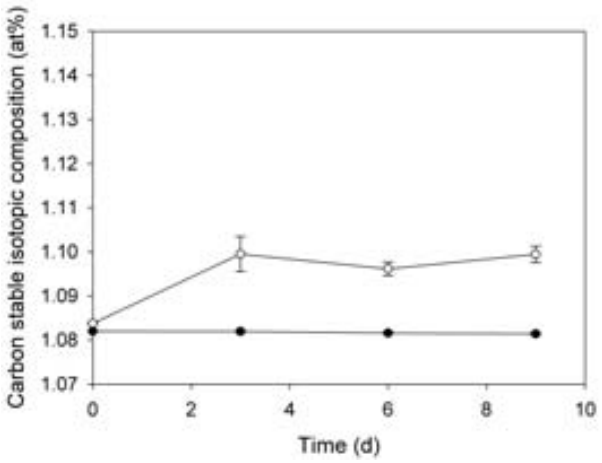



**Figure 6.10.** Carbon stable isotopic composition (atom%) of the fungal biomass in experimental cultures spiked with either (○) $^{13}C$-BP3 or (●) $^{12}C$-BP3.

Previous indirect evidences using specific inhibitors suggested a role to cytochrome P450 system in the primary oxidation of BP3 (see Chapter 5). This mechanism would imply an active uptake of contaminants through the fungal cell wall and membranes and their further intracellular transformation. In addition, the role of cytochrome P450 in BP3 degradation by *T. versicolor* was underpinned by the previous identification of hydroxylated and demethylated transformation products typically catalyzed by this intracellular enzymatic. Both hydroxylated and conjugated byproducts of BP3 disappeared from the medium after two weeks of incubation. Therefore, the observed biomass labelling could be attributed to the intracellular presence of labelled untransformed BP3 or associated byproducts without implying a final incorporation into anabolic products.

Amino acids-SIP was used to unequivocally demonstrate the transformation of BP3 into anabolic products. In Fig. 6.11, the amino acid enrichment (as atom % $^{13}C$) in cultures of *T. versicolor* spiked with $^{13}C$-BP3 compared with controls spiked with $^{12}C$-BP3 are presented. Incorporation of $^{13}C$ was observed in all the target amino acids since the third day of incubation, except for serine that did

not show significant enrichment (0.016 at%) until the sixth day of incubation. Fluctuations in the isotope content over time were attributed to the continuous turnover of amino acids to precursor intermediates that was reflected in the labelling patterns. The extent of enrichment varies between amino acids. The highest $^{13}C$ incorporation (>0.05%) was observed for alanine, glutamate and aspartate, which can be biosynthesized by transamination of pyruvate and tricarboxylic acid (TCA) cycle intermediates α-ketoglutarate and oxaloacetate respectively. A common degradation pathway of aromatic compounds by white-rot fungi involves the ring cleavage to produce β-ketoadipate and finally succinate and acetyl-CoA, essential compounds of TCA cycle (Wells and Ragauskas, 2012). Therefore, an hypothetical metabolic pathway of BP3 would include i) the ring oxidation by the action of cytochrome P450 system (as previously reported in Chapter 5), ii) catechol formation and ring cleavage by ortho-fission generating an intradiol, and iii) anabolism precursors formation via β-ketoadipate pathway (Fig. 6.12). Nuclear magnetic resonance analyses of media from experimental cultures were performed in an attempt to find some of those intermediate metabolites, trying to take advantage of the $^{13}C$ labelling. However, none of them were detected, probably due to their low concentration in the medium and rapid transformation (data not shown). Interestingly, $^{13}C$ incorporation of amino acids are in the same range than the global incorporation observed in bulk biomass (0.02 at%), except for glutamate, aspartate, alanine, threonine and proline that showed higher values.

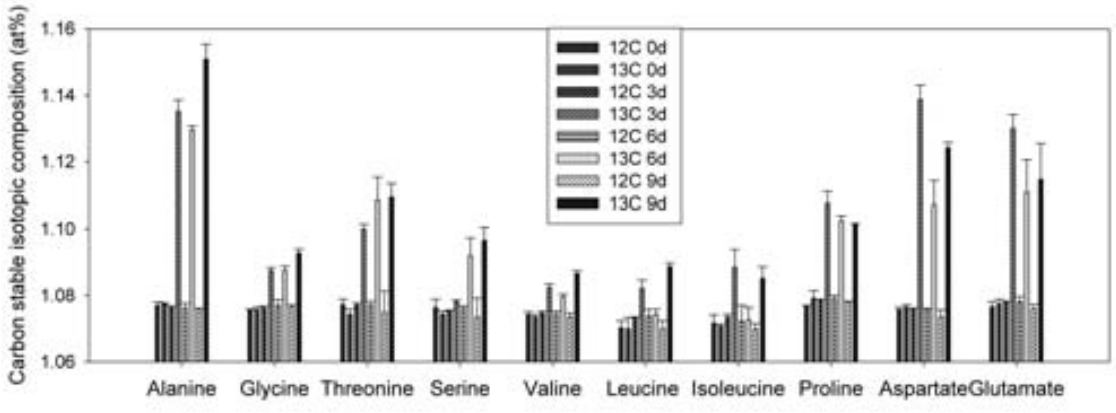



**Figure 6.11.** Carbon stable isotopic composition (atom%) of the amino acids in cultures spiked with 12C-BP3 and 13C-BP3 at different experimental times. Values were corrected for the carbon introduced during derivatiztion.

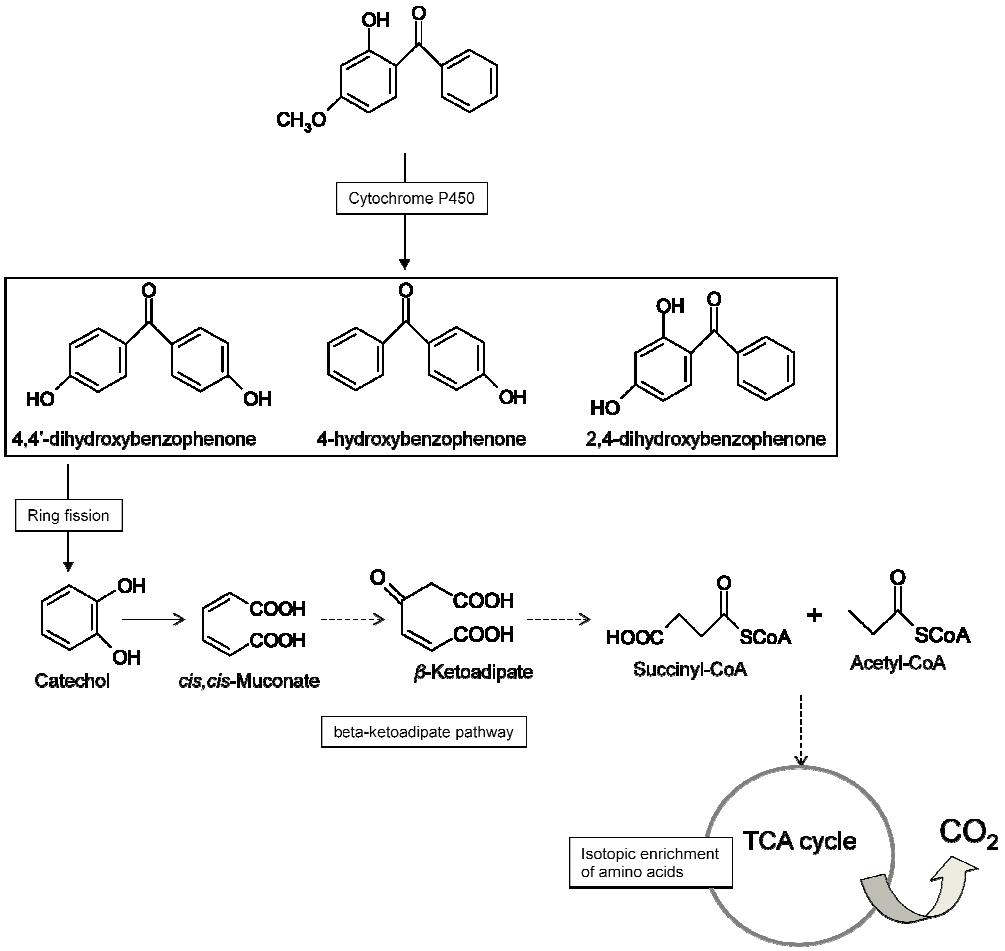



**Figure 6.12.** Degradation pathway of benzophenone-3 (BP-3) by *T. versicolor*. Metabolites put in a frame were previously identified (see Chapter 5). Identification of aromatic ring fission products of BP-3 were not detected in this study by nuclear magnetic resonance analysis probably due to their low concentration. However, the observed isotopic enrichment of amino acids and $CO_2$ production from BP-3 quantified in the present work suggest amino acid formation via beta-ketoadipate as depicted.

Finally, a $^{13}C$-mass balance of labelled BP3 was performed across four different sections over time i) $^{13}C$ remaining in the liquid media as non-transformed parent compound, ii) $^{13}C$ newly formed byproducts in the media, iii) $^{13}C$ mineralised as carbon dioxide, iv) $^{13}C$ incorporated into fungal biomass (Fig. 6.13). Estimation of byproducts was calculated by subtracting to the initial amount of $^{13}C$ added in form of parent compound, the remaining non-transformed parent compound, the amount accumulated into biomass and

mineralisation. With regards to the $^{13}C$ calculated as incorporated into fungal biomass, it is not possible to distinguish between the $^{13}C$ assimilated into biomass and $^{13}C$ deriving from the accumulation of labelled parent compounds and/or byproducts into the fungus cell. However, amino acid-SIP allows us to confirm that BP3 was used as carbon source. As observed in Fig. 6.13, mineralisation of BP3 did not reach a plateau, suggesting that higher mineralisation rates could be reached if cultures were incubated longer. The mass balance fits well with previous evidences of BP3 degradation by *T. versicolor*, since 30% of the initial BP3 was reported to be in the medium as glycoconjugate the ninth day of incubation (see Chapter 5).

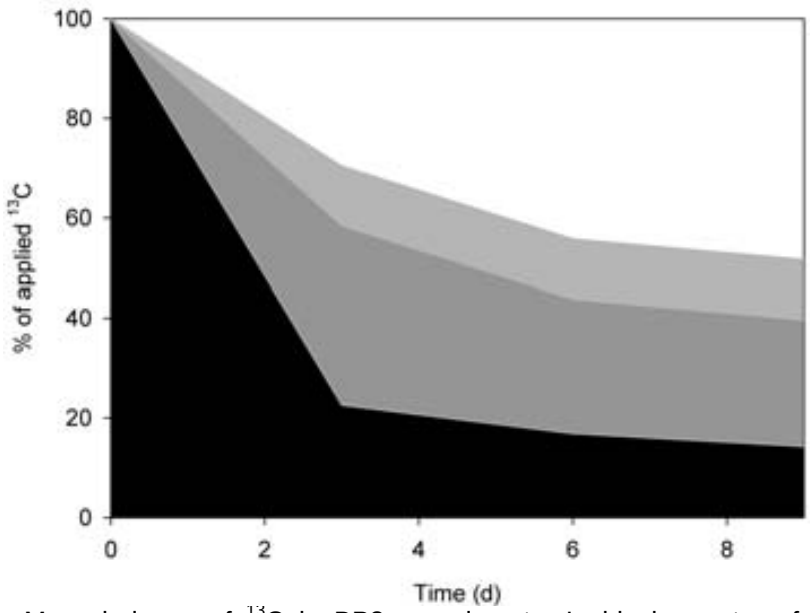



**Figure 6.13.** Mass balance of $^{13}C$ in BP3 experiments. In black non-transformed $^{13}C$-BP3 remaining in the media, in dark grey estimated $^{13}C$ remaining in the media in form of by-products to complete the mass balance, in light grey $^{13}C$ into fungal biomass, and in white $^{13}C$ mineralised ($^{13}CO_2$).

## 6.2. Diclofenac

### *6.2.1. Mineralisation of diclofenac (DCF)*

Then, studies of mineralisation and carbon incorporation for the analgesic and anti-inflammatory diclofenac (DCF) were also carried out. DCF was chosen due

to its importance as high toxic and low biodegradable emerging contaminant. Moreover, Marco-Urrea et al. (2010) pointed out to the possible mineralisation by *T. versicolor* after monitoring the apearance and disapearance of metabolites during the fungal treatment. Taking into account those previous studies of DCF degradation by *T. versicolor,* DCF transformation was expected to be faster than BP3 one and, thus, samples were planed to be taken at shorter time expecting a faster mineralisation too. Therefore, the experiment was prepared in a nearer facility to the analytical equipment instead of our laboratory for an extended monitoring of $CO_2$. Then, nitrogen and oxygen gas (for evaporating the solvent of the DCF stock and for oxygenating the samples, respectively) could not be used. As shown at Fig. 6.14, degradation without oxygen addition was tested to be successful in a prior experiment, although slower than previously reported (Marco-Urrea et al., 2010b). Nevertheless, acetonitrile of the stock solution of DCF could not be totally evaporated in the experiment with $^{13}$C-DCF and it was oxydized to $CO_2$ by *T. versicolor,* increasing the amount of $CO_2$ produced and, thus, masking the possible $^{13}CO_2$ produced by the mineralisation of DCF (Fig. 6.15).

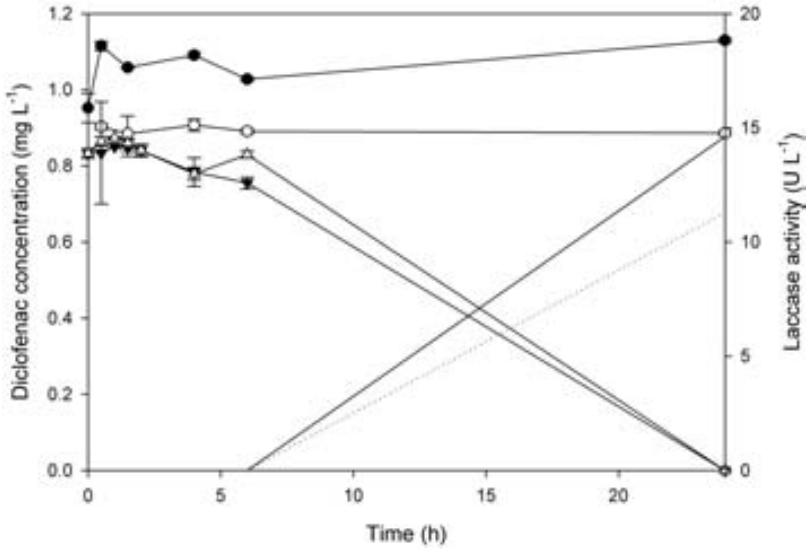



**Figure 6.14.** Time course degradation of DCF during the exploratory experiment. Symbols: (●) uninoculated controls, (○) controls containing sodium azide, (▼) experimental cultures with oxygen supply and (△) experimental cultures without extra oxygen supply. Laccase activity detected at experimental cultures (—) with and (···) without extra oxygen is also plotted.

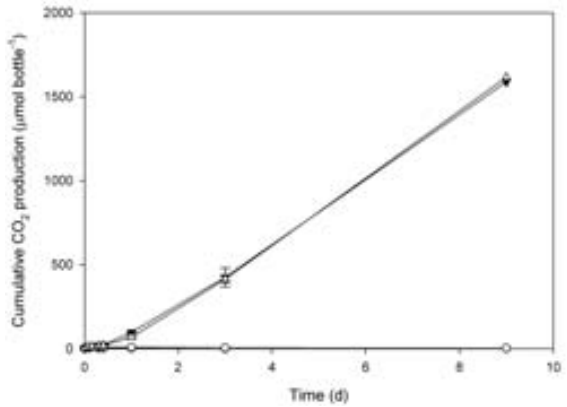



**Figure 6.15.** Cumulative $CO_2$ produced in the first experiment with $^{13}$C-DCF. Symbols: (●) uninoculated control, (○) inactivated control with sodium azide, (▼) experimental bottles spiked with $^{12}$C-DCF and (△) experimental bottles spiked with $^{13}$C-DCF.

Therefore, taking into account those results, the final DCF experiment was performed under the same conditions than BP3 one (totally evaporating the acetonitrile of the stock solution, enriching the gas phase with oxygen and taking samples every 3 days). Then, DCF was totally removed from the solution within 3 d (Fig. 6.16). Negligible removal was observed in the inactivated controls, indicating that removal was not due to sorption. Glucose consumption rate and laccase activity showed the previously observed profiles, concluding that the fungus was active. Glucose was totally consumed during the first 3 days and laccase maximum activity was around 30 U L$^{-1}$.

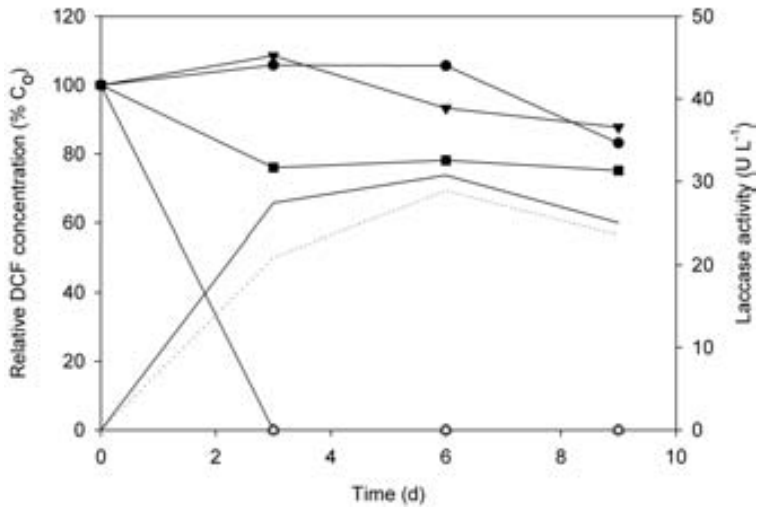



**Figure 6.16.** Time course degradation of DCF and laccase activity. Symbols: (●) uninoculated controls with $^{12}$C-DCF, () heat-killed controls with $^{13}$C-DCF, (▼) controls containing sodium

azide with $^{13}$C-DCF, (○) experimental cultures with $^{12}$C-DCF, (△) experimental cultures with $^{13}$C-DCF, (—) laccase activity at experimental cultures with $^{13}$C-DCF and (···) laccase activity at experimental cultures with $^{12}$C-DCF.

However, an unexpected $^{13}$C isotopic enrichment of $CO_2$ was observed in controls inactivated with sodium azide plus $^{13}$C-DCF that reached $\delta^{13}$C values up to 25.9 ± 2.1 at 9 d. However, the percentage of initial $^{13}$C-DCF mineralised in these controls was negligible when referred to the low $CO_2$ emitted in these microcosms. This enrichment was probably produced by the oxidative action of extracellular laccase, not fully deactivated by sodium azide, which was able to remove rapidly DCF from the medium in previous *in vitro* experiments (Marco-Urrea et al., 2010b). To confirm this aspect, thermically inactivated controls with $^{13}$C-DCF were also included and resulted in $\delta^{13}$C values comparable to inactivated controls containing $^{12}$C-DCF.

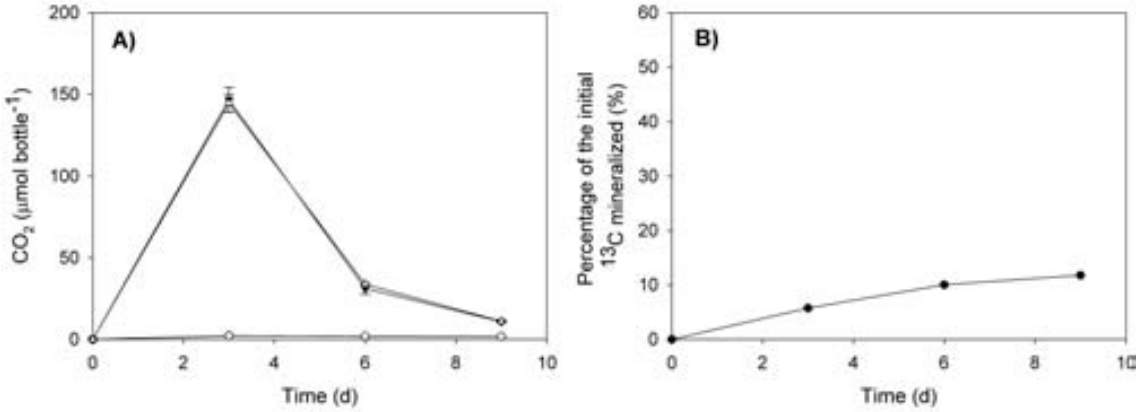



**Figure 6.17. A)** Carbon dioxide produced every three days in cultures of *T. versicolor* spiked with DCF. Symbols: controls containing sodium azide with (●) $^{12}$C-DCF and (○) $^{13}$C-DCF; experimental cultures with (▼) $^{12}$C-DCF and (△) $^{13}$C-DCF. **B)** Cumulative $^{13}CO_2$ production in experimental cultures spiked with $^{13}$C-DCF.

As shown in Fig. 6.17, mineralisation of $^{13}$C from DCF reached a final percentage of only 11% the ninth day with respect to the initial $^{13}$C. Higher mineralisation values were expected since degradation rate and removal of identified transformation products occurred more rapidly than in the case of BP3 (Gago-Ferrero et al., 2012; Marco-Urrea et al., 2010b). Biological mineralisation of [$^{14}C_1$-carboxyl]-DCF was previously shown (Al-Rajab et al., 2010) in soils by the indigenous microbiota although it should be noted that the $^{14}$C labelled

carbon was in the carboxyl group, probably easier to convert to $CO_2$ than the aromatic ring carbons. In the present work, although only one aromatic ring of each target compound was labelled, the fact that both aromatic rings are equally oxidized leads to think that ring cleavage would occur similarly to the whole molecule.

### *6.2.2. $^{13}$C incorporation into fungal biomass by EA-IRMS and amino acid-SIP analyses*

The bulk analysis of the $\delta^{13}C$ values of fungal biomass by EA-IRMS showed $\delta^{13}C$ enrichment in cultures spiked with $^{13}$C-DCF in comparison with the $\delta^{13}C$ values observed in the unlabeled controls which remained constant over time. A continuous incorporation of $^{13}$C into biomass was observed for cultures containing $^{13}$C-DCF, with a $\delta^{13}C$ increasing from -6.4 ± 1.2 at time zero to +28.4 ± 3.9 at 9 days. The carbon isotopic signature in heat killed and sodium azide killed controls spiked with either labelled or unlabelled DCF resulted in $\delta^{13}C$ values statistically identical, indicating that $^{13}$C-incorporation in live cultures was not due to passive uptake associated to physico chemical processes such as sorption. The time-course of $^{13}$C enrichment into biomass is depicted in Fig. 6.18 as percentage of $^{13}$C atom (at%).

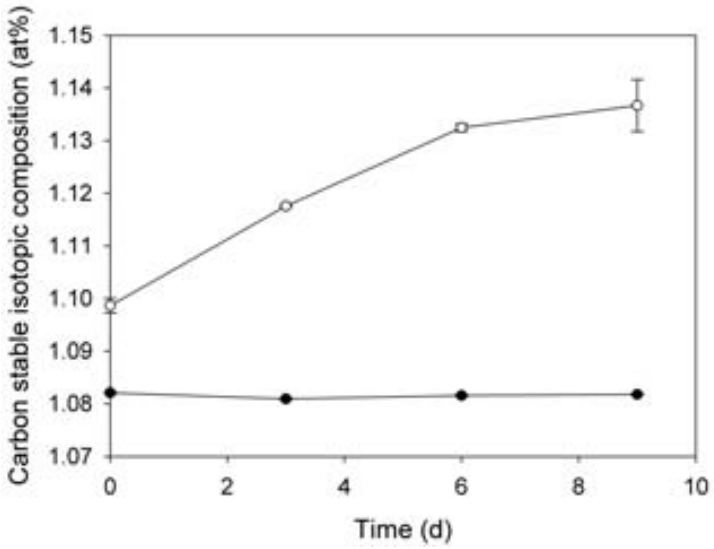



**Figure 6.18.** Carbon stable isotopic composition (atom%) of the fungal biomass in experimental cultures spiked with either (○) $^{13}$C-DCF or (●) $^{12}$C-DCF.

In previous studies (Marco-Urrea et al., 2010b), DCF was rapidly transformed to 4'-hydroxydiclofenac and 5-hydroxydiclofenac by *T. versicolor* (Fig. 6.19) and these intermediates disappeared after 24 h from the medium. Therefore, the observed biomass labelling could be attributed to the intracellular presence of labelled untransformed DCF or associated byproducts without implying a final incorporation into anabolic products.

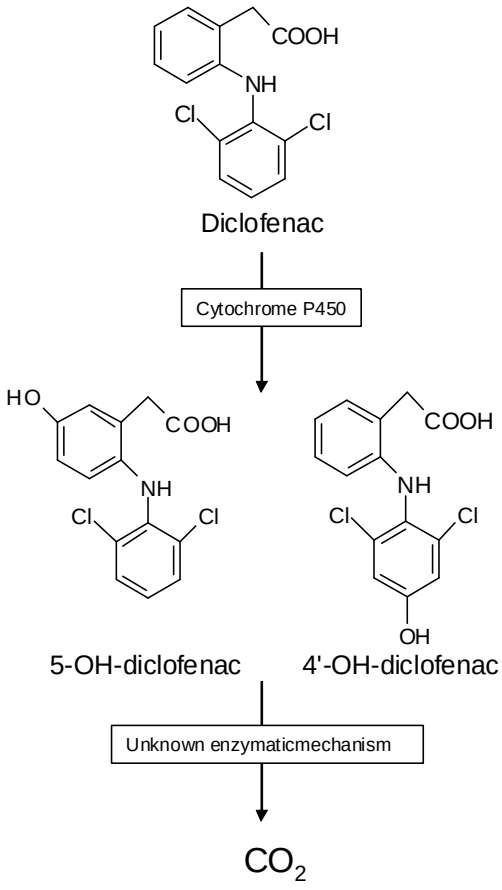



**Figure 6.19.** Degradation pathway of diclofenac by *T. versicolor*. Metabolites 4′-hydroxydiclofenac and 5-hydroxydiclofenac were previously identified (Marco-Urrea et al., 2010b). Production of carbon dioxide ($CO_2$) from diclofenac was demonstrated in the present work.

Regarding amino acids-SIP in DCF experiment, no significant $^{13}C$ incorporation was detected in the amino acids. These results may suggest that DCF (or byproducts) can be accumulated into the cell for being further transformed by the cytochrome P450 system, as previous inhibitory experiments indicated (Marco-Urrea et al., 2010b) and thus contributing to the high isotopic

enrichment observed in the bulk biomass. Therefore, our results indicate that DCF would be mineralised mainly by cometabolic pathways instead of being used as carbon source by *T. versicolor*.

A $^{13}$C-mass balance of labelled DCF was also performed as described at Section 6.1.4. As observed in Fig. 6.20, DCF could be rapidly transformed to intermediates that would be slightly removed from the media with a gradual accumulation into the fungal biomass. At the end of the incubation, ~ 90% of DCF remained either as byproduct or accumulated into the cells.

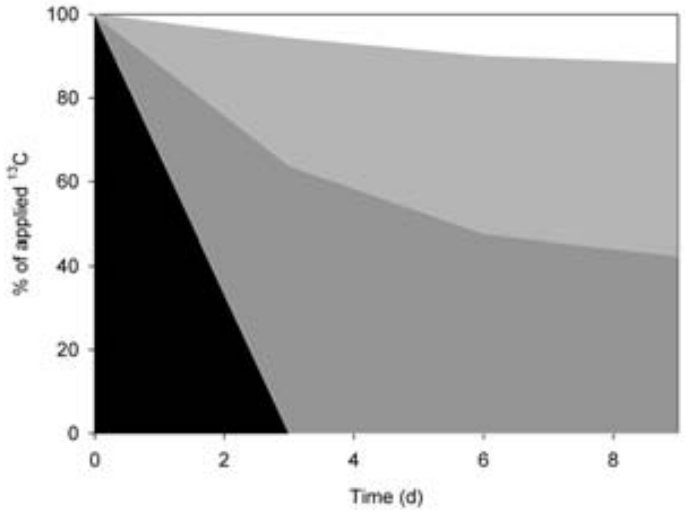



**Figure 6.20.** Mass balance of $^{13}$C in DCF experiments. In black non-transformed $^{13}$C-DCF remaining in the media, in dark grey estimated $^{13}$C remaining in the media in form of by-products to complete the mass balance, in light grey $^{13}$C into fungal biomass, and in white $^{13}$C mineralised ($^{13}CO_2$).

## CONCLUSIONS

From the results derived from this chapter, mineralisation of BP3 and DCF by the action of *T. versicolor* was confirmed. It was shown that mineralisation rate and yield can not be inferred from the removal rate of the parent compound and the main metabolites and experiments on $CO_2$ isotopic signature are needed to unequivocally confirm mineralisation. As an example, DCF was transformed faster by the fungus but it achieved lower mineralisation yield than BP3.

The use of amino acid-SIP and EA-IRMS techniques allowed us to determine whether the fungus used the tested emerging contaminants as carbon source for e.g. amino acid biosynthesis or, on the contrary, the elimination process occurred via alternative mechanisms. In the present study, amino acid-SIP allowed us to confirm that BP3 was used as carbon source whereas DCF degradation might proceed via cometabolism or detoxification mechanisms. The use of these techniques is relevant for white-rot fungi due to the complexity of the enzymatic system involved in the transformation of contaminants that include: i) cometabolism (ligninolytic enzymes, i.e. laccases and peroxidases), ii) detoxification mechanism (i.e. cytochorme P450), and iii) metabolism (i.e. via lipid or amino acid synthesis). By using amino acid-SIP, unequivocal use of xenobiotics in metabolism can be proved, and it is more reliable than indirect evidences such as the removal of the target compound when added as sole carbon source (Prenafeta-Boldú et al., 2001), mineralisation of xenobiotics by measuring isotopic signature of emitted $CO_2$ (Tuomela et al., 1999), or disappearance of both parent compounds and byproducts (Marco-Urrea et al., 2010b). Nonetheless, as emerging contaminants are usually at low concentrations, probably some external addition of nutrients would be likewise necessary. All in all, the use of amino acid-SIP can shed light on the fate of xenobiotics during white-rot fungi degradation and help to establish bioremediation strategies using these organisms.

# Chapter 7

## Treatment of a reverse osmosis concentrate by *Trametes versicolor*: study of the degradation of UV filters, PhACs and EDCs

## INTRODUCTION

Very few studies can be found in the literature dealing with the fungal treatment of real effluents. Until now, most of the published data refer to the demonstration of the ability of fungi to degrade selected contaminants (e.g. dyes, PhACs and so on) spiked in synthetic medium (Jelic et al., 2012; Rodarte-Morales et al., 2012a). Some studies also treated synthetic medium or real effluents under non-sterile conditions but with the addition of spiked contaminants (Yang et al., 2013a; Zhang and Geissen, 2012). However, only few recent articles report the fungal treatment of non-sterile real effluents with the aim of degrading emerging contaminants (Cruz-Morató et al., 2014, 2013a).

On the other hand, for a proper efficiency, fungal treatment requires hydraulic residence time (HRT) of 24-48 hours at least (Rodarte-Morales et al., 2012a; Yang et al., 2013a; Zhang and Geissen, 2012). Taking into account that, at the moment, the main cost of the fungal treatment is due to the fungal pellet production (Borràs et al., 2008; Gabarrell et al., 2012), this can only be achieved at a reasonable cost with small volumes of concentrated effluents. Therefore, rather than the entire WWTP, effluents such as the reverse osmosis concentrate (ROC) from a tertiary treatment of a WWTP, used in the experiments of this chapter, or treatments at source point of veterinary hospital wastewater (Chapter 8) are the most suitable ones for the treatment with WRF.

In order to evaluate the treatment of a real effluent, some factors affecting the operation need to be studied, such as the fungal survival in the effluent, the search for the optimal conditions, like the need of an extra supply of nutrients, some other aspects related to the correct quantification of micropollutants, and the effect of the growth of other microorganisms on the activity of the inoculated fungus, among others.

In this chapter, first of all, the study of the possible negative effects of ROC effluent (e.g. salinity) on the fungus is presented. To this end, different treatments in serum bottles were carried out, with and without addition of extra

carbon and nitrogen sources, to have a preliminar idea of the survival of *T. versicolor.* Moreover, maintenance of the degradation capacity was tested in different conditions as well, with the UV filter BP3 as model compound.

Furthermore, most of the fungal biodegradation studies at lab-scale, as some of the experiments described in this Chapter, are carried out under sterile conditions. However, autoclaving can alter the effluent, as it has been reported by Citulski et al. (2012), who showed changes in the toxicity of the samples before and after heat-sterilisation. To date, nobody has reported how the sterilisation process can affect micropollutants concentrations. In this Chapter, a comparison in the concentration of PhACs before and after the autoclaving process is provided. The effect of the sampling process during bioreactor experiments (from a sampling system or directly from inside the bireactor) on PhACs quantification was also evaluated.

Then, although some authors have already reported carbon and nitrogen requirements for fungal survival and degradation (Casas et al., 2013; Zhang and Geissen, 2012), the amount of nutrients required by the fungus for treating real wastewater still needs to be optimised. Thinking of a further application, it would be optimal that the effluent would provide the fungus with all it needs. Therefore, in the present work, the need of an extra supply of nutrients for treating ROC was assessed. The study was performed in a 1.5 L fungal biomass air-pulsed fluidised glass bioreactor under sterile conditions and operating in batch mode. Sterile conditions were applied in order to discriminate the degradation by the inoculated fungi from the one by bacteria that might grow in the reactor and also degrade the contaminants. Two treatments were compared, one without extra nutrients (ROC1) and the other one with continuous addition of glucose and ammonium tartrate (ROC2). Treatments were assessed by means of the achieved removal percentage of PhACs and EDCs already present in the effluent. Moreover, at ROC2, two bioreactors were set in parallel under the same theoretical conditions (replicates). However, differences in the removal percentages allowed us to discuss the possible effect of aeration on processes affecting the increase in the concentration of some compounds. UV filters were quantified too and their removal was also compared

between treatments with and without extra nutrients, but at Erlenmeyer-scale, due to their high adsorption in the walls of the reactor reported in Chapter 5.

Finally, ROC3 experiment consisted of a non-sterile bioreactor operated in continuous mode with a non-inoculated control reactor set up in parallel. The aim of this last experiment was to study the effect of the non-sterile conditions in the fungal survival and degradation capacity. However, operational conditions were still not optimised as it was only a proof-of-concept assay to show that fungus can degrade emerging contaminants in a real effluent during continuous operation in non-sterile conditions. In Chapter 9, results derived from the analyses of the microbial community in the ROC3 treatment are presented. Moreover, toxicity analyses were performed before and after all the treatments.

Regarding analytical methods, monitoring of the parent compounds as performed in the present work (although some carbamazepine metabolites were already included in the analytical method) is important to assess a treatment for the degradation of emerging contaminants. Nonetheless, sometimes this is not enough. If metabolites generated during the treatment are not further degraded, they can also represent an environmental problem because of their possible persistence as well as any possible associated toxicity. Sometimes they might even lead to the generation of the initial compound, resulting in an increase of the concentration of some compounds after a treatment instead of their removal. Therefore, for an exhaustive and complete degradation assessment, parent and transformation products should be included in the analytical methods. In this study, an attempt to include in the analytical method the fungal metabolites of BTZ and TTZ identified in Chapter 5 was performed. Quantification of emerging contaminants at environmental concentrations was performed by the environmental analitical chemistry groups of IDAEA-CSIC and ICRA, with whom we collaborate. Quantification of UV filters was performed by Pablo Gago, supervised by Dr. Sílvia Díaz of IDAEA-CSIC. Quantification of PhACs and EDCs was performed by Daniel Lucas, Dr. Meritxell Gros and Dr. Marta Llorca at ICRA, supervised by Dr. Sara Rodríguez. Both groups are led by Dr. Damià Barceló.

# 7.1. Study of factors affecting fungal treatment of ROC

### *7.1.1. Survival of* Trametes versicolor *in the ROC*

ROC effluent comes from a RO unit acting as tertiary treatment in a pilot plant treating urban wastewater in a MBR. Every sampling of ROC effluent was characterised for common physical-chemical parameters. Table 7.1 shows the results, that are in the same range as other ROC from urban WWTP effluents followed by RO treatment (Pérez et al., 2010; Radjenovic et al., 2011; Zhou et al., 2011).

**Table 7.1**. Physicochemical characterisation of reverse osmosis concentrate (ROC).

| Experiment[a] | ROC1 | ROC2 | ROC3 |
|---|---|---|---|
| Sampling data | 03/2011 | 09/2011 | 04/2013 |
| pH | 8.49 | 8.13 | 8.05 |
| Conductivity (mS $cm^{-1}$) | 1.40 | 2.76 | 3.28 |
| Chloride (mg Cl $L^{-1}$) | 217 | 425 | 479 |
| Sulfate (mg S $L^{-1}$) | 162 | 237 | 146 |
| Nitrate (mg N $L^{-1}$) | 132.9 | 1.68 | 28 |
| Phosphate (mg P $L^{-1}$) | 7.75 | 4.54 | 0.7 |
| Ammonia (mg N $L^{-1}$) | n.d. | 0.17 | n.d. |
| TSS (mg $L^{-1}$) | 14.5 | 1.4 | 9.8 |
| Soluble COD (mg $O_2$ $L^{-1}$) | 42.7 | 57.6 | 64.7 |
| DIC (mg $L^{-1}$) | 24 | 74 | 127 |
| DOC (mg $L^{-1}$) | 148 | 93 | 70 |

[a] Experiments details can be found at section 4.7.3

ROC is usually characterised for having higher conductivity (1.7-5.1 mS $cm^{-1}$) and ions concentration than urban wastewater (0.03-1.02 mS $cm^{-1}$) (Cruz-Morató et al., 2013a; Odjadjare and Okoh, 2010; Pérez et al., 2010; Zhou et al., 2011). However, in this case, values were in the low part of the range and, thus, quite similar to those found in urban wastewaters. Nevertheless, prior to assessing pharmaceuticals and EDCs degradation, preliminary experiments were done in order to assure fungal survival in the ROC. Those experiments

were performed in serum bottles with heat-sterilised ROC to assure that the only parameters that could affect the fungus were the characteristics of the effluent but not the growth of other microorganisms. Different conditions were included to preliminarly assessing the need of nutrients, either extra C or N source: without extra addition of nutrients (ROC), with addition of N source (ROC_N), with addition of N and C source (ROC_CN) and controls with defined media (DM), which also included micro and macronutrients (for more detailed description see Section 4.7.1 of Materials and Methods). Conditions of adjusting or not the pH were also studied. Fungal dry weight, glucose consumption, laccase activity and pH were monitored as indicators of fungal survival and activity.

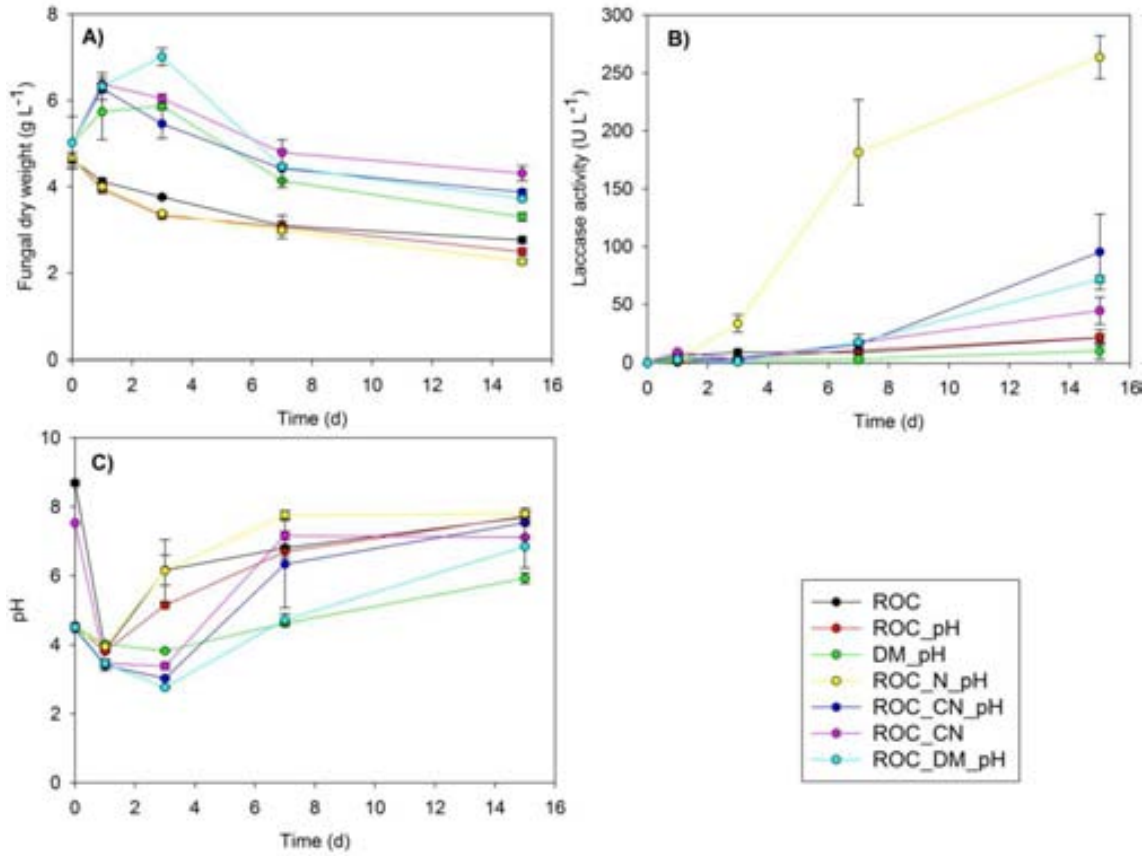



**Figure 7.1. A)** Fungal dry weight, **B)** laccase activity and **C)** pH values during 14 day experiment at serum bottles at different experimental conditions (see legend in the Figure).

Results are shown at Fig. 7.1. Thus, initial fungal biomass was 4.77 ± 0.37 and it increased in all the media containing glucose and it decreased in the media without glucose (Fig. 7.1A). Glucose in the former ones was totally consumed

after 3 days, being the consumption rate between 633 and 903 mg $g^{-1}$ DCW $d^{-1}$. Ammonia consumption rate was around 9.6 mg N $g^{-1}$ DCW $d^{-1}$ in the DM experiment (data not shown). All treatments presented laccase activity, even those without extra nutrients and at the experimental bottes ROC_N_pH, laccase activity even reached 264 U $L^{-1}$ (Fig. 7.1B). As previously reported (Freitag and Morrell, 1992), high nitrogen source (low C/N ratio) might promote laccase activity, although no fungal growth was obtained. As can be seen at Fig. 7.1C, fungus acidified the media during the first 24-48 hours, indicating active fungal activity (Tavares et al., 2006), and then, when nutrients in the media were consumed, it basified. Therefore, in those media without glucose, pH started to increase earlier than in the other ones. Little differences can be found in those treatments were pH was previously adjusted from those where pH was not adjusted. Treatments where pH was maintained in acid conditions longer time were those with defined medium (DM), that 2,2-dimethyl succinic acid was added as buffer.

Taking into account those results, ROC_pH, ROC_N_pH and ROC_CN_pH (and DM_pH as control) were chosen for a test of the degradation capacity of the fungus in those media. BP3 was selected to evaluate the degradation capacity of the fungus and, thus, it was spiked at a final concentration of around 10 mg $L^{-1}$. Results of that experiment are shown at Fig. 7.2. Like in the previous experiment, fungal dry weight in those cultures with glucose increased whereas in those without glucose it slightly decreased (Fig. 7.2A). Regarding laccase activity, no significant differences were found among treatments, not even for ROC_N_pH that exhibited high laccase activity in the previous experiment (Fig. 7.2B). Those differences in laccase activity were probably due to differences in the metabolic state of the inoculated fungus rather than due to experimental conditions. As shown at Fig 7.2C, pH was maintained acid at all cultures on the contrary that at the previous experiment, where, at day 3, ROC_N_pH and ROC_pH treatments had already started to increase the pH. TOC was reduced in all the treatments (Fig. 7.2D). However it has to be taken into account that TOC of the ROC was 17 mg $L^{-1}$. It means that the addition of fungi and, obviously, nutrients increased the initial TOC at each treatment. Therefore, although it decreased, the value was always above the initial TOC of the ROC.

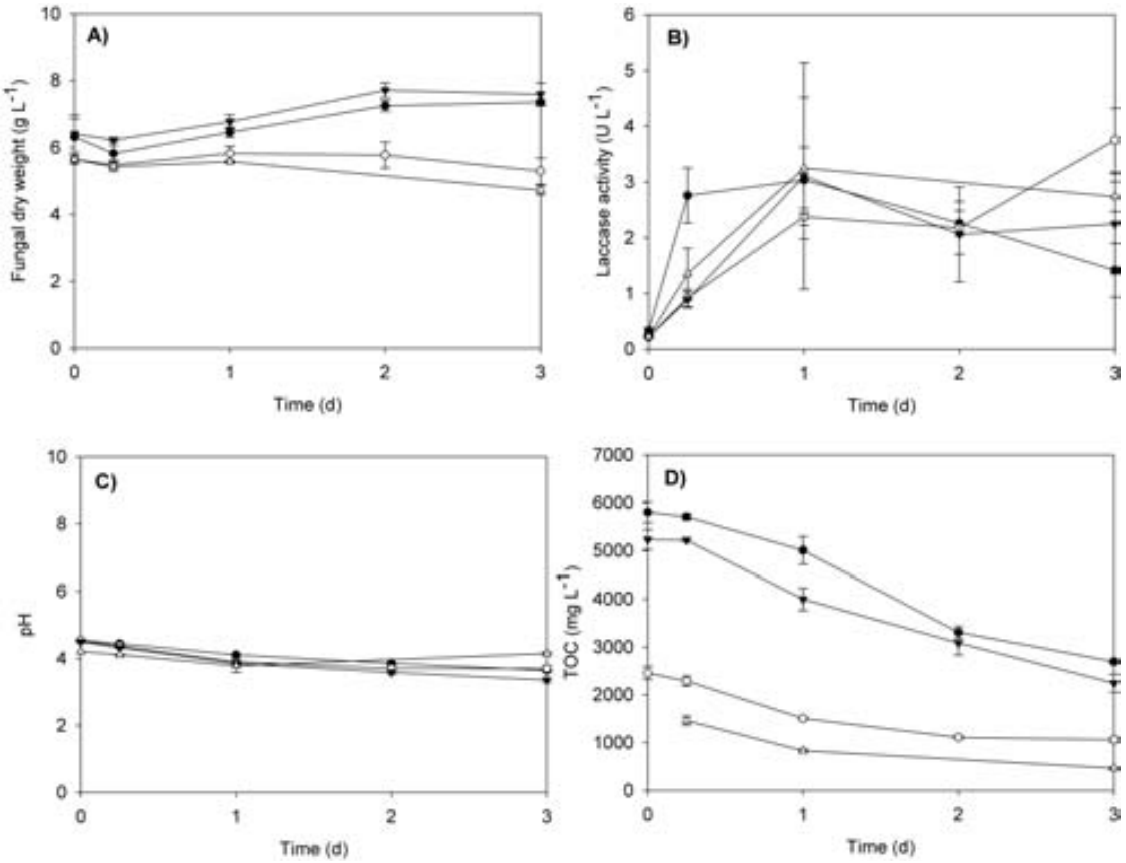



**Figure 7.2. A)** Fungal dry weight, **B)** laccase activity, **C)** pH and **D)** TOC values during 3 day experiment at serum bottles at different experimental conditions spiked with BP3. Symbols: (●) DM_pH, (○) ROC_N_pH, (▼) ROC_CN_pH and (△) ROC_pH.

Regarding BP3 degradation (Fig. 7.3A), it was satisfactory achieved at all treatments, even at ROC-pH, where no extra nutrients were added. The only difference was that in those cultures where no glucose was added, lower amount of the glucose conjugated metabolite (M2, see Chapter 5) was produced (Fig. 7.3C).

Summarising, *T. versicolor* can survive in ROC suplemented or not with extra nutrients. Degradation of BP3 was achieved in a medium without extra nutirents as well. Therefore, for future experiments both treatments, ROC supplemented with glucose and ammonia and ROC without extra nutrients, were tested (see section 7.1.3).

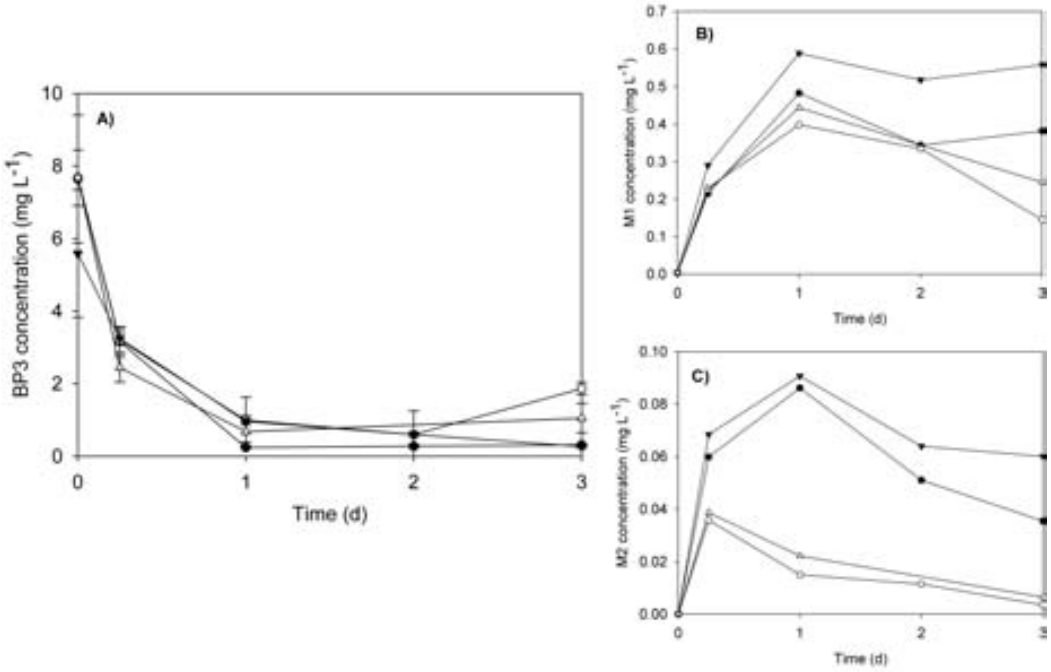



**Figure 7.3.** Concentrations of **A)** BP3, **B)** M1 metabolite (pentose conjugate) and **C)** M2 metabolite (glucose conjugate) during 3 day experiment at serum bottles at different experimental conditions spiked with BP3. Symbols: (●) DM_pH, (○) ROC_N_pH, (▼) ROC_CN_pH and (△) ROC_pH.

### *7.1.2. Evaluation of factors affecting emerging contaminants quantification*

Then, two aspects that could alter the quantification of emerging contaminants in experiments at lab-scale (sterilisation process and sampling mode) were studied. PhACs were quantified as an example that might be extrapolated to other emerging contaminants.

*Differences in pharmaceuticals concentration after heat-sterilisation*

Many lab-scale studies of PhACs removal are performed under sterile conditions (Jelic et al., 2012; Rodarte-Morales et al., 2012a). However, as they are performed with synthetic media and/or spiked selected contaminants, none of them analysed the changes in micropollutants composition that autoclaving could entail when working with real wastewater. Therefore, a test for assessing possible alterations on pharmaceuticals concentration during the sterilisation process was performed. In Table 7.2, pharmaceuticals concentrations, measured before and after autoclaving are indicated. There was some

statistically significant variation (either increasing or decreasing) in 48 out of 57 compounds present in ROC. There were huge variations in ibuprofen, epoxycarbamazepine, irbesartan, salicylic acid, iopromide and acetaminophen. Other studies also observed different behaviours in the toxicity of samples before and after autoclaving (Citulski and Farahbakhsh, 2012) but to the authors knowledge, there are no studies that quantified changes in micropollutants concentrations after autoclaving. The concentration decrease of some compounds (i.e. 10,11-epoxycarbamazepine and irbesartan) was probably due to the same well-known physico-chemical degradation processess occurring during long storage periods but accelerated. This mechanism may account for the increase of some compounds (i.e. salicylic acid, acetaminophen) if deconjugation of conjugated metabolites occurs. Concentration increses might be due to desorption from particulate matter too, as higher temperatures favour desorption kinetics (Cornelissen et al., 1997). The high increase of ibuprofen from non-detectable levels up to 21.2 µg $L^{-1}$ after heat-sterilisation is, however, quite unexplicable. Adsorption of ibuprofen accounts for only 9-28% of the total concentration (Duan et al., 2013) and human conjugated metabolites represents around 15% of the total excreted ibuprofen metabolites (Ternes, 1998). The formation of conjugated compounds by activated sludge is reported by Jewell et al. (2014), although it is still a topic barely studied. Therefore, taking into account that there was a biological MBR treatment previously to the RO unit, part of those high removal percentages recorded for ibuprofen and other easily degradable compounds at WWTP (Verlicchi et al., 2012) might be only due to microbial conjugations and not total biodegradation.

On the other hand, taking into account those variations in the concentrations, pharmaceuticals concentrations taken as initial time values for removal calculations in the experiments under sterile conditions (ROC1 and ROC2) were those quantified after sterilisation process.

**Table 7.2.** Pharmaceutical concentration and their varation before and after sterilisation process

| | Non-sterile | | Sterile | | | |
|---|---|---|---|---|---|---|
| Compound | C (µg $L^{-1}$) | SD | C (µg $L^{-1}$) | SD | Removal (%) | Variation (µg $L^{-1}$) |
| Ibuprofen | n.d. | - | 21.166 | 2.521 | n.q. | 21.166 |
| 10,11-EpoxyCBZ | 12.499 | 0.613 | 3.330 | 0.234 | 73 | 9.169 |
| Ibersartan | 6.881 | 1.012 | 0.048 | 0.003 | 99 | 6.833 |
| Salicylic acid | n.d. | - | 3.523 | 0.091 | n.q. | 3.523 |
| Iopromide | 0.676 | 0.079 | 2.409 | 0.327 | -256 | 1.733 |
| Acetominophen | BQL | - | 1.327 | 0.000 | -6072 | 1.306 |
| Valsartan | 4.287 | 0.623 | 3.086 | 0.184 | 28 | 1.201 |
| Azithromycin | 0.838 | 0.073 | n.d. | - | 100 | 0.838 |
| Atenolol | 2.467 | 0.205 | 3.230 | 0.212 | -31 | 0.763 |
| Furosemide[a] | 0.422 | 0.101 | 0.988 | 0.341 | -134 | 0.566 |
| Lorazepam | 0.447 | 0.009 | n.d. | 0.001 | 100 | 0.447 |
| Olanzapine | 0.394 | 0.139 | n.d. | - | 100 | 0.394 |
| Ciprofloxacin[a] | BQL | - | 0.631 | 0.190 | -152 | 0.381 |
| Indomethacin | 0.374 | 0.112 | n.d. | - | 100 | 0.374 |
| Venlafaxine | 1.710 | 0.109 | 1.356 | 0.035 | 21 | 0.354 |
| Citalopram | 0.470 | 0.046 | 0.768 | 0.043 | -63 | 0.298 |
| Ranitidine | 0.468 | 0.026 | 0.171 | 0.001 | 63 | 0.297 |
| Losartan | 0.020 | 0.001 | 0.267 | 0.049 | -1235 | 0.247 |
| Ketoprofen | 0.241 | 0.05 | 0.484 | 0.057 | -101 | 0.243 |
| Levamisol | 0.494 | 0.013 | 0.252 | 0.004 | 49 | 0.242 |
| Ofloxacin | 0.292 | 0.028 | 0.067 | 0.029 | 77 | 0.225 |
| Norfluoxetine | n.d. | - | 0.223 | 0.001 | n.q. | 0.223 |
| Oxycodone | n.d. | - | 0.216 | 0.019 | n.q. | 0.216 |
| Hydrochlorotiazide | 1.037 | 0.003 | 0.851 | 0.022 | 18 | 0.186 |
| Fluoxetine | BQL | - | 0.221 | 0.021 | -338 | 0.171 |
| Gemfibrozil | 0.878 | 0.053 | 1.027 | 0.002 | -17 | 0.149 |
| Metoprolol | 0.086 | 0.005 | 0.216 | 0.019 | -151 | 0.130 |
| Tetracyclin | n.d. | - | BQL | - | n.q. | 0.130 |
| Sotalol[a] | 0.350 | 0.100 | 0.479 | 0.002 | -37 | 0.129 |
| Carbamazepine[a] | 0.412 | 0.018 | 0.539 | 0.151 | -31 | 0.127 |
| Acridone | n.d. | - | 0.123 | 0.003 | n.q. | 0.123 |
| Dimetridazole | BQL | - | n.d. | - | 100 | 0.107 |
| Glibenclamide | 0.099 | 0.116 | n.d. | - | 100 | 0.099 |
| Phenazone | BQL | - | 0.154 | 0.001 | -168 | 0.097 |
| Amlodipine | n.d. | - | BQL | - | n.q. | 0.093 |
| Diltiazem | 0.101 | 0.008 | 0.008 | 0.002 | 92 | 0.093 |
| Nadolol | n.d. | - | 0.088 | 0.019 | n.q. | 0.088 |
| Torasemide | n.d. | - | 0.082 | 0.005 | n.q. | 0.082 |
| Codeine | BQL | - | 0.115 | 0.004 | -174 | 0.073 |
| Metronidazole | n.d. | - | BQL | - | n.q. | 0.073 |
| Metronidazole-OH[a] | 0.266 | 0.055 | 0.195 | 0.009 | 27 | 0.071 |
| Propanolol | BQL | - | 0.141 | 0.016 | -100 | 0.071 |
| Carazolol | 0.066 | 0.003 | n.d. | 0.001 | 100 | 0.066 |
| Desloratadine | 0.058 | 0.020 | n.d. | 0.002 | 100 | 0.058 |
| Naproxen | n.d. | - | 0.058 | 0.003 | n.q. | 0.058 |
| Cimitidine | 0.052 | 0.001 | n.d. | - | 100 | 0.052 |
| Piroxicam | n.d. | - | 0.049 | 0.003 | n.q. | 0.049 |
| Ronidazole | n.d. | - | 0.049 | 0.023 | n.q. | 0.049 |
| Thiabe0azole | 0.083 | 0.002 | 0.124 | 0.009 | -49 | 0.041 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Propyphenazone | n.d. | - | BQL | - | n.q. | 0.041 |
| Diclofenac[a] | 1.214 | 0.337 | 1.176 | 0.257 | 3 | 0.038 |
| Sertraline[a] | 0.142 | 0.067 | 0.179 | 0.012 | -26 | 0.037 |
| Fluvastatin | n.d. | - | BQL | - | n.q. | 0.032 |
| Norverapamil | n.d. | - | BQL | - | n.q. | 0.022 |
| Diazepam | 0.052 | 0.002 | 0.038 | 0.008 | 27 | 0.014 |
| Alprazolam[a] | BQL | - | BQL | - | 0 | 0 |
| Verapamil[a] | BQL | - | BQL | - | 0 | 0 |

[a] No statistical difference in the concentration between before and after sterilisation process ($p<0.01$).
n.d.: not detected; n.q.: not quantifiable; BQL: below quantification limit

*Differences between sampling modes*

All monitoring samples of batch bioreactors were taken using the sampling system described in section 4.7.3 but, in addition, in one of the treatments (ROC1), samples were also directly taken from inside the reactor at the end of the treatment to test any potential difference in pharmaceutical quantification. Significant differences in pharmaceutical concentration between sampling modes were only found for hydrochlorothiazide, propranolol, xylazine and diazepam (see Table 7.3). The highest difference was identified for propranolol, but the four were was detected in higher amount in the samples taken directly from the reactor than in the ones taken using the sampling system due to adsorption at the silicone tubes of the sampling system, as reported for plastic surfaces (Fukazawa et al., 2010). Therefore, as sampling from inside the reactor is not possible at intermediate time points and samples were taken through the sampling system, removal of these compounds (mainly propranolol and hydrochlorothiazide) could be overestimated in all treatments due to adsorption effects instead of biodegradation itself. However, in general, no adsorption during the sampling was found for the vast majority of compounds.

**Table 7.3.** Pharmaceuticals concentrations and their varation between samples from the sampling site (S) and directly from inside the reactor (R).

| Compounds | Initial time | | 7 d (S) | | 7 d (R) | | Rem. S (%) | Rem. R (%) | S-R (µg $L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| | C (µg $L^{-1}$) | SD | C (µg $L^{-1}$) | SD | C (µg $L^{-1}$) | SD | | | |
| Ketoprofen | 0.975 | 0.177 | 1.638 | 0.298 | 1.358 | 0.247 | -68 | -39 | 0.280 |
| Carbamazepine | 1.172 | 0.199 | 4.977 | 0.847 | 5.186 | 0.882 | -325 | -343 | -0.209 |
| Ibuprofen | 12.059 | 1.314 | 13.807 | 1.505 | 13.626 | 1.485 | -14 | -13 | 0.181 |
| Ciprofloxacin | 0.702 | 0.301 | 0.366 | 0.157 | 0.521 | 0.223 | 48 | 26 | -0.154 |
| Salicylic acid | 2.046 | 0.344 | 0.627 | 0.105 | 0.778 | 0.131 | 69 | 62 | -0.151 |
| Hydrochlorothiazide[a] | 0.217 | 0.012 | 0.043 | 0.002 | 0.189 | 0.010 | 80 | 13 | -0.146 |
| Propranolol[a] | 0.037 | 0.004 | n.d. | - | 0.128 | 0.015 | 100 | -247 | -0.128 |
| Atenolol | 1.044 | 0.473 | 1.752 | 0.793 | 1.698 | 0.768 | -68 | -63 | 0.054 |
| Xylazine[a] | n.d. | - | 0.039 | 0.003 | 0.069 | 0.005 | n.a. | n.a. | -0.030 |
| Ofloxacin | 0.177 | 0.028 | 0.076 | 0.012 | 0.103 | 0.016 | 57 | 42 | -0.028 |
| Sotalol | 0.082 | 0.013 | 0.127 | 0.020 | 0.115 | 0.018 | -55 | -39 | 0.013 |
| Trimethoprim | 0.059 | 0.006 | 0.037 | 0.004 | 0.046 | 0.005 | 36 | 22 | -0.008 |
| Iopromide | 0.056 | 0.006 | 0.053 | 0.006 | 0.061 | 0.007 | 4 | -9 | -0.007 |
| Thiabendazole | 0.071 | 0.010 | 0.107 | 0.015 | 0.112 | 0.016 | -51 | -58 | -0.005 |
| Venlafaxine | 0.218 | 0.023 | 0.217 | 0.023 | 0.215 | 0.023 | 1 | 2 | 0.002 |
| Diazepam[a] | 0.009 | 0.000 | 0.010 | 0.000 | 0.012 | 0 | -4 | -28 | -0.002 |
| Torasemide | 0.021 | 0.002 | 0.011 | 0.001 | 0.009 | 0.001 | 49 | 58 | 0.002 |
| Tamsulosin | 0.019 | 0.003 | 0.025 | 0.004 | 0.026 | 0.004 | -34 | -41 | -0.001 |
| Clarithromycin | 0.070 | 0.010 | 0.018 | 0.002 | 0.018 | 0.003 | 75 | 74 | -0.001 |
| Levamisol | 0.037 | 0.005 | 0.038 | 0.005 | 0.037 | 0.005 | -2 | -1 | 0 |
| Citalopram | 0.128 | 0.011 | 0.148 | 0.013 | 0.148 | 0.013 | -15 | -15 | 0 |
| Acetaminophen | 0.632 | 0.052 | n.d. | - | n.d. | - | 100 | 100 | 0 |
| Diclofenac | 0.550 | 0.075 | n.d. | - | n.d. | - | 100 | 100 | 0 |
| Valsartan | 0.520 | 0.101 | n.d. | - | n.d. | - | 100 | 100 | 0 |
| Furosemide | 0.277 | 0.045 | n.d. | - | n.d. | - | 100 | 100 | 0 |
| Naproxen | 0.259 | 0.024 | n.d. | - | n.d. | - | 100 | 100 | 0 |
| Gemfibrozil | 0.137 | 0.017 | n.d. | - | n.d. | - | 100 | 100 | 0 |
| Sulfamethoxazole | 0.100 | 0.004 | n.d. | - | n.d. | - | 100 | 100 | 0 |
| Phenazone | 0.042 | 0.003 | n.d. | - | n.d. | - | 100 | 100 | 0 |
| Atorvastatin | 0.040 | 0.009 | n.d. | - | n.d. | - | 100 | 100 | 0 |
| Codeine | 0.031 | 0.000 | n.d. | - | n.d. | - | 100 | 100 | 0 |
| Irbesartan | 0.014 | 0.001 | n.d. | - | n.d. | - | 100 | 100 | 0 |
| Alprazolam | 0.008 | 0.000 | n.d. | - | n.d. | - | 100 | 100 | 0 |
| Bezafibrate | 0.006 | 0.000 | n.d. | - | n.d. | - | 100 | 100 | 0 |
| Metoprolol | BQL | - | BQL | - | BQL | - | 0 | 0 | 0 |

[a] Statistical difference between both methods of sampling ($p$<0.01).
n.d.: not detected; BQL: below quantification limit

### *7.1.3. Sterile batch treatments: study of nutrients addition*

Pharmaceuticals and EDCs degradation by *T. versicolor* was assessed in 1.5 L air pulsed fluidised bioreactors, operating under sterile conditions to evaluate the percentage of degradation attributed to *T. versicolor* without interference of any possible degradation by indigenous microorganisms. The amount of

nutrients needed in a growth-limiting synthetic media for *T. versicolor* was previously calculated to be 120 mg C g $DCW^{-1}$ $d^{-1}$ and 0.3 mg N g $DCW^{-1}$ $d^{-1}$ (Casas et al., 2013). However, when working with real effluents, nutrients already present in the wastewater have to be taken into account. Therefore, although ammonia and DOC concentrations in the ROC (Table 7.1) were lower than the established needed amount of nutrients, as preliminar experiments were also satisfactory for ROC-pH treatment (section 7.1), a fungal batch bioreactor (ROC1) was performed for confirmation. After that, two bioreactors (duplicates) were operated in parallel with external addition of nutrients (ROC2A and ROC2B).

*Monitoring of bioreactors performance*

Parameters monitored during treatments are shown in Fig. 7.4. In sterile batch fungal reactor (ROC1), where no extra nutrients were added and only pH and temperature were controlled, 22% of biomass was lost after the 7 days of operation, depite the apparent increase in the concentration due to a concentration factor of sampling (Fig. 7.4A). The lysis of *T. versicolor* was probably because of the lack of enough assimilable nutrients in the ROC. COD increased from 47.2 to 881.5 mg $O_2$ $L^{-1}$ after fungal addition and then it decreased to around 300 mg $O_2$ $L^{-1}$ until the end of the treatment (Fig. 7.4C), which confirms that *T. versicolor* cannot eliminate COD already present in the ROC as Cruz-Morató et al. (2013) described for urban wastewater. Low laccase activity was detected, with a maximum of 15 U $L^{-1}$ at 5 days (Fig. 7.4B).

In the sterile batch treatment with external addition of nutrients (ROC2), there were two reactors in parallel under aproximately the same conditions. Glucose and ammonia tartrate were added as C and N sources respectively. Although all parameters were set up to have the same conditions at both bioreactors, some experimental differences were observed: aeration turned out to be higher in the ROC2B because of preferential pathways in the system and glucose addition was slightly lower at ROC2A and incidentally stopped at ROC2B during the $4^{th}$ day (Fig. 7.4D). As a consequence, ROC2B showed more brownish color and foam than B2A and acidification of the media was observed in ROC2A between 1-2 days whereas basification was noticed in the remaining period and in all

treatment at ROC2B. Acidification is usually indicative of fungal activity (Tavares et al., 2006) although sometimes fungal acids are being produced by active *T. versicolor* but acidification was not observed (Milagres et al., 2002). In our case, fungus was active as fungal biomass increased from 2 g DCW $L^{-1}$ to over 3 g DCW $L^{-1}$ in both reactors (Fig. 7.4A). Therefore, basification might be due to the ROC characteristics. Laccase activity had a peak of around 20 U $L^{-1}$ at day 2 and, after that, dissimilar values were detected for both reactors (Fig. 7.4B). COD increased until 3 g $O_2$ $L^{-1}$ (Fig. 7.4C) because of the addition of glucose to the medium. Glucose concentration was always mantained below 2 g $L^{-1}$ (Fig. 7.4D).

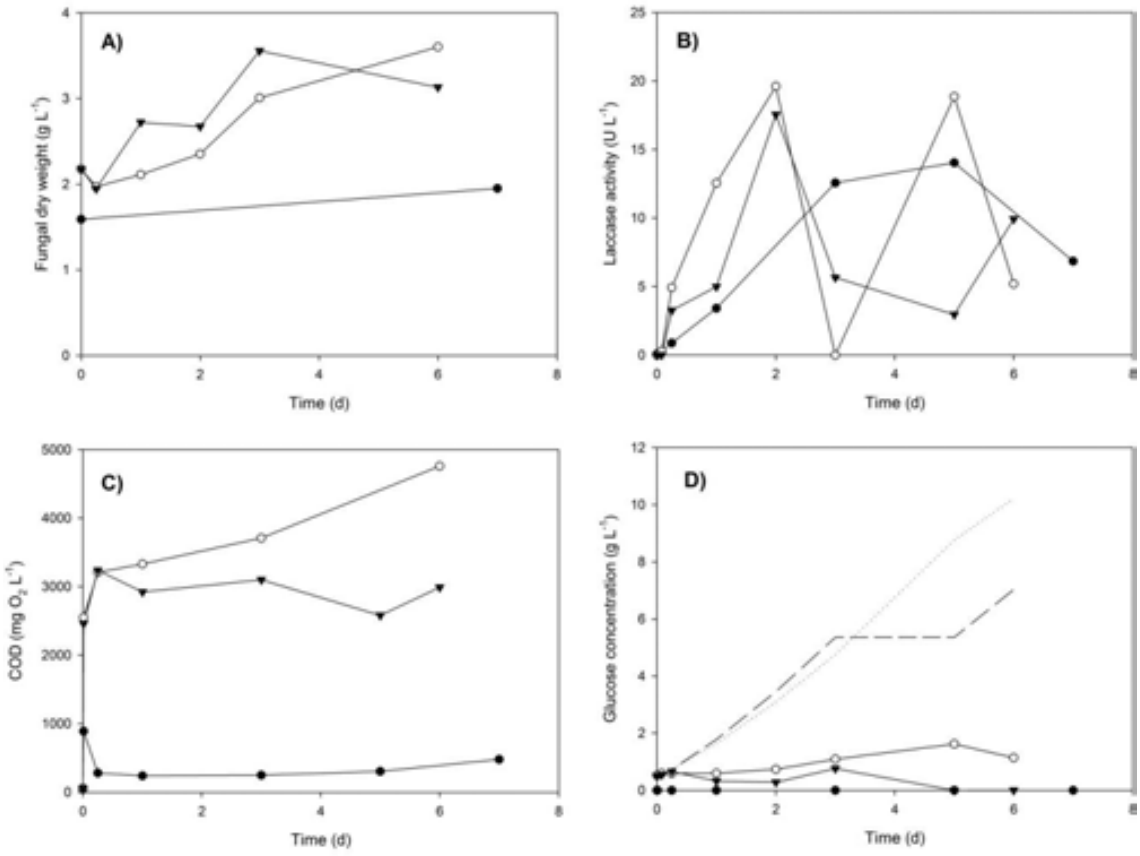



**Figure 7.4.** Evolution of **A)** fungal dry weight, **B)** laccase activity, **C)** COD and **D)** glucose concentration during (●) ROC1, (○) ROC2A and (▼) ROC2B batch reactors. Dotted line represents total glucose added at ROC2A and dashed line total glucose added at ROC2B.

ROC effluents presented no toxicity by Microtox analyses before and after the treatment (Table 7.4), which means that no toxic metabolites were produced or at least not at enough concentration to be toxic. Estrogenic and dioxin-like

activities of ROC2 experiment were also analysed by means of RYA assay. The former was found to be below detection limit either at ROC wastewater or after the fungal treatment. Regarding dioxin-like activity, initial value for ROC was 5.8 ± 2.1 μg $L^{-1}$ NFQ at it slightly increased to 11.4 ± 1.7 μg $L^{-1}$ NFQ after the fungal treatment. It has to be taken into account that $EC_{50}$ of β-naftoflavone is 188 μg $L^{-1}$. Thus, dioxin-like activity is low.

**Table 7.4**. Acute toxicity measured by Microtox analysis. Results are in toxic units (TU).

| | ROC1 | ROC2A | ROC2B |
|---|---|---|---|
| Initial | 0.7 | n.d. | n.d. |
| Final | 0.3 | 0.3 | 2.2 |

*Removal of PhACs*

Initial concentration of PhACs can be found in Table 7.5. Pharmaceuticals concentrations were in good agreement with previous studies, ranging from ng $L^{-1}$ to few μg $L^{-1}$ (Justo et al., 2013; Snyder et al., 2007). Analgesics and anti-inflammatories are the family with the highest concentrations detected in the ROC (16.594 μg $L^{-1}$ in ROC1 and 28.306 μg $L^{-1}$ in ROC2), mainly due to the contribution of ibuprofen. Psychiatric drugs were the second group with higher concentrations (1.535 μg $L^{-1}$ at ROC1 and 6.616 μg $L^{-1}$ at ROC2), led by carbamazepine in B1 and by 10,11-epoxycarbamazepine and venlafaxine at B2. β-blocker atenolol was found at high concentrations as well (1.044 μg $L^{-1}$ at ROC1 and 3.230 μg $L^{-1}$ at ROC2). Antibiotics, which are usually found at remarkable concentrations in the WWTPs effluent, were detected at concentrations around 1 μg $L^{-1}$ in both sampling campaigns. Evolution of individual pharmaceuticals concentration during fungal treatment can be found at Fig. 7.5 and 7.6 for ROC1 and ROC2, respectively. Evolution of pharmaceuticals concentration, grouped by therapeutic classes, is shown in Fig. 7.7 and final removal percentage for each compound is listed at Table 7.5.

**Table 7.5**. Pharmaceuticals initial concentration and removal after the treatments (7 days for ROC1 and 6 days for ROC2).

| Therapeutical group | Compound | ROC1 | | ROC2 | | | |
|---|---|---|---|---|---|---|---|
| | | $C_o$ (µg $L^{-1}$) | Rem. (%) | $C_o$ (µg $L^{-1}$) | Rem. (%) | Rem. ROC2A (%) | Rem. ROC2B (%) |
| Analgesics and anti-inflammatories | Ibuprofen | 12.059 | -14 | 21.160 | 67* | 64*† | 57*† |
| | Salicylic Acid | 2.046 | 69* | 3.523 | -141* | 70* | -146*† |
| | Ketoprofen | 0.975 | -68 | 0.484 | -152* | 46 | -449*† |
| | Acetaminophen | 0.632 | 100* | 1.327 | 95* | 69* | 73* |
| | Diclofenac | 0.550 | 100* | 1.176 | 93* | 94*† | 85*† |
| | Naproxen | 0.259 | 100* | BQL | 100* | 100*† | 100*† |
| | Phenazone | 0.042 | 100* | 0.154 | 100* | 100*† | 100*† |
| | Codeine | 0.031 | 100* | 0.115 | *63** | *63*† | *63*† |
| | Oxycodone | n.d. | - | 0.216 | 57* | 55*† | 59*† |
| | Piroxicam | n.d. | - | BQL | 100* | 100*† | -182* |
| | Propyphenazone | n.d. | - | BQL | 100* | *0* | 100*† |
| | TOTAL | 16.594 | 3 | 28.306 | 40* | 66* | 25* |
| Psychiatric drugs | Carbamazepine | 1.172 | -325* | 0.539 | -155 | -202* | -34† |
| | Venlafaxine | 0.218 | 1 | 1.356 | 23* | 29* | 49* |
| | Citalopram | 0.128 | -15 | 0.768 | 52* | 12* | 92* |
| | Diazepam[a] | 0.009 | -4 | 0.038 | *51** | *51*† | *51*† |
| | Alprazolam | 0.008 | 100* | BQL | 100* | *0* | 100*† |
| | 10,11-EpoxyCBZ | n.d. | - | 3.330 | -18 | 100* | -71* |
| | Fluoxetine | n.d. | - | 0.221 | 13 | -16† | 12† |
| | Acridone | n.d. | - | BQL | *0* | *0*† | *0*† |
| | Sertraline | n.d. | - | 0.179 | -6 | -6† | -2† |
| | Norfluoxetine | n.d. | - | 0.060 | 13* | 0 | 2 |
| | TOTAL | 1.535 | -249* | 6.616 | -10 | 41* | -16† |
| β-blockers | Atenolol | 1.044 | -68 | 3.230 | 81* | 91* | 82*† |
| | Sotalol | 0.082 | -55 | 0.479 | 67* | 86* | 66*† |
| | Propranolol[a] | 0.037 | 100* | 0.141 | *50** | *50*† | *50*† |
| | Metoprolol | BQL | 0 | 0.216 | 57* | 55*† | 59*† |
| | Nadolol | n.d. | - | 0.088 | 16 | 16† | 16† |
| | TOTAL | 1.182 | -62 | 4.154 | 76* | 85* | 76*† |
| Antibiotics | Ciprofloxacin | 0.702 | 48 | 0.631 | 100* | 100*† | 100*† |
| | Ofloxacin | 0.177 | 57* | 0.067 | 100* | 100*† | 100*† |
| | Sulfamethoxazole | 0.100 | 100* | BQL | 100* | 100*† | 100*† |
| | Clarithromycin | 0.070 | 75* | n.d. | - | - | - |
| | Trimethoprim | 0.059 | 36 | n.d. | - | - | - |
| | Metronidazole-OH | n.d. | - | 0.195 | -9 | 3† | -1† |
| | Metronidazole | n.d. | - | BQL | *0* | *0*† | *0*† |
| | Tetracyclin | n.d. | - | BQL | -354* | *0* | -481* |
| | Dimetridazole | n.d. | - | 0.078 | 4* | 4*† | 1*† |
| | Ronidazole | n.d. | - | 0.049 | 24 | 29† | 31† |
| | TOTAL | 1.108 | 55 | 1.226 | 26 | 65* | 14† |
| Antihyper-tensives | Valsartan | 0.520 | 100* | 3.086 | 94* | 93*† | 99*† |
| | Irbesartan | 0.014 | 100* | 0.048 | *71** | *71*† | 100* |
| | Losartan | n.d. | - | 0.267 | 100* | *91*† | 100*† |
| | Amlodipine | n.d. | - | BQL | *0* | *0*† | *0*† |
| | TOTAL | 0.534 | 100* | 3.494 | 91* | 90*† | 96*† |
| Diuretics | Furosemide | 0.277 | 100* | 0.988 | 100* | 100*† | 100*† |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Hydrochlorotiazide[a] | 0.217 | 80* | 0.851 | 46* | 52* | 60* |
| | Torasemide | 0.021 | 49* | 0.082 | *68** | 24* | *68**† |
| | TOTAL | 0.515 | 90* | 1.921 | 75* | 76*† | 81*† |
| Contrast media | Iopromide | 0.056 | 4 | 2.409 | 67* | 86*† | 79*† |
| | TOTAL | 0.056 | 4 | 2.409 | 67* | 86*† | 79*† |
| Lipid regulators | Gemfibrozil | 0.137 | 100* | 1.027 | *98** | *98**† | *98**† |
| | Atorvastatin | 0.040 | 100* | n.d. | - | - | - |
| | Bezafibrate | 0.006 | 100* | n.d. | - | - | - |
| | Fluvastatin | n.d. | - | BQL | 100* | 100*† | 100*† |
| | TOTAL | 0.183 | 100* | 1.059 | 98* | 98*† | 98*† |
| Anti-helmintics | Thiabendazole | 0.071 | -51 | 0.124 | *80** | 57* | *80**† |
| | Levamisol | 0.037 | -2 | 0.252 | 50* | 63* | *90** |
| | TOTAL | 0.108 | -34 | 0.376 | 60* | 61*† | 87* |
| Ca channels blocker | Verapamil | n.d. | - | BQL | 100* | 100*† | 100*† |
| | Norverapamil | n.d. | - | BQL | 100* | 100*† | 100*† |
| | Diltiazem | n.d. | - | 0.008 | 25 | -13† | 25† |
| | TOTAL | n.d. | - | 0.060 | 90* | 85*† | 90*† |
| H2 antagonist | Ranitidine | n.d. | - | 0.171 | 29* | 29*† | 29*† |
| | TOTAL | n.d. | - | 0.171 | 29* | 29*† | 29*† |
| Drug against prostatic hiperplasia | Tamsulosin | 0.019 | -34 | n.d. | - | - | - |
| | TOTAL | 0.019 | -34 | n.d. | - | - | - |
| Sedative | Xylazine[a] | n.d. | n.q.* (↑) | n.d. | - | - | - |
| | TOTAL | n.d. | n.q.* (↑) | n.d. | - | - | - |
| TOTAL | | 21.830 | -11 | 49.788 | 44* | 68* | 35*† |

[a] Compounds adsorbed in the sampling system; * Statistically significant with respect the entrance ($p$<0.01); † Not statistically different than the mixture. BQL: below quantification limit; n.d.: not detected; n.q.: not quantifiable; ↑: increase of the concentration Removal percentages calculated with values BQL are in italics.

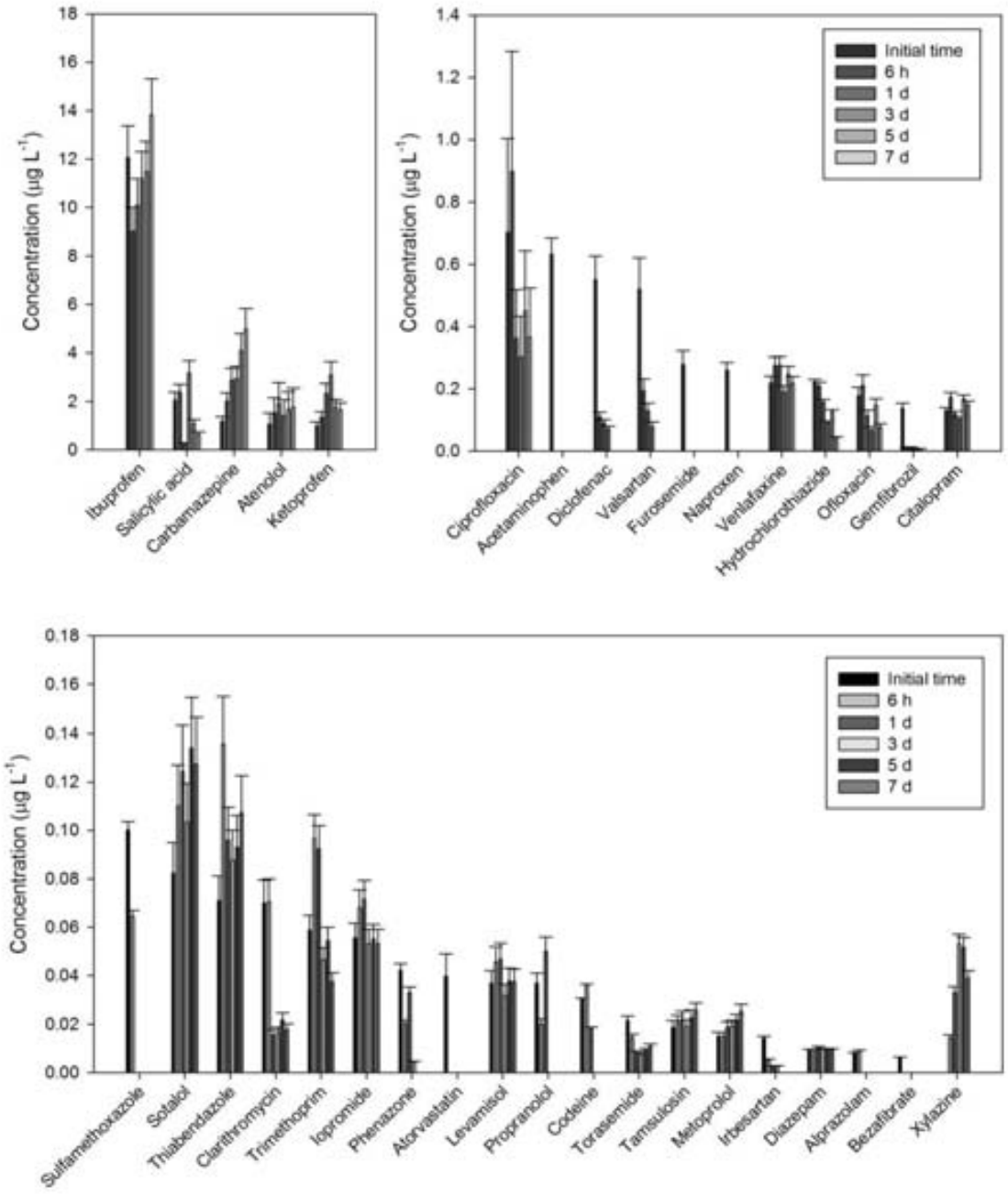



**Figure 7.5.** Evolution of pharmaceuticals concentration during the sterile batch reactor without extra nutrient (ROC1)

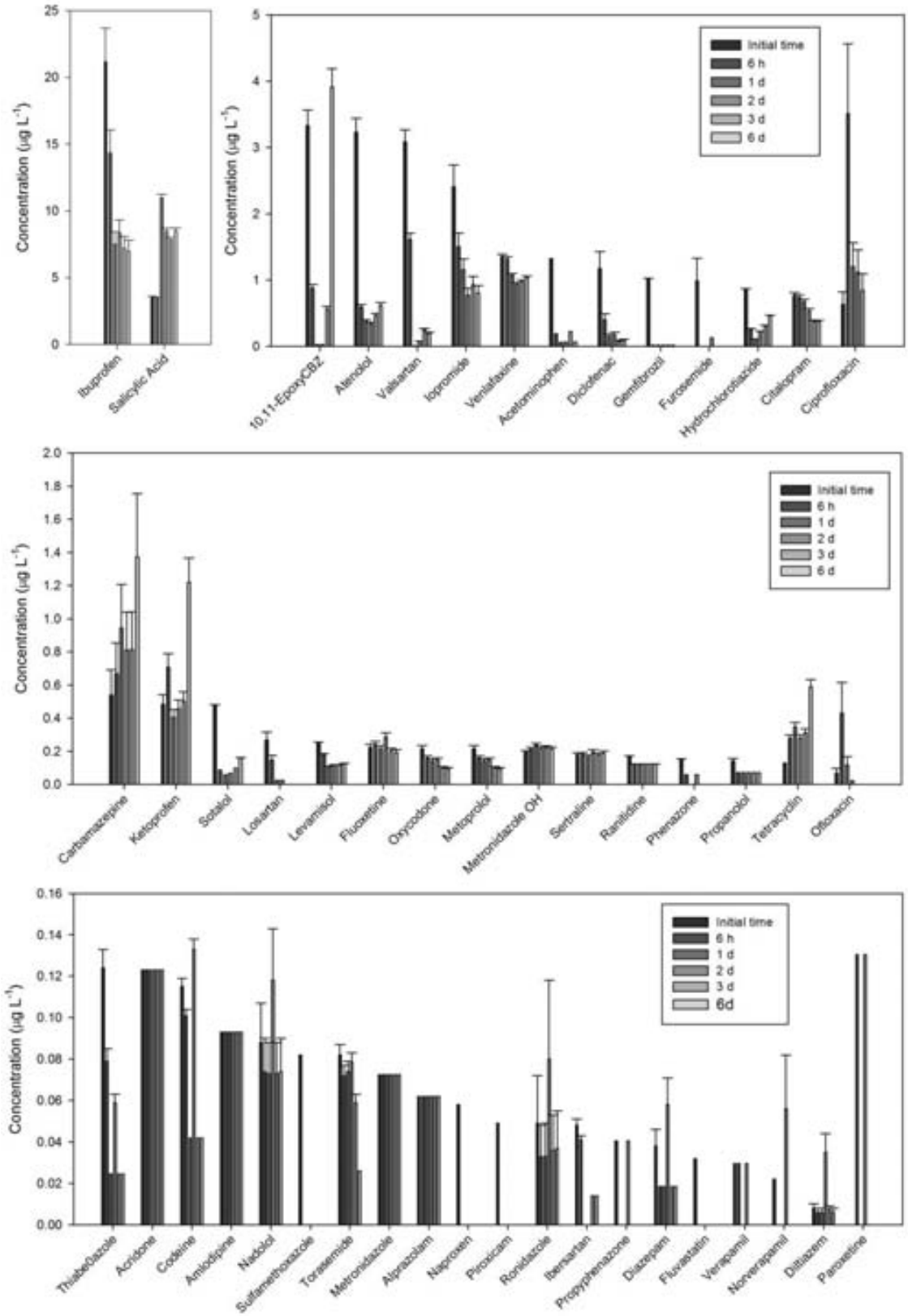



**Figure 7.6.** Evolution of pharmaceuticals concentration during the sterile batch reactor with extra nutrient addition (ROC2)

In ROC1 bioreactor (experiment without nutrients addition), small decrease in the global concentration of pharmaceuticals was found after 6 hours of treatment (19%) (Fig. 7.7A) probably due to some fungal activity while *T. versicolor* consumed the nutrients present in ROC. However, no significant overall degradation and even a small increase were detected at the end of the treatment (7 days) although some compounds were totally eliminated (Table 7.5). Those are mainly easily degradable compounds, such as analgesics and anti-inflammatories acetaminophen, naproxen, phenazone and codeine, but also diclofenac was totally removed. The antihypertensive valsartan, the diuretic furosemide and the antibiotic sulfamethoxazole, among others, were also thoroughly removed. Those compounds, as they are degraded without sufficient nutrients, are candidates to be used as carbon sources by *T. versicolor,* such as the UV filter benzophenone-3 (see Chapter 6). β-blocker propranolol was not actually removed as shown in section 7.3.2. In fact, its concentration increased, as well as for carbamazepine, xylazine and diazepam, which mask the above mentioned removal of some compounds in the overall removal calculations. Those increases could be due to deconjugation processes, which are further discussed below.

In contrast, in the experiment peformed with nutrients addition (ROC2 treatment), 44% removal of total pharmaceuticals was achieved after 6 days, being already 33% after 6 hours of treatment, with a maximum removal of 53% after 3 days (Fig. 7.7B). The same compounds that were totally removed in ROC1 were also highly degraded in this experiement. Additionally, other pharmaceuticals such as antibiotic ciprofloxacin and the antihypertensive losartan showed 100% removal in ROC2. Some compounds also increased their concentration after the treatment, such as salicylic acid, ketoprofen, carbamazepine, 10,11-epoxycarbamazepine and tetracyclin, mostly in the mixture and ROC2B but not at ROC2A. Differences between ROC2A and ROC2B are further discussed in section 7.4.3.

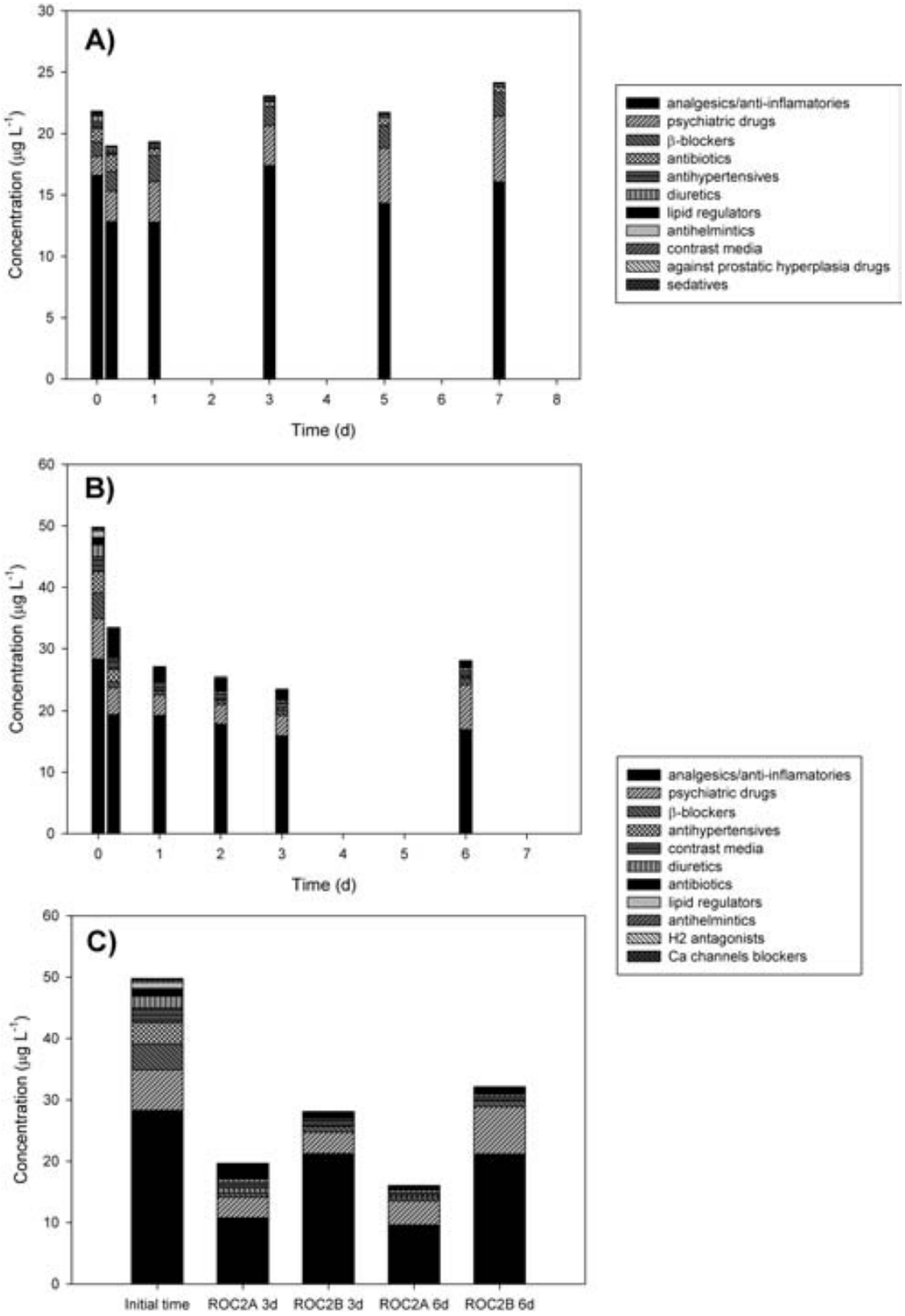



**Figure 7.7.** Evolution of total pharmaceuticals concentration during **A)** ROC1, **B)** ROC2 (mixture of ROC2A and ROC2B replicates) and **C)** ROC2 (separate concentrations of ROC2A and ROC2B replicates) treatments.

The overall increase in the concentration of pharmaceutical compounds from day 3 to day 6 at ROC2 can be attributed to the increase of 10,11-epoxycarbamazepine, that could be due to both oxidation of the parent compound or deconjugation processes in the fungal bioreactor. However, the amount was higher than the initial concentration of carbamazepine and its metabolite summed. Therefore, increase can be attributed to some deconjugation or other transformation processes taking place. These results highlight the importance of the addition of extra nutrients (i.e. glucose and ammonia) for a good degradation by *T. versicolor*, as most of the pollutants are degraded co-metabolically (Harms et al., 2011).

When evaluating a treatment, it is important to determine the overall degradation of contaminants, but it is also important to pay attention to those compounds with higher toxicity or lower biodegradability. In this study, high removal percentages were achieved with the fungal treatment for some recalcitrant compounds, such as diclofenac and antibiotics. The anti-inflammatory diclofenac is usually poorly degraded at conventional activated sludge but also in MBR systems (Kovalova et al., 2012). However, in our fungal bioreactors more than 90% degradation was observed even in the bioreactor without extra nutrient addition. All the other analgesics and anti-inflammatories detected (with exception of salicylic acid and ketoprofen) were highly degraded. Some antibiotics are usually poorly degraded by conventional WWTP as well. For example, ciprofloxacin, ofloxacin and sulfamethoxazole, which are removed in average 70, 60 and 52% respectively in WWTP (Verlicchi et al., 2012), can be completely removed by *T. versicolor* bioreactors. Increases in sulfamethoxazole were reported as well (Göbel et al., 2005) even in rivers (Osorio et al., 2012), but in the present study it was 100% removed. Ofloxacin concentration was one of the highest detected and it was not degraded at all in the MBR unit in a previous sampling in the same MBR/RO pilot plant (Dolar et al., 2012). However, in our study, its concentrations in the ROC were lower and it was 57-100% removed in the fungal bioreactors. Psychiatric drugs are not well degraded either in the WWTP or by fungal treatment of the present study. β-blockers are also usually not well degraded at WWTPs, with removal percentages below 40% in conventional activated sludge (CAS) plants

(Verlicchi et al., 2012). Atenolol, which was highly detected in our study, was removed up to 81% in ROC2. Moreover, the majority of detected β-blockers were more than 50% removed. Hydrochlorothiazide is not well removed either in some studies (Radjenović et al., 2009). In our treatments, 46-80% degradation by *T. versicolor* was achieved. However, possible partial adsorption in the system should be considered (see section 7.3.2). Another hardly degradable diuretic, furosemide, was totally removed after only 6 hours of treatment at both reactors. Contrast media are also hardly biodegradable compounds and an increasing concern for their persistence and toxicity after chlorination processes exists (Kormos et al., 2010). However, *T. versicolor* was able to degrade iopromide by 67% after 7 days of treatment from an initial concentration of 2.4 μg $L^{-1}$.

On the other hand, when working with known spiked concentration of selected contaminants, it is straight forward to determine removal percentages because initial concentrations are known and no conjugated compounds or other metabolites are present in the media. However, when working with real effluents at pre-existent pharmaceuticals concentrations some issues need to be addressed, such as increases in the concentration of some compounds after treatments. In our experiments, we observed the increase of some PhACs (i.e. carbamazepine and its epoxy metabolite, propanolol, tetracycline, salicylic acid, ketoprofen, xylazine and diazepam). Carbamazepine, propranolol and tetracycline had already been reported to increase in WWTP. This has been attributed to deconjugation of human phase II metabolites, parent compound formation by enzymatic reactions from phase I metabolites or desorption from particulate matter (Jelic et al., 2011; Kovalova et al., 2012; Verlicchi et al., 2012). For the rest of above mentioned compounds, concentration increase after the biological treatment, although no reported so far, could be attributed to deconjugation processes. In fact, some of the compounds are known to form conjugates (Kovalova et al., 2012; Pérez and Barceló, 2007) whereas the others may form them because of their chemical structure (presence of electrophilic groups (Parkinson, 2001)).

Among those compounds, there are some of them usually removed in WWTP, such as ketoprofen and salicylic acid, with average removals of 56% and 99% respectively (Verlicchi et al., 2012). To the author's knowledge, no increase has been reported during any CAS or MBR wastewater treatment, and only increases in salicylic acid were already reported in a *T. versicolor* treatment of urban wastewater in a similar reactor under sterile conditions (Cruz-Morató et al., 2013a). Carbamazepine, tetracycline and propranolol, on the contrary, are pharmaceutical compounds that usually pass through WWTP without being altered. Due to its low biodegradability, they constitute compounds of major concern. Previous studies reported the ability of ligninolytic fungi to degrade them (Jelic et al., 2012; Suda et al., 2012; Zhang and Geissen, 2012). However, when working with real effluents, increase in carbamazepine concentration is already reported (Cruz-Morató et al., 2013a). Surprisingly, in a previous work, high removal rates of carbamazepine (51%) and propranolol (67%) were found in the MBR system placed before the RO unit (Dolar et al., 2012).

Therefore, considering that fungal treatment was applied after MBR microbial process, our preliminar hypotheses for increase of certain PhACs are that:

i) removal percentages at WWTPs were partly only due to microbial conjugations or simple transformations (Jewell et al., 2014; Khunjar et al., 2011; Marvalin et al., 2012) reverted by *T. versicolor* under certain conditions;

ii) that fungus, but not microorganism at WWTP, revert certain human conjugates;

iii) PhACs are the transformation products of other unidentified micropollutants as happened with phenazone in a MBR (Kovalova et al., 2012); or

iv) desorption from particulate matter was occurring (Plósz et al., 2010).

Consequently, observed removal percentages account for the fungal ability to degrade certain pollutants but also depend on the transformation rates of related unidentified compounds. Therefore it might be the reason of why some compounds are badly removed when working with real effluents despite their high degradation in synthetic media. Moreover, fungal degradation can also generate conjugated metabolites. Thus, all the considerations suggested above lead to a high uncertainty about reliable degradation quantification during

biological processess with the available analytical methods. Therefore, research should make progresses in different fields, as in the treatment technologies but also in the analytical environmental chemistry.

*Differences between parallel bioreactors at ROC2 treatment*

The reported results for B2 experiment were the mean of duplicates, as parallel bioreactors under the same theoretical conditions were set up for the treatment with addition of nutrients. However, glucose addition and aeration end up being slightly different between both reactors: at ROC2A there was higher glucose and lower aeration (although always in excess) and at ROC2B there was higher aeration but also a period of nutrient shortage (see section 7.4.1). These differences were initially thought to be irrelevant and bioreactors could be taken as replicates. However, they led to totally different PhACs removal for some compounds (Table 7.5 and Fig. 7.7C), especially among analgesics/anti-inflammatories and psychiatric drugs. Thus, global removal after 6 days at ROC2B (35%) was lower than at ROC2A (68%) since some compounds increased their concentration at ROC2B, whereas they decreased at ROC2A. Removal percentages without taking into account those compounds were 69% for ROC2A and 68% for ROC2B. As differences were both on days 3 and 6 and nutrient shortage was on day 4, our preliminar hypothesis is that the higher aeration is related to the deconjugation of certain compounds (except for carbamazepine, whose increase occurred in ROC2A). In the literature, there are few works analysing aeration effect on PhACs fungal removal. Rodarte-Morales et al. (2012b), for example, found that oxygen supply led to slightly better diclofenac degradation percentages than air. However, there is no study that reports that aereation causes deconjugation of such compounds because those experiments were done with spiked concentrations instead of pre-existing ones. Only temperature has been reported to increase deconjugation rates in activated sludge (Gomes et al., 2009). Based on our results, we can conclude that aeration control is crutial, even with an excess of it as in the present work, because it might affect deconjugation processes. Further studies should be done to optimise aeration in bioreactors treating real wastewater and to confirm this hypothesis.

*Removal of EDCs*

Degradation of endocrine dirupting compounds by the ligninolytic fungus *T. versicolor* was also analysed at ROC2 treatment. The first screening confirmed the presence of BTZ, TTZ, TCEP, $OP_2EO$, NP and estriol-16-glucuronide, and the biomarker caffeine (Fig. 7.8). Other compounds such as estradiol-17-glucuronide, estrone, estrone-3-glucuronide, estriol-3-sulfate, TBEP and $NP_1EO$ were detected in some samples along the treatment without being present at the beginning of the experiment.

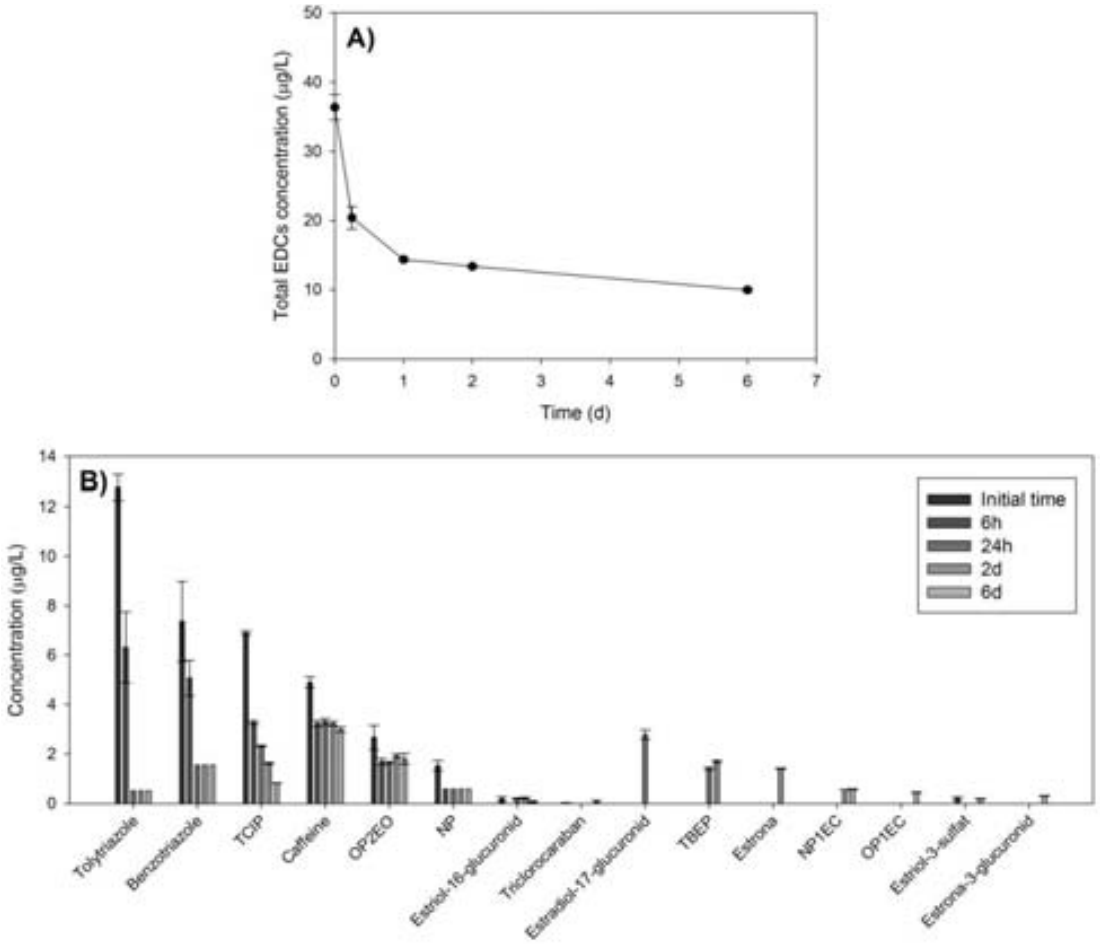



**Figure 7.8.** Evolution of **A)** total EDCs concentration and **B)** individual compounds in ROC2 treatment.

Removal of 73% of total EDCs was achieved at the end of the treatment with a removal of already 44% after 6 hours (Fig. 7.8A). The compounds detected at higher concentrations were the BTs (with maximum of 12.8 for BTZ and 7.4 µg $L^{-1}$ for TTZ) which were degraded by *T. versicolor* until concentrations below limit of quantification. They were selected for individual degradation analysis

(see Chapter 5) in order to find the fungal metabolites and that they could be incorporated in the analytical method and monitored in further fungal treatments.

*Removal of UV filters*

Quantification of degradation by *T. versicolor* of UV filters present in ROC was wanted to be studied as well. However, as shown in Chapter 5, their high adsorption at bioreactor walls recommends to performe the experiments in bottles, where unitary samples can be taken, instead of the bioreactor. Thus, uninoculated (UNI) and dead-fungi (HK) controls could be also included for the assessment of possible abiotic degradation and adsorption additionally to the experimental bottles (EB). Experiment was performed under sterile conditions as well and included EB without extra nutrients and EB supplemented with glucose and ammonium tartrate. As previously described, fungal dry weight increased in those cultures supplemented with extra nutrient and maintained stable in those without them (Fig. 7.9A). Laccase activity was around 20-40 U $L^{-1}$ in both treatments (Fig. 7.9B).

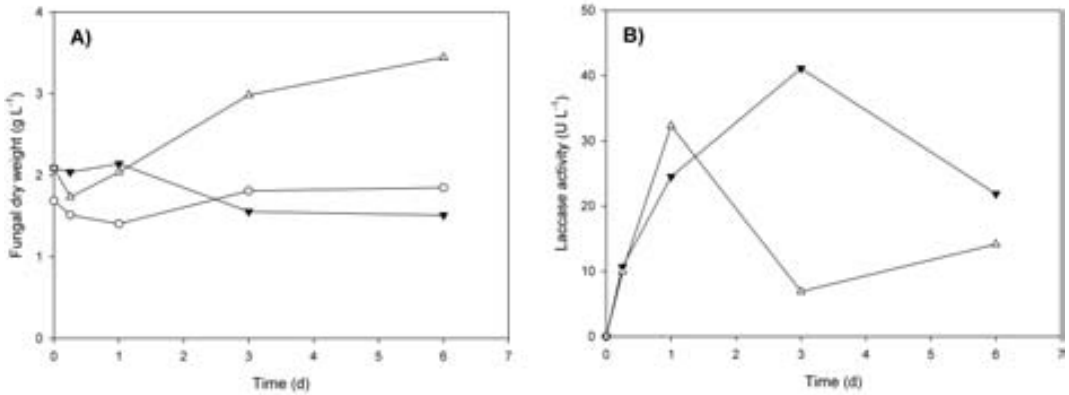



**Figure 7.9. A)** Fungal dry weight and **B)** laccase activity during UV filters degradation experiment in bottles. Symbols: (○) heat killed inactivated control, (▼) experimental culture without nutrient addition and (△) experimental culture with addition of glucose and ammonia.

As UV filters were analysed at their pre-existent concentrations, a solid-phase extraction (SPE) step was necessary. It was performed in our laboratory as described in Chapter 5. Table 7.6 shows the recovery percentages of the SPE method for each type of treatment. Values are very different from those obtained during the SPE optimisation, highlighting the strong matrix

interferences in the analysis. Low recoveries were found for some compounds, such as BP2, and high differences in the percentages of recovery were obtained between treatments for other compounds, such as BP1. However, generally, recoveries at HK were lower than at UNI and EB treatments.

**Table 7.6.** Percentage of recovery of the analysed UV filters in the SPE.

| | Recovery (%) | | |
|---|---|---|---|
| | UNI | HK | EB |
| BP1 | 139.8 | 30.1 | 101.0 |
| 4DHB | 72.8 | 33.5 | 70.6 |
| 4HB | 154.0 | 55.5 | 124.1 |
| BP3 | 131.7 | 60.0 | 113.0 |
| 4-MBC | 86.3 | 38.7 | 65.9 |
| OC | 78.8 | 58.8 | 86.9 |
| OD-PABA | 66.4 | 32.5 | 41.2 |
| EMC | 36.8 | 56.6 | 100.9 |
| DHMB | 51.3 | 21.1 | 63.9 |
| BP2 | 37.3 | 12.0 | 31.4 |
| Et-PABA | 55.1 | 20.5 | 29.7 |

Those UV filters detected at ROC were BP3, BP1, EMC and OC and the BP3 metabolite 4HB. Their removal profiles during the treatment are shown at Fig. 7.10. For those compounds less hydrophobic, almost no adsorption was found in the bottles. Taking into account the adsorption in the fungus (calculated from the HK controls), at least 55% of BP3 and 72% of 4HB were degraded by *T. versicolor*. For BP3, faster degradation was found for those cultures with extra nutrients addition than for those without them. Regarding BP1, higher adsorption at HK controls was found in comparison with the experiments in spiked synthetic media (Chapter 5). On the other hand, regarding those compounds more lipophilics (EMC and OC), no concluding results were obtained as lower concentrations were found in the UNI samples than in the EB. The reason probably was the high variability between the samples due to their adsorption. Those results highlight the difficulties in the removal assessment of those compounds that are more hydrophobic, taking into account that $\log K_{ow}$ of EMC and OC are 5.8 and 7.35, respectively (Díaz-Cruz et al., 2008). That high dispersion of the concentrations might also occur in the quantification of those compounds in reals samples. Therefore, evaluation of the efficiency of treatments might be difficult when working with hydrophobic compounds.

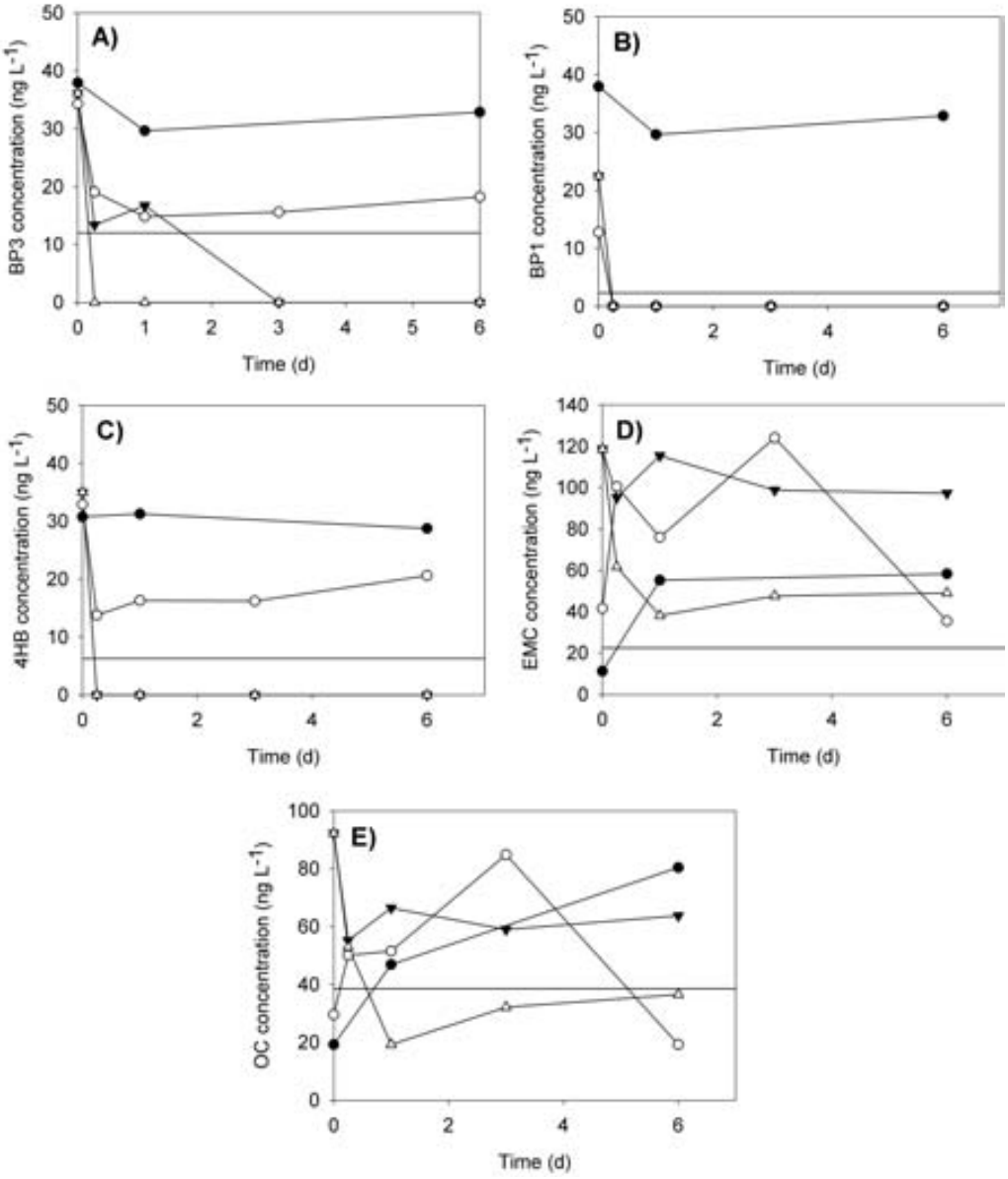



**Figure 7.10.** Concentrations of UV filters **A)** BP3, **B)** BP1, **C)** 4HB, **D)** EMC and **E)** during 6 day experiment at serum bottles at different experimental conditions. Symbols: (●) uninoculated control, (○) heat killed inactivated control, (▼) experimental bottle without nutrients addition and (△) experimental bottle with nutrients addition. Values are mean of duplicate samples. Solid lines mark the LOQ of each compound.

## 7.2. Fungal treatment of ROC in bioreactors under non-sterile continuous operational mode

Then, a bioreactor operated in continuous mode for treating ROC under non-sterile conditions was performed. Two 1.5 L air-pulsed reactors were set up in paralel, one inoculated with *T. versicolor* (ROC3-I) and the other one non-inoculated as control (ROC3-NI). The main operational parameters of the continuous experiment were: HRT of 3 days and changed to 2d at day 15, CRT was mantained at 15 days as it was calculated from previous studies (Blánquez et al., 2006), glucose feed rate was fixed initially at 192 mg $g^{-1}$ $d^{-1}$, ammonia feed rate at 0.43 mg $g^{-1}$ $d^{-1}$ (Casas et al., 2013) and initial fungal biomass was 3.6 g $L^{-1}$. Glucose and ammonia feed rate were changed according to monitoring results (laccase activity, glucose consumption and fungal dry weight among others) and visual evolution of bioreactors, after verifying that the amount calculated for sterile treatments (Casas et al., 2013) was not sufficient.

### *7.2.1. Monitoring of bioreactors performance*

Extracellular enzymes activity has been used as an indicative of fungal activity, although some works refute the need of them for the achievement of good removal percentages (Blánquez et al., 2004; Yang et al., 2013a). Laccase activity (Fig. 7.11A) in this study was quite low (maximum of 10 U $L^{-1}$) and it was not mantained stable along the time. It was lower than expected for a mixed culture, where laccase activity has been reported to be induced (Baldrian, 2004; Freitag and Morrell, 1992). Nonetheless, activity was described to be lower in low-N media (Freitag and Morrell, 1992), such as in the present study. Additionally, at HRT of 2 days, laccase activity was most of the time below 1 U $L^{-1}$. Therefore, at a first glance, results can suggest that lowering HRT from 3 to 2 days was detrimental for extracellular laccase activity and some washing out could be taking place. Some laccase activity (<5 U $L^{-1}$) was observed at ROC3-NI control bioreactor from the day 16 until the end of the operation (Fig 7.11B). This aspect will be discussed afterwards in the Chapter 9.

As shown in Fig 7.11A and B, glucose was totally consumed at fungal bioreactor (ROC3-I) and accumulated in the media during the first 9 days of

operation at control bioreactor (ROC3-NI) until microbial contamination insided the reactor was grown. Therefore, COD was higher at ROC3-NI (reaching almost 3500 mg $O_2$ $L^{-1}$) than at ROC3-I because of the accumulation of metabolic products in the media (Fig. 7.11C and D). COD was also higher in the bioreactors effluent than in the initial wastewater, what means that *T. versicolor* treatment cannot remove wastewater COD as already reported Cruz-Morató et al. (2013), being the fungal treatment more suitable as a pre-treatment than as a final process.

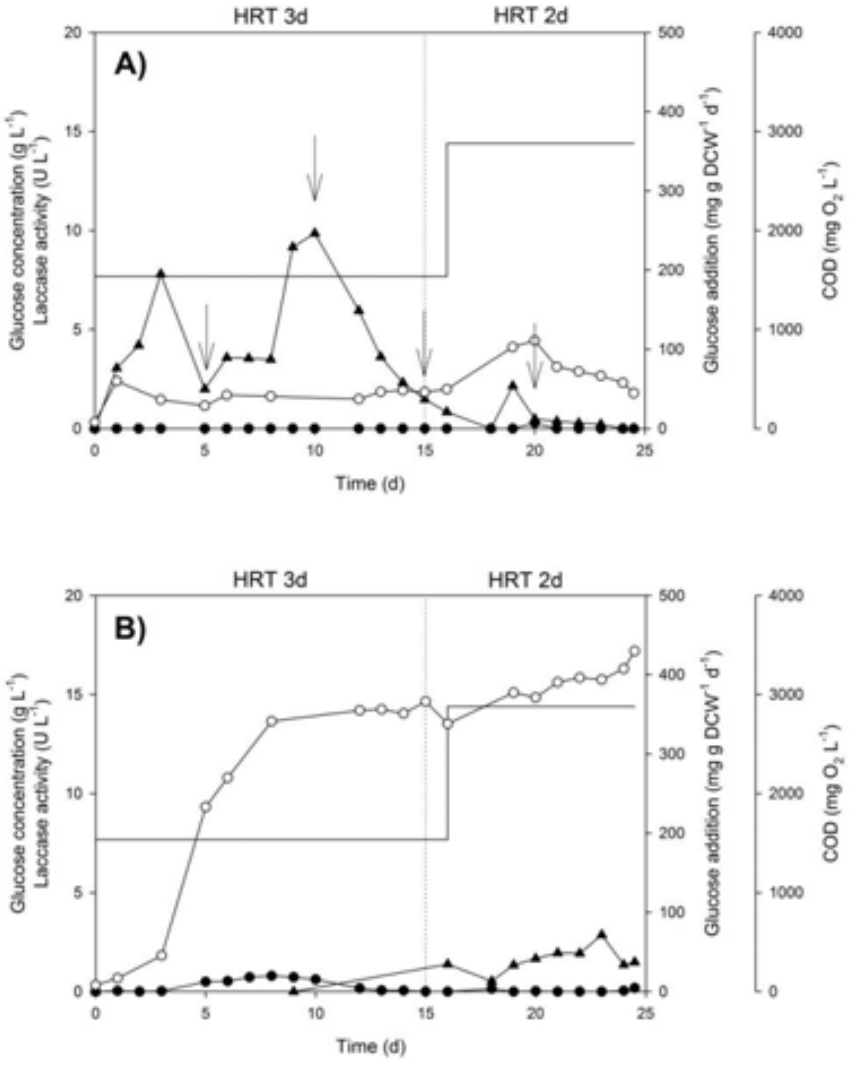



**Figure 7.11.** Evolution of (●) glucose concentration, (▲) laccase activity and (○) COD during the **A)** non-sterile fungal inoculated continous reactor (ROC3-I) and **B)** non-sterile non-inoculated control reactor (ROC3-NI). Arrows shows the days where 1/3 old fungal biomass was changed by fresh one. Dotted vertical line marks the change of HRT from 3 to 2 days. Continuous line shows the gluccose addition rate.

Regarding fungal biomass, it decreased until 1.4 g DCW $L^{-1}$ between days 15 and 20 at ROC3-I for unknown reasons (data not shown). Increase in the nutrients feed to 360 mg glucose $g^{-1}$ $d^{-1}$ at day 16 (increasing also N addition to maintain C/N ratio constant) and partial renewal of biomass at day 20 seemed to recover fungal biomass. Visual aspect of bioreactors biomass changed along the time (Fig. 7.12). At ROC3-I, pink and green biofilm in the reactor walls apeared whereas, at ROC3-NI, biofilm was brown and white.

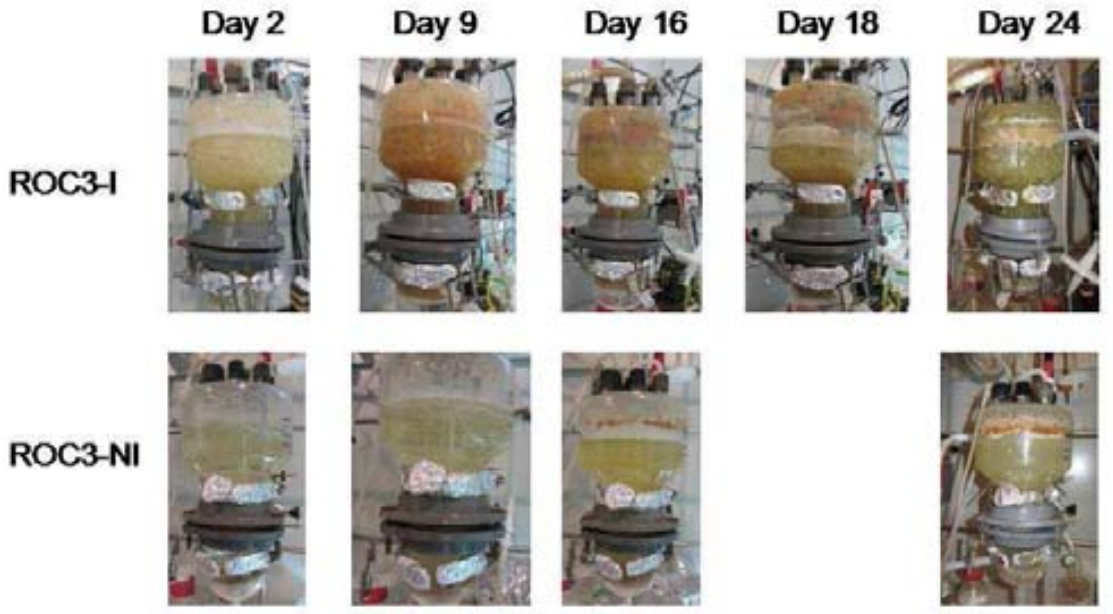



**Figure 7.12.** Visual evolution of I and NI bioreactors of ROC3 treatment

Microtox analysis of acute toxicity showed no toxicity at all of the reverse osmosis concentrate and ROC3-I effluent. However, some increase in the toxicity (5 T.U.) was found at ROC3-NI at both HRT. That means that some toxic compounds were produced at the control bioreactor. However, in any case, values were always below 25 T.U., when an effluent is considered as toxic (Generalitat de Catalunya, 2003). Therefore, those compounds were not very toxic or those possible toxic compounds were at low concentration.

Finally, estrogenicity and anti-estrogenicity were tested for the samples from the bioreactors. No estrogenic activity was detected at any sample but possible anti-estrogenic activity was found for samples of non inoculated continuous bioreactor for both HRT. This could indicate that, even though bacteria can

degrade analysed EDCs, they or other non analysed compounds present in the effluent can be transformed to more toxic compounds while in the fungal bioreactor it did not happen.

### *7.2.2. Removal of PhACs*

For pharmaceuticals removal evaluation at ROC3 experiment, mean values of 4 samples for each HRT (at 12, 13, 14 and 15 days for HRT 3d; and at 21, 22, 23 and 24 for HRT 2d) were compared with the ROC concentration in the influent at initial time. It has to be noted that concentration of most of the pharmaceuticals already decreased in the influent storage tank, probably because of adsorption to the plastic, abiotic processes or biological transformation after some microbial growing. Antibiotics were the only group that their concentrations were maintained or even increased along the storage period at 25ºC (3-5 days) (data not shown). Therefore, removal percentages were calculated with respect the concentrations of wastewater at initial time, as removal in influent storage tank equally affected both fungal and control bioreactors because it was the same tank.

PhACs concentration in the ROC and degradation percentages of specific compounds can be found at Table 7.7. Unlike in the previous campaigns, analgesics and anti-inflammatories were not the compounds at higher concentration, probably due to the much lower levels of ibuprofen in non-sterile ROC compared to the increase in the concentration in sterilised ROC (Table 7.3)

Similar degradation percentages of global PhACs than in sterile batch bioreactor were achieved under non-sterile conditions in the continous fungal inoculated bioreactor. At Fig. 7.13 significant differences can be observed at fungal bioreactor at HRT 3d with respect to the non-inoculated control (degradation of 52% at I and 25% in the NI), showing that degradation was due to fungal action. At HRT 2d there were not statistically significant differences between treatments as much from a decrease in the degradation percentages at ROC3-I (43% removal) as much as from an increase at ROC3-NI (38%). However, there were always significant differences with respect the influent at

both reactors. The decrease in the removal at ROC3-I at HRT 2d can be either due to the decrease in the HRT or the biomass conditions.

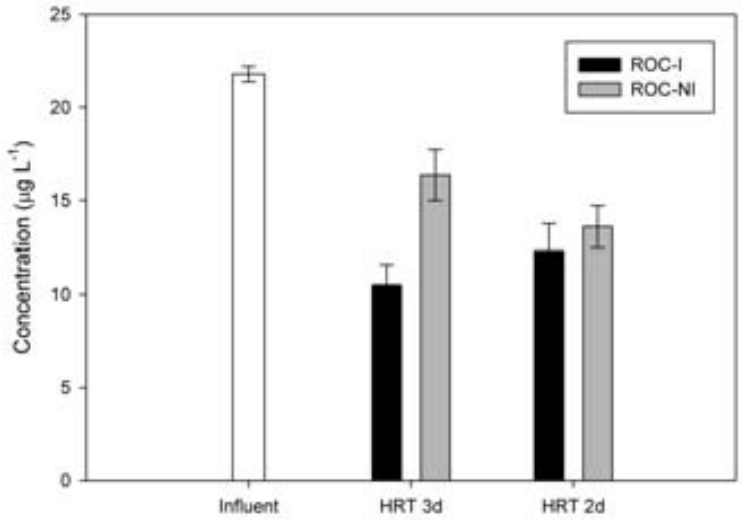



**Figure 7.13.** PhACs concentration at the wastewater (influent) and at every HRT for each bioreactor (ROC3-I and ROC3-NI). Error barrs represents standard deviation of 4 samples taken at hydraulic steady state.

Analgesics and anti-inflammatories were highly degraded except for ketoprofen (maximum removal of 45% at HRT 2d). However, at least its concentration did not increase such as in the sterile batch treatments (section 7.4). Psychiatric drugs were removed 45% at HRT 3d, although carbamazepine was not significantly degraded. However, neither its concentration increased as usually happened in other treatments (see section 7.4 and (Cruz-Morató et al., 2013a)). In fact, in the continuous treatment, fewer compounds increased their concentrations, only metronidazole-OH and ofloxacin at ROC3-I and sulfamethoxazole, ofloxacin and ketoprofen at ROC3-NI. Among β-blockers and antihypertensive drugs, no statistical significant differences were found between ROC3-I and ROC3-NI. On the other hand, compounds such as diuretics furosemide and hydrochlorothiazide, antibiotic ciprofloxacin, psychiatric drugs venlafaxine and citalopram and tranquilizer azaperol were better removed at the fungal bioreactor.

**Table 7.7.** Pharmaceuticals initial concentration and their removal percentages at non-sterile continous ROC3 bioreactors

| Therapeutic group | Compound | Influent | | ROC3-I | | | | | | ROC3-NI | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | HRT 3d | | | HRT 2d | | | HRT 3d | | | HRT 2d | | |
| | | C (µg $L^{-1}$) | SD | C (µg $L^{-1}$) | SD | Rem (%) | C (µg $L^{-1}$) | SD | Rem (%) | C (µg $L^{-1}$) | SD | Rem (%) | C (µg $L^{-1}$) | SD | Rem (%) |
| Antibiotics | Azithromycin | 1.725 | 0.058 | 3.080 | 1.188 | -79 | 2.762 | 1.120 | -60 | 3.075 | 1.411 | -78 | 1.881 | 0.745 | -9 |
| | Tetracycline | 0.909 | 0.018 | 0.192 | 0.050 | 79* | 0.092 | 0.016 | 90*† | 0.153 | 0.080 | 83* | 0.021 | 0.041 | 98* |
| | Ciprofloxacin | 0.632 | 0.128 | 0.361 | 0.019 | 43*† | 0.423 | 0.022 | 33* | 0.715 | 0.095 | -13 | 0.521 | 0.031 | 18 |
| | Metronidazole-OH | 0.454 | 0.013 | 0.624 | 0.122 | -37*† | 1.737 | 0.273 | -283*† | 0.126 | 0.023 | 72* | 0.318 | 0.155 | 30 |
| | Sulfamethoxazole | 0.200 | 0.020 | ND | - | 100*† | 0.023 | 0.012 | 88† | 2.761 | 0.484 | -1280* | 1.442 | 0.268 | -621* |
| | Ofloxacin | 0.197 | 0.026 | 0.110 | 0.066 | 44† | 0.500 | 0.036 | -154* | 0.643 | 0.137 | -227* | 0.535 | 0.057 | -172* |
| | Erythromicin | 0.155 | 0.031 | ND | - | 100* | ND | - | 100* | ND | - | 100* | ND | - | 100* |
| | Metronidazole | 0.129 | 0.013 | ND | - | 100* | ND | - | 100* | 0.020 | 0.041 | 84* | ND | - | 100* |
| | Ronidazole | 0.110 | 0.002 | 0.103 | 0.112 | 6 | ND | - | 100* | 0.027 | 0.000 | 75 | ND | - | 100* |
| | Clarithromycin | 0.046 | 0.003 | 0.018 | 0.000 | 61* | 0.014 | 0.000 | 70* | 0.018 | 0.000 | 61* | 0.014 | 0.000 | 70* |
| | TOTAL | 4.557 | 0.150 | 4.461 | 0.998 | 2† | 5.537 | 1.378 | -22 | 7.493 | 1.159 | -64* | 4.717 | 0.850 | -4 |
| Analgesics and anti-inflamma-tories | Diclofenac | 1.614 | 0.173 | 0.028 | 0.019 | 98* | 0.127 | 0.119 | 92* | 0.198 | 0.223 | 88* | 0.061 | 0.026 | 96* |
| | Ketoprofen | 1.144 | 0.125 | 0.915 | 0.220 | 20† | 0.633 | 0.143 | 45*† | 1.311 | 0.127 | -15 | 1.662 | 0.100 | -45* |
| | Indomethacine | 0.320 | 0.043 | 0.112 | 0.029 | 65* | 0.094 | 0.044 | 70* | 0.093 | 0.055 | 71* | 0.034 | 0.022 | 90* |
| | Codeine | 0.207 | 0.008 | 0.043 | 0.035 | 79*† | 0.122 | 0.042 | 41 | 0.293 | 0.085 | -42 | 0.549 | 0.665 | -165 |
| | Acetaminophen | 0.128 | 0.002 | 0.022 | 0.026 | 82* | ND | - | 100* | 0.038 | 0.044 | 71* | ND | - | 100* |
| | Piroxicam | 0.071 | 0.003 | 0.011 | 0.021 | 85*† | ND | - | 100*† | 0.053 | 0.005 | 25 | 0.062 | 0.003 | 13* |
| | Naproxen | 0.065 | 0.009 | ND | - | 100* | ND | - | 100* | ND | - | 100* | ND | - | 100* |
| | Phenazone | 0.062 | 0.003 | 0.012 | 0.008 | 81*† | 0.001 | 0.003 | 98* | 0.028 | 0.008 | 54* | 0.004 | 0.008 | 93* |
| | TOTAL | 3.611 | 0.218 | 1.132 | 0.243 | 69*† | 0.945 | 0.279 | 74*† | 2.015 | 0.408 | 44* | 2.372 | 0.564 | 34* |
| Psychiatric drugs | Venlafaxine | 0.971 | 0.023 | 0.288 | 0.058 | 70*† | 0.495 | 0.046 | 49*† | 0.613 | 0.087 | 37* | 0.588 | 0.070 | 39* |
| | Carbamazepine | 0.769 | 0.038 | 0.656 | 0.063 | 15 | 0.755 | 0.012 | 2 | 0.804 | 0.148 | -5 | 0.814 | 0.078 | -6 |
| | Citalopram | 0.563 | 0.005 | 0.017 | 0.027 | 97*† | 0.080 | 0.031 | 86* | 0.091 | 0.051 | 84* | 0.125 | 0.059 | 78* |
| | Lorazepam | 0.543 | 0.040 | 0.522 | 0.138 | 4 | 0.476 | 0.141 | 12 | 0.560 | 0.103 | -3 | 0.426 | 0.096 | 22 |
| | Olanzapine | 0.486 | 0.004 | 0.473 | 0.002 | 3 | 0.478 | 0.005 | 2 | 0.492 | 0.024 | -1 | 0.502 | 0.039 | -3 |
| | Fluoxetine | 0.219 | 0.006 | ND | - | 100* | ND | - | 100* | ND | - | 100* | ND | - | 100* |
| | Alprazolam | 0.058 | 0.010 | 0.024 | 0.005 | 58*† | 0.024 | 0.005 | 59* | 0.040 | 0.007 | 30* | 0.026 | 0.005 | 56* |
| | TOTAL | 3.609 | 0.061 | 1.981 | 0.269 | 45*† | 2.307 | 0.204 | 36* | 2.601 | 0.335 | 28* | 2.480 | 0.279 | 31* |
| Diuretics | Furosemide | 1.628 | 0.103 | 0.018 | 0.013 | 99*† | 0.232 | 0.073 | 86* | 0.530 | 0.415 | 67* | 0.506 | 0.295 | 69* |
| | HCTZ | 1.149 | 0.071 | 0.807 | 0.165 | 30*† | 1.046 | 0.050 | 9 | 1.069 | 0.077 | 7 | 0.983 | 0.067 | 14* |
| | Torasemide | 0.384 | 0.026 | 0.052 | 0.011 | 86*† | 0.080 | 0.025 | 79* | 0.116 | 0.027 | 70* | 0.072 | 0.016 | 81* |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TOTAL | 3.161 | 0.128 | 0.877 | 0.166 | 72*† | 1.359 | 0.113 | 57* | 1.715 | 0.463 | 46* | 1.561 | 0.314 | 51* |
| β-blockers | Atenolol | 2.120 | 0.227 | 0.918 | 0.064 | 57* | 0.696 | 0.124 | 67* | 1.025 | 0.073 | 52* | 0.789 | 0.110 | 63* |
| | Sotalol | 0.288 | 0.012 | 0.179 | 0.055 | 38 | 0.222 | 0.049 | 23 | 0.273 | 0.121 | 5 | 0.307 | 0.055 | -7 |
| | Metropolol | 0.242 | 0.011 | 0.209 | 0.010 | 14*† | 0.192 | 0.009 | 21*† | 0.137 | 0.007 | 43* | 0.211 | 0.012 | 13* |
| | Propanolol | 0.124 | 0.002 | 0.042 | 0.021 | 66* | 0.060 | 0.029 | 51* | 0.036 | 0.008 | 71* | 0.052 | 0.019 | 58* |
| | Nadolol | 0.052 | 0.002 | 0.052 | 0.005 | 0† | 0.046 | 0.003 | 11 | 0.038 | 0.003 | 26* | 0.041 | 0.007 | 21* |
| | TOTAL | 2.826 | 0.228 | 1.400 | 0.031 | 50* | 1.217 | 0.120 | 57* | 1.510 | 0.172 | 47* | 1.400 | 0.136 | 50* |
| Antihyper-tensives | Irbesartan | 1.307 | 0.151 | 0.090 | 0.021 | 93* | 0.024 | 0.027 | 98* | 0.198 | 0.052 | 85* | 0.071 | 0.065 | 95* |
| | Valsartan | 0.207 | 0.007 | 0.059 | 0.010 | 72* | 0.068 | 0.021 | 67* | 0.045 | 0.009 | 78* | 0.053 | 0.004 | 75* |
| | Losartan | 0.156 | 0.017 | 0.024 | 0.016 | 85* | 0.041 | 0.026 | 74* | 0.039 | 0.018 | 75* | 0.053 | 0.016 | 66* |
| | Amlodipine | 0.098 | 0.011 | 0.092 | 0.016 | 6 | 0.081 | 0.005 | 17* | 0.096 | 0.026 | 2 | 0.084 | 0.004 | 14* |
| | TOTAL | 1.768 | 0.153 | 0.265 | 0.040 | 85* | 0.212 | 0.035 | 88* | 0.378 | 0.081 | 79* | 0.261 | 0.075 | 85* |
| H2 receptor antagonists | Ranitidine | 0.835 | 0.055 | ND | - | 100* | 0.241 | 0.036 | 71* | 0.035 | 0.050 | 96* | 0.226 | 0.047 | 73* |
| Anti-helmintics | Levamisol | 0.561 | 0.019 | 0.401 | 0.092 | 29* | 0.512 | 0.033 | 9 | 0.444 | 0.065 | 21 | 0.484 | 0.047 | 14 |
| Tranquilizers | Azaperol | 0.423 | 0.030 | ND | - | 100*† | ND | - | 100* | 0.099 | 0.129 | 76* | ND | - | 100* |
| Lipid regulators | Gemfibrozil | 0.265 | 0.017 | 0.001 | 0.002 | 100* | 0.001 | 0.003 | 99* | 0.023 | 0.039 | 91* | 0.003 | 0.005 | 99* |
| | Bezafibrate | 0.000 | 0.000 | ND | - | 0 | ND | - | 0 | ND | - | 0 | 0.089 | 0.081 | n.q. |
| | TOTAL | 0.265 | 0.017 | 0.001 | 0.002 | 100*† | 0.001 | 0.003 | 99* | 0.023 | 0.039 | 91* | 0.092 | 0.086 | 65* |
| Antidiabetics | Glibenclamide | 0.103 | 0.007 | 0.002 | 0.000 | 98*† | 0.002 | 0.000 | 98* | 0.037 | 0.022 | 64 | ND | - | 100 |
| Ca channels blockers | Diltiazem | 0.058 | 0.002 | ND | - | 100*† | 0.001 | - | 98* | 0.018 | 0.006 | 69* | 0.006 | 0.011 | 90* |
| Antiplatelet agents | Clopidogrel | 0.012 | 0.002 | ND | - | 100* | ND | - | 100* | ND | - | 100* | ND | - | 100* |
| TOTAL | | 21.789 | 0.412 | 10.519 | 1.080 | 52*† | 12.335 | 1.431 | 43* | 16.369 | 1.378 | 25* | 13.599 | 1.119 | 38* |

ND: not detected; n.q. not quantifiable. HCTZ: hydrochlorotiazide

* Difference statistically significant ($p<0.05$) with the entrance ROC (In)

† Difference statistically significant ($p<0.05$) with the control bioreactor (ROC-NI)

### *7.2.3. Removal of EDCs*

The results of the study of the 27 EDCs and related compounds are shown in Table 7.8. BTZ and TTZs were detected at ROC at lower concentration (1.57 µg $L^{-1}$ and 1.60 µg $L^{-1}$, respectively) than in the previous sampling study (for the sterile batch bioreactor ROC2). Regarding the total amount of detected EDCs, fewer compounds were also detected in ROC than in the previous sampling campaign. In this case, the global removal of EDCs at the hydraulic stationary state whithin HRT of 3d (samples from days 12-15) was 74%, and within HRT of 2d (samples from days 21-24) this was mantained at 65%. However, removal percentages were not statistically different than those obtained at the uninoculated control bioreactor.

**Table 7.8.** Initial concentration of EDCs and removal percentages during non-sterile continuous experiment. The treatments included a bioreactor inoculated with *T. versicolor* (Fungal treatment) and a non-inoculated control bioreator. Removal percentages of HRT of 3 days are the mean of samples at 12, 13, 14 and 15 days and HRT of 2 days results are the mean of 21, 22, 23 and 24 days.

| | Initial time concentration (µg $L^{-1}$) | SD | ROC3-I | | ROC3-NI | |
|---|---|---|---|---|---|---|
| | | | Rem. HRT 3d (%) | Rem. HRT 2d (%) | Rem. HRT 3d (%) | Rem. HRT 2d (%) |
| TCPP | 5.439 | 0.006 | *99** | *99** | *99** | *99** |
| Tolyltriazole (5MBTZ) | 1.598 | 0.233 | 55* | 45* | 49* | 57* |
| Benzotriazole | 1.571 | 0.495 | 35 | *68** | *68** | *68** |
| TCEP | BQL | - | *0* | *0* | *0* | *0* |
| Methylparaben | BQL | - | 50 | -5 | 100* | 100* |
| TBEP | BQL | - | *0* | *0* | -320 | -364 |
| Estriol-16-glucuronide | 0 | - | n.a. | n.a. | increase | increase |
| Estrone-3-sulfat | 0 | - | n.a. | increase | n.a. | n.a. |
| Estrone-3-glucuronide | 0 | - | n.a. | increase | n.a. | n.a. |
| Estrone | 0 | - | n.a. | increase | n.a. | n.a. |
| Ethynilestradiol | 0 | - | n.a. | increase | n.a. | n.a. |
| Diethylbestol | 0 | - | n.a. | increase | n.a. | n.a. |
| TOTAL | 9.387 | 0.548 | 74* | 65* | 75* | 79* |

BQL: Below Quantification Limit; n.a.: not apply; * Statistically significant removal; † Statistically different from control bioreactor. Removal percentages calculated with values BQL are in italics.

Tris (1-chloro-2-propyl) phosphate (TCPP), a flame retardant, was the EDC detected at higher concentration at ROC (5.4 µg $L^{-1}$). However it was almost totally degraded at both reactors. Regarding BTZ and TTZ, the former was

detected BLQ in the majority of the treatment samples, even in the uninoculated control, and the last was partially degraded (around 50%) in both reactors during both HRT (Table 7.8). In that non-sterile continuous bioreactor, degradation rates of both BTZ and TTZ were lower than those observed in synthetic media but also lower than those observed in sterile ROC batch treatment. Therefore, probably competition with indigenous microorganisms lowers the capacity of *T. versicolor* to degrade those compounds. However, when the HRT was decreased to 2d, degradation percentages were maintained for BTZ, TTZ and also the global EDCs (Table 7.8).

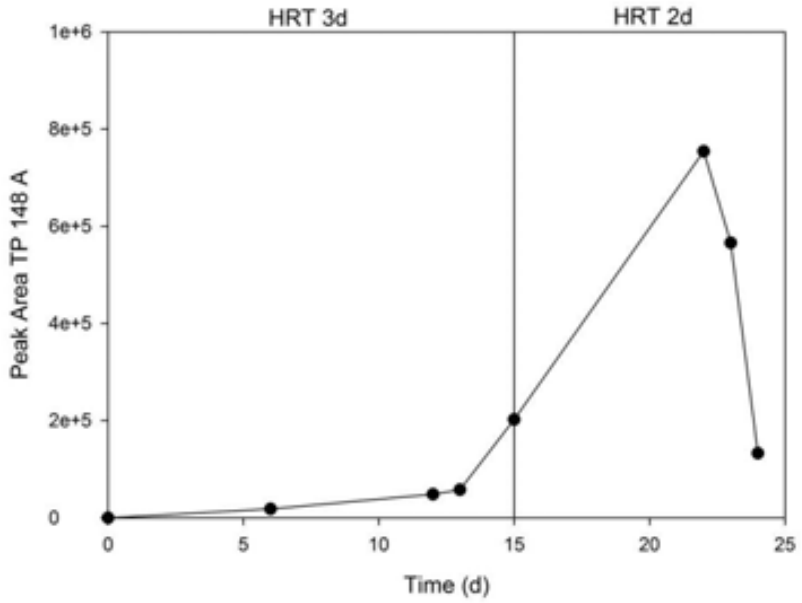



**Figure 7.14.** Peak area for TP148A along the ROC3 treatment in the fungal bioreactor (I). Results obtained by TFC-LTQ Orbitrap working in SRM approximation.

Finally, the screening for determining the presence of any metabolite of BTZ and TTZ was carried out according to the results obtained in synthetic media (see section 5.2). After data processing, just one compound was identified as a potential metabolite generated during the treatment of ROC by fungi, TP148A. TP222, which was detected in higher amount in the spiked experiment of Chapter 5 was not detected in the treatment of real effluent. As can be appreciated in Figure 7.14, the proposed metabolite was not present in the wastewater (data at initial time) and it increased along the time reaching its maximum the 21$^{st}$ day of the experiment in the fungal bioreactor whereas it was

not detected in the control reactor. However, the detection of this compound should be confirmed by comparison with a standard using a SRM method in a more sensitive instrument (*i.e.* LC-MS/MS used for the quantification of 27 EDCs and related compounds in this work) in order to assure the presence of this compound as well as its concentration. Concentration of metabolite TP148A presents a peak because although bireactor was operated in continuous mode, only hydraulic steady state was achieved because other parameter, such as nutrient addition rate or composition of influent wastewater changed during the experiment.

## CONCLUSIONS

Removal of emerging contaminants from wastewater, and among them PhACs and EDCs, is a scientific and technological challenge. The use of reverse osmosis could be a solution for decontamination of effluents, mainly for reusing purposes. However, an extra treatment is needed to deal with the concentrated effluent obtained. Fungal degradation by *T. versicolor* could be used to this purpose, with further improvements in the operation, since it already achieved 73% removal of total EDCs and 44% of total PhACs in bioreactors with some operational conditions still under ongoing optimisation (i.e. aeration and optimal nutrient addition). Those removal percentages were maintained in a continuous bioreactor.

In parallel, we found that heat-sterilisation changes considerably the concentration of the vast majority of the compounds. It could be due to abiotic deconjugation or desorption from particulate matter. It is an important point to take into account in lab-scale experiments dealing with sterile effluents at pre-existent concentrations of contaminants. On the other hand, changes in the concentration for propranolol, hydrochlorotiazide, diazepam and xylazine were also detected due to their sorption in the sampling system, in contrast with direct sampling inside the bioreactor. Therefore, these factors have to be taken into account for proper removal calculations.

Another aspect that should be pointed out is the difficulty that exists for a reliable assessment of degradation of highly hydrophobic compounds, such as some UV filters, due to their high adsorption coefficients. This fact is what actually happens in the WWTP, where these hydrophobic compounds tend to adsorb to sludge.

Degradation performance of *T. versicolor* was enhanced by external addition of nutrients. High degradation percentages were achieved for hardly biodegradable compounds such as the anti-inflammatory diclofenac (93%), the antibiotics ciprofloxacin (100%), ofloxacin (100%) and sulfamethoxazole

(100%), the diuretic furosemide (100%), the β-blocker atenolol (81%), the antihypertensive valsartan (94%) and the lipid regulator gemfibrozil (98%) under sterile conditions, which means that degradation was due to fungal action. Psychiatric drugs were more difficult to degrade even for the fungus.

Then, when non-sterile continuous bioreactors were performed, the majority of the hardly biodegradable compounds discussed above, were also well degraded: diclofenac, furosemide, ranitidine and gemfibrozil >90%, atenolol 57% and valsartan 72% among others. However, all of them except for atenolol and valsartan were equally removed at the non-inoculated control. This fact could indicate that microorganisms present or developed at ROC were also able to degrade or transform these compounds.

Parallel bioreactors under similar conditions led to the same removal percentage of total compounds (68 and 69% of degradation) when compounds whose concentration increased were not taken into account. However, in the bioreactor with higher aeration, compounds such as salicylic acid, ketoprofen, 10,11-epoxycarbamazepine and tetracyclin increased their concentration. Further studies about how aeration affects aerobic biodegradation processes should be done. These increases could be probably due to different factors such as deconjugation of human and/or microbial metabolites, backward transformation, transformation from another compound or desorption from particulate matter. Summarising, the preliminar hypothesis is that high aeration might decrease overall degradation percentages by promoting deconjugation processes. Therefore, when treating real wastewater, conjugation and deconjugation reactions have to be taken into account for degradation calculations, as they can easily be reverted.

Since it is impossible to analyse all parent compounds and transformation products present in the real effluent, degradation experiments with these effluents are notably more difficult to evaluate than those spiked at known concentration. However, they highlight the complexity of processes taking place during water treatment. Nevertheless, steps in that direction should be made, such as incorporation of more metabolites in the analytical methods. To that

respect, fungal metabolites of BTZ and TTZ were monitored in the continuous treatment apart from the parent compounds and other EDCs. BTZ and TTZ were removed by the fungal bioreactor as well as in the non-inoculated control. Additionally, one metabolite coming from the degradation of BTZ was identified in the fungal bioreactor but not initially or in the non-inoculated control, clearly showing that it was due to the action of the fungus.

Analysis of a broad range of toxicities can help in the evaluation of the treatments. In the present study, acute toxicity, estrogenic, anti-estrogenic and dioxin-like activities were determined. ROC was not found to be toxic, neither after fungal treatment, although dioxin-like activity slightly increased. Regarding non-sterile treatment, ROC3-I bioreactor effluent was not toxic at all whereas ROC3-NI effluent increased its acute toxicity and presented possible antiestrogenic activity. Therefore, although some degradation of emerging contaminants was found at control bioreactor, metabolites produced (from those analysed compounds but also from others present in the wastewater) could be more toxic than those generated during the fungal treatment.

# Chapter 8

## Degradation of PhACs present in veterinary hospital wastewater by *Trametes versicolor*

# INTRODUCTION

In Chapter 7, PhACs and EDCs degradation was reported for a reverse osmosis concentrate (ROC) treatment under non-sterile conditions to study some of the factors affecting the treatment of real effluents by fungi. Only few works treating synthetic or real wastewater under non-sterile conditions can be found in the literature (Cruz-Morató et al., 2013a; Nguyen et al., 2013; Zhang and Geissen, 2012) and they are still far from the optimal operational conditions because some drawbacks, such as bacterial growth and extracellular enzymes washout in continuous treatment, are not yet solved. The aim of the present work was to determine if PhACs degradation was possible under non-sterile conditions for veterinary hospital wastewater (VHW) in batch and continuous mode and to point out some operational parameters that could improve it. Some conclusions from Chapter 7 could be applied to these treatments. However, many of the studies were performed in parallel, without the knowledge of the results of previous experiments. To that respect, the amount of external nutrients added and cellular residence time (CRT) were changed along the treatments in search for the conditions where *T. versicolor* was able to survive and actively degrade the emerging contaminants.

Thus, all experiments in this Chapter were performed under non-sterile conditions in a closer approach to a possible real treatment. Firstly, 1.5 L batch treatment was carried out with its respective non-inoculated bioreactor as a control (VHW1). Then, further operation in a 10 L bioreactor was performed, also operating in batch mode (VHW2). Finally, 1.5 L bioreactors were operated in continuous mode, also with a non-inoculated control bioreactor (VHW3 and VHW4). Results of PhACs and EDCs removal are presented and discussed together with toxicity data and different operational conditions such as changes in nutrient addition rate between VHW3 and VHW4. Quantification of PhACs and EDCs at environmental concentrations was performed by Daniel Lucas and Dr. Meritxell Gros, respectively, at ICRA, supervised by Dr. Sara Rodríguez and Dr. Damià Barceló, under the frame of the joint project CTQ 2010-21776-C02.

Microbial communities of all reactors were also analysed but results are presented in Chapter 9.

# 8.1. Non-sterile batch treatment in 1.5 L bioreactors

That veterinary hospital wastewater (VHW) was though to be quite similar to urban wastewater or human hospital wastewater, as shown by the results presented at Table 8.1. Therefore, as fungal survival tests had already been performed for urban wastewater (Cruz-Morató et al., 2013a) and ROC (Chapter 7) with successful results, *T. versicolor* survival at VHW was assumed and no further tests were performed.

**Table 8.1**. Physicochemical characterisation of veterinary hospital wastewater (VHW).

| Experiment[a] | VHW1 | VHW2 | VHW3[b] | VHW4[b] |
|---|---|---|---|---|
| Sampling data | 09/2012 | 09/2012 | 04/2013 | 07/2013 |
| pH | 9.1 | 10.0 | 7.95 ± 0.15 | 7.9 ± 0.3 |
| Conductivity (mS $cm^{-1}$) | 0.98 | 1.75 | 0.80 ± 0.27 | 2.75 ± 0.91 |
| Chloride (mg Cl $L^{-1}$) | 245 | 418 | 117 ± 89 | 652 ± 289 |
| Sulfate (mg S $L^{-1}$) | 20.4 | 122 | 22 ± 7 | 18 ± 4 |
| Nitrate (mg N $L^{-1}$) | n.d. | 1.5 | 2.4 ± 1.6 | 0.97 ± 0.15 |
| Phosphate (mg P $L^{-1}$) | 2.82 | 12 | 2.7 ± 0.9 | 0.5 ± 0.1 |
| Ammonia (mg N $L^{-1}$) | 2.2 | n.d. | 2.2 ± 1.9 | 3.2 ± 1.8 |
| TSS (mg $L^{-1}$) | 170 | 226 | 88 ± 69 | 97 ± 57 |
| Soluble COD (mg $O_2$ $L^{-1}$) | 270 | 444 | 245 ± 71 | 264 ± 18 |
| DIC (mg $L^{-1}$) | 47 | 47 | 28.2 ± 2.6 | 43 ± 4 |
| DOC (mg $L^{-1}$) | 84 | 139 | 93 ± 28 | 153 ± 31 |

[a] Experiments details can be found at section 4.7.3
[b] Values are the mean of 3-4 samplings during continuous operation

Two parallel 1.5 L bioreactors, one inoculated with *T. versicolor* (VHW1-I) and the other one without fungi as a control (VHW1-NI) were set up to assess fungal biodegradation of PhACs in VHW under non-sterile batch conditions. Glucose and ammonia tartrate were continuously added at theorical consumption rate, as in the Chapter 7 was reported to be necessary because the nutrients present in the effluent were not enough. Results are presented below.

### *8.1.1. Monitoring of bioreactors performance*

In order to evaluate the fungal performance during the treatment, monitoring of the production of laccase and glucose consumption among other parameters were analysed. A peak of laccase activity (around 70 U $L^{-1}$) on the fungal

bioreactor at day 3 was firstly observed but, after 7 days, no activity was detected (Fig. 8.1A), which led us to think that *T. versicolor* could not be active. Initially, glucose was added at 276 mg g $DCW^{-1}$ $d^{-1}$ as previously calculated (Casas et al., 2013) and totally consumed (Fig. 8.1). At day 5 glucose addition was increased to 1453 mg g $DCW^{-1}$ $d^{-1}$ when pellets started to disaggregate in parallel to the laccase activity reduction. However, glucose addition rate was reduced again at day 8 when it was observed that glucose started to accumulate in the media. COD was maintained around 400 mg $O_2$ $L^{-1}$ until day 7, reaching 2930 mg $O_2$ $L^{-1}$ after glucose input was augmented (Fig. 8.1A). However, it was far below the 11560 mg $O_2$ $L^{-1}$ of COD accumulated in the control reactor at the end of the experiment (Fig. 8.1B), which indicates that metabolic products accumulated in it.

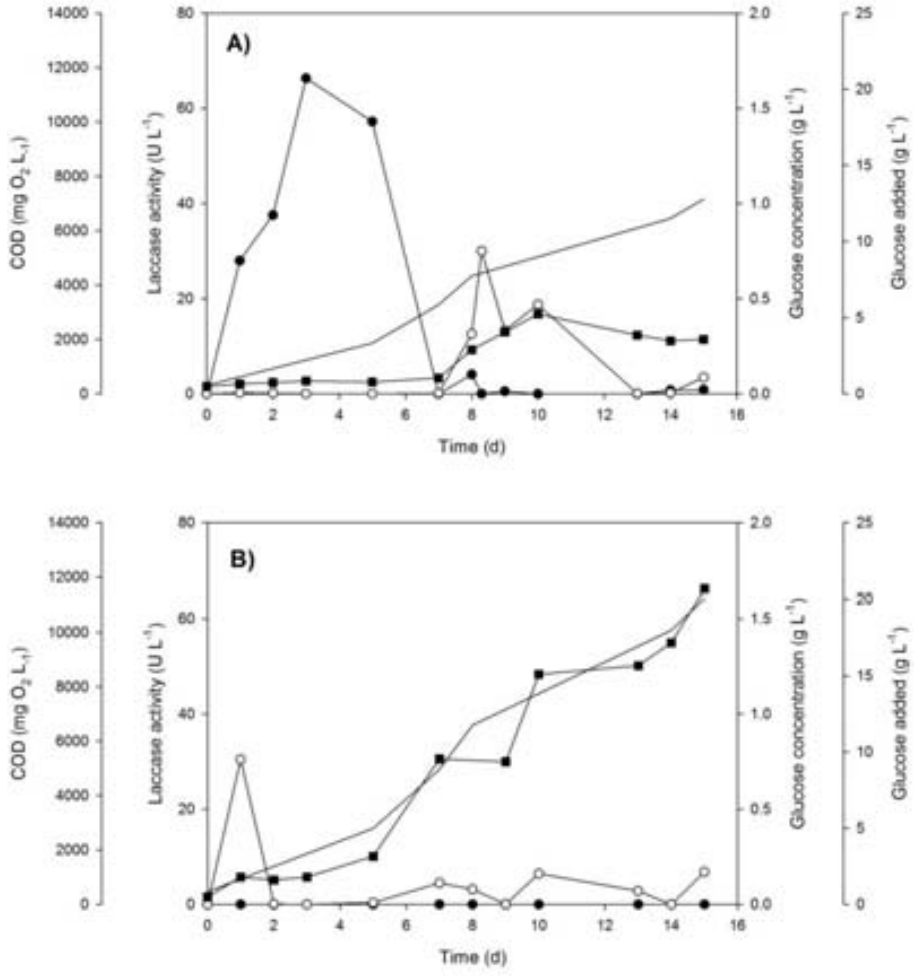



**Figure 8.1.** Profiles of (●) laccase activity, (○) glucose concentration, (■) COD and (—) the sum of glucose added i **A)** *T. versicolor* inoculated batch bioreactor (VHW1-I) and **B)** non-inoculated control batch bioreactor (VHW1-NI).

### *8.1.2. Removal of PhACs*

Regarding PhACs, 76 pharmaceutical compounds and some of their metabolites were analysed in both bioreactors at 3 time points: at the beginning, after 7 days and at the end of the treatment (15 d). Antibiotics, anti-inflammatories and analgesics were the compounds detected at higher concentrations (Fig. 8.2, Initial time), which is in agreement with the type of drugs mostly prescribed at the hospital, according to the veterinary hospital staff. However, although not prescribed for veterinary treatment, psychiatric drugs were also found among the highest concentrations, even though lower than those reported in hospital and urban wastewaters (Cruz-Morató et al., 2014, 2013a; Gros et al., 2012; Santos et al., 2013).

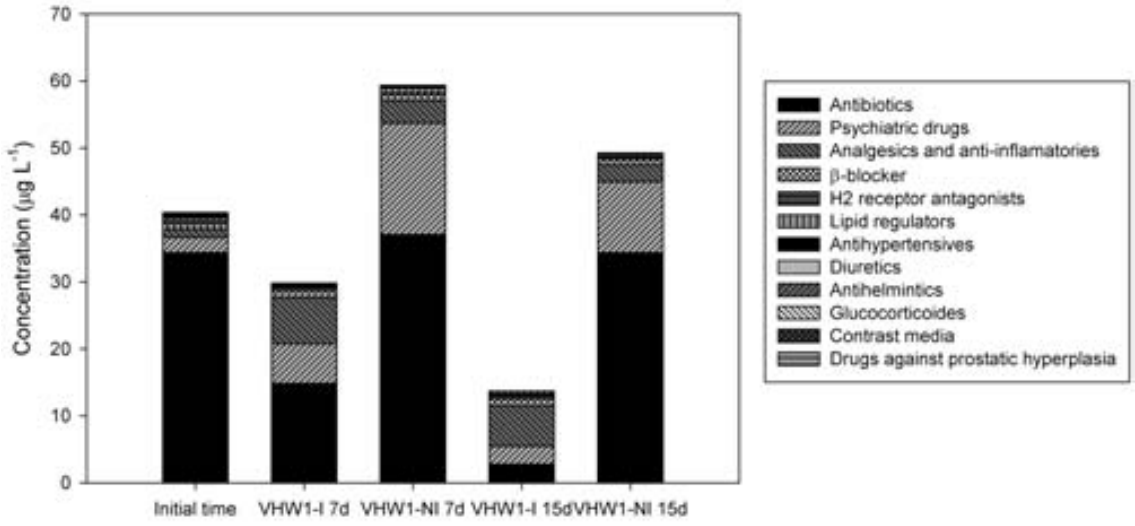



**Figure 8.2.** Evolution of total PhACs grouped by therapeutic classes in the batch bioreactor inoculated with *T. versicolor* (B-I) and in the non-inoculated control bioreactor (B-NI) at 7 and 15 days of treatment in comparison with the initial concentration (the same for both reactors).

Overall antibiotics were found at 34.4 µg $L^{-1}$, in the same order of magnitude than it is reported for human hospitals, with an average value of 11 µg $L^{-1}$. The antibiotics found at highest concentration were cefalexin, ciprofloxacin, metronidazole, trimethoprim and tetracyclin. Ciprofloxacin, metronidazole and trimethoprim are also among those highly detected at hospital wastewaters (Santos et al., 2013; Verlicchi et al., 2010). Analgesics and anti-inflammatories were at lower concentration than expected; they were found at 1.16 µg $L^{-1}$ whereas they are reported to be at concentrations around 100 µg $L^{-1}$ in hospital

wastewaters (Cruz-Morató et al., 2014; Verlicchi et al., 2010), It might be because some highly prescribed veterinary PhACs were not included in the analytical method, such as some morphine derived drugs. Overall PhACs levels were in general lower than those reported for hospital wastewaters (Kovalova et al., 2012; Verlicchi et al., 2010) probably due to the higher use of water (and therefore dilution of wastewater) for cleaning the veterinary facilities and the fact that urine from big animals (i.e. horses) was collected with straw and disposed separately.

Table 8.2 shows initial concentration and removal percentages of detected PhACs. In the VHW1-I reactor, removal of 66% of total analysed compounds was achieved at the end of the treatment, whereas in the control VHW1-NI reactor, no removal was observed (Fig. 8.2). Therefore, fungi constituted an efficient treatment for the removal of some compounds. That is the case of some antibiotics (with an overall removal of 57% after 7 days and until 92% after 14 days at VHW1-I, and negligible removal at VHW1-NI) such as ciprofloxacin, metronidazole and its hydroxilated metabolite, β-blocker carazolol, psychiatric drug diazepam and anti-inflammatory naproxen. Other compounds were equally degraded at VHW1-I and VHW1-NI, among which were the antibiotics cefalexin and tetracyclin, psychiatric drugs sertraline and paroxetine, lipid regulator gemfibrozil, antihypertensive amlodipine and diuretic furosemide. Some PhACs, like the antibiotics dimetridazole, azythromycin and ronidazole and psychiatric drug olanzapine were not statistically removed in any of the reactors. For some compounds, such as anti-inflammatories ketoprofen and piroxicam and β-blockers metoprolol, propranolol and nadolol, their concentration increased higher at VHW1-I bioreactor than at VHW1-NI. Ketoprofen increase (up to 5.7 µg $L^{-1}$ and 2.3 µg $L^{-1}$ after 15 days in VHW1-I and VHW1-NI reactors respectively) even masks degradation of other anti-inflammatories in the overall removal calculation. The concentration of psychiatric drug carbamazepine and its metabolite acridone (detected already up to 1.07 µg $L^{-1}$ initially), as well as lipid regulators atorvastatin and pravastatin and the antibiotic ciprofloxacin increased preferentially at VHW1-NI. Deconjugation or other transformation reactions were postulated as the main factor for the increase of carbamazepine and ketoprofen among other

compounds in the ROC fungal treatment (Chapter 7). Taking into account that those increases also occurred in VHW1-NI, other factors beyond *T. versicolor,* such as transformation by means of bacterial or other fungus might be responsible for it. Finally, only desloratadine was better removed at VHW1-NI than at VHW1-I after 7 days.

**Table 8.2.** PhACs initial concentration and removal at the batch fungal treatment (VHW1-I) and non-inoculated control (VHW1-NI) after 7 and 15 days.

| Therapeutical class | Compound | Co ($\mu g\ L^{-1}$) | SD | % Rem I 7 | % Rem I 15 | % Rem NI 7 | % Rem NI 15 |
|---|---|---|---|---|---|---|---|
| Antibiotics | Cefalexin | 15.941 | 0.468 | 97* | 100* | 98* | 98* |
| | Ciprofloxacin | 9.846 | 0.412 | 75*† | 99*† | -68* | -189* |
| | Metronidazole | 4.387 | 0.268 | -23† | 76*† | -202* | 13 |
| | Trimethoprim | 1.759 | 0.166 | -231* | 37 | -239* | 45 |
| | Tetracyclin | 1.324 | 0.006 | 100* | 100* | 96* | *98** |
| | Metronidazole-OH | 0.612 | 0.032 | 22 | 60* | -3 | 14 |
| | Dimetridazole | 0.192 | 0.138 | *70* | *70* | *70* | *70* |
| | Azyhtromycin | 0.159 | 0.071 | 40 | 47 | 50 | 53 |
| | Ronidazole | 0.123 | 0.007 | 22 | 23 | 19 | 27 |
| | Ofloxacin | 0.030 | 0.003 | 100* | 100* | 100* | 100* |
| | TOTAL | 34.374 | 0.716 | 57*† | 92*† | -8 | 0 |
| Psychiatric drugs | Acridone | 1.071 | 0.068 | -393*† | -73*† | -911* | -497* |
| | Diazepam | 0.244 | 0.018 | 50*† | 88*† | 13 | 46* |
| | Sertraline | 0.240 | 0.012 | 42* | 60* | 57* | 64* |
| | Carbamazepine | 0.238 | 0.011 | 21† | -143*† | -2104* | -1489* |
| | Olanzapine | 0.192 | 0.198 | 88 | *96* | *96* | *96* |
| | Paroxetine | 0.160 | 0.006 | 48* | 64* | 44* | 61* |
| | Lorazepam | 0.060 | 0.002 | 20† | 30* | -45* | 17 |
| | Alprazolam | 0.037 | 0.003 | 18† | 31*† | -26* | 10 |
| | Citalopram | 0.015 | 0.001 | 28 | 57 | -10 | 59 |
| | TOTAL | 2.257 | 0.211 | -163*† | -20† | -637* | -368* |
| Analgesics and anti-inflammatories | Ketoprofen | 0.752 | 0.038 | -740*† | -652*† | -224* | -201* |
| | Naproxen | 0.128 | 0.009 | -25 | 30* | -24 | 25 |
| | Ibuprofen | BQL | 0.001 | *0*† | 100 | -621* | 100 |
| | Tenoxicam | 0.084 | 0.004 | -18 | 6 | -15 | -12 |
| | Acetaminophen | 0.051 | 0.003 | 100*† | 100* | 17* | 100* |
| | Piroxicam | BQL | 0.001 | -315*† | -204*† | *0* | *0* |
| | Oxycodone | 0.032 | 0.003 | -5† | *61* | -116* | *61* |
| | Codeine | BQL | 0.003 | *0* | *0* | 100 | 100 |
| | Hydrocodone | 0.017 | 0.001 | *56* | *56* | *56* | *56* |
| | TOTAL | 1.161 | 0.040 | -484*† | -410*† | -184*† | -115*† |
| β-blockers | Propanolol | 0.335 | 0.003 | -56*† | -63*† | 10 | 13 |
| | Nadolol | 0.160 | 0.001 | -46* | -43*† | -42* | -17 |
| | Carazolol | 0.145 | 0.004 | 100*† | 100*† | -12 | -7 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Metropolol | 0.143 | 0.002 | -90* | -89*† | -91* | -20* |
| | Atenolol | 0.031 | 0.000 | 14 | 10 | 22 | 52 |
| | TOTAL | 0.813 | 0.005 | -29* | -32*† | -21* | -1 |
| H2 receptor antagonists | Desloratadine | 0.435 | 0.047 | 9† | -4 | 64* | 24 |
| Lipid regulators | Gemfibrozil | 0.302 | 0.021 | *97** | *97** | 90* | *97** |
| | Atorvastatin | 0.078 | 0.001 | -41*† | -24*† | -122* | -107* |
| | Pravastatin | 0.032 | 0.002 | 100† | 100† | -1567* | -349* |
| | TOTAL | 0.412 | 0.021 | 71*† | 74*† | -78* | 24* |
| Antihypertensives | Amlodipine | 0.347 | 0.021 | 32* | 53* | 38* | 56* |
| | Irbesartan | 0.036 | 0.005 | 100* | 100* | 100* | 100* |
| | TOTAL | 0.382 | 0.022 | 39* | 57* | 44* | 60* |
| Diuretics | Furosemide | 0.146 | 0.012 | 100* | 100* | 100* | 100* |
| Antihelmintics | Levamisol | 0.091 | 0.011 | 16 | 27 | 3 | 23 |
| | Tiabendazol | BQL | 0.000 | 100* | 100* | 100* | 100* |
| | TOTAL | 0.096 | 0.011 | 21 | 31 | 8 | 27 |
| Glucocorticoides | Dexamethasone | 0.079 | 0.004 | -180*† | -330*† | -29 | 10 |
| Contrast media | Iopromide | BQL | 0.000 | *0* | 100 | 100 | 100 |
| Drugs against prostatic hyperplasia | Tamsulosin | 0.025 | 0.002 | 16 | 37 | 14 | 28 |
| TOTAL | | 40.210 | 0.750 | 26*† | 66*† | -47* | -22* |

In italics removal data calculated with values BQL; * Statistical difference with the entrance value; † Statistical difference with the NI bioreactor

On the other hand, as previously observed by Blánquez et al. (2008), absence of laccase activity does not seem to affect PhACS degradation as there was still a significant rate of degradation between days 7 and 15. Therefore, degradation could be assigned to other enzymes such as cytochrome P450 (Marco-Urrea et al., 2010a) or to other microorganisms growing in the bioreactor.

### *8.1.3. Toxicity*

Veterinary hospital wastewater had very low acute toxicity, with a TU value in the Microtox® analysis of 1.64. After the treatment, VHW1-NI bioreactor had a toxicity of 0.66 TU whereas the effluent after fungal treatment had no toxicity at all, which means that *T. versicolor* was able to degrade toxic compounds present in the water and no toxic metabolites were produced or, at least, not at significant concentrations.

## 8.2. Non-sterile batch treatment in 10 L bioreactor

A 10 L bioreactor was set up for treating veterinary hospital wastewater in batch mode (VHW2 experiment). As bioreactor was larger, more frequent samples were planned to be taken in order to have a better monitoring of the removal of PhACs and also EDCs.

### *8.2.1. Monitoring of bioreactors performance*

In that treatment, laccase activity achieved during the experiment was higher than at 1.5 L bioreactor, reaching more than 400 U $L^{-1}$ (Fig. 8.3). After 10 days it started to decrease but, in contrast to VHW1-I, some laccase activity was recorded during all the treatment. Ammonium tartrate was constantly added at 0.74 mg g $DCW^{-1}$ $d^{-1}$ and glucose at 330 mg g $DCW^{-1}$ $d^{-1}$ and totally consumed after a short period of small accumulation. When glucose was totally consumed, COD was maintained around 1000 mg O2 L, again higher than the initial COD of VHW, confirming the inability of *T. versicolor* to remove COD of treated wastewaters.

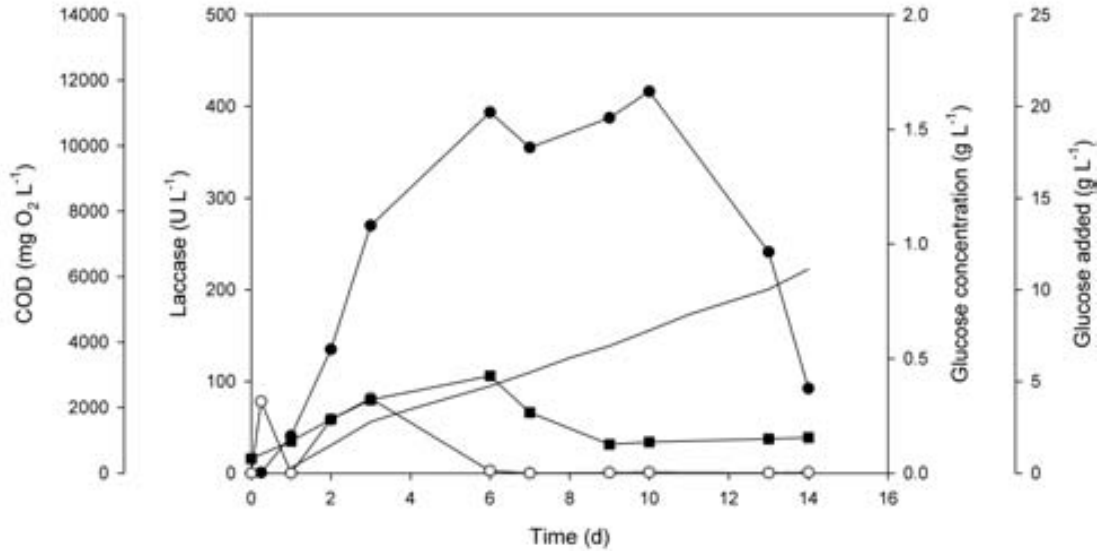



**Figure 8.3.** Profiles of (●) laccase activity, (○) glucose concentration, (■) COD and (—) the sum of glucose added at 10 L fungal batch bioreactor (VHW2).

### *8.2.2. Removal of PhACs*

The VHW sampled for VHW2 experiment was found to have a lower amount of analyzed PhACs. As shown at Fig. 8.4, overall PhACs concentration was only 1.9 µg $L^{-1}$. Psychiatric drugs, antibiotics and analgesics and anti-inflammatories were those detected at higher concentrations as well as in the VHW1 sampling. In the present experiment, total removal of norfluoxetine, ibuprofen, dimetridazole, carazolol, glibenclamide and valsartan was obtained. However, increase of overall PhACs concentration was observed during the treatment, mainly due to the increase of the antibiotic ciprofloxacin and the analgesic and anti-inflammatory ketoprofen (see Table 8.3 and Fig. 8.5). Ketoprofen and ciprofloxacin have been shown to be degraded by *T. versicolor* in spiked experiments (Marco-Urrea et al., 2010c; Prieto et al., 2011). However, as discussed in the section 7.1.3, when treating real effluens, transformations to the parent compound from related compounds (i.e. deconjugations) might have an important role. Therefore, despite *T. versicolor* was totally active as shown by laccase activity (Fig. 8.3), when contaminants concentration is lower, increase of some PhACs totally masks the degradation of the other compounds.

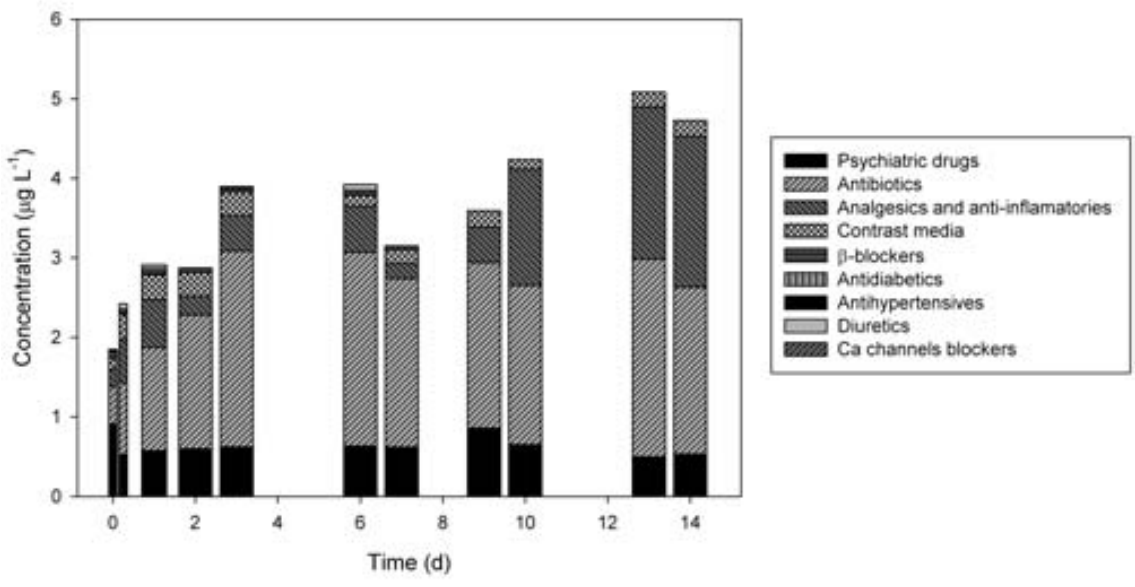



**Figure 8.4.** Evolution of total PhACs grouped by therapeutic clases in the 10 L batch bioreactor inoculated with *T. versicolor* (VHW2).

**Table 8.3.** PhACs initial concentration and removal at the 10 L batch fungal treatment (VHW2) after 7 and 14 days.

| Therapeutical class | Compound | $C_o$ (µg $L^{-1}$) | SD | % Rem 7d | % Rem 14d |
|---|---|---|---|---|---|
| Psychiatric drugs | Norfluoxetine | 0.315 | 0.033 | 100* | 100* |
| | Paroxetine | 0.265 | 0.030 | -5 | -9 |
| | Diazepam | 0.211 | 0.010 | 75* | 67* |
| | Olanzapine | 0.080 | 0.004 | -13 | -60* |
| | Fluoxetine | 0.039 | 0.004 | -405* | -23 |
| | TOTAL | 0.910 | 0.046 | 32* | 42* |
| Antibiotics | Azythromycin | 0.137 | 0.004 | 4 | -7 |
| | Metronidazole | 0.119 | 0.021 | -166* | -48 |
| | Ciprofloxacin | 0.117 | 0.014 | -1256* | -1066* |
| | Ofloxacin | 0.099 | 0.014 | 14 | -317* |
| | Dimetridazole | BQL | 0.000 | 100* | 100* |
| | TOTAL | 0.542 | 0.029 | -291* | -287* |
| Analgesics and anti-inflammatories | Ibuprofen | 0.135 | 0.004 | 100* | 100* |
| | Diclofenac | 0.078 | 0.004 | 47* | 35* |
| | Ketoprofen | BQL | 0.000 | -2350* | -30717* |
| | TOTAL | 0.219 | 0.006 | 14 | -768* |
| Contrast media | Iopromide | 0.136 | 0.008 | -30 | -46 |
| β-blocker | Carazolol | 0.047 | 0.006 | 2 | 100* |
| Antidiabetic | Glibenclamide | 0.036 | 0.002 | 100* | 100* |
| Antihypertensive | Valsartan | 0.034 | 0.004 | 100* | 100* |
| Ca channels blocker | Diltiazem | BQL | - | *0* | 100* |
| Diuretic | Torasemide | 0.000 | 0.000 | increase | n.a. |
| TOTAL | | 1.928 | 0.056 | -64* | -145* |

In italics removal data calculated with values BQL; n.a.: not apply; * Statistical difference with the entrance value ($p<0.01$).

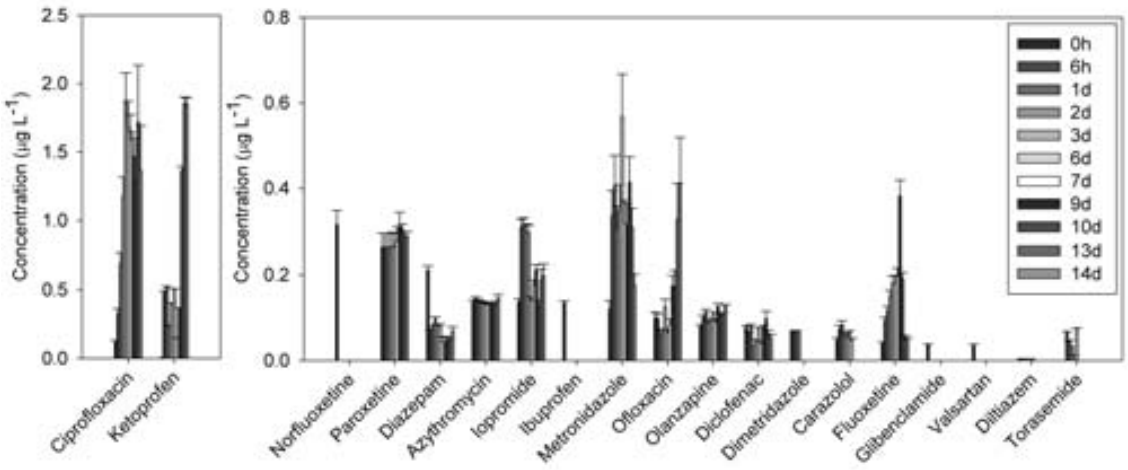



**Figure 8.5.** Evolution of individual PhACs at VHW2 treatment.

### *8.2.3. Removal of EDCs*

27 EDCs and related compounds were analysed for their presence in VHW and their removal after fungal treatment. As shown at Fig. 8.6, almost all analysed compounds were BLQ except for the caffeine. Therefore, VHW effluent was considered to have low concentration of EDCs and they were not further analysed in the continuous treatment.

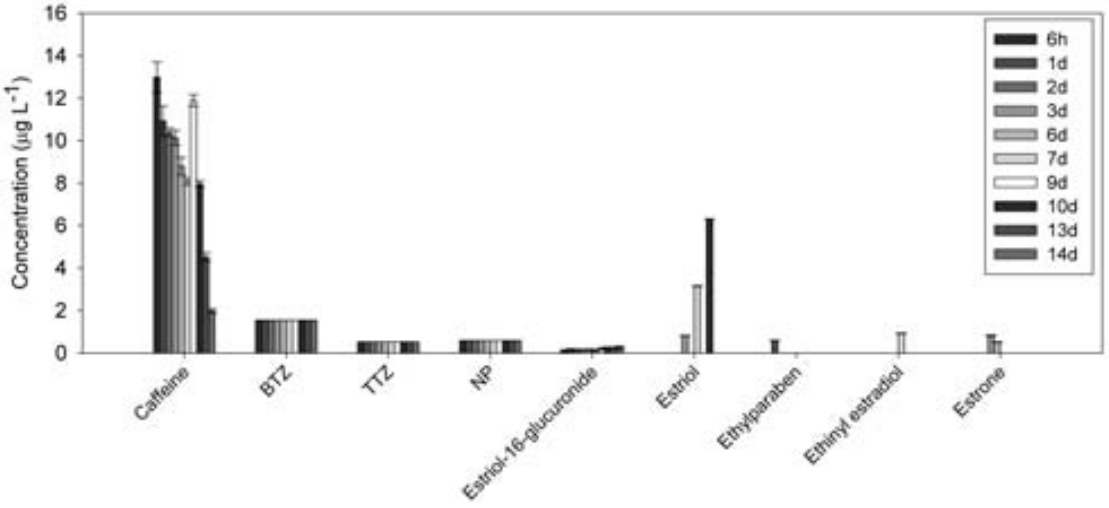



**Figure 8.6.** Evolution of individual EDCs at VHW2 treatment.

### *8.2.4. Toxicity*

Although PhACs detected at VHW2 were lower than at VHW1, toxicity of VHW sampled for VHW2 was higher than the previous one. Therefore, acute toxicity was due to some unidentified contaminant. Initial TU obtained with Microtox analysis were 25. However, after 14 days of fungal treatment, acute toxicity of the wastewater was reduced to 1.2 TU, meaning that fungus was able to degrade that or those compounds more toxic. Those results highlight the importance of monitoring toxicity as well due to the limitation of analytical methods.

## 8.3. Non-sterile continuous bioreactors

### *8.3.1. Bioreactors operation and monitoring*

Results obtained in batch experiments, encouraged us to further treat veterinary hospital wastewater in continuous mode also under non-sterile conditions. The

same setting of VHW1, with two parallel 1.5 L bioreactors, one inoculated with pellets of *T. versicolor* (I) and the other one non-inoculated as control (NI), was performed.

Firstly, VHW3 experiment was carried out with the aim of studying the degradation of PhACs already present in the VHW by the fungus and to evaluate the survival of the fungus and trying to find some operational parameters that could improve fungal performance. As continuous bioreactors were operated during almost one month, partial biomass renewal was applied. Therefore, cellular residence time (CRT) was maintained at 15 days as it was previously calculated (Blánquez et al., 2006) while two HRT (3d and 2d) were studied. Glucose and ammonium tartrate addition rates were 200 and 0.45 mg g $DCW^{-1}$ $d^{-1}$, respectively, during all the treatment, as Casas et al. (2013) calculated to be the optimal amount and ratio for *T. versicolor* treatment.

As shown at Fig. 8.7, glucose was totally consumed at fungal bioreactor (I) whereas at control bioreactor (NI) it accumulated in the media during the first 5 days and also between days 11 and 13 when pH increased until 8 due to malfunctioning of the controller (data not shown). Thus, COD values were also higher at NI reactor, reaching 3.4 g $O_2$ $L^{-1}$ at HRT 3d and decreasing to 1.0-1.5 g $O_2$ $L^{-1}$ at HRT 2d. COD at I bioreactor at HRT 2d was only of 118 mg $O_2$ $L^{-1}$. Extracellular enzymes activity has been used as an indicative of fungal activity, although some works refute the need of them for the achievement of good removal percentages (Blánquez et al., 2004; Yang et al., 2013a). Laccase activity (Fig. 8.7A) was not maintained stable along the time, presenting peaks of activity after fungal partial renewal, with a maximum of 215 U $L^{-1}$ at day 6. Additionally, at HRT of 2 days, laccase activity was most of the time below 1 U $L^{-1}$, as happened at ROC3 experiment as well (see section 7.5.1). Therefore, at a first glance, results can suggest that lowering HRT from 3 to 2 days was detrimental for extracellular laccase activity and some washing out could be taking place or, on the other hand, we were unable to maintain *T. versicolor* active until the end of the experiment.

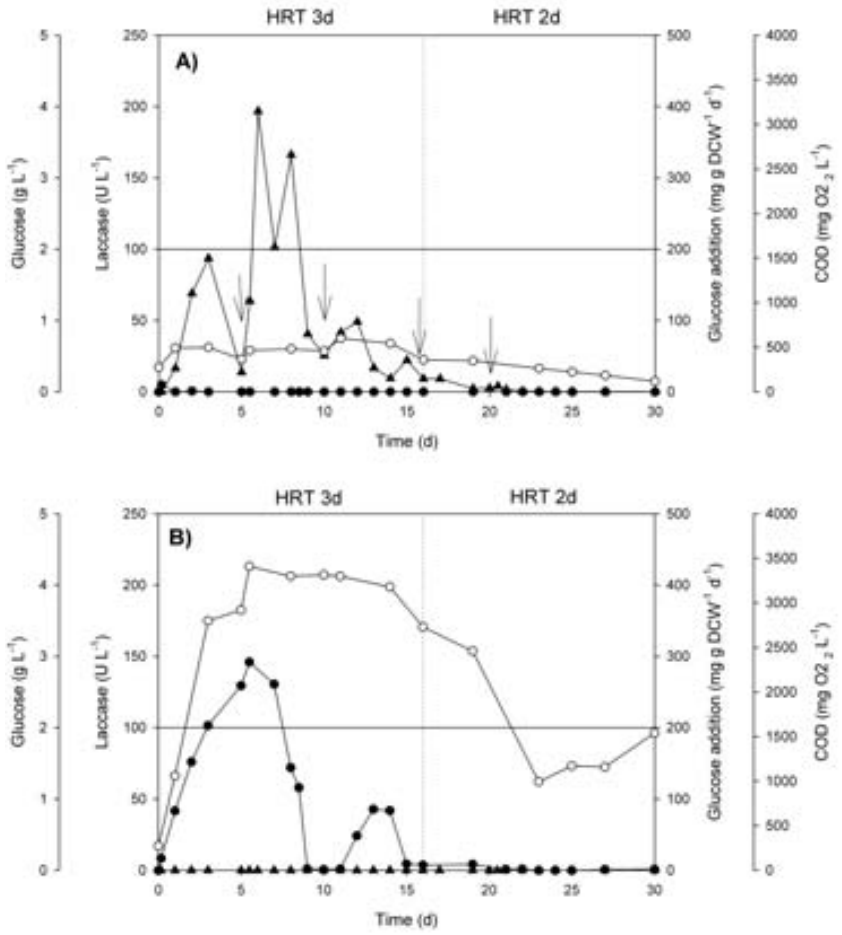



**Figure 8.7.** Evolution at **A)** non-sterile fungal inoculated continous reactor (VHW3-I) and **B)** non-sterile non-inoculated control reactor (VHW3-NI) of (●) glucose concentration, (▲) laccase activity and (○) COD. Arrows shows the days where 1/3 old fungal biomass was changed by fresh one. Dotted vertical line marks the change of HRT from 3 to 2 days. Continuous line shows the gluccose addition rate.

Visual aspect of bioreactors biomass changed along the time. As can be seen at Fig. 8.8, at VHW3-NI between days 23 and 29 a black biofilm was developed in the bioreactor inner walls. Fungal pellets of VHW3-I became also black at the end of the treatment, what made us to suspect the failure of *T. versicolor* to succeed against contamination.

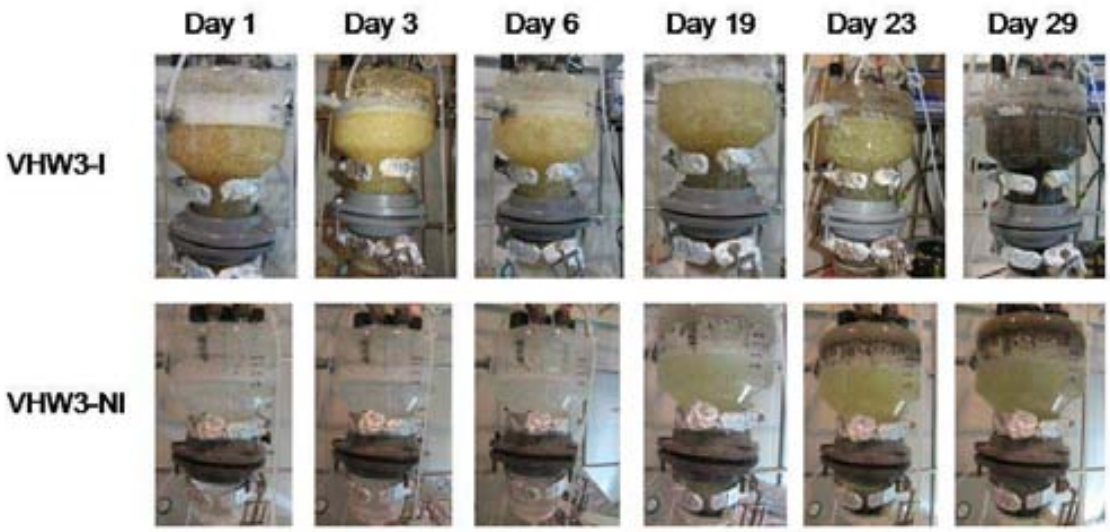



**Figure 8.8.** Visual evolution of I and NI bireactors of VHW3 treatment

Then, as VHW3 seemed to have been not very successful, another experiment was set up for treating VHW at non-sterile conditions under continuous operational mode (VHW4) but with some modifications. There were also two 1.5 L bioreactors in parallel: the fungal inoculated (I) and the control (NI) ones. In that case, HRT was maintained at 3.3 d to avoid it as a variable for simplifying results interpretation. Taking into account the results of VHW3 experiment, nutrient addition rate was increased to 343 mg glucose $g^{-1}$ $d^{-1}$ after realizing that those values previously calculated under sterile conditions were not enough for *T. versicolor* survival under non-sterile effluents. However amount of nutrients supplied and also CRT were changed along the experiment (Fig. 8.9) according to monitoring results (laccase activity, glucose consumption and fungal dry weight among others) and visual evolution of bioreactors. Therefore, addition of glucose was increased until 1040 mg $g^{-1}$ $d^{-1}$ at day 9 trying to avoid fungal lysis (Fig. 8.9C). However, fungal biomass was not stabilized until the addition of a pulse of 4.9 g of ammonia tartrate at day 12 and the following decrease in the C/N ratio in the nutrient stock after the day 19, when ammonia from the pulse was depleted. Ammonia tartrate amount used for the pulse and C/N ratio in the feeding nutrient stock corresponded to the one used when working in synthetic media, as some authors pointed out that higher nitrogen stimulated fungal growht in detriment of bacterial one (Rousk and Bååth, 2007). Therefore, glucose was added at 480-1040 mg $g^{-1}$ $d^{-1}$ and ammonia tartrate at 217-433 mg $g^{-1}$ $d^{-1}$ from day 19 onward.

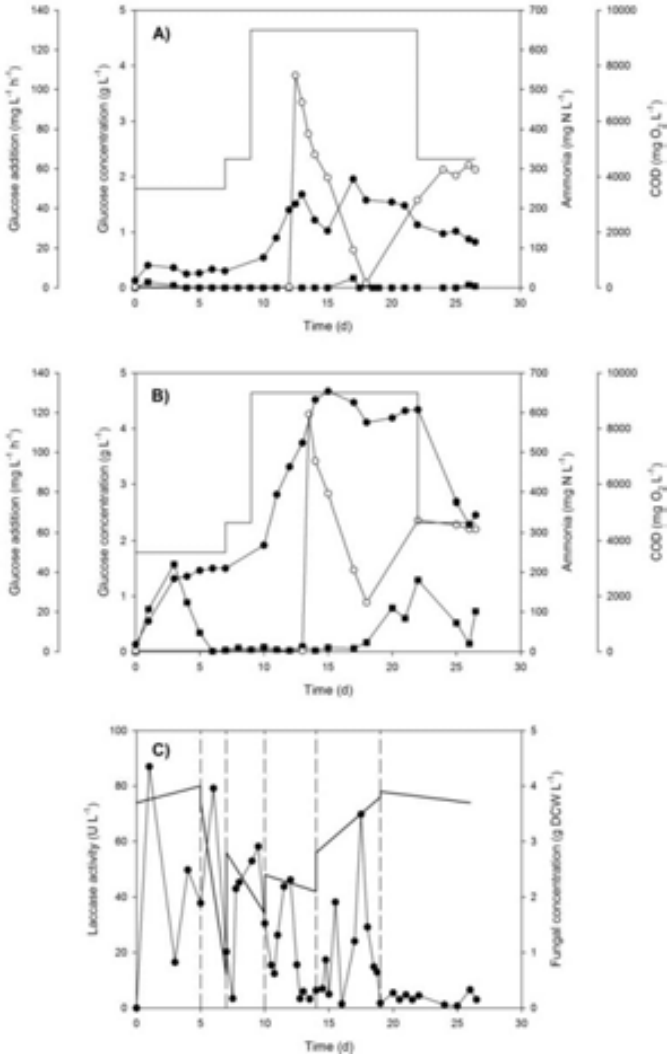



**Figure 8.9.** Profiles of (●) soluble COD, (○) ammonia concentration, (■) glucose concentration and (—) glucose addition rate at **A)** *T. versicolor* inoculated continouous bioreactor (VHW4-I) and **B)** non-inoculated control continuous bioreactor (VHW4-NI). **C)** Profiles of (●) laccase activity and (—) fungal pellets concentration at *T. versicolor* inoculated continouous bioreactor (VHW4-I). Vertical dashed lines mark renewal points of 1/3 of the old pellets by fresh biomass.

As shown at Fig. 8.9, glucose was totally consumed at fungal bioreactor (I), even when higher supply was provided, and accumulated in the media during the first 3 days of operation at control bioreactor (NI) until microbial contamination inside the reactor was grown and also at the end of the treatment, when C/N ratio of nutrients was changed. Therefore, COD was also higher at NI reactor, reaching almost 10 g $O_2$ $L^{-1}$, than at I reactor because of the accumulation of metabolic products in the media. However, COD was also higher in the bioreactors effluent than in the initial wastewater, what means that

*T. versicolor* treatment cannot remove wastewater COD as already reported Cruz-Morató et al. (2013), being the fungal treatment more suitable as a pre-treatment at source-point, as already established in many industrial effluents, than as a final process.

Laccase activity oscillated between 0 and 90 U $L^{-1}$ until day 19 (Fig. 8.9C). From that moment onwards, laccase activity remained below 10 U $L^{-1}$, despite being the period when fungal pellets biomass was maintained, confirming the decoupling between fungal degradation and production of extracellular enzymes (Blánquez et al., 2008). Whashing out of laccase at low HRT as pointed out before was also discarded. Therefore, at VHW3 and ROC3 experiments, laccase activity might be more related with pellets aging despite biomass renewal due to an insuficient turnover ratio or the lack of the optimal conditions for the longer survival of *T. versicolor*.

At Fig. 8.10, it is clearly shown how *T. versicolor* pellets lysed at day 6 of VHW4-I treatment but it was recovered probably due to the change in the nutrient addition. Later, bioreactor media became brownish for the production of melanin-like pigments due to the higher supply of nutrients in that reactor from day 10 (Song, 1999; Temp and Eggert, 1999). VHW4-NI bioreactor became more and more whitish and cloudy as the treatment progressed.

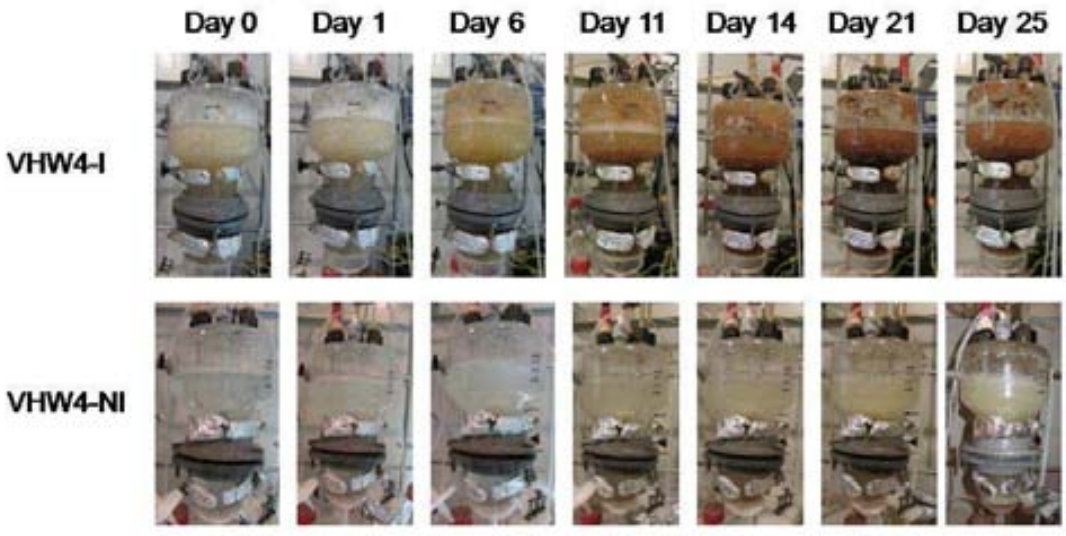



**Figure 8.10.** Visual evolution of I and NI bireactors of VHW4 treatment

### *8.3.2. Degradation of pharmaceuticals*

PhACs were not analysed for VHW3 treatment due to the suspicion of poor fungal performance; the main purpose was only to compare the microbial analysis with those of VHW4 (Chapter 9).

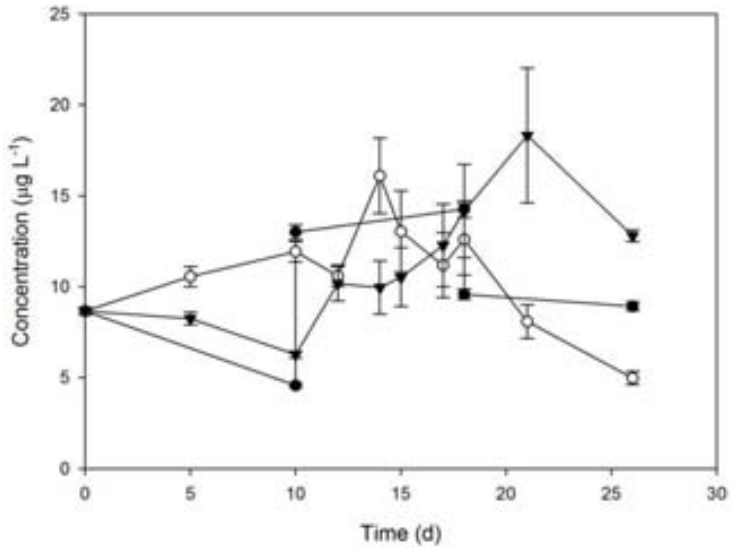



**Figure 8.11.** Total PhACs concentration in the (●) feed wastewater and the effluent of (○) VHW4-I and (▼) VHW4-NI.

Regarding PhACs degradation at VHW4, statistical significant removal was detected at the last part of the continuous treatment (from day 21 to 26), when removal in VHW4-I bioreactor was 44% whereas in the VHW4-NI global concentration of PhACs increased (Fig. 8.11). It is noteworthy to highlight that no stationary state was achieved because different nutrient additions were being tested during the process to determine favorable conditions for fungal survival. Therefore, removal data for continuous treatments were calculated at the best performance point of fungal bioreactors, which was at the end of the operation (26 days). However, the decreasing tendency in PhACs concentration at VHW4-I can be observed already from the 14$^{th}$ day, just after ammonia pulse, when lysis of *T. versicolor* was stopped, highlighting the relationship of *T. versicolor* with the PhACs degradation. Analgesics and anti-inflammatories were the PhACs detected at higher concentration in the samplings for continuous treatment (Table 8.4). Concentration decrease after ammonia addition can be observed as well in their profile (Fig. 8.12). However,

concentration in the VHW4-NI bioreactor was always lower than VHW4-I except for the last point. That profile is dominated by salycilic acid, the compound detected at highest concentration. Regarding antibiotics, their concentration was much lower than in the previous sampling campaign for batch bioreactors. The compounds detected at higher concentrations were metronidazole and its hydroxylated metabolite, mainly in the wastewater fed during days 18 to 26. When concentration in the entrance was lower (days 0-18), no statistically significant degradation was observed neither in VHW4-I nor VHW4-NI bioreactors. However, after the day 21, around 40-50% degradation was detected for VHW4-I bioreactor whereas an increase in the concentration was observed in VHW4-NI.

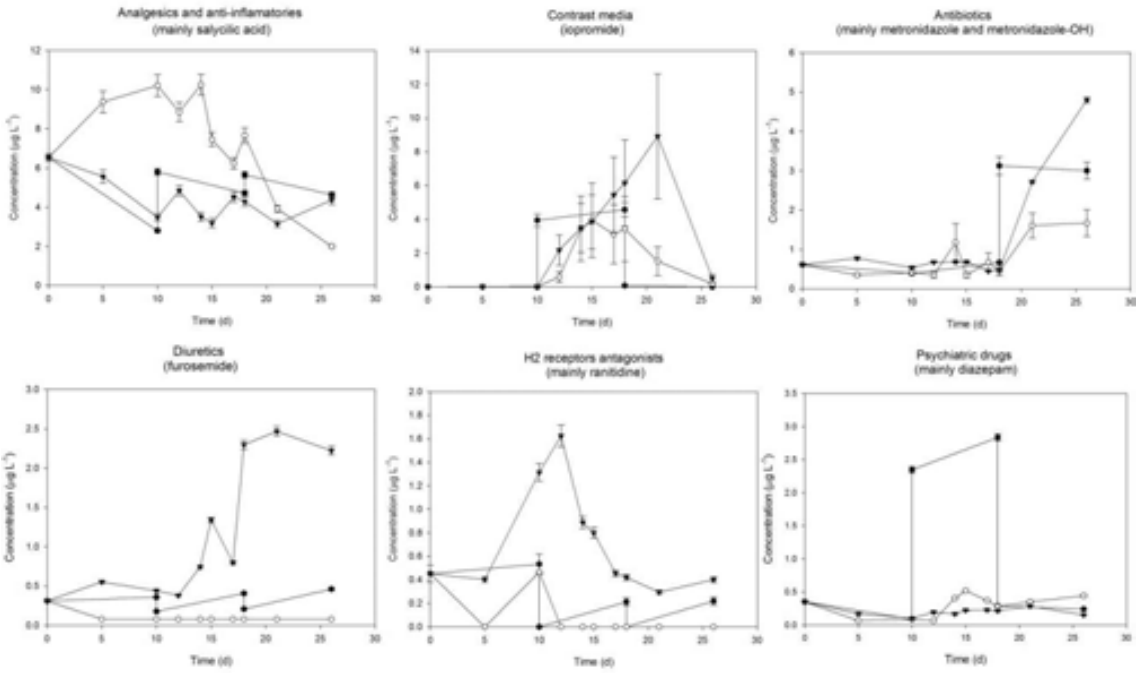



**Figure 8.12.** PhACs concentration grouped by families present at higher concentrations in the (●) entrance and the effluent of (○) VHW4-I and (▼) VHW4-NI.

The low biodegradable contrast media iopromide was only detected in the entrance wastewater from day 10 to 18. The concentration of iopromide in the effluent of VHW4-I and VHW4-NI increased progressively from the day 10 to 15, followed by a decrease in VHW4-I due to fungal degradation whereas in VHW4-NI it continued to increase (Fig. 8.12). Psychiatric drugs had the same concentration profile in the entrance as iopromide, with a peak of aproximately

2.5 µg $L^{-1}$ between days 10 and 18 due to the increase of diazepam in the veterinary hospital wastewater. That increase was not observed in the effluent of the reactors, which shown a diazepam concentration of around 0.1-0.3 µg $L^{-1}$. However, diazepam degradation can not be assured as adsoption in the sampling system was observed previously (Chapter 7.3). Carbamazepine was never detected in the fed wastewater, however it was detected in the effluent of the bioreactors due to deconjugation reactions or other processes as previously described (Cruz-Morató et al., 2013a) combined with low degradation rates (Kovalova et al., 2012).

**Table 8.4.** Fresh and old wastewater entrance concentrations of PhACs in the last period of the continuous treatment and removal at the fungal treatment (VHW4-I) and non-inoculated control (VHW4-NI). As global stationary state was not reached due to the change in nutrients addition despite of working at hydraulic stationary state, removal percentages were calculated with the concentrations at 26 days with respect to entrance concentrations at that day.

| Therapeutic class | | Fresh 18 d | | Old 26 d | | % Rem. | % Rem. |
|---|---|---|---|---|---|---|---|
| | | C (µg $L^{-1}$) | SD | C (µg $L^{-1}$) | SD | C-I | C-NI |
| Analgesics and anti-inflammatories | Salycilic acid | 4.591 | 0.154 | 3.730 | 0.125 | 81*† | 19* |
| | Ketoprofen | 0.419 | 0.037 | 0.320 | 0.028 | -57 | -95 |
| | Ibuprofen | 0.351 | 0.029 | 0.212 | 0.018 | 30* | 36* |
| | Piroxicam | 0.154 | 0.003 | 0.136 | 0.002 | -59*† | -17 |
| | Diclofenac | 0.102 | 0.004 | 0.123 | 0.004 | -177*† | -122* |
| | Naproxen | BQL | 0.000 | 0.085 | 0.002 | *71*† | -13* |
| | Indomethacine | BQL | 0.000 | 0.034 | 0.000 | -79*† | *59** |
| | Acetaminophen | ND‡ | 0.000 | ND‡ | 0.000 | n.a.† | *Incr.** |
| | TOTAL | 5.655 | 0.161 | 4.640 | 0.129 | 57*† | 6 |
| Antibiotics | Metronidazole | 1.718‡ | 0.140 | 1.736‡ | 0.142 | 40*† | -56* |
| | Metrondiazole-OH | 1.410‡ | 0.180 | 1.222‡ | 0.156 | 49† | -70* |
| | Ciprofloxacin | ND | 0.000 | 0.042 | 0.001 | 100* | *54* |
| | TOTAL | 3.127‡ | 0.228 | 3.000‡ | 0.211 | 44*† | -60* |
| Psychiatric drugs | Diazepam | 0.289 | 0.006 | 0.236 | 0.005 | 83*† | 63* |
| | Citalopram | ND | 0.000 | BQL | 0.000 | *0* | *0* |
| | Carbamazepine | ND‡ | 0.000 | ND‡ | 0.000 | Incr.*† | Incr.* |
| | Olanzapine | ND‡ | 0.000 | ND‡ | 0.000 | n.a.† | *Incr.** |
| | TOTAL | 0.289 | 0.006 | 0.240 | 0.005 | -84*† | 37* |
| Diuretics | Furosemide | 0.208 | 0.007 | 0.462 | 0.015 | *83*† | -381* |
| Lipid | Fluvastatin | 0.043‡ | 0.001 | 0.044‡ | 0.001 | -64*† | -10 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| regulators | Gemfibrozil | 0.040 | 0.000 | 0.035 | 0.000 | *55**† | -6 |
| | Atorvastatin | 0.030‡ | 0.001 | 0.030‡ | 0.001 | -8 | 1 |
| | TOTAL | 0.113 | 0.001 | 0.110 | 0.001 | -11 | -6 |
| Antiplatelette agents | Clopidrogel | 0.112 | 0.002 | 0.247 | 0.004 | 100*† | 78* |
| Contrast media | Iopromide | 0.071 | 0.007 | ND | 0.000 | Incr. | Incr.* |
| Antihelmin-tics | Albendazole | BQL | 0.000 | 0.008 | 0.001 | 45*† | -142* |
| Antihyper-tensives | Amlodipine | ND‡ | 0.000 | ND‡ | 0.000 | Incr.*† | Incr.* |
| H2 receptor antagonists | Ranitidine | ND | 0.000 | 0.219 | 0.035 | 100*† | -83* |
| TOTAL | | 9.577 | 0.279 | 8.926 | 0.250 | 44*† | -43* |

In italics removal data calculated with values BQL; * Statistical difference with the entrance value; † Statistical difference with the NI bioreactor; ‡ No statistical difference in PhACs concentration between fresh and old wastewater effluent; Inc.: increase in the concentration

Diuretic furosemide was highly removed in the fungal bioreactor (83% at 26d) whereas its concentration increased in the VHW4-NI reactor in the second half of the treatment. The H2 receptors antagonist ranitidine was never detected in the fresh water but its concentration increased in the storage tank and also in the VHW4-NI bioreactor where some deconjugations could occur. However in the VHW4-I bioreactor it was totally removed. Other changes in PhACs concentration were detected between fresh and old wastewater in the storage tank (Table 8.4). In the antihypertensives amlodipine and irbesartan, similar deconjugation processes might occur.

Taking into account removal percentages at day 26 (Table 8.4), PhACs can be grouped as compounds better removed in VHW4-I than in VHW4-NI (i.e. salicylic acid, metronidazole, diazepam, furosemide, clopidrogel and ranitidine), equally removed in both reactors (ibuprofen) and not statistically significant removed in any of them (ketoprofen). Among whose concentration increased, piroxicam, diclofenac and carbamazepine increased more at VHW4-I and metronidazole-OH increased more at VHW4-NI.

### *8.3.3. Toxicity*

Acute toxicity was analysed by Microtox® and toxicity of feed wastewater for VHW3 was between 1 and 3 TU. TU for VHW4 veterinary hospital fresh wastewater were 10, 11 and 20 for samples at initial time, 10 d and 18 d respectively. Old wastewater stored in the storage tank decreased their toxicity to 1-2 TU, denoting some degradation activity in the storage tank of feed wastewater. As shown at Table 8.5, toxicity in the reactors at days between 10 and 13 was also 1-2 TU while at the end of the operation, after 24-30 days, acute toxicity was totally removed.

**Table 8.5**. Acute toxicity by Microtox analysis of VHW3 and VHW4 treatments expressed as toxic units (TU).

| | VHW3 | | VHW4 | |
|---|---|---|---|---|
| | I | NI | I | NI |
| Influent wastewater | 1-3 | | 1-20 | |
| Effluent 10-13 d | 3 | 1 | 1 | 2 |
| Effluent 24-30 d | 0 | 2 | 0 | 0 |

However all of the toxicity values were below the value of 25 TU when an effluent is considered as toxic (Generalitat de Catalunya, 2003).

# CONCLUSIONS

Non-sterile batch treatment of veterinary hospital wastewater with *T. versicolor* reached 66% removal of PhACs after 15 days of operation. In continuous treatment at HRT of 3 days, 44% removal was achieved when nutrients were increased with respect to the previously calculated for sterile conditions. Therefore, further optimisation on nutrient requirements under non-sterile conditions is needed, although promising results arise from the present work.

Laccase activity decreased after 7 days at batch reactor and after 15-19 days at continuous reactor despite partial biomass renewal in the latter. Taking into account that high degradation was still achieved at those periods, laccase might not be a good indicator of fungal activity as Blánquez et al. (2004) and Yang et al. (2013) already postulated.

In the VHW studied, the analysed EDCs were at low concentrations, being most of them BLQ in all samples. PhACs detected at higher concentrations were antibiotics, analgesics and anti-inflammatories and psychiatric drugs. Contrast media iopromide was also occasionally found at high concentration. Metronidazole and diazepam, that are two examples of hardly biodegradable PhACs, are present at all sampled wastewaters at considerable concentration and better removed by the fungal bioreactor in both treatments (batch and continuous).

It has been shown that fungal degradation was less effective when initial PhACs concentration was lower because deconjugation of some compounds masked the degradation of others. Moreover, no clear pattern for PhACs degradation was observed among the different treatments applied due to the variability that deconjugation and other transformation processes imply in real effluents.

Taking into account the above mentioned results, further experiments should be focused in optimising the amount and the C/N ratio of the nutrients supplied, considering that our preliminary results pointed out that a decrease in the C/N

ratio favoured the removal of PhACs. Change in the operational conditions might also help to reduce the COD after the treatments. However, from the results obtained in this thesis and as Cruz-Morató et al. (2013) already pointed out, fungal treatment would be more suitable as a pre-treatment because *T. versicolor* is not able to remove the COD of wastewater.

# Chapter 9

## Bacterial and fungal communities assessment during *Trametes versicolor* treatment of wastewater effluents

# INTRODUCTION

In Chapters 7 and 8, non-sterile fungal treatments of reverse osmosis concentrate (ROC) and veterinary hospital wastewater (VHW) have been presented. One of the main drawbacks of working in non-sterile conditions is the growing of other microorganisms that could compete with the inoculated fungus and even overtake and kill it. However, little is known about fungi and bacteria interactions in liquid media (Weber et al., 2007; Yang et al., 2011), as the vast majority of studies are in soils (Mikesková et al., 2012; Rousk and Bååth, 2007). Moreover, some operational parameters, such as nutrients addition, were modified during some experiments trying to find the proper conditions for the survival of *Trametes versicolor* and hence, different results were obtained regarding PhACs and EDCs removal in the different reactors. In this Chapter, results of diverse molecular analyses performed in order to characterise the microbial populations are presented. So, the main aim of the study was to determine the microbial communities that developed in the bioreactors and try to correlate micropollutants removal and fungal survival with the operational parameters or the data from traditional monitoring methods (laccase activity, glucose consumption, visual aspect and so on).

Firstly, denaturing gradient gel electrophoresis (DGGE) analyses of VHW batch treatments (experiments VHW1 and VHW2) were performed in a research stay at Centre of Biological Engineering (University of Minho) supervised by Dr. Alcina Pereira and Dr. Madalena Alves. Some clone libraries of bacterial 16S rDNA and fungal ITS rDNA were generated for certain samples, which allowed the subsequent obtention of long sequences for a good phylogenetic study. With the results obtained, continuous treatments were planned.

Then, after performing the experiments in non-sterile continuous operation (VHW3, VHW4 and ROC3), bacterial and fungal communities assessment of these treatments was performed in another research stay at the Finnish Forest Research Institute (METLA) under the supervision of Dr. Taina Pennanen and Dr. Hannu Fritze. In that case, a broader array of molecular techniques was performed: i) phospholipid fatty acids analysis (PLFA) to determine the

variations in the global community of the liquid samples; ii) real-time PCR (qPCR) to obtain the amount of *Trametes versicolor* in relation to all the fungi in liquid samples but also in the fungal pellets; and iii) DGGE analysis and further sequencing of the bands to perform a phylogenetic study of the bacteria and fungi present during the treatments. Finally, statistical analysis of Principal Component Analysis (PCA) and Detrended Correspondence Analysis (DCA) were performed for the results of PLFA and DGGE, respectively, in order to try to find some grouping of the samples or correlation with some operational parameters.

# 9.1. Microbial community assessment of veterinary hospital wastewater batch treatments

DGGE analysis of PCR-amplified bacterial 16S rDNA and fungal ITS fragments were performed for samples of the batch treatments of VHW (VHW1 and VHW2). As explained in Chapter 8, VHW1 consisted of two parallel 1.5 L bioreactors, one inoculated with *T. versicolor* (I) and the other one non-inoculate as control (NI). VHW2 experiment consisted of a batch fungal treatment in a 10 L bioreactor. Samples for DGGE were taken before (at initial time, bacteria) or after (day 1, fungi) inoculation of *T. versicolor*, after 7 days and at the end of the treatment (14 or 15 days) from both I and NI reactors. The obtained DGGE profiles are shown in Fig. 9.1. As can be seen at a first glance, *T. versicolor* (bands F10 and F17) seems to lose predominance in favour of other fungi after 15 days of operation at VHW1 treatment whereas it is still detected at the end of the VHW2 experiment. Moreover, bacterial and fungal diversity is generaly lower in the non-inoculated reactors or when *T. versicolor* was not predominant, as bacterial profiles of NI and I at the end of VHW1 experiment are identical. Bacterial and fungal communities developed inside the bioreactors differed significantly from those of the initial VHW (profiles at initial time). Identification of the microorganisms which those bands correspond allows a better discussion of the results. Hence, libraries of clones were performed for those samples that, a priori, would allow obtaining the highest diversity of microorganisms. Thus, for bacteria, samples of VHW1-I at 7 days, VHW1-NI at 15 days and VHW2 at 14 days were chosen, whereas samples of VHW1-I at 15 days, VHW1-NI at 15 days and VHW2 at 14 days were taken for fungi. Phylogenetic affiliation of the selected clones that were sequenced can be found at Tables 9.1 and 9.2.

Sequencing confirmed that the bands F10 and F17 corresponded to the inoculated fungus *T. versicolor.* When comparing results between the 1.5 L bioreactor (VHW1) and the 10 L bioreactor (VHW2), it can be seen that, despite lower overall PhACs removal in the later due to the lower concentration of contaminants (see section 8.2), *T. versicolor* survival is enhanced in the larger bioreactor. Previous experiments already pointed to this option due to its better

oxygen transfer (Borràs et al., 2008). Unfortunately, continuous treatments were not performed in that reactor because a control treatment was wanted to be included in the experiments. Those results are in agreement with laccase activity detected along the treatment, which decreased after 7 days at VHW1-I reactor (Fig. 8.1) but was still detected after 14 days at VHW2 (Fig. 8.3). Therefore, from those first microbilogical results, it could be drawn that laccase activity might be a good indicator of the survival of the fungus. However, as discussed later (section 9.2.3), that fact might not be true. On the other hand, in the VHW1-NI reactor less diversity of bacteria and fungi was found, being predominant yeasts affiliated to *Galactomyces* and *Trichosporon* and γ- and α-proteobacteria (*Enterobacteriales* and *Rhodospirillales,* respectively). Generally, higher diversity was found at fungal bioreactors. It should be noted that higher diversity does not imply higher concentration. In the present work, microbial populations have only been identified but not quantified. In the VHW1-I bioreactor, bacteria belonging to the orders *Burkholderiales* (β-proteobacteria), *Xanthomonadales* (γ-proteobacteria) and *Rhizobiales* (α-proteobacteria) were also detected. Regarding fungus, sequences related to the Ascomycota *Fusarium, Trichoderma, Rhinocladiella* and *Phialemonium* were found instead of those related to the Basidiomycota *Trichosporon*. At VHW2 bioreactor, similar fungi than those obtained at VHW1-I were found: a fungus related to *Trichoderma* was predominant at 7 days and after 15 days, also *Phialemonium, Exophiala* and *Rhinocladiella* were grown, apart from *T. versicolor.* Therefore, the presence of *T. versicolor* seems to inhibit the growth of any other basidiomycete. However, other fungi were favoured, such as *Trichoderma* spp., some strains of which were previously reported to inhibit *T. versicolor* (Bruce and Highley, 1991) and increase laccase activity on *Pleurotus ostreatus* (Flores et al., 2009). On the other hand, whereas a work points towards the dominance of *T. versicolor* over *Fusarium oxysporum* (Ruiz-Dueñas and Martínez, 1996), in the present study, related fungi were detected in presence of *T. versicolor.* This could imply that depending on the media conditions and interaction with other microorganisms, dominant species can vary. However, there are few studies about fungal interactions and, as stated before, with contradictory results. Thus, more works should be done regarding multiple interactions between fungi and bacteria in fungal treatments.

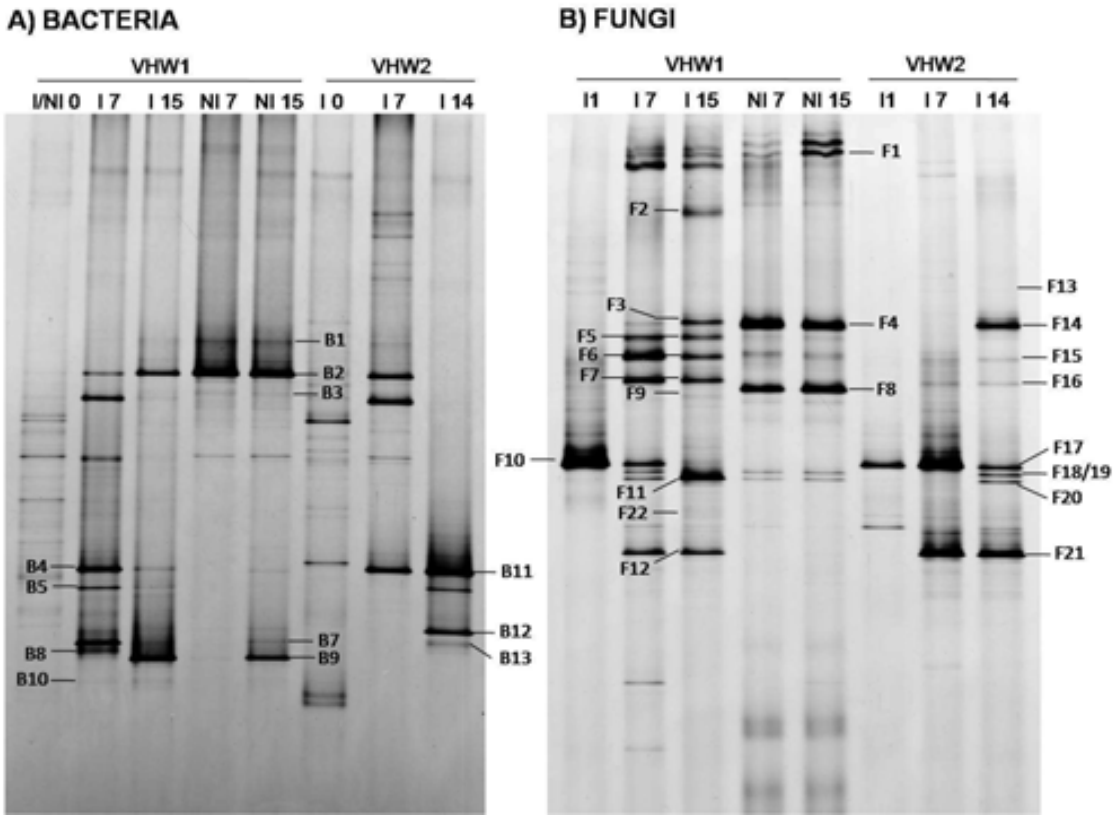



**Figure 9.1.** DGGE profiles of PCR amplified **A)** bacterial 16S rRNA fragment and **B)** fungal ITS fragment. Samples are from the non-sterile batch bioreactor. I correspond to bioreactor inoculated with *T. versicolor* and NI to non-inoculated reactor. 0, 1, 7 and 15 correspond to the time of sampling.

**Table 9.1.** Phylogenetic affiliation of 16S rRNA bacterial sequences corresponding to the different bands of DGGE patterns.

| DGGE Band | Accession number | Seq. length | Closest relative[a] | Covery | Identity (%) | Class/Order[b] |
|---|---|---|---|---|---|---|
| VHW1-B1 | KM392022 | 1464 | *Klebsiella pneumoniae* strain Kp52.145 (FO834906) | 1460/1464 | 99 | γ-Proteobacteria/ Enterobacteriales |
| VHW1-B2 | KM392028 | 1465 | Enterobacter sp. LRC22 (JF772056)<br>*Raoultella ornithinolytica* strain B6 (CP004142) | 1463/1465<br>1462/1465 | 99 | γ-Proteobacteria/ Enterobacteriales |
| VHW1-B3 | KM392031 | 1463 | *Klebsiella pneumoniae* strain MBR11 (JX966429) | 1462/1464 | 99 | γ-Proteobacteria/ Enterobacteriales |
| VHW1-B4 | KM392024 | 1469 | Luteibacter sp. enrichment culture clone 35Fe215 (KF287733)<br>*Dyella yeojuensis* strain rif200829 (FJ527678) | 1464/1469<br>1453/1469 | 99 | γ-Proteobacteria/ Xanthomonadales |
| VHW1-B5 | KM392033 | 1409 | Uncultured agrobacterium sp. clone CBXB23 (GU569105)<br>Agrobacterium tumefaciens strain NBRC 15293 (AB680824) | 1395/1409<br>1392/1409 | 99 | α-Proteobacteria/ Rhizobiales |
| VHW1-B7 | KM392030 | 1414 | *Acidomonas methanolica* strain: NBRC 104435 (AB682176) | 1406/1414 | 99 | α-Proteobacteria/ Rhodospirillales |
| VHW1-B8 | KM392032 | 1457 | *Burkholderia gladioli* strain CACua-73 (HQ023278) | 1456/1457 | 99 | β-Proteobacteria/ Burkholderiales |
| VHW1-B9 | KM392025 | 1414 | *Acidomonas methanolica* strain: NBRC 104435 (AB682176) | 1410/1414 | 99 | α-Proteobacteria/ Rhodospirillales |
| VHW1-B10 | KM392027 | 1457 | *Burkholderia gladioli* strain CACua-73 | 1438/1457 | 99 | β-Proteobacteria/ |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | (HQ023278) | | | Burkholderiales |
| VHW2-B11 | KM392026 | 1470 | Luteibacter sp. enrichment culture clone 35Fe215 (KF287733)<br>*Dyella jeojuensis* strain rif200829 (FJ527678) | 1464/1470<br>1457/1470 | 99 | γ-Proteobacteria/ Xanthomonadales |
| VHW2-B12 | KM392029 | 1459 | Uncultured bacterium clone AKIW679 (DQ129596)<br>*Burkholderia cepacia* strain NBRC 15124 (AB680771) | 1454/1459<br>1453/1459 | 99 | β-Proteobacteria/ Burkholderiales |
| VHW2-B13 | KM392023 | 1457 | Uncultured bacterium clone AKIW679 (DQ129596)<br>*Burkholderia cepacia* strain NBRC 15124 (AB680771) | 1456/1457<br>1455/1457 | 99 | β-Proteobacteria/ Burkholderiales |

[a] Closest organism at GenBank and, when possible, cultured closest match. In parenthesis, Accession Number.

[b] Classified using the Ribosomal Database Project (RDP)

**Table 9.2.** Phylogenetic affiliation of ITS fungal sequences corresponding to the different bands of DGGE patterns.

| DGGE band | Accession number | Seq. length | Closest relative[a] | Covery | Identity (%) |
|---|---|---|---|---|---|
| VHW1-F1 | KM392045 | 368 | *Galactomyces candidum* strain CBS 180.33 (JN974289) | 366/368 | 99 |
| VHW1-F2 | KM392048 | 603 | *Pichia guilliermondii* strain S0201 (EF643576) | 601/603 | 99 |
| VHW1-F3 | KM392046 | 552 | *Phialemonium curvatum* strain: CBS 490.82 (AB278180) | 547/552 | 99 |
| VHW1-F4 | KM392035 | 527 | Uncultured fungus isolate RFLP27 (AF461588)<br>*Trichosporon laibachii* strain CBS 5790 (EU559348) | 527/527<br>511/511 | 100 |
| VHW1-F5 | KM392042 | 565 | *Fusarium solani* strain 6.M8 (FJ224382) | 559562 | 99 |
| VHW1-F6 | KM392037 | 563 | Uncultured *Fusarium* sp. clone PA1912 (GQ280338)<br>*Fusarium solani* culture-collection UOA/HCPF:12649 (KC254048) | 562/563<br>557/565 | 99 |
| VHW1-F7 | KM392044 | 565 | *Fusarium solani* culture-collection UOA/HCPF:12649 (KC254048) | 565/565 | 100 |
| VHW1-F8 | KM392040 | 524 | Uncultured eukaryote clone N414T_268 (GU941914)<br>*Trichosporon dermatis* culture-collection UOA/HCPF (KC254108) | 521/524<br>519/524 | 99 |
| VHW1-F9 | KM392039 | 523 | *Trichosporonales* sp. LM88 (EF060462)<br>*Trichosporon jirovecii* strain ATCC 34499 (HM802131) | 523/524<br>510/510 | 99<br>100 |
| VHW1-F10 | KM392055 | 619 | *Trametes versicolor* sample ID: MQN028 (AB811868) | 619/619 | 100 |
| VHW1-F11 | KM392034 | 612 | *Rhinocladiella similis* culture-collection UOA/HCPF:11700 (KC254071) | 610/612 | 99 |
| VHW1-F12 | KM392054 | 599 | *Trichoderma asperellum* strain ZJSX5001 (JQ040323) | 598/599 | 99 |
| VHW2-F13 | KM392043 | 551 | *Phialemonium curvatum* strain CBS 490.82 (AB278180) | 547/551 | 99 |
| VHW2-F14 | KM392041 | 551 | *Phialemonium curvatum* strain CBS 490.82 (AB278180) | 547/551 | 99 |
| VHW2-F15 | KM392036 | 537 | *Trichosporon asahii* strain IFM 48429 (AB369919) | 535/537 | 99 |
| VHW2-F16 | KM392053 | 565 | *Fusarium solani* culture-collection UOA/HCPF:12649 (KC254048) | 564/565 | 99 |
| VHW2-F17 | KM392052 | 619 | *Trametes versicolor* sample ID: MQN028 (AB811868) | 619/619 | 100 |
| VHW2-F18 | KM392051 | 628 | *Exophiala oligosperma* strain IP 69.52 (DQ836792) | 626/628 | 99 |
| VHW2-F19 | KM392049 | 627 | *Exophiala jeanselmii* isolate SC1201_05 (KC311520)<br>*Exophiala oligosperma* strain JM13 (JQ686670) | 627/627<br>624/628 | 100<br>99 |
| VHW2-F20 | KM392050 | 612 | *Rhinocladiella similis* culture-collection UOA/HCPF:11700 (KC254071) | 611/612 | 99 |
| VHW2-F21 | KM392038 | 599 | *Trichoderma asperellum* strain ZJSX5001 (JQ040323) | 598/599 | 99 |
| VHW2-F22 | KM392047 | 612 | *Rhinocladiella similis* culture-collection UOA/HCPF:11700 (KC254071) | 608/612 | 99 |

[a] Closest organism at GenBank and, when possible, cultured closest match. In parenthesis, Accession Number.

An interesting fact is that almost all genera of fungi detected in both I and NI bioreactors are reported to degrade organic pollutants. For example, *Fusarium* and *Trichoderma* can degrade PAHs (Verdin et al., 2005) and *Trichosporon* can degrade dyes (Saratale et al., 2009). *Fusarium* is an ubiquitous plant pathogen fungi and was found, for example, in urban WWTP (More et al., 2010) and olive-mill wastewater lagoons (Millán et al., 2000). *Rhinocladiella* was also found in the latter but its growth was limited; however, in another study, some signs of organic micropollutants transformation were provided (Fujii and Kikuchi, 2005). Therefore, taking into account that *T. versicolor* was overtaken at VHW1-I by other fungi and/or bacteria between days 7 and 15, further degradation of PhACs observed in the fungal bioreactor (Fig. 8.4) might be due to the activity of fungi belonging to the genera *Fusarium*, *Trichoderma* and/or *Rhinocladiella*. In fact, *Trichoderma* spp. seem to have some strong competitive advantages in wastewater treatment bioreactors because Lu et al. (2009) reported a biofilm inoculated with a microbial consortium with decolorizing strains of *Fusarium oxysporum* and *Candida tropicalis* among others, that, after 4 months in operation, the main fungi turned out to be *Candida* and a non-decolorizing specie of *Trichoderma* too.

Regarding bacteria, VHW1-I profile at the end of the treatment was similar to the VHW1-NI one (dominated by *Enterobacter* sp. and *Acidomonas* sp.) because there was almost no *T. versicolor* at that point and bacteria that might be inhibited by *T. versicolor* could develop in the VHW1-I bioreactor as well. On the other hand, bacteria found in the presence of *T. versicolor* at both VHW1-I and VHW2 were *Luteibacter* sp. and *Burkholderia* sp. All the bacteria found, either at the fungal bioreactors or at non-inoculated control, belong to the phylum *Proteobacteria*. Results are coherent because proteobacteria are generally the main bacteria found in urban wastewater (Miura et al., 2007). Regarding microbial interactions between *T. versicolor* (or WRF in general) and bacteria there is few data in the literature. In the work of Lu et al. (2009), the main bacteria found after 4 months of operation of a biofilm inoculated with a mixed consortium of fungi and bacteria were α-proteobacteria (*Rhizobiales* and *Rhodobacteriales*).

Results derived from those analyses reinforced us the need for partial fungal renewals when working under non-sterile conditions as some authors already pointed out before based on other parametres (Blánquez et al., 2006; Dhouib et al., 2006). Therefore, as explained in Chapters 7 and 8, continuous treatments of ROC and VHW were performed with partial renewal of *T. versicolor*, i.e. with a cellular retention time (CRT) of around 15 d as derived from DGGE results.

## 9.2. Microbial community assessment of continuous treatments

After performing the non-sterile continuous bioreactors presented in the Chapters 7 and 8 (VHW3, VHW4 and ROC3), the microbial communities developed during those treatments were analysed by means of different molecular analysis: phospholipid fatty acids analysis (PLFA), denaturing gradient gel electrophoresis (DGGE) of PCR-amplified fragments of ribosomal DNA (rDNA) and real-time or quantitative PCR (qPCR). Each technique allowed determining different aspects of the populations, explained in detail at the corresponding section.

### 9.2.1. Fungal/Bacterial ratio by PLFA analysis

PLFA analyses are based in the differences in some PLFAs between different groups of microorganisms. They were performed in order to quantify bacteria and fungi at different times of each experiment and to determine variations at community-level. For results discussion, it has to be taken into account that *T. versicolor* pellets were immobilized inside the reactor and samples were taken from the effluent and, therefore, only fungi in the liquid phase (not in the pellets) were quantified. Results were obtained in nmols of PLFA and converted to bacterial and fungal carbon with conversion factors obtained from the literature (Frostegård and Bååth, 1996; Klamer and Bååth, 2004). It has to be noted that those values are aproximate as they were calculated for soil biomass, but they are the only ones available.

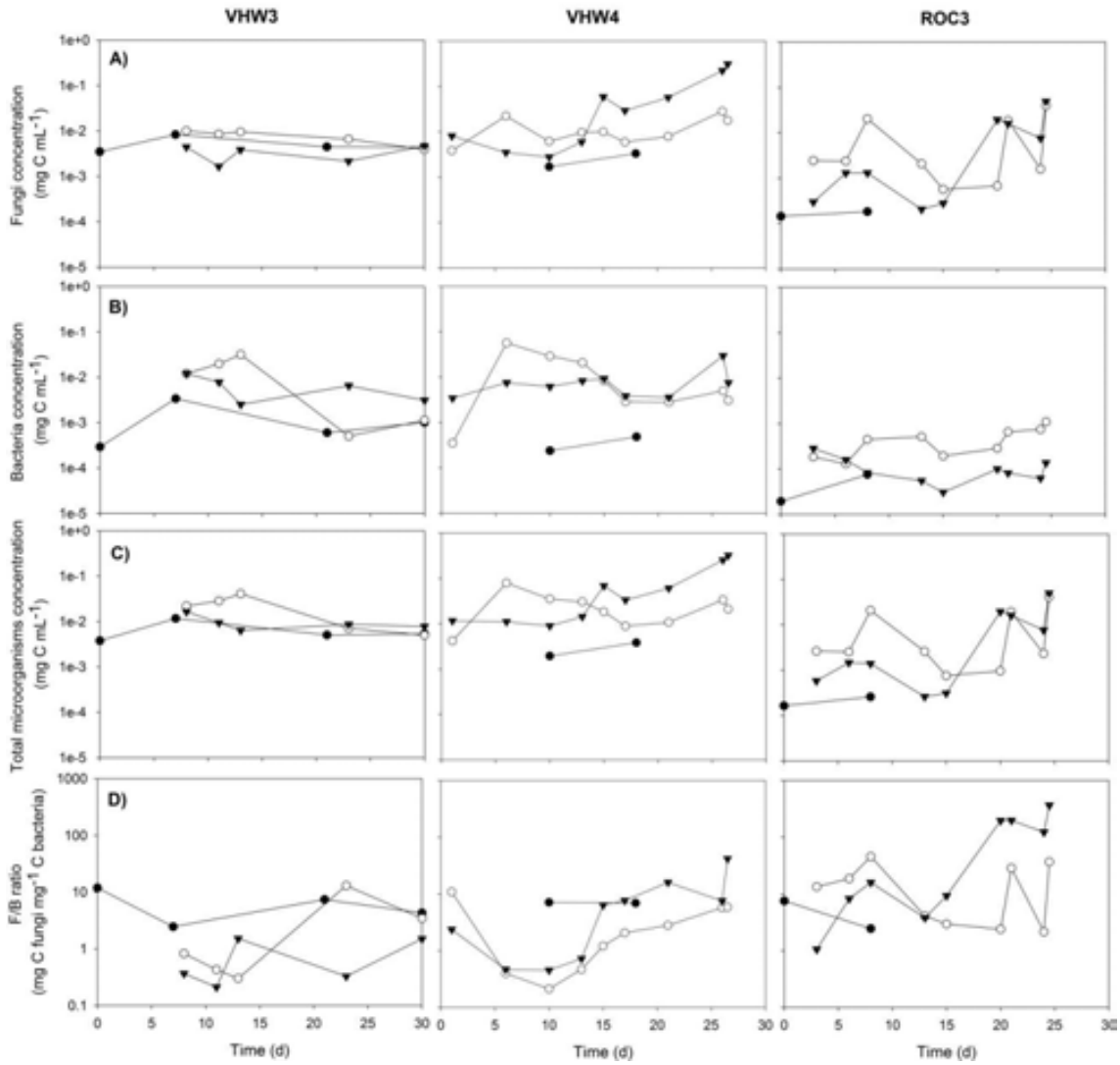



**Figure 9.2.** Evolution of **A)** fungal carbon, **B)** bacterial carbon, **C)** total biological carbon and **D)** ratio fungi/bacteria in the three experiments (VHW3, VHW4 and ROC3). Symbols: (●) influent wastewater, (○) fungal inoculated bioreactor (I) effluent and (▼) non-inoculated control bioreactor (NI) effluent.

In the first column of Fig. 9.2, results for VHW3 are shown. As can be seen, fungal and bacterial concentrations in the effluent were maintained similar to the feed one (Fig. 9.2, first column). At VHW4 (Fig. 9.2, second column), microorganisms growth was more notable due to the higher supply of nutrients. A peak of bacteria at day 6 of VHW4-I, when *T. versicolor* was lysed, was observed. However, the increase in the F/B ratio from day 10 at both I and NI bioreactors shows that the decrease in the C/N ratio might favour the growth of fungi. C/N ratio effect on fungal and bacterial population is controvertial as some studies reported no correlation (Wymore et al., 2013) while other studies

found that N stimulated fungal growth and decreased bacterial one (Rousk and Bååth, 2007). Zhang and Geissen (2012) also found that glucose addition was not enough to recover *Phanerochaetes chrysosporium* activity after fungal lysis and, only after N addition, carbamazepine degradation was achieved. Regarding ROC (Fig. 9.2, last column), microorganisms concentration in the influent was considerably lower than at VHW as it was expected due to the origin of each wastewater. Inside the bioreactors, those concentrations increased but bacteria neither reached the values obtained with VHW bioreactors. Therefore, at ROC3 treatment, F/B ratio was the highest.

### 9.2.2. qPCR of *T. versicolor* and total fungi

Quantitative PCR (qPCR) with primers targeting total fungi and specific for *T. versicolor* were performed at liquid samples of VHW3, VHW4 and ROC3 experiments and also to the fungal pellets of experiments VHW4 and ROC.

As shown in Fig. 9.3A, C and E, other fungi than *T. versicolor* developed significantly in the liquid of all fungal bioreactors except at VHW3. During all VHW3 treatment, *T. versicolor* amount was similar to the total fungi. On the other hand, at ROC3, *T. versicolor* drop off and even disappeared at day 24 from the liquid sample and decreased by more than 4 orders of magnitude at pellet samples. Therefore, other fungi were growing in the original pellets of *T. versicolor*. The increase of *T. versicolor* observed at 21 days in the liquid was probably due to the 1/3 pellets renewal of the day 20. When comparing fungal bioreactors (I) with the controls (NI) (Fig. 9.3B, D and F), fungal concentration at I was maintained stable, whereas at NI it increased along the treatment until reaching the concentration of I. In general, results from qPCR analyses are in accordance with those obtained in the PLFA.

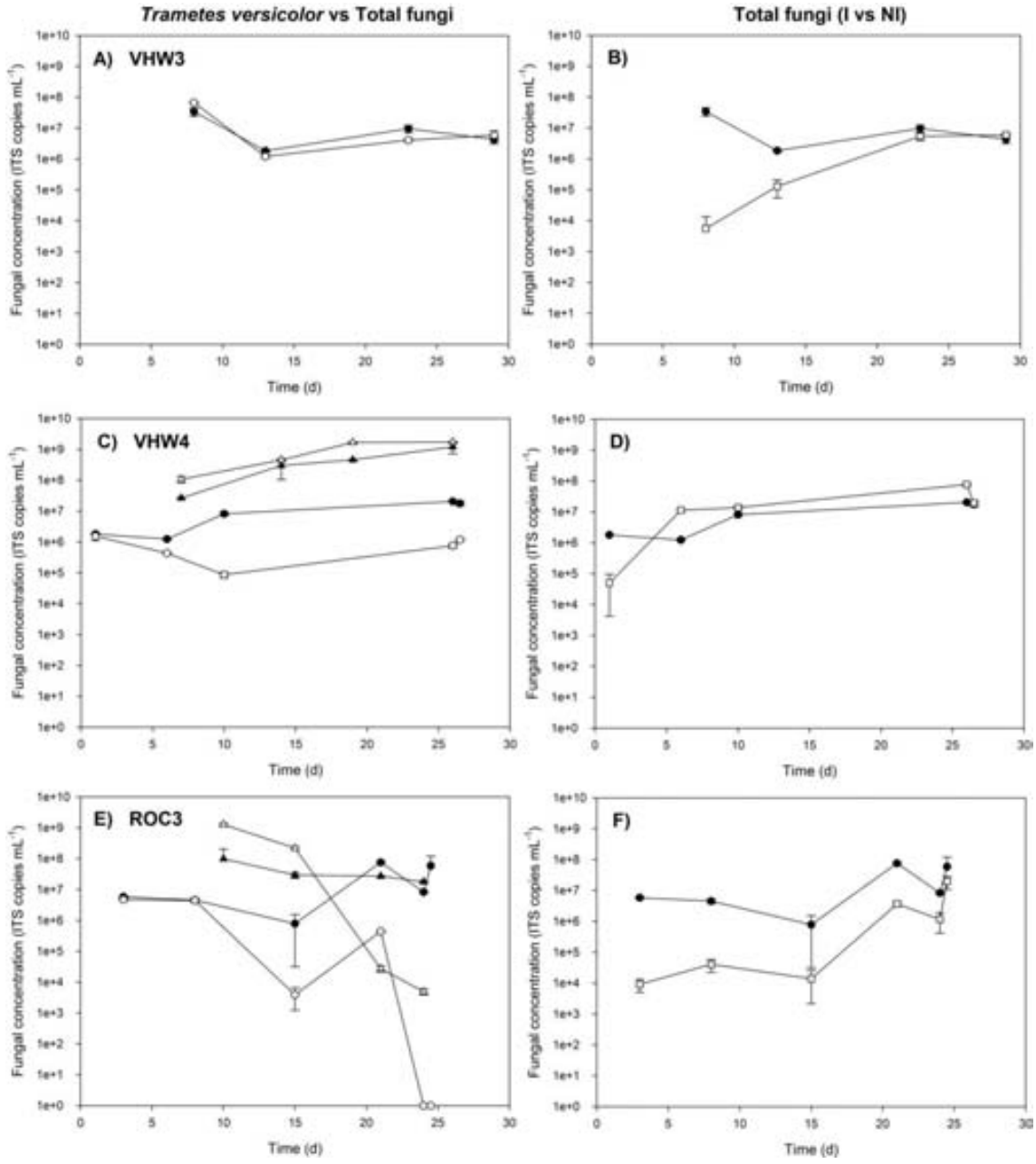



**Figure 9.3.** Total fungi and *T. versicolor* quantification by qPCR at treatments **A)** and **B)** VHW3, **C)** and **D)** VHW4 and **E)** and **F)** ROC3. Symbols: (●) Total fungi at liquid sample of fungal (I) reactor, (○) *T. versicolor* at liquid sample of I reactor, (▲) Total fungi at pellets sample of I reactor, (△) *T. versicolor* at pellets sample of I reactor and (□) Total fungi at liquid sample of non-inoculated (NI) control reactor.

### 9.2.3. Microbial diversity by DGGE

Results derived from the visual analysis of fungal DGGE profiles for *T. versicolor* are in agreement with those of qPCR. *T. versicolor* was found at every sample of I bioreactors except for ROC experiment from day 15 onwards (Fig. 9.4). Therefore, the decrease of laccase activity in ROC3-I in the second half of the treatment can be explained by the absence of *T. versicolor*. However, at VHW3 and VHW4, where laccase activity also decreased the last days of operation, *T. versicolor* was still present at both pellets and liquid samples (Fig, 9.5). Therefore, on the contrary of what was pointed out with the results obtained in the batch, laccase activity might not be so good indicator of fungal activity.

After performing the phylogenetic analysis of the sequences corresponding to the selected and cleaned DGGE bands, fungal DGGE profiles indicated that the fungus that competed with *T. versicolor* at ROC3-I and finally prevailed was most *Trichoderma asperellum* (Fig. 9.4, Table 9.3). It was probably responsible for the green colour of the biofilm and the bioreactor (Fig. 7.12). *Trichoderma* spp. are filamentous soil fungus used as wood decaying inhibitors (Freitag and Morrell, 1992) and as biocontrol of several plant pathogens as well (Fernandes Qualhato et al., 2013). *T. asperellum* and *T. harzianum* are the most effective antagonists of pathogens in agar plate studies by means of nutrient competition or direct mycoparasitism with antifungal metabolites or cell-wall degrading enzymes (Fernandes Qualhato et al., 2013). In fact, it has been reported that *T. harzianum* consumes glucose at higher rate than *T. versicolor* (Freitag and Morrell, 1992), what gives it a clear competitive advantage in the bioreactor. However, *Trichoderma* spp. were more effective against brown-rot fungi than against white-rot fungi (Bruce and Highley, 1991) as overgrowing occurred without lysis of the last (Baldrian, 2004; Bruce and Highley, 1991; Freitag and Morrell, 1992), considered as the main factor for an efficient biological control (Fernandes Qualhato et al., 2013). Nonetheless, there are huge interspecies and interstrain variability in the level of antagonism of *Trichoderma* spp. (Bruce and Highley, 1991; Fernandes Qualhato et al., 2013) and although it remains unclear the mechanism of antagonism of *T. asperellum* in the present study, it is evident that it was able to kill it and grow on the own *T. versicolor* pellets. In a

study of Lu et al. (2009), *Trichoderma* spp. were also developed and became the dominant species, together with *Candida* spp., in a microbial consortia biofilm reactor. At the same time at ROC3-NI, the predominant fungus was a *Penicillium sp* (probably responsible for the brown and white biofilm of the reactor inner wall (Fig. 7.12)) whereas at the beginning of the experiment (until day 15) the major fungus at NI bioreactor was *Fusarium oxysporum* with presence of *Rhodotorula sp.* as well. Taking into account that some laccase activity was detected from day 16 onwards, it was apparently produced by the Ascomycete *Penicillium sp.* Penicillium spp. are known for they ability to degrade some PAHs and the production of extracellular enzymes such as laccases (Rodríguez et al., 1996). *Trichoderma* spp. and *Fusarium* spp. are described to degrade organic pollutants as well (Cobas et al., 2013; Machín-Ramírez et al., 2010; Rafin et al., 2000). Thus, high degradation percentages also achieved at ROC3-NI and at ROC3-I at HRT 2d might be assigned to those three fungi. Ascomycetes were found to be dominating (98%) in a RO plant (Al Ashhab et al., 2014) as happened in the bioreactors communities developed from ROC as well.

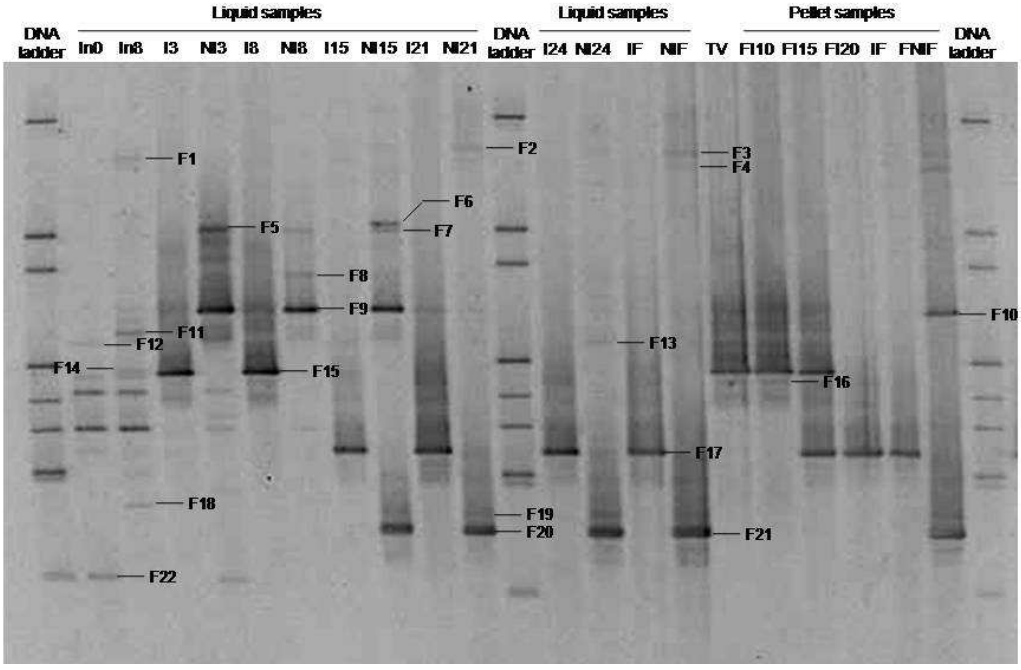



**Figure 9.4.** DGGE gel of ITS1F-ITS2 fungal fragments PCR amplified from samples of ROC experiment. F9: *Fusarium oxysporum*; F15: *Trametes versicolor*; F17: *Trichoderma* sp.; F20 and F21: *Penicillium* sp. Closest relatives for the other bands can be found at Table 9.3.

**Table 9.3**. Phylogenetic affiliation of ITS fungal sequences obtained from the different bands of DGGE patterns of ROC experiment.

| DGGE Band | Accession number | Seq. lenght | Closest relative[a] | Covery | Identity (%) |
|---|---|---|---|---|---|
| ROC3-F4 | KM361323 | 205 | Ascomycota sp. PIMO_170 (JF705941)<br>Zalerion arborícola MF5771 (AF169307) | 203/205 | 99 |
| ROC3-F5 | - | 188 | Uncultured Basidiomycota clone 1513n25 (KJ461426)<br>Rhodotorula dairenensis strain PYCC 4897 (AF444684) | 188/188 | 100 |
| ROC3-F6 | - | 179 | Uncultured Rhodotorula clone 234 (KC785592)<br>Rhodotorula mucilaginosa strain AUMC 7782 (JQ425399) | 178/179 | 99 |
| ROC3-F7 | - | 178 | Uncultured eukaryote clone NS31T_257 (KJ182680)<br>Rhodotorula mucilaginosa strain ATCC 4056 (KC881070) | 178/178 | 100 |
| ROC3-F8 | - | 160 | Trichosporon sp. R018 (KC252965)<br>Trichosporon montevideense CBS 6721 (NR_073245) | 160/160<br>156/160 | 100<br>98 |
| ROC3-F9 | - | 183 | Uncultured soil fungus isolate DGGE gel band F11 (KJ562389)<br>Fusarium oxysporum strain ZJLM001 (KJ544916) | 183/183 | 100 |
| ROC3-F10 | - | 186 | Uncultured soil fungus isolate DGGE gel band F11 (KJ562389)<br>Fusarium oxysporum strain ZJLM001 (KJ544916) | 186/186 | 100 |
| ROC3-F11 | KM361324 | 227 | Uncultured fungus clone J054 (JX974803) | 181/192 | 94 |
| ROC3-F12 | - | 199 | Uncultured fungus isolate DGGE gel band 209_1 (HQ441887) | 197/199 | 99 |
| | | | Hyphodontia alutacea strain GEL 2291 (EU583419) | 193/199 | 97 |
| ROC3-F13 | - | 199 | Uncultured fungus isolate DGGE gel band 209_1 (HQ441887) | 197/199 | 99 |
| | | | Hyphodontia alutacea strain GEL 2291 (EU583419) | 193/199 | 97 |
| ROC3-F14 | - | 149 | Uncultured fungus clone M8D-35 (HE605274) | 130/151 | 86 |
| ROC3-F15 | KM361325 | 222 | Trametes versicolor culture-collection ICMP:19973 (KF727428) | 222/222 | 100 |
| ROC3-F16 | KM361326 | 220 | Trametes versicolor culture-collection ICMP:19973 (KF727428) | 220/220 | 100 |
| ROC3-F17 | KM361327 | 221 | Trichoderma asperellum strain Tr85 (KF737410)<br>Trichoderma asperellum strain CGMCC 6422 (KF425754) | 221/221 | 100 |
| ROC3-F18 | KM361328 | 203 | Without similarities at BLAST | - | - |
| ROC3-F19 | KM361329 | 214 | Penicillium sp. CCF2941 (FJ430758)<br>Talaromyces helicus strain NRRL 2106 (AF033396) | 214/214<br>212/215 | 100<br>99 |
| ROC3-F20 | KM361330 | 213 | Penicillium sp. FZ101 (KF848942)<br>Talaromyces pinophylus strain IBRC-M 30027 (KF471076) | 213/213<br>211/213 | 100<br>99 |
| ROC3-F21 | KM361331 | 213 | Penicillium sp. FZ101 (FJ430758)<br>Penicillium aculeatum strain NRRL 2129 (AF033397) | 213/213<br>212/213 | 100<br>99 |
| ROC3-F22 | - | 168 | Lulwoana sp. MH630 (FJ430722) | 157/171 | 92 |

[a] Closest organism at GenBank and, when possible, cultured closest match. In parenthesis, Accession Number.

Fungal DGGE profiles of VHW3 and VHW4 and the corresponding phylogenetic affiliation of the selected bands can be found at Fig. 9.5 and Table 9.4. In the previous batch bioreactors treating VHW (Section 9.1), *Trichoderma* sp. was also found among other fungi overcoming *T. versicolor* at VHW1-I and at VHW2 but not at the control reactor (VHW1-NI). However, in the continuous treatments, *Trichoderma* spp. were not significantly found at any VHW

bioreactor. At VHW3-I, *Candida sp.*, *Exophiala equina* and *Scytalidium lignicola* grew in the bioreactor whereas *Exophiala oligosperma* and also *Fusarium* sp*,* developed at VHW3-NI. *Exophiala* spp. were probably the cause for the black colour of bioreactors the last days of operation (Fig. 8.8). Although the aspect was worst, the effect was shown to be not as severe as the one of *Trichoderma* sp. *Exophiala* spp. belong to the so-called black yeasts, causing superficial mycoses in humans. However, potential biological hazards of dark biofilms in domestic water taps were regarded as low because the majority of the species found were only oportunistic pathogens in immunocompromised humans (Heinrichs et al., 2013). In a study of Isola et al. (2013), some *Exophiala* spp, *E. oligosperma* among them, resulted positive in a toluene degradation test, but not *E. equina*. At VHW4 fungi detected at I and NI bioreactors were similar, mainly *Candida spp.* and *Fusarium spp.* At VHW4-NI an unknown fungi (88% of identity with *Ophiostoma*) was grown as well. Therefore, fungi that grew in the bioreactors were mainly Ascomycetes, unlike in the batch treatment of VHW where Basidiomycetes were predominant in the control reactor.

*Fusarium* spp*.* were very ubiquitous as they developed at 4 out of the 6 bioreactors. The lack of *Fusarium* spp. at ROC3-I and VHW3-I reactors could be explainted by the antagonistic behaviour reported by Ruiz-Dueñas and Martínez (Ruiz-Dueñas and Martínez, 1996). However, Fusarium was found at VHW4-I and in the fungal batch treatment of VHW, under the presence of *T. versicolor* as well. Therefore, it might have a weak antagonism or be specie-specific. *Candida* spp*.* were present at 3 out of the 4 VHW bioreactors. *Candida* spp. was found to be one of the main genera at urban WWTP, together with *Rhodotorula* spp., *Trichosporon* spp. and other 5 unidentified genera (Yang et al., 2011). At VHW treatments, higher diversity was generally found at the samples of the last days of the treatment.

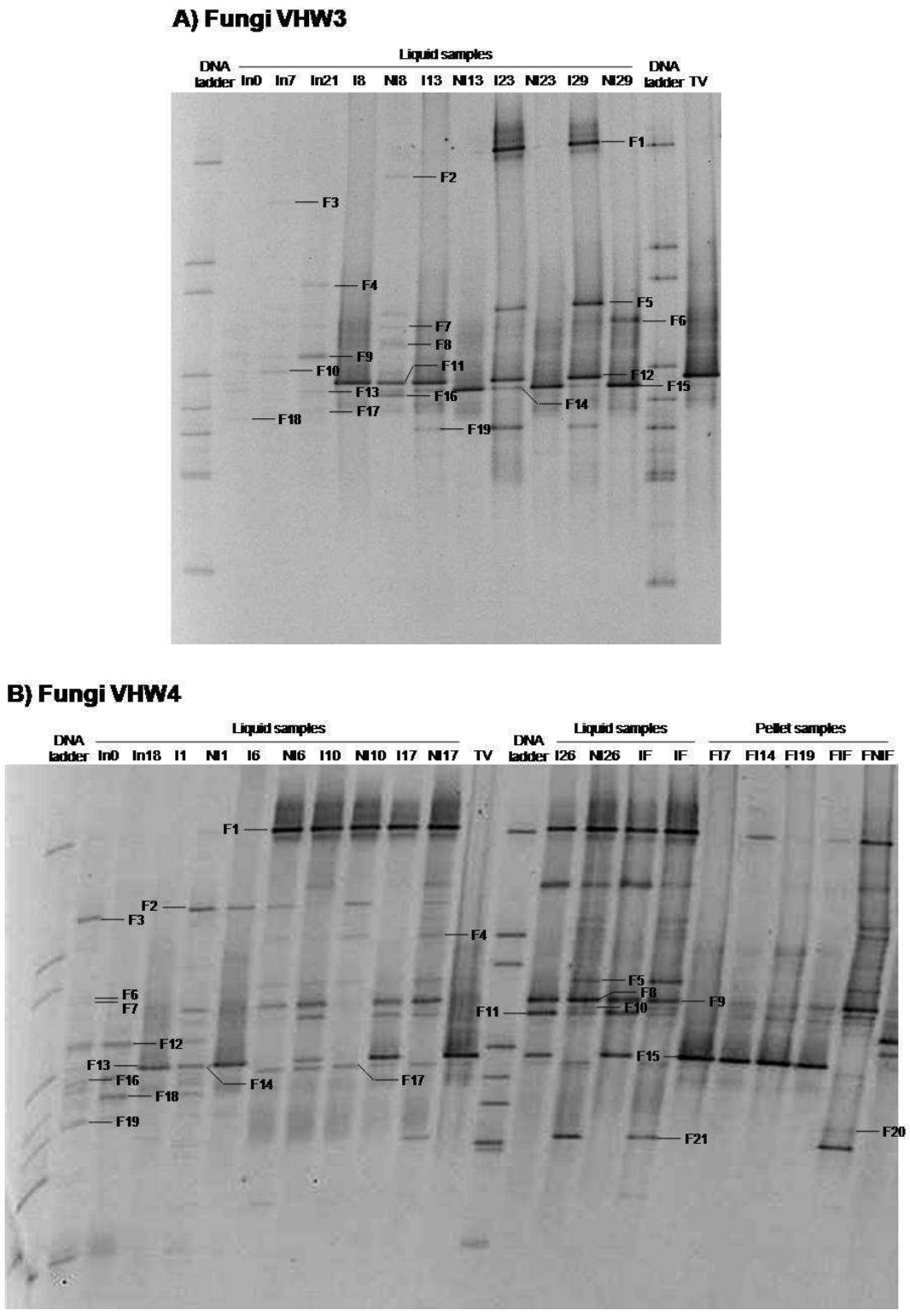



**Figure 9.5.** DGGE gel of ITS1F-ITS2 fungal fragments PCR amplified from samples of A) VHW3 experiment and B) VHW4 experiment. Closest relatives for the labeled bands can be found at Table 9.4.

**Table 9.4**. Phylogenetic affiliation of ITS fungal sequences obtained from the different bands of DGGE patterns at VHW3 and VHW4 experiments.

| DGGE Band | Accession number | Seq. lenght | Closest relative[a] | Covery | Identity (%) |
|---|---|---|---|---|---|
| VHW3-F1 | - | 168 | Candida sp. NRRL Y-27161 (DQ911459) | 164/64 | 100 |
| VHW3-F2 | KM361332 | 229 | Debaryomyces sp. CLIB 1277 (FR693386) | 229/229 | 100 |
| VHW3-F4 | KM361333 | 263 | Without similarities at BLAST | - | - |
| VHW3-F5 | KM361334 | 228 | Exophiala sp. CCFEE 5933 (JX681050)<br>Exophiala equina CBS 119.23 (NR_111627) | 228/228 | 100 |
| VHW3-F6 | - | 182 | Uncultured Ascomycota genomic DNA (FR682427)<br>Fusarium oxysporum strain ATCC 52557 (GU256752) | 182/182<br>181/182 | 100<br>99 |
| VHW3-F8 | KM361335 | 218 | Uncultured fungus clone J054 (JX974803) | 181/192 | 94 |
| VHW3-F9 | - | 135 | Uncultured fungus clone OTU45 (EF521247) | 88/101 | 87 |
| VHW3-F11 | KM361336 | 220 | Trametes versicolor culture-collection ICMP:19973 (KF727428) | 220/220 | 100 |
| VHW3-F12 | KM361337 | 222 | Trametes versicolor culture-collection ICMP:19973 (KF727428) | 222/222 | 100 |
| VHW3-F13 | KM361338 | 239 | Exophiala oligosperma strain MH2012-17 (AB777520) | 238/239 | 99 |
| VHW3-F14 | KM361339 | 239 | Exophiala oligosperma strain MH2012-17 (AB777520) | 239/239 | 100 |
| VHW3-F15 | KM361340 | 239 | Exophiala oligosperma strain MH2012-17 (AB777520) | 239/239 | 100 |
| VHW3-F18 | KM361341 | 254 | Uncultured fungus clone Unisequence#31-3440_1744 (GQ525142)<br>Knufia petricola CBS 726.95 (NR_111871) | 218/255<br>192/214 | 85<br>90 |
| VHW3-F19 | KM361342 | 209 | Scytalidium lignicola strain KACC 41229 (GQ272635) | 209/209 | 100 |
| VHW4-F1 | - | 179 | Candida sp. NRRL Y-27161 (DQ911459) | 164/164 | 100 |
| VHW4-F2 | KM361343 | 227 | Candida palmioleophila strain ATCC 96299 (KC479687) | 227/227 | 100 |
| VHW4-F4 | - | 188 | Uncultured eukaryote clone NS31T_257 (KJ182680)<br>Rhodotorula mucilaginosa strain ATCC 4056 (KC881070) | 188/188 | 100 |
| VHW4-F5 | - | 172 | Xylariales sp. HP-2011b (HE599293)<br>Phialemonium curvatum strain UTHSC R-3447 (AY818323) | 172/172<br>171/172 | 100<br>99 |
| VHW4-F6 | - | 172 | Xylariales sp. HP-2011b (HE599293)<br>Phialemonium curvatum strain UTHSC R-3447 (AY818323) | 172/172<br>171/172 | 100<br>99 |
| VHW4-F7 | KM361344 | 213 | Exophiala sp. CCFEE 5933 (JX681050)<br>Exophiala equina CBS 119.23 (NR_111627) | 213/213 | 100 |
| VHW4-F8 | - | 188 | Fusarium solani voucher CEQCA-O0484 (KC771504) | 188/188 | 100 |
| VHW4-F9 | - | 188 | Fusarium solani voucher CEQCA-O0484 (KC771504) | 188/188 | 100 |
| VHW4-F10 | - | 188 | Uncultured soil fungus isolate DGGE gel band F3 (KJ562397)<br>Fusarium solani strain DAOM 215455 (JN942906) | 188/188 | 100 |
| VHW4-F13 | KM361345 | 220 | Trametes versicolor culture-collection ICMP:19973 (KF727428) | 220/220 | 100 |
| VHW4-F14 | KM361346 | 202 | Trametes versicolor culture-collection ICMP:19973 (KF727428) | 202/202 | 100 |
| VHW4-F15 | KM361347 | 222 | Trametes versicolor culture-collection ICMP:19973 (KF727428) | 222/222 | 100 |
| VHW4-F16 | KM361348 | 241 | Exophiala oligosperma strain MH2012-17 (AB777520)<br>Exophiala oligosperma CBS 725.88 (NR_111134) | 239/239<br>239/240 | 100<br>99 |
| VHW4-F17 | KM361349 | 239 | Exophiala oligosperma strain MH2012-17 (AB777520)<br>Exophiala oligosperma CBS 725.88 (NR_111134) | 239/239<br>239/240 | 100<br>99 |
| VHW4-F18 | KM361350 | 212 | Uncultured exophiala clone CBB3.14 (KC876175)<br>Exophiala moniliae CBS 520.76 (NR_111448) | 200/212<br>200/215 | 94<br>93 |
| VHW4-F19 | KM361351 | 202 | Heterobasidion sp. NL-2013 isolate PFC 5320 (KC492958)<br>Heterobasidion parviporum strain B1142 (GQ162421) | 202/202 | 100 |
| VHW4-F20 | KM361352 | 201 | Trichoderma asperellum strain TR08 (AB935955) | 201/201 | 100 |
| VHW4-F21 | - | 132 | Ophiostoma stenoceras strain CBS103.78 (AF484449) | 106/121 | 88 |

[a] Closest organism at GenBank and, when possible, cultured closest match. In parenthesis, Accession Number.

Regarding bacterial communities, some difficulties were found to amplify ribosomal DNA, especially at ROC samples, due to the lower amount of bacteria and maybe other factors related to PCR optimisation. At VHW4 and ROC3 experiments, samples from pellets were also tryied to amplify with bacterial primers but no PCR products were obtained, what means that pellets were not contaminated with bacteria. As shown in the Fig. 9.6 and Table 9.5, in the liquid samples, only an *Enterobacter* sp. and *Clostridia* could be identified at ROC3-NI and *Clostridia, γ.proteobacteria* (*Enterobacteriales* and *Xanthomonadales*) and some unidentified bacteria at ROC3-I. Enterobacteriales could be responsible for the pink color of the biofilm at ROC3-I (Fig. 7.12). At VHW3, initally, some *Enterobacter sp.* developed in both reactors (I and NI). Proteobacteria *Luteibacter* and *Burkholderia* also grew at VHW3-I whereas at VHW3-NI another *Enterobacter* replaced the previous one. In the feed water and in some isolated VHW reactor samples, some *Clostridiales* were present but, taking into account that *Clostridia* spp. are anaerobic bacteria, they probably did not grow inside the bioreactors but their spores were present in the feed water. At VHW4 bioreactors, similar bacterial communities were found at I and NI as happened with fungi. Thus, many unclassified proteobacteria, *Enterobacteriales sp.*, *Burkholderiales sp.* and *Verrucomicrobiales sp.* were detected. *Chlamydiales sp.* and *Rhodospirillales* were also found at day 26 at VHW4-NI. No specific bacteria can be related with the dead of *T. versicolor* around day 6 of VHW4 because all the bacteria detected at that moment were also present during all the treatment, also when *T. versicolor* recovered (Fig. 9.6C). As can be noted, *Enterobacteria spp.* were present at each bioreactor, independently of the wastewater origin and if it was inoculated by *T. versicolor* or not. *Burkholderia spp.* were also present at 3 out of 4 VHW bioreactors and also in the batch bioreactors (section 9.1). Results are consistent because Burkholderia belong to β-proteobacteria, which are the major populations in environmental samples and also aerobic MBRs and RO systems (Ayache et al., 2013) and Enterobacteriales are also typical of urban wastewaters.

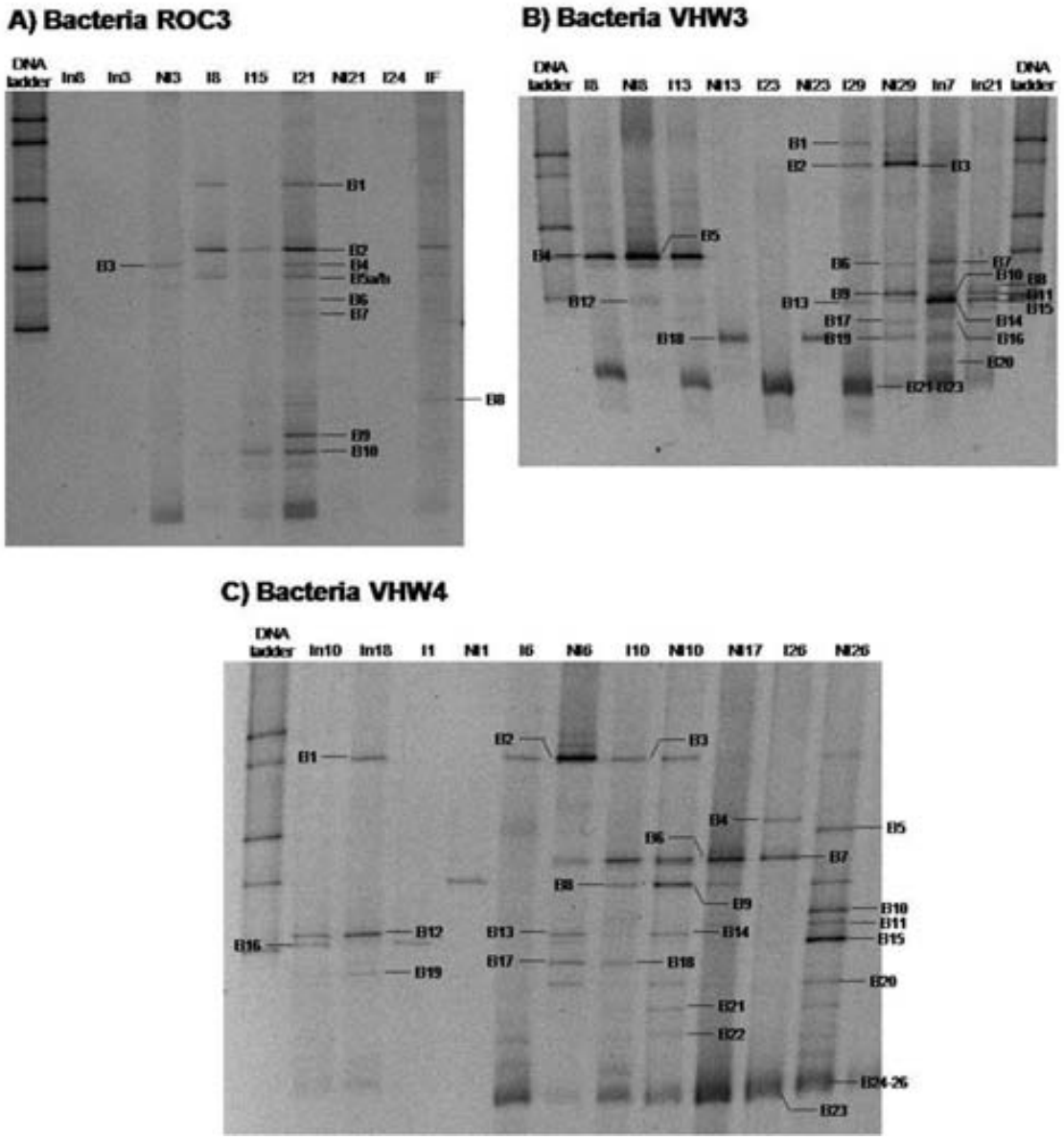



**Figure 9.6.** DGGE profiles of PCR amplified bacterial 16S rDNA fragments from samples of **A)** ROC3, **B)** VHW3 and **C)** VHW4 experiments. Closest relatives for the labeled bands can be found at Table 9.5.

**Table 9.5**. Phylogenetic affiliation of 16S bacterial sequences obtained from the different bands of DGGE patterns.

| DGGE Band | Accession number | Seq. lenght | Closest relative[a] | Covery | Identity (%) | Class/Order[b] |
|---|---|---|---|---|---|---|
| VHW3-B1 | KM355631 | 344 | Uncultured Alphaproteobacteria bacterium (CU926719) | 342/344 | 99 | Unclassified Bacteria |
| VHW3-B2 | KM355632 | 296 | Uncultured bacterium clone ncd2574c06c1 (JF226179) | 289/296 | 98 | Unclassified Bacteria |
| VHW3-B3 | KM355633 | 361 | Uncultured bacterium clone ncd2574c06c1 (JF226179) | 353/361 | 98 | Unclassified α-Proteobacteria |
| VHW3-B4 | KM355634 | 346 | Enterobacter asburiae strain BD (KF772923) | 344/344 | 100 | γ-Proteobacteria/ Enterobacteriales |
| VHW3-B5 | KM355635 | 379 | Enterobacter asburiae strain BD (KF772923) | 379/379 | 100 | γ-Proteobacteria/ Enterobacteriales |

| | | | | | | |
|---|---|---|---|---|---|---|
| VHW3-B6 | KM355636 | 355 | Uncultured bacterium clone ncd2610e03c1 (JF228379) | 346/355 | 97 | Unclassified Proteobacteria |
| VHW3-B7 | KM355637 | 337 | Uncultured bacterium clone DolRc_B586 (JQ204250) Clostridium perfringens strain ATCC 13124 (NR_112169) | 337/337 | 100 | Clostridia/ Clostridiales |
| VHW3-B8 | | 184 | Uncultured Lachnospiraceae bacterium clone D-05Biofilm019 (KJ600442) | 182/184 | 99 | Clostridia/ Clostridiales |
| | | | Bacteroides xylanolyticus strain X5-1 (NR_104899) | 181/184 | 98 | |
| VHW3-B10 | KM355638 | 333 | Bacterium NLAE-zl-P34 (JQ606884) Clostridium sticklandii str. DSM 519 (FP565809) | 332/333 | 99 | Clostridia/ Clostridiales |
| VHW3-B11 | KM355639 | 343 | Acetoanaerobium sp. WJDL-Y2 (KF176997) Clostridium sticklandii str. DSM 519 (FP565809) | 341/343 | 99 | Clostridia/ Clostridiales |
| VHW3-B14 | KM355640 | 341 | Pseudomonas sp. CL-1 (FJ594992) | 338/340 | 99 | γ-Proteobacteria/ Pseudomonadales |
| | | | Pseudomonas alcaligenes strain ATCC 14909 (NR_114472) | 339/342 | 99 | |
| VHW3-B15 | KM355641 | 339 | Uncultured bacterium clone J70 (KF233654) | 338/338 | 100 | Clostridia/ Clostridiales |
| VHW3-B17 | KM355642 | 331 | [Clostridium] sticklandii strain DSM 519 (NR_102880) | 330/331 | 99 | Clostridia/ Clostridiales |
| VHW3-B19 | KM355643 | 340 | Uncultured Massilia sp. clone D-05Catholyte072 (KJ600107) Enterobacter aerogenes strain KCTC 2190 (NR_102493) | 340/340 | 100 | γ-Proteobacteria/ Enterobacteriales |
| VHW3-B20 | KM355644 | 308 | Shewanella sp. GLY-1210 (KC953871) Shewanella putrefaciens CN-32 (CP000681) | 308/308 | 100 | γ-Proteobacteria/ Alteromonadales |
| VHW3-B21 | KM355645 | 279 | Luteibacter sp. enrichment culture clone 35Fe23 (KF287734) | 277/279 | 99 | γ-Proteobacteria/ Xanthomonadales |
| VHW3-B22 | | 120 | Burkholderia sp. B8 (JN975047) | 119/119 | 100 | β-Proteobacteria/ Burkholderiales |
| VHW4-B2 | KM355646 | 334 | Uncultured bacterium clone ncd2574c06c1 (JF226179) | 329/334 | 99 | Unclassified proteobacteria |
| VHW4-B4 | KM355647 | 339 | Uncultured soil bacterium clone SM11 (EU339599) | 334/339 | 99 | Unclassified proteobacteria |
| | | | Micavibrio aeruginosavorus EPB (CP003538) | 298/325 | 92 | |
| VHW4-B5 | KM355648 | 337 | Uncultured alpha proteobacterium (AB809968) | 337/337 | 100 | α-Proteobacteria/ Rhodospirillales |
| | | | Acidocella aluminiidurans strain NBRC 104303 (NR_114266) | 336/337 | 99 | |
| VHW4-B6 | KM355649 | 351 | Klebsiella sp. Enrichment culture clone F-2 (KJ465989) Klebsiella pneumoniae str. Kp52.145 (FO834906) | 351/351 | 100 | γ-Proteobacteria/ Enterobacteriales |
| VHW4-B7 | KM355650 | 377 | Klebsiella sp. Enrichment culture clone F-2 (KJ465989) Klebsiella pneumoniae str. Kp52.145 (FO834906) | 377/377 | 100 | γ-Proteobacteria/ Enterobacteriales |
| VHW4-B8 | KM355651 | 346 | Uncultured bacterium clone ZBAF2-82 (HQ682040) | 346/347 | 99 | Unclassified Proteobacteria |
| VHW4-B9 | KM355652 | 317 | Uncultured bacterium clone ZBAF2-82 (HQ682040) | 317/317 | 100 | Unclassified Proteobacteria |
| VHW4-B10 | KM355653 | 340 | Uncultured bacterium clone UVaBiofilter-V26 (KJ002532) | 335/340 | 99 | Verrucomicrobiae/ Verrucomicrobiales |
| | | | Prosthecobacter fluviatilis strain HAQ-1 (NR_041608) | 327/341 | 96 | |
| VHW4- | KM355654 | 335 | Uncultured bacterium clone eff4 | 331/335 | 99 | Unclassified |

| | | | | | | |
|---|---|---|---|---|---|---|
| B11 | | | (JN245720) | | | α-Proteobacteria |
| VHW4-B12 | KM355655 | 341 | Bacterium NLAE-zl-P34 (JQ606884)<br>Acetoanaerobium noterae strain NOT-3 (NR_104848) | 341/341 | 100 | Clostridia/ Clostridiales |
| VHW4-B13 | KM355656 | 341 | Acetoanaerobium sp. WJDL-Y2 (KF176997)<br>Acetoanaerobium noterae strain NOT-3 (NR_104848) | 340/341 | 99 | Clostridia/ Clostridiales |
| VHW4-B14 | KM355657 | 353 | Uncultured bacterium A1Q1_fos_291 (JX649891)<br>Acetoanaerobium noterae strain NOT-3 (NR_104848) | 353/353<br>352/353 | 100<br>99 | Clostridia/ Clostridiales |
| VHW4-B15 | KM355658 | 335 | Parachlamydia acanthamoebae UV-7 strain UV-7 (NR_074972) | 335/335 | 100 | Chlamydiae/ Chlamydiales |
| VHW4-B16 | KM355659 | 336 | Uncultured Peptostreptococcaceae bacterium clone CatInokulum014 (KJ600240) | 336/336 | 100 | Clostridia/ Clostridiales |
| VHW4-B17 | KM355660 | 336 | Micavibrio aeruginosavorus EPB (CP003538) | 336/336 | 100 | Unclassified proteobacteria |
| VHW4-B18 | KM355661 | 327 | Micavibrio aeruginosavorus EPB (CP003538) | 327/327 | 100 | Unclassified proteobacteria |
| VHW4-B19 | KM355662 | 338 | Uncultured bacterium isolate DGGE gel band MFC7-1 (EU272913)<br>Clostridium sticklandii str. DSM 519 (FP565809) | 337/338<br>335/338 | 99 | Clostridia/ Clostridiales |
| VHW4-B21 | KM355663 | 319 | Uncultured alpha proteobacterium clone S-30-48 (KF827415) | 313/319 | 98 | Unclassified Proteobacteria |
| VHW4-B22 | KM355664 | 305 | Uncultured bacterium clone ncd1306f09c1 (JF107942)<br>Dongia mobilis strain LM22 (NR_116676) | 272/307<br>263/300 | 89<br>88 | Unclassified Proteobacteria |
| VHW4-B23 | KM355665 | 331 | Burkholderia sp. WR43 (AB365791)<br>Burkholderia nodosa strain Br3470 (AM284972) | 327/331<br>325/331 | 99<br>98 | β-Proteobacteria/ Burkholderiales |
| VHW4-B25 | KM355666 | 304 | Uncultured Verrucomicrobiaceae bacterium clone A03-06C (FJ542838) | 292/304 | 96 | Verrucomicrobiae/ Verrucomicrobiales |
| VHW4-B26 | KM355667 | 333 | Burkholderia sp. WR43 (AB365791)<br>Burkholderia nodosa strain Br3470 (AM284972) | 330/333<br>328/333 | 99<br>98 | β-Proteobacteria/ Burkholderiales |
| ROC3-B1 | KM355623 | 336 | Uncultured bacterium clone MG19 (KC000046)<br>Bacteroides xylanolyticus strain X5-1 (NR_104899) | 336/336 | 100 | Clostridia/ Clostridiales |
| ROC3-B2 | KM355624 | 336 | Uncultured organism clone ELU0144-T169-S-IIPCRAMgANa_000554 (HQ799871)<br>Bacteroides xylanolyticus strain X5-1 (NR_104899) | 335/336<br>333/336 | 99 | Clostridia/ Clostridiales |
| ROC3-B3 | KM355625 | 326 | Enterobacter sp. RA192 (KJ534455) | 326/326 | 100 | γ-Proteobacteria/ Enterobacteriales |
| ROC3-B4 | KM355626 | 335 | Uncultured bacterium clone 16slp117-1h05.p1k (GQ157904) | 335/335 | 100 | γ-Proteobacteria/ Enterobacteriales |
| ROC3-B5a | KM355627 | 335 | Uncultured bacterium clone SHCB0927 (JN698054)<br>Bacteroides xylanolyticus strain X5-1 (NR_104899) | 333/335<br>331/335 | 99 | Clostridia/ Clostridiales |
| ROC3-B5b | - | 127 | Enterobacter cloacae subsp. cloacae ATCC 13047 (CP001918) | 122/127 | 96 | Unclassified root |
| ROC3-B6 | - | 168 | Enterobacter cloacae subsp. cloacae ATCC 13047 (CP001918) | 161/168 | 96 | Unclassified root |
| ROC3-B8 | KM355628 | 332 | Verrucomicrobiaceae bacterium CHC12 (FN554390) | 330/332 | 99 | Verrucomicrobiae/ Verrucomicrobiales |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | Luteolibacter yonseiensis strain EBTL01 (NR_109549) | 329/332 | | |
| ROC3-B9 | KM355629 | 341 | Stenotrophomonas sp. enrichment culture clone CCNWSP15 (KF735811) | 341/341 | 100 | γ-Proteobacteria/ Xanthomonadales |
| ROC3-B10 | KM355630 | 340 | Uncultured bacterium clone I13 (DQ926681) | 340/340 | 100 | γ-Proteobacteria/ Xanthomonadales |

[a] Closest organism at GenBank and, when possible, cultured closest match. In parenthesis, Accession Number.

[b] Classified using the Ribosomal Database Project (RDP)

### 9.2.4. PCA and DCA analysis of PLFA and DGGE results

In an attempt to find possible trends or grouping of samples, Principal Component Analysis (PCA) was performed for PLFA and Detrended Correspondence Analysis (DCA) for DGGE results. They led to different grouping of samples because of the differences between the approaches: data in the former is a quantitative pattern of certain community-level changes of microbes whereas in the last the data gives a qualitative estimation of presence or absence of microbial taxa in the sampled systems. For PCA analysis, data of 43 quantified PLFAs was considered for every sample. Analyses were performed for each experiment separately but also grouped. At Fig. 9.7 only the most relevant PCA results are ploted. Those are the ones from: i) all the three experiments grouped, ii) only the two treating VHW and, iii) ROC3 alone. Data for DCA analysis was obtained through performing binary matrixes (presence/absence of band) for all the DGGE gels with the program GelCompar II, manually checked to avoid errors. Then, DCA analysis for the joined binary matrixes of bacteria and fungi were performed for the same groups of experiments than those presented for PCA to facilitate the comparison of both analyses. The only difference is that samples for DCA are from liquid samples but also from the fungal pellets at bioreactors I and from the formed biofilm at the end of the bioreactors NI whereas for PCA analysis only liquid samples were taken. DCA analyses of other groupings were performed as well for possible different patterns but no extra information could be obtained. Finally, DCA analysis for bacteria and fungi separately were also performed to determine if similar distributions were found or not. Those results are not presented, as they did not provide with new relevant information. VHW3-NI8 and VHW4-NI1 samples were not taken into account for DCA analysis due to suspicion of contamination as they presented a band corresponding to *T.*

*versicolor* (Fig. 9.5) Environmental and operational parameters such as pH, COD, conductivity, glucose concentration and addition rate, laccase activity, bacterial and fungal concentration, F/B ratio, *T. versicolor* concentration and day of operation were assessed for their relation with the samples distribution at all PCA and DCA analyses.

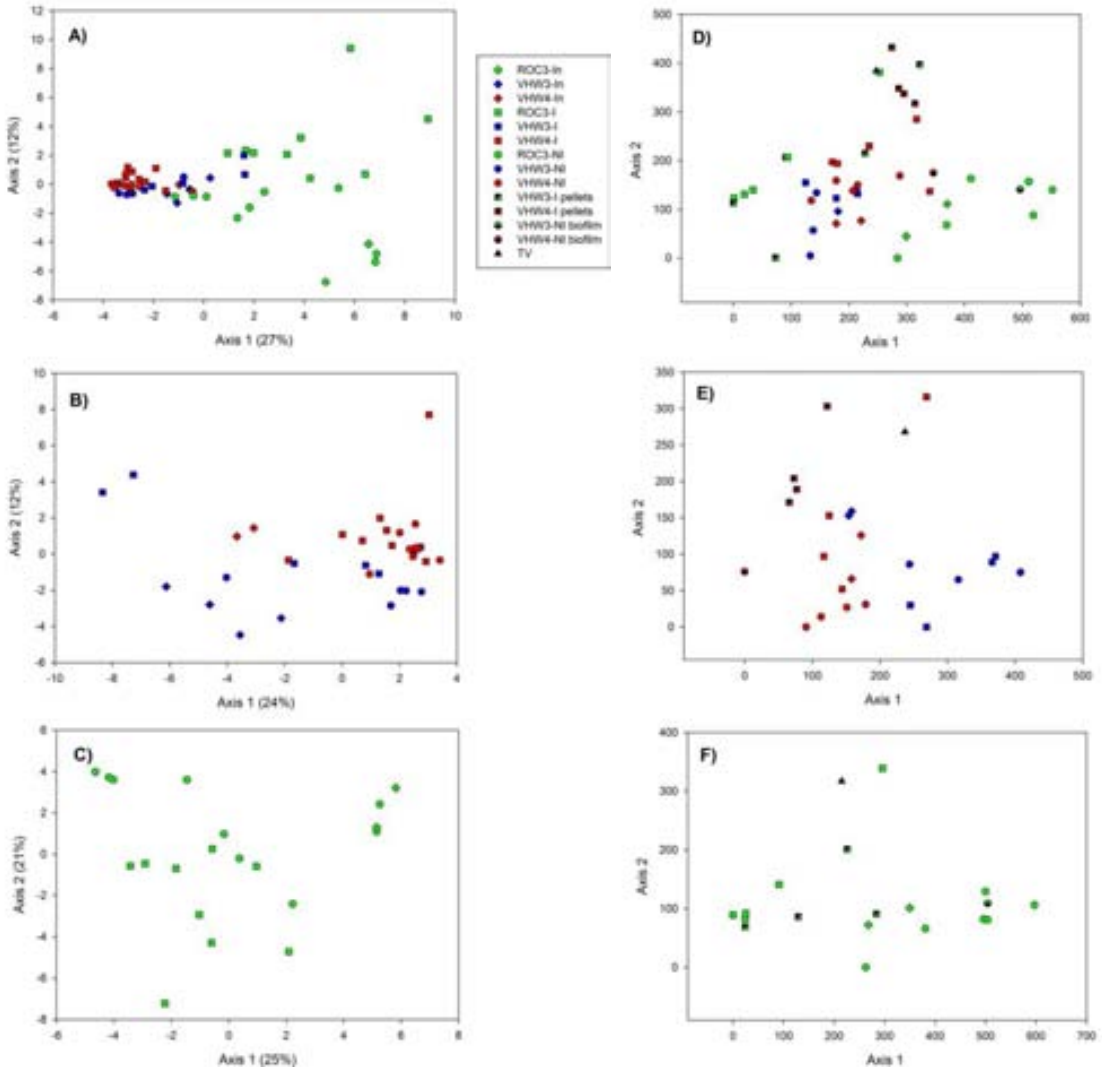



**Figure 9.7.** Principal Component Analysis (PCA) of PLFA results:**A)** Total samples, **B)** VHW samples and **C)** ROC samples; and Detrended Correspondence Analysis (DCA) of DGGE results: **D)** Total samples, **E)** VHW samples and **F)** ROC samples.Samples can be grouped in: influent samples (In), samples from the effluent of the fungal inoculated (I) or the non-inoculated control (NI) bioreactors, or from whashed pellets of *T. versicolor* from inside the I reactor.

When examining the general distribution of all the samples, it can be seen that in PCA, there was a clear separation between microbial communities of ROC

and VHW systems (Fig. 9.7A) but not so clear at DCA graph (Fig. 9.7D). This indicated that the differences would be in the relative abundances of certain microbial groups rather than in the species-level composition of microorganisms. In the last, only VHW pellet samples and samples from the first days of operation of fungal reactors, and influent and non-inculated ROC samples (ROC3-In/NI) can be grouped but systematic big differences between ROC and VHW samples were not observed. Differences in pellets samples are clearly due to the only presence of *T. versicolor* in them. On the other hand, divergence in ROC3-In and ROC3-NI samples with respect to the other samples is probably due to the different origins of the wastewater treated, such as Yang et al. (2011) also found for yeasts analysis. Then, as ROC3-I samples are closer to VHW ones, it is suggested that inoculation with *T. versicolor* can lead to similar microbial communities independently of the wastewater origin.

Regarding VHW experiments (Fig. 9.7B and E), at PCA graph (Fig 9.7B) only influent wastewater and some samples from the first days of operation were differentiated from the other samples. Three samples, VHW4-I6 and VHW3-I23 and VHW3-I30 were seen as out layers: VHW4-I6 probably for its high bacterial concentration (high correlation with 17:0 bacterial phospholipid) and the others for unknown reasons. It has to be taken into account that at day 6 of VHW4 experiment was when *T. versicolor* was dying. No clear grouping of samples from VHW4 when C/N ratio was changed can be found. At DCA, separation between VHW3 and VHW4 can be observed indicating that the microbial taxa in the two treatments were mostly different. Surprisingly, inoculation of *T. versicolor* did not affect as much on the development of microbial communities.

In ROC systems (Fig. 6C and 6F) there was quite clear separation between In, NI and I samples. Specifically, NI and I samples evolved different in both community- and taxa level. Even when *T. versicolor* disapeared at ROC3-I, microbial communities were totally different between I and NI. At DCA analysis, COD is related to Axis 1 with an $r^2$ of 0.47 because NI samples had higher COD and laccase activity is related to Axis 2 by an $r^2$ of 0.35.

Summarising, inoculation of *T. versicolor* strongly affected the development of microbial communities in the ROC bioreactors but not in the VHW ones. The last were more homogeneous, with fewer differences between VHW-I and VHW-NI than between VHW3 and VHW4. Therefore, the feeding water has a major importance in the developing community of the bioreactors as already pointed Yang et al. (2011). Time is not an explanatory variable for samples distribution.

## CONCLUSIONS

The results obtained in the batch bioreactors treating VHW, where *T. versicolor* lost predominance between 7 and 15 days in the 1.5 L reactor (but not in the 10 L bioreactor), confirmed the previous insights that partial renewal of fungal biomass is needed for long-term operations.

Derived from the results of the continuous treatment, it seems that the concentration of microorganisms in the wastewater determines their concentration inside the bioreactors. Even so, total concentration of microorganisms seems to be independent of *T. versicolor* survival because it was overtaken just in the bioreactor with less microbial load.

Discussion about microbial competition with the inoculated fungi in non-sterile bioreactors has focused mainly on bacterial growth, faster than fungi. Indeed, at VHW4 experiment, bacteria growth due to the faster consumption of glucose than fungi probably caused the lysis of *T. versicolor* on day 6. However, in the present work, results show that other fungi can also successfully compete with the inoculated one. Particularly, at ROC3 treatment, *Trichoderma* sp. overtook *T. versicolor* between days 15 and 20.

On the other hand, higher degradation of micropollutants can be achieved when certain fungi are favoured instead of bacteria. For instance, at control reactor of the ROC treatment (ROC-NI), a *Penicillium* sp. with laccase activity was detected when removal of PhACs was up to 38%. Moreover, an increase in the fungal/bacterial (F/B) ratio (at VHW4-I and NI) and the subsequent increase in the degradation percentage (only at VHW4-I) were detected when C/N ratio of the added nutrients was decreased. Therefore, further experiments changing C/N ratio should be performed to confirm those as fungal-favouring conditions. Moreover, other approaches trying to minimise bacteria and enhance fungal survival should be also tested, such as using some lignocellulosic materials as nutrient source and support for their growth.

DCA and PCA analysis show that the feeding water and/or the different operational conditions applied to each experiment had more importance in the community that developed in the bioreactors than the inoculation or not with the fungus.

Finally, in the attempt of finding a parameter to monitor the survival of the inoculated fungus, laccase activity does not seem to be a good one, as its presence or absence is not directly correlated with the activity of the fungus. Therefore, traditional methods to monitor inoculated fungi survival and activity, such as dry cell weight, laccase activity or visual aspect of the pellets, can help in giving a clue about bioreactor performance but they cannot be a determinant factor for operation evaluation. Complementary tools such as molecular analyses should be performed for every experiment for a deeper understanding of the processes taking place until optimal conditions for the treatment of non-sterile effluents will be achieved.

# Chapter 10

## General conclusions and future perspectives

# GENERAL CONCLUSIONS

In the present thesis, some relevant factors in the fungal degradation of emerging contaminants have been assessed. The obtained conclusions can be grouped in the three blocks presented in the objectives Chapter:

Factors related to individual degradation of contaminants:

- *Trametes versicolor* was able to degrade all EDCs studied in serum bottles with spiked defined medium. However, degradation rates were different for each compound (even between isomers): UV filters (BP3, BP1 and 4-MBC) were transformed faster than benzotriazoles.

- During the degradation of the emerging contaminants studied in this thesis, conjugated metabolites constituted an important percentage of the detected metabolites. Therefore, if the contaminant lacked suitable groups (i.e. hydroxyl groups), the action of the oxidative enzyme cytochrome P450 constituted the first step in the degradation pathway followed by its conjugation and, usually, further deconjugation and subsequent degradation.

- This work highlights the importance of identifying the transformation products because sometimes removal of the parent compounds only implies partial but not complete degradation, as happened with BTZ, which turned out to be the most recalcitrant among the studied contaminants.

- The selected contaminants presented acute toxicity and endocrine disrupting activity, measured by means of *Vibrio fisheri* and RYA assays, at concentrations above those which they are usually detected in the environment. Despite fungal metabolites sometimes presented higher toxicity (e.g. 4HB), calculated $EC_{50}$ were still in the range of mg $L^{-1}$ or high µg $L^{-1}$.

- Use of combined techniques using contaminants labelled with stable isotopes allowed us to determine their fate (i.e. mineralization, incorporation or only partial degradation) during fungal degradation. Fungal incorporation of PPCPs as carbon source is reported for the first time.

Factors related to operational conditions of bioreactors treating real wastewater:

- When working with real effluents, addition of certain amount of external nutrients was found to be necessary, because nutrients supplied by the wastewater were not enough.

- Conjugation and deconjugation processes might be relevant during the fungal treatments but also in the biological processes of WWTPs. This issue makes it difficult to monitor the treatments.

- Aeration might be an important factor for the deconjugation of some compounds in the fungal treatment and, thus, requires to be controlled.

- Reverse osmosis concentrate and veterinary hospital wastewater were successfully treated, with maximum removal percentages of PhACs achieved of 53% for ROC and 66% for VHW. EDCs were not significantly detected in VHW and removed around 74% in ROC (both sterile batch and non-sterile continuous at HRT 3d).

- Some identified fungal metabolites of benzotriazoles were subsequently detected in the fungal treatment of reverse osmosis concentrate, closing the circle of individual study of metabolites of detected compounds in certain wastewater and further introduction in the analytical methods to monitor them in the fungal treatment of that wastewater.

- Better results regarding emerging contaminants removal were always obtained in the fungal inoculated bioreactors. Despite certain removal

was sometimes observed in the non-inoculated control, global degradation rates and yields were always higher in the fungal treatment.

Factors related to interactions of the inoculated fungus with other microorganisms developed in the bioreactors:

- PLFA analysis pointed out that lower C/N ratios in the extra nutrients supplied might be related with the increase in the fungal/bacterial (F/B) ratio. Therefore, C/N ratio might be important for the maintenance of the fungal predominance when working under non-sterile conditions.

- Fungal competition was observed to be at least as much detrimental as the bacterial one, because a fungus belonging to the genus *Trichoderma* completelly killed *T. versicolor* and colonised the pellets.

- DGGE results confirmed the need for partial biomass renovation to work with a CRT of approximately 10-15 days.

- PCA and DCA analyses showed that *T. versicolor* did not affect microbial community as much as the different feed wastewater and the operational conditions applied did.

- Laccase does not seem to be a good indicator to monitor the survival of the inoculated fungus as its presence or absence was not directly correlated with the activity of the fungus.

# FUTURE PERSPECTIVES

From the results obtained in the present thesis and with the final aim of successfully treating real effluents with fungi in the most efficient way, we think that future studies should focus on the following issues. They can be classified into the two aspects in need for improvement: analytical methods and operational parameters of the process.

On one hand, fungal treatment for emerging contaminant removal is still not optimized and, thus, some operational parameters of the process still need to be improved.

- To study and further optimize either the total amount of nutrients supplied as well as their C/N ratio.

- To study the effect of aeration on the increase of certain compounds and optimize it to avoid that increase.

- To study other strategies trying to promote the advantage of the inoculated fungus over the other competing microorganisms. Some of these strategies might be the operation in sequencing batches to minimize the bacterial growth or the growth of fungal pellets on lignocellulosic materials, reducing at the same time the supply of extra nutrients, in order to minimize the readily available nutrients for the competing microorganisms.

- To find a suitable and useful parameter for the control and monitoring of the fungal treatment.

On the other hand, analytical methods would allow a better monitorisation of the process or understanding of the biological processes taking place.

- To study in depth the conjugation and deconjugation processes taking place in the biological treatments of wastewater. This implies the following: inclusion of conjugated metabolites in the analytical methods, to ensure that the extraction methods include the conjugated compounds and/or incorporation of a step of hydrolysis before the analysis.

- To perform PLFA-SIP, DNA-SIP or RNA-SIP analyses to non-sterile treatments in order to determine the fate of the contaminants among the microbial community.

- To keep analysing the treatments microbiologically in order to broaden the knowledge regarding the relationship between operational conditions and the microorganisms developed.

# Chapter 11

## References

Al Ashhab, A., Herzberg, M., Gillor, O., 2014. Biofouling of reverse-osmosis membranes during tertiary wastewater desalination: microbial community composition. Water Res. 50, 341–9.

Al-Rajab, A.J., Sabourin, L., Lapen, D.R., Topp, E., 2010. The non-steroidal anti-inflammatory drug diclofenac is readily biodegradable in agricultural soils. Sci. Total Environ. 409, 78–82.

APHA-AWWA-WEF, 1995. Standard Methods for the Examination of Water and Wastewater, 19th ed. American Public Association/AmericaWaterWorks Association/Water Environment Federation, Washington, DC.

Archibald, F., Paice, M.G., Jurasek, L., 1990. Decolorization of kraft bleachery effluent chromophores by Coriolus (Trametes) versicolor. Enzyme Microb. Technol. 12, 846–853.

Ash, M., Ash, I., 2004. Handbook of green chemicals, 2nd ed. Synapse Information Resources Inc., New York.

Asha, S., Vidyavathi, M., 2009. Cunninghamella – A microbial model for drug metabolism studies – A review. Biotechnol. Adv. 27, 16–29.

Asimakopoulos, A.G., Ajibola, A., Kannan, K., Thomaidis, N.S., 2013. Occurrence and removal efficiencies of benzotriazoles and benzothiazoles in a wastewater treatment plant in Greece. Sci. Total Environ. 452-453, 163–71.

Auriol, M., Filali-meknassi, Y., Adams, C.D., Tyagi, R.D., Noguerol, T., Piña, B., 2008. Removal of estrogenic activity of natural and synthetic hormones from a municipal wastewater : Efficiency of horseradish peroxidase and laccase from Trametes versicolor. Chemosphere 70, 445–452.

Ayache, C., Manes, C., Pidou, M., Croué, J.P., Gernjak, W., 2013. Microbial community analysis of fouled reverse osmosis membranes used in water recycling. Water Res. 47, 3291–9.

Baccar, R., Sarrà, M., Bouzid, J., Feki, M., Blánquez, P., 2012. Removal of pharmaceutical compounds by activated carbon prepared from agricultural by-product. Chem. Eng. J. 211-212, 310–317.

Bachelot, M., Li, Z., Munaron, D., Le, P., Casellas, C., Fenet, H., Gomez, E., 2012. Organic UV filter concentrations in marine mussels from French coastal regions. Sci. Total Environ. 420, 273–279.

Badia-Fabregat, M., Caminal, G., Vicent, T., Blanquez, P., Gago-Ferrero, P., Olivares, A., Piña, B., Díaz-Cruz, M.S., Barceló, D., 2013. UV Filters Biodegradation by Fungi , Metabolites Identification and Biological Activity Assessment, in: Vicent, T., Caminal, G., Eljarrat, E., Barceló, D. (Eds.), Emerging Organic Contaminants in Sludges. Springer, pp. 215–240.

Badia-Fabregat, M., Rodríguez-Rodríguez, C.E., Gago-Ferrero, P., Olivares, A., Piña, B., Díaz-Cruz, M.S., Vicent, T., Barceló, D., Caminal, G., 2012. Degradation of UV filters in sewage sludge and 4-MBC in liquid medium by the ligninolytic fungus Trametes versicolor. J. Environ. Manage. 104, 114–20.

Baldrian, P., 2004. Increase of laccase activity during interspecific interactions of white-rot fungi. FEMS Microbiol. Ecol. 50, 245–53.

Balmer, M.E., Buser, H., Müller, M.D., Poiger, T., 2005. Occurrence of Some Organic UV Filters in Wastewater , in Surface Waters , and in Fish from Swiss Lakes. Environ. Sci. Technol. 39, 953–962.

Banitz, T., Johst, K., Wick, L.Y., Schamfuß, S., Harms, H., Frank, K., 2013. Highways versus pipelines: contributions of two fungal transport mechanisms to efficient bioremediation. Environ. Microbiol. Rep. 5, 211–8.

Barr, D.P., Aust, S.D., 1994. Mechanisms white rot fungi use to degrade pollutants. Environ. Sci. Technol. 28, 78A–87A.

Bastida, F., Jechalke, S., Bombach, P., Franchini, A.G., Seifert, J., Von Bergen, M., Vogt, C., Richnow, H.H., 2011. Assimilation of benzene carbon through multiple trophic levels traced by different stable isotope probing methodologies. FEMS Microbiol. Ecol. 77, 357–369.

Bastida, F., Rosell, M., Franchini, A.G., Seifert, J., Finsterbusch, S., Jehmlich, N., Jechalke, S., Bergen, M. Von, Richnow, H.H., 2010. Elucidating MTBE degradation in a mixed consortium using a multidisciplinary approach. FEMS Microbiol. Ecol. 73, 370–384.

Bayramoğlu, G., Yakup Arica, M., 2007. Biosorption of benzidine based textile dyes “Direct Blue 1 and Direct Red 128” using native and heat-treated biomass of Trametes versicolor. J. Hazard. Mater. 143, 135–43.

Blánquez, P., Caminal, G., Sarrà, M., Vicent, T., 2007. The effect of HRT on the decolourisation of the Grey Lanaset G textile dye by Trametes versicolor. Chem. Eng. J. 126, 163–169.

Blánquez, P., Casas, N., Font, X., Gabarrell, X., Sarrà, M., Caminal, G., Vicent, T., 2004. Mechanism of textile metal dye biotransformation by Trametes versicolor. Water Res. 38, 2166–72.

Blánquez, P., Guieysse, B., 2008. Continuous biodegradation of 17beta-estradiol and 17alpha-ethynylestradiol by Trametes versicolor. J. Hazard. Mater. 150, 459–62.

Blánquez, P., Sarrà, M., Vicent, M.T., 2006. Study of the cellular retention time and the partial biomass renovation in a fungal decolourisation continuous process. Water Res. 40, 1650–6.

Blánquez, P., Sarrà, M., Vicent, T., 2008. Development of a continuous process to adapt the textile wastewater treatment by fungi to industrial conditions. Process Biochem. 43, 1–7.

Boas, M., Feldt-rasmussen, U., Main, K.M., 2011. Thyroid effects of endocrine disrupting chemicals. Mol. Cell. Endocrinol. 355, 240–248.

Borchert, M., Libra, J.A., 2001. Decolorization of reactive dyes by the white rot fungus Trametes versicolor in sequencing batch reactors. Biotechnol. Bioeng. 75, 313–21.

Borràs, E., Blánquez, P., Sarrà, M., Caminal, G., Vicent, T., 2008. Trametes versicolor pellets production: Low-cost medium and scale-up. Biochem. Eng. J. 42, 61–66.

Borràs, E., Caminal, G., Sarrà, M., Novotný, Č., 2010. Effect of soil bacteria on the ability of polycyclic aromatic hydrocarbons (PAHs) removal by Trametes versicolor and Irpex lacteus from contaminated soil. Soil Biol. Biochem. 42, 2087–2093.

Bourbonnais, R., Paice, M.G., 1990. Oxidation of non-phenolic subsstrates. An expanded of non-phenolic substrates role for laccase in lignin biodegradation. FEBS Lett. 267, 99–102.

Brausch, J.M., Rand, G.M., 2011. A review of personal care products in the aquatic environment : Environmental concentrations and toxicity. Chemosphere 82, 1518–1532.

Bruce, A., Highley, T.L., 1991. Control of growth of wood decay Basidiomycetes by Trichoderma spp . and other potentially antagonistic fungi. For. Prod. J. 41, 63–67.

Buser, H.-R., Balmer, M.E., Schmid, P., Kohler, M., 2006. Occurrence of UV filters 4-methylbenzylidene camphor and octocrylene in fish from various Swiss rivers with inputs from wastewater treatment plants. Environ. Sci. Technol. 40, 1427–31.

Buser, H.-R., Müller, M.D., Balmer, M.E., Poiger, T., Buerge, I.J., 2005. Stereoisomer composition of the chiral UV filter 4-methylbenzylidene camphor in environmental samples. Environ. Sci. Technol. 39, 3013–9.

Cajthaml, T., Kresinová, Z., Svobodová, K., Möder, M., 2009. Biodegradation of endocrine-disrupting compounds and suppression of estrogenic activity by ligninolytic fungi. Chemosphere 75, 745–50.

Calafat, A.M., Wong, L.-Y., Ye, X., Reidy, J. a, Needham, L.L., 2008. Concentrations of the sunscreen agent benzophenone-3 in residents of the United States: National Health and Nutrition Examination Survey 2003--2004. Environ. Health Perspect. 116, 893–7.

Campoy, S., Alvarez-Rodríguez, M.L., Recio, E., Rumbero, A., Coque, J.-J.R., 2009. Biodegradation of 2,4,6-TCA by the white-rot fungus Phlebia radiata is initiated by a phase I (O-demethylation)-phase II (O-conjugation) reactions system: implications for the chlorine cycle. Environ. Microbiol. 11, 99–110.

Cancilla, D. a, Baird, J.C., Geis, S.W., Corsi, S.R., 2003. Studies of the environmental fate and effect of aircraft deicing fluids: detection of 5-methyl-1H-benzotriazole in the fathead minnow (Pimephales promelas). Environ. Toxicol. Chem. 22, 134–40.

Cancilla, D. a., Martinez, J., van Aggelen, G.C., 1998. Detection of Aircraft Deicing/Antiicing Fluid Additives in a Perched Water Monitoring Well at an International Airport. Environ. Sci. Technol. 32, 3834–3835.

Cañas, A.I., Camarero, S., 2010. Laccases and their natural mediators: biotechnological tools for sustainable eco-friendly processes. Biotechnol. Adv. 28, 694–705.

Carballa, M., Omil, F., Lema, J.M., 2008. Comparison of predicted and measured concentrations of selected pharmaceuticals, fragrances and hormones in Spanish sewage. Chemosphere 72, 1118–23.

Carballeira, J.D., Quezada, M.A., Hoyos, P., Simeó, Y., Hernaiz, M.J., Alcantara, A.R., Sinisterra, J. V, 2009. Microbial cells as catalysts for stereoselective red-ox reactions. Biotechnol. Adv. 27, 686–714.

Carey, R.O., Migliaccio, K.W., 2009. Contribution of wastewater treatment plant effluents to nutrient dynamics in aquatic systems: a review. Environ. Manage. 44, 205–17.

Casas, N., Blánquez, P., Vicent, T., Sarrà, M., 2013. Laccase production by Trametes versicolor under limited-growth conditions using dyes as inducers. Environ. Technol. 34, 113–119.

Cerniglia, C.E., 1997. Fungal metabolism of polycyclic aromatic hydrocarbons: past, present and future applications in bioremediation. J. Ind. Microbiol. Biotechnol. 19, 324–33.

Cerniglia, C.E., Freeman, J.P., Mitchum, R.K., 1982. Glucuronide and sulfate conjugation in the fungal metabolism of aromatic hydrocarbons. Appl. Environ. Microbiol. 43, 1070–5.

Cheng, Z., Xiang-hua, W., Ping, N., 2013. Continuous Acid Blue 45 decolorization by using a novel open fungal reactor system with ozone as the bactericide. Biochem. Eng. J. 79, 246–252.

Christen, V., Zucchi, S., Fent, K., 2011. Effects of the UV-filter 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC) on expression of genes involved in hormonal pathways in fathead minnows (Pimephales promelas) and link to vitellogenin induction and histology. Aquat. Toxicol. 102, 167–176.

Christensen, F.M., 1998. Pharmaceuticals in the environment--a human risk? Regul. Toxicol. Pharmacol. 28, 212–21.

Citulski, J., Farahbakhsh, K., 2012. Overcoming the toxicity effects of municipal wastewater sludge and biosolid extracts in the Yeast Estrogen Screen (YES) assay. Chemosphere 87, 498–503.

Cobas, M., Ferreira, L., Tavares, T., Sanromán, M.A., Pazos, M., 2013. Development of permeable reactive biobarrier for the removal of PAHs by Trichoderma longibrachiatum. Chemosphere 91, 711–6.

Collins, P.J., Dobson, A.D.W., 1997. Regulation of Laccase Gene Transcription in Trametes versicolor. Appl. Environ. Microbiol. 63, 3444–3450.

Comerton, A.M., Andrews, R.C., Bagley, D.M., Hao, C., 2008. The rejection of endocrine disrupting and pharmaceutically active compounds by NF and RO membranes as a function of compound and water matrix properties. J. Memb. Sci. 313, 323–335.

Coplen, T.B., Brand, W.A., Gehre, M., Gröning, M., Meijer, H.A.J., Toman, B., Verkouteren, R.M., 2006. New Guidelines for δ 13 C Measurements. Anal. Chem. 78, 2439–2441.

Cornelissen, G., van Noort, P.C.M., Parsons, J.R., Govers, H. a. J., 1997. Temperature Dependence of Slow Adsorption and Desorption Kinetics of Organic Compounds in Sediments. Environ. Sci. Technol. 31, 454–460.

Coronado, M., De Haro, H., Deng, X., Rempel, M.A., Lavado, R., Schlenk, D., 2008. Estrogenic activity and reproductive effects of the UV-filter oxybenzone (2-hydroxy-4-methoxyphenyl-methanone) in fish. Aquat. Toxicol. 90, 182–187.

Cruz-Morató, C., Ferrando-Climent, L., Rodriguez-Mozaz, S., Barceló, D., Marco-Urrea, E., Vicent, T., Sarrà, M., 2013a. Degradation of pharmaceuticals in non-sterile urban wastewater by Trametes versicolor in a fluidized bed bioreactor. Water Res. 47, 5200–10.

Cruz-Morató, C., Lucas, D., Llorca, M., Rodriguez-Mozaz, S., Gorga, M., Petrovic, M., Barceló, D., Vicent, T., Sarrà, M., Marco-Urrea, E., 2014. Hospital wastewater treatment by fungal bioreactor: Removal efficiency for pharmaceuticals and endocrine disruptor compounds. Sci. Total Environ. 493, 365–376.

Cruz-Morató, C., Rodríguez-Rodríguez, C.E., Marco-Urrea, E., Sarrà, M., Caminal, G., Vicent, T., Jelic, A., García-Galán, M.J., Pérez, S., Díaz-Cruz, M.S., Petrovic, M., Barceló, D., 2013b. Biodegradation of Pharmaceuticals by Fungi and Metabolites Identification, in: Vicent, T., Caminal, G., Eljarrat, E., Barceló, D. (Eds.), Emerging Organic Contaminants in Sludges. Springer, pp. 165–213.

D'Annibale, a, Crestini, C., Vinciguerra, V., Sermanni, G.G., 1998. The biodegradation of recalcitrant effluents from an olive mill by a white-rot fungus. J. Biotechnol. 61, 209–18.

D'Annibale, A., Quaratino, D., Federici, F., Fenice, M., 2006. Effect of agitation and aeration on the reduction of pollutant load of olive mill wastewater by the white-rot fungus Panus tigrinus. Biochem. Eng. J. 29, 243–249.

Daughton, C.G., Ternes, T.A., 1999. Pharmaceuticals and personal care products in the environment: agents of subtle change? Environ. Health Perspect. 107, 907–38.

Del Pino, M.P., Durham, B., 1999. Wastewater reuse through dual-membrane processes : opportunities for sustainable water resources. Desalination 124, 271–277.

Desai, C., Pathak, H., Madamwar, D., 2010. Advances in molecular and "-omics" technologies to gauge microbial communities and bioremediation at xenobiotic/anthropogen contaminated sites. Bioresour. Technol. 101, 1558–69.

Dhouib, A., Aloui, F., Hamad, N., Sayadi, S., 2006. Pilot-plant treatment of olive mill wastewaters by Phanerochaete chrysosporium coupled to anaerobic digestion and ultrafiltration. Process Biochem. 41, 159–167.

Díaz-Cruz, M.S., Barceló, D., 2009. Chemical analysis and ecotoxicological effects of organic UV-absorbing compounds in aquatic ecosystems. TrAC Trends Anal. Chem. 28, 708–717.

Díaz-Cruz, M.S., Gago-Ferrero, P., Llorca, M., Barceló, D., 2012. Analysis of UV filters in tap water and other clean waters in Spain. Anal. Bioanal. Chem. 402, 2325–2333.

Díaz-Cruz, M.S., Llorca, M., Barceló, D., 2008. Organic UV filters and their photodegradates , metabolites and disinfection by-products in the aquatic environment. Trends Anal. Chem. 27, 873–887.

Dietrich, D.R., Prietz, A., 1999. Fish embryotoxicity and teratogenicity of pharmaceuticals, detergents and pesticides regularly detected in sewage treatment plants effluents and surface waters. Toxicol. 38, 151.

Dolar, D., Gros, M., Rodriguez-Mozaz, S., Moreno, J., Comas, J., Rodriguez-Roda, I., Barceló, D., 2012. Removal of emerging contaminants from municipal wastewater with an integrated membrane system, MBR-RO. J. Hazard. Mater. 239-240, 64–9.

Duan, Y.-P., Meng, X.-Z., Wen, Z.-H., Ke, R.-H., Chen, L., 2013. Multi-phase partitioning, ecological risk and fate of acidic pharmaceuticals in a wastewater receiving river: the role of colloids. Sci. Total Environ. 447, 267–73.

Eibes, G., Debernardi, G., Feijoo, G., Moreira, M.T., Lema, J.M., 2011. Oxidation of pharmaceutically active compounds by a ligninolytic fungal peroxidase. Biodegradation 22, 539–50.

Eikenes, M., Hietala, A.M., Alfredsen, G., Gunnar Fossdal, C., Solheim, H., 2005. Comparison of quantitative real-time PCR, chitin and ergosterol assays for monitoring colonization of Trametes versicolor in birch wood. Holzforschung 59, 568.

Ellis, J.B., 2006. Pharmaceutical and personal care products (PPCPs) in urban receiving waters. Environ. Pollut. 144, 184–9.

EPA, 2000. Assigning values to non-detected/non-quantified pesticide residues in human health food exposure assessments. Washington, DC.

Esplugas, S., Bila, D.M., Krause, L.G.T., Dezotti, M., 2007. Ozonation and advanced oxidation technologies to remove endocrine disrupting chemicals (EDCs) and pharmaceuticals and personal care products (PPCPs) in water effluents. J. Hazard. Mater. 149, 631–42.

European Commission, 2002. COMMISSION DIRECTIVE 2002/72/EC of 6 August 2002 relating to plastic materials and articles intended to come into contact with foodstuffs.

European Commission, 2007. Commission Staff Working Document on the implementation for the "Community Strategy for Endocrine Disrupters"- a range of substances suspected of interfering with the hormone systems of humans and wildlife. SEC (2007) 1635, Framework.

European Commission, 2012. Report from the Commission to the European Parliament and the Council on the outcome of the review of Annex X to Directive 2000/60/EC of the European Parliament and of the Council on priority substances in the field of water policy. COM (2011) 875 final.

European Parliament, 2000. Directive 2000/60/CE of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy., Official Journal of the European Communities L 327.

Eyre, C., Muftah, W., Hiscox, J., Hunt, J., Kille, P., Boddy, L., Rogers, H.J., 2010. Microarray analysis of differential gene expression elicited in Trametes versicolor during interspecific mycelial interactions. Fungal Biol. 114, 646–660.

Farré, M.L., Ferrer, I., Ginebreda, a, Figueras, M., Olivella, L., Tirapu, L., Vilanova, M., Barceló, D., 2001. Determination of drugs in surface water and wastewater samples by liquid chromatography-mass spectrometry: methods and preliminary results including toxicity studies with Vibrio fischeri. J. Chromatogr. A 938, 187–97.

Felix, T., Hall, B.J., S. Brodbelt, J., 1998. Determination of benzophenone-3 and metabolites in water and human urine by solid-phase microextraction and quadrupole ion trap GC–MS. Anal. Chim. Acta 371, 195–203.

Fent, K., Kunz, P.Y., Zenker, A., Rapp, M., 2010a. A tentative environmental risk assessment of the UV-filters. Mar. Environ. Res. 3–5.

Fent, K., Weston, A. a, Caminada, D., 2006. Ecotoxicology of human pharmaceuticals. Aquat. Toxicol. 76, 122–59.

Fent, K., Zenker, A., Rapp, M., 2010b. Widespread occurrence of estrogenic UV-filters in aquatic ecosystems in Switzerland. Environ. Pollut. 158, 1817–1824.

Fernandes Qualhato, T., Cardoso Lopes, F.A., Stecca Steindorff, A., Silva Brandão, R., Amorim Jesuino, R.S., Ulhoa, C.J., 2013. Mycoparasitism studies of Trichoderma species against three phytopathogenic fungi: evaluation of antagonism and hydrolytic enzyme production. Biotechnol. Lett. 35, 1461–8.

Ferrari, B., Paxéus, N., Lo Giudice, R., Pollio, A., Garric, J., 2003. Ecotoxicological impact of pharmaceuticals found in treated wastewaters: study of carbamazepine, clofibric acid, and diclofenac. Ecotoxicol. Environ. Saf. 55, 359–70.

Ferreira, B., Jelic, A., López-serna, R., Mozeto, A.A., Petrovic, M., Barceló, D., 2011. Occurrence and distribution of pharmaceuticals in surface water, suspended solids and sediments of the Ebro river basin, Spain. Chemosphere 85, 1331–1339.

Flores, C., Vidal, C., Trejo-Hernández, M.R., Galindo, E., Serrano-Carreón, L., 2009. Selection of Trichoderma strains capable of increasing laccase production by Pleurotus ostreatus and Agaricus bisporus in dual cultures. J. Appl. Microbiol. 106, 249–57.

Font, X., Caminal, G., Gabarrell, X., Romero, S., Vicent, M.T., 2003. Black liquor detoxification by laccase of Trametes versicolor pellets. J. Chem. Technol. Biotechnol. 78, 548–554.

Freitag, M., Morrell, J.J., 1992. Changes in selected enzyme activities during growth of pure and mixed cultures of the white-rot decay fungus Trametes versicolor and the potential biocontrol fungus Trichoderma harzianum. Can. J. Microbiol. 38, 317–23.

Frostegård, A., Bååth, E., 1996. The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. Biol. Fertil. Soils 22, 59–65.

Frostegård, Å., Bååth, E., Tunlio, A., 1993. Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty acid analysis. Soil Biol. Biochem. 25, 723–730.

Frostegard, A., Tunlid, A., Baath, E., 1991. Microbial biomass measured as total lipid phosphate in soils of different organic content. J. Microbiol. Methods 14, 151–163.

Fujii, K., Kikuchi, S., 2005. Degradation of benzophenone, a potential xenoestrogen, by a yeast isolated from the activated sludge of a sewage treatment plant in Hokkaido. World J. Microbiol. Biotechnol. 21, 1311–1315.

Fukazawa, T., Yamazaki, Y., Miyamoto, Y., 2010. Reduction of non-specific adsorption of drugs to plastic containers used in bioassays or analyses. J. Pharmacol. Toxicol. Methods 61, 329–33.

Gabarrell, X., Font, M., Vicent, T., Caminal, G., Sarrà, M., Blánquez, P., 2012. A comparative life cycle assessment of two treatment technologies for the Grey Lanaset G textile dye: biodegradation by Trametes versicolor and granular activated carbon adsorption. Int. J. Life Cycle Assess. 17, 613–624.

Gago-Ferrero, P., Badia-Fabregat, M., Olivares, A., Piña, B., Blánquez, P., Vicent, T., Caminal, G., Díaz-Cruz, M.S., Barceló, D., 2012. Evaluation of fungal- and photo-degradation as potential treatments for the removal of sunscreens BP3 and BP1. Sci. Total Environ. 427-428, 355–63.

Gago-Ferrero, P., Díaz-Cruz, M.S., Barceló, D., 2011a. Occurrence of multiclass UV filters in treated sewage sludge from wastewater treatment plants. Chemosphere 84, 1158–1165.

Gago-Ferrero, P., Díaz-Cruz, M.S., Barceló, D., 2011b. Fast pressurized liquid extraction with in-cell purification and analysis by liquid chromatography tandem mass spectrometry for the determination of UV filters and their degradation products in sediments. Anal. Bioanal. Chem. 400, 2195–2204.

Garcia, H.A., Hoffman, C.M., Kinney, K.A., Lawler, D.F., 2011. Laccase-catalyzed oxidation of oxybenzone in municipal wastewater primary effluent. Water Res. 45, 1921–1932.

García-Galán, M.J., Rodríguez-Rodríguez, C.E., Vicent, T., Caminal, G., Díaz-Cruz, M.S., Barceló, D., 2011. Biodegradation of sulfamethazine by Trametes versicolor: Removal from sewage sludge and identification of intermediate products by UPLC-QqTOF-MS. Sci. Total Environ. 409, 5505–12.

Gardes, M., Bruns, T.D., 1993. ITS primers with enhanced specificity for basidiomycetes--application to the identification of mycorrhizae and rusts. Mol. Ecol. 2, 113–8.

Generalitat de Catalunya, 2003. DECRET 130/2003, de 13 de maig, pel qual s'aprova el Reglament dels serveis públics de sanejament. DOGC.

Gesell, M., Hammer, E., Mikolasch, A., Schauer, F., 2004. Oxidation and ring cleavage of dibenzofuran by the filamentous fungus Paecilomyces lilacinus. Arch. Microbiol. 182, 51–59.

Giger, W., Schaffner, C., Kohler, H.P.E., 2006. Benzotriazole and Tolyltriazole as Aquatic Contaminants . 1 . Input and Occurrence in Rivers and Lakes. Environ. Sci. Technol. 40, 7186–7192.

Gilbride, K.A., Lee, D.Y., Beaudette, L.A., 2006. Molecular techniques in wastewater: Understanding microbial communities, detecting pathogens, and real-time process control. J. Microbiol. Methods 66, 1–20.

Giokas, D., Salvador, a, Chisvert, a, 2007. UV filters: From sunscreens to human body and the environment. TrAC Trends Anal. Chem. 26, 360–374.

Glenn, J.K., Gold, M.H., 1985. Purification and characterization of an extracellular Mn(II)-dependent peroxidase from the lignin-degrading basidiomycete, Phanerochaete chrysosporium. Arch. Biochem. Biophys. 242, 329–341.

Glenn, J.K., Morgan, M.A., Mayfield, M.B., Kuwahara, M., Gold, M.H., 1983. An extracellular H2O2-requiring enzyme preparation involved in lignin biodegradation by the white rot basidiomycete Phanerochaete chrysosporium. Biochem. Biophys. Res. Commun. 114, 1077–1083.

Göbel, A., Thomsen, A., McArdell, C.S., Joss, A., Giger, W., 2005. Occurrence and sorption behavior of sulfonamides, macrolides, and trimethoprim in activated sludge treatment. Environ. Sci. Technol. 39, 3981–9.

Gomes, R.L., Scrimshaw, M.D., Lester, J.N., 2009. Fate of conjugated natural and synthetic steroid estrogens in crude sewage and activated sludge batch studies. Environ. Sci. Technol. 43, 3612–8.

Gorga, M., Petrovic, M., Barceló, D., 2013. Multi-residue analytical method for the determination of endocrine disruptors and related compounds in river and waste water using dual column liquid chromatography switching system coupled to mass spectrometry. J. Chromatogr. A 1295, 57–66.

Gros, M., Rodríguez-Mozaz, S., Barceló, D., 2012. Fast and comprehensive multi-residue analysis of a broad range of human and veterinary pharmaceuticals and some of their metabolites in surface and treated waters by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem. J. Chromatogr. A 1248, 104–121.

Haarmann-Stemmann, T., Bothe, H., Abel, J., 2009. Growth factors, cytokines and their receptors as downstream targets of arylhydrocarbon receptor (AhR) signaling pathways. Biochem. Pharmacol. 77, 508–20.

Hai, F.I., Yamamoto, K., Nakajima, F., Fukushi, K., 2008. Factors governing performance of continuous fungal reactor during non-sterile operation – The case of a membrane bioreactor treating textile wastewater Tap water. Chemosphere 74, 810–817.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Hammer, E., Schoefer, L., Schäfer, A., Hundt, K., Schauer, F., 2001. Formation of glucoside conjugates during biotransformation of dibenzofuran by Penicillium canescens SBUG-M 1139. Appl. Microbiol. Biotechnol. 57, 390–4.

Harms, H., Schlosser, D., Wick, L.Y., 2011. Untapped potential: exploiting fungi in bioremediation of hazardous chemicals. Nat. Rev. Microbiol. 9, 177–92.

Harris, C.A., Routledge, E.J., Schaffner, C., Brian, J. V., Giger, W., Sumpter, J.P., 2007. Benzotriazole is antiestrogenic in vitro but not in vivo. Environ. Toxicol. Chem. 26, 2367–2372.

Hart, D.S., Davis, L.C., Erickson, L.E., Callender, T.M., 2004. Sorption and partitioning parameters of benzotriazole compounds. Microchem. J. 77, 9–17.

Hata, T., Kawai, S., Okamura, H., Nishida, T., 2010. Removal of diclofenac and mefenamic acid by the white rot fungus Phanerochaete sordida YK-624 and identification of their metabolites after fungal transformation. Biodegradation 21, 681–9.

He, Z., Gentry, T.J., Schadt, C.W., Wu, L., Liebich, J., Chong, S.C., Huang, Z., Wu, W., Gu, B., Jardine, P., Criddle, C., Zhou, J., 2007. GeoChip: a comprehensive microarray for investigating biogeochemical, ecological and environmental processes. ISME J. 1, 67–77.

Heinrichs, G., Hübner, I., Schmidt, C.K., de Hoog, G.S., Haase, G., 2013. Analysis of black fungal biofilms occurring at domestic water taps. I: compositional analysis using Tag-Encoded FLX Amplicon Pyrosequencing. Mycopathologia 175, 387–97.

Heneweer, M., Muusse, M., Berg, M. Van Den, Sanderson, J.T., 2005. Additive estrogenic effects of mixtures of frequently used UV filters on pS2-gene transcription in MCF-7 cells. Toxicol. Appl. Pharmacol. 208, 170 – 177.

Henriksson, G., Zhang, L., Li, J., Ljungquist, P., Reitberger, T., Pettersson, G., Johansson, G., 2000. Is cellobiose dehydrogenase from Phanerochaete chrysosporium a lignin degrading enzyme? Biochim. Biophys. Acta 1480, 83–91.

Hernández Leal, L., Vieno, N., Temmink, H., Zeeman, G., Buisman, C.J.N., 2010. Occurrence of Xenobiotics in Gray Water and Removal in Three Biological Treatment Systems. Environ. Sci. Technol. 44, 6835–6842.

Hernández-macedo, M.L., Ferraz, A., Rodríguez, J., Ottoboni, L.M.M., Mello, M.P. De, 2002. Iron-regulated proteins in Phanerochaete chrysosporium and Lentinula edodes : Differential analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis and two-dimensional polyacrylamide gel electrophoresis profiles. Electrophoresis 23, 655–661.

Hernando, M.., Mezcua, M., Fernández-Alba, A.R., Barceló, D., 2006. Environmental risk assessment of pharmaceutical residues in wastewater effluents, surface waters and sediments. Talanta 69, 334–42.

Herrero, P., Borrull, F., Pocurull, E., Marcé, R.M., 2014. An overview of analytical methods and occurrence of benzotriazoles, benzothiazoles and benzenesulfonamides in the environment. TrAC Trends Anal. Chem. 62, 46–55.

Ho, Y.B., Zakaria, M.P., Latif, P.A., Saari, N., 2012. Simultaneous determination of veterinary antibiotics and hormone in broiler manure, soil and manure compost by liquid chromatography-tandem mass spectrometry. J. Chromatogr. A 1262, 160–8.

Hofrichter, M., Ullrich, R., Pecyna, M.J., Liers, C., Lundell, T., 2010. New and classic families of secreted fungal heme peroxidases. Appl. Microbiol. Biotechnol. 87, 871–97.

Hörsing, M., Ledin, A., Grabic, R., Fick, J., Tysklind, M., Jansen, J. la C., Andersen, H.R., 2011. Determination of sorption of seventy-five pharmaceuticals in sewage sludge. Water Res. 45, 4470–4482.

Huber, M.M., Canonica, S., Park, G.-Y., von Gunten, U., 2003. Oxidation of pharmaceuticals during ozonation and advanced oxidation processes. Environ. Sci. Technol. 37, 1016–24.

Huerta, B., Jakimska, A., Gros, M., Rodríguez-Mozaz, S., Barceló, D., 2013. Analysis of multi-class pharmaceuticals in fish tissues by ultra-high-performance liquid chromatography tandem mass spectrometry. J. Chromatogr. A 1288, 63–72.

Hughes, S.R., Kay, P., Brown, L.E., 2013. Global synthesis and critical evaluation of pharmaceutical data sets collected from river systems. Environ. Sci. Technol. 47, 661–77.

Hundt, K.A.I., Martin, D., Hammer, E., Jonas, U., Kindermann, M.K., Schauer, F., 2000. Transformation of Triclosan by Trametes versicolor and Pycnoporus cinnabarinus. Appl. Environ. Microbiol. 66, 4157–4160.

Huntscha, S., Hofstetter, T.B., Schymanski, E.L., Spahr, S., Hollender, J., 2014. Biotransformation of benzotriazoles: Insights from transformation product identification and compound-specific isotope analysis. Environ. Sci. Technol. 48, 4435–4443.

Ingerslev, F., Vaclavik, E., Halling-Sørensen, B., 2003. Pharmaceuticals and personal care products - A source of endocrine disruption in the environment? Pure Appl. Chem. 75, 1881–1893.

Isola, D., Selbmann, L., de Hoog, G.S., Fenice, M., Onofri, S., Prenafeta-Boldú, F.X., Zucconi, L., 2013. Isolation and screening of black fungi as degraders of volatile aromatic hydrocarbons. Mycopathologia 175, 369–79.

Iwamoto, T., Nasu, M., 2001. Current bioremediation practice and perspective. J. Biosci. Bioeng. 92, 1–8.

Iwamoto, T., Tani, K., Nakamura, K., Suzuki, Y., Kitagawa, M., Eguchi, M., Nasu, M., 2000. Monitoring impact of in situ biostimulation treatment on groundwater bacterial community by DGGE. FEMS Microbiol. Ecol. 32, 129–141.

Jakobs-Schönwandt, D., Mathies, H., Abraham, W.-R., Pritzkow, W., Stephan, I., Noll, M., 2010. Biodegradation of a biocide (Cu-N-cyclohexyldiazenium dioxide) component of a wood preservative by a defined soil bacterial community. Appl. Environ. Microbiol. 76, 8076–83.

Janna, H., Scrimshaw, M.D., Williams, R.J., Churchley, J., Sumpter, J.P., 2011. From Dishwasher to Tap ? Xenobiotic Substances Benzotriazole and Tolyltriazole in the Environment. Environ. Sci. Technol. 45, 3858–3864.

Janzen, N., Banzhaf, S., Scheytt, T., Bester, K., 2009. Vertical flow soil filter for the elimination of micro pollutants from storm and waste water. Chemosphere 77, 1358–65.

Jarosz-Wilkolazka, A., Malarczyk, E., Pierszel, J., Skowronski, T., Leonowicz, A., 2002. Uptake of Cadmium Ions in White-Rot Fungus Trametes Versicolor: Effect of Cd (Ii) Ions on the Activity of Laccase. Cell Biol. Int. 26, 605–613.

Jechalke, S., Franchini, A.G., Bastida, F., Bombach, P., Rosell, M., Seifert, J., von Bergen, M., Vogt, C., Richnow, H.H., 2013. Analysis of structure, function, and activity of a benzene-degrading microbial community. FEMS Microbiol. Ecol. 85, 14–26.

Jelic, A., Cruz-Morató, C., Marco-Urrea, E., Sarrà, M., Perez, S., Vicent, T., Petrović, M., Barcelo, D., 2012. Degradation of carbamazepine by Trametes versicolor in an air pulsed fluidized bed bioreactor and identification of intermediates. Water Res. 46, 955–964.

Jelic, A., Gros, M., Ginebreda, A., Cespedes-Sánchez, R., Ventura, F., Petrovic, M., Barcelo, D., 2011. Occurrence , partition and removal of pharmaceuticals in sewage water and sludge during wastewater treatment. Water Res. 5, 1165–1176.

Jeon, H.-K., Chung, Y., Ryu, J.-C., 2006. Simultaneous determination of benzophenone-type UV filters in water and soil by gas chromatography-mass spectrometry. J. Chromatogr. A 1131, 192–202.

Jeon, H.-K., Sarma, S.N., Kim, Y.-J., Ryu, J.-C., 2008. Toxicokinetics and metabolisms of benzophenone-type UV filters in rats. Toxicology 248, 89–95.

Jewell, K.S., Wick, A., Ternes, T.A., 2014. Comparisons between abiotic nitration and biotransformation reactions of phenolic micropollutants in activated sludge. Water Res. 48, 478–89.

Jonkers, N., Laane, R.W.P.M., de Voogt, P., 2005. Sources and fate of nonylphenol ethoxylates and their metabolites in the Dutch coastal zone of the North Sea. Mar. Chem. 96, 115–135.

Joss, A., Zabczynski, S., Göbel, A., Hoffmann, B., Löffler, D., Mcardell, C.S., Ternes, T.A., Thomsen, A., Siegrist, H., 2006. Biological degradation of pharmaceuticals in municipal wastewater treatment: Proposing a classification scheme. Water Res. 40, 1686– 1696.

Jurado, A., Gago-Ferrero, P., Vàzquez-Suñé, E., Carrera, J., Pujades, E., Díaz-Cruz, M.S., Barceló, D., 2014. Urban groundwater contamination by residues of UV filters. J. Hazard. Mater. 271, 141–9.

Jurado, A., Vàzquez-Suñé, E., Carrera, J., López de Alda, M., Pujades, E., Barceló, D., 2012. Emerging organic contaminants in groundwater in Spain: a review of sources, recent occurrence and fate in a European context. Sci. Total Environ. 440, 82–94.

Justo, A., González, O., Aceña, J., Pérez, S., Barceló, D., Sans, C., Esplugas, S., 2013. Pharmaceuticals and organic pollution mitigation in reclamation osmosis brines by UV/H2O2 and ozone. J. Hazard. Mater. 263, 268–74.

Kaal, E.E.J., de Jong, E., Field, J.A., 1993. Stimulation of ligninolytic peroxidase activity by nitrogen nutrients in the white rot fungus Bjerkandera sp. Strain BOS55. Appl. Environ. Microbiol. 59, 4031–4036.

Kaiser, D., Sieratowicz, A., Zielke, H., Oetken, M., Hollert, H., Oehlmann, J., 2012. Ecotoxicological effect characterisation of widely used organic UV filters. Environ. Pollut. 163, 84–90.

Kapley, A., Purohit, H.J., 2009. Genomic tools in bioremediation. Indian J. Microbiol. 49, 108–13.

Kasprzyk-Hordern, B., Dinsdale, R.M., Guwy, A.J., 2008. The occurrence of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs in surface water in South Wales, UK. Water Res. 42, 3498–518.

Kerley-Hamilton, J.S., Trask, H.W., Ridley, C.J. a, Dufour, E., Lesseur, C., Ringelberg, C.S., Moodie, K.L., Shipman, S.L., Korc, M., Gui, J., Shworak, N.W., Tomlinson, C.R., 2012. Inherent and benzo[a]pyrene-induced differential aryl hydrocarbon receptor signaling greatly affects life span, atherosclerosis, cardiac gene expression, and body and heart growth in mice. Toxicol. Sci. 126, 391–404.

Khunjar, W.O., Mackintosh, S. a, Skotnicka-Pitak, J., Baik, S., Aga, D.S., Love, N.G., 2011. Elucidating the relative roles of ammonia oxidizing and heterotrophic bacteria during the biotransformation of 17α-Ethinylestradiol and Trimethoprim. Environ. Sci. Technol. 45, 3605–12.

Kim, S.D., Cho, J., Kim, I.S., Vanderford, B.J., Snyder, S.A., 2007. Occurrence and removal of pharmaceuticals and endocrine disruptors in South Korean surface , drinking , and waste waters. Sci. Technol. 41, 1013 – 1021.

Kirk, T.K., Connors, W.J., Zeikus, J.G., 1976. Requirement for a Growth Substrate During Lignin Decomposition by Two Wood-Rotting Fungi Requirement for a Growth Substrate During Lignin Decomposition by Two Wood-Rotting Fungi. Appl. Environ. Microbiol. 32, 192–194.

Kirk, T.K., Schultz, E., Connors, W.J., Lorenz, L.F., Zeikus, J.G., 1978. Influence of culture parameters on lignin metabolism by Phanerochaete chrysosporium. Arch. Microbiol. 117, 277–285.

Kirk, T.K., Yang, H.H., 1979. Partial delignification of unbleached kraft pulp with ligninolytic fungi. Biotechnol. Lett. 1, 347–352.

Klamer, M., Bååth, E., 2004. Estimation of conversion factors for fungal biomass determination in compost using ergosterol and PLFA 18:2ω6,9. Soil Biol. Biochem. 36, 57–65.

Klamerth, N., Rizzo, L., Malato, S., Maldonado, M.I., Agüera, A., Fernández-Alba, A.R., 2010. Degradation of fifteen emerging contaminants at µg/L initial concentrations by mild solar photo-Fenton in MWTP effluents. Water Res. 44, 545–54.

Klimek-Ochab, M., Brzezińska-Rodak, M., Żymańczyk-Duda, E., Lejczak, B., Kafarski, P., 2011. Comparative study of fungal cell disruption — scope and limitations of the methods. Folia Microbiol. (Praha). 56, 469–475.

Kondo, R., Yamagami, H., Sakai, K., 1993. Xylosylation of Phenolic Hydroxyl Groups of the Monomeric Lignin Model Compounds 4-Methylguaiacol and Vanillyl Alcohol by Coriolus versicolor. Appl. Environ. Microbiol. 59, 438–41.

Kormos, J.L., Schulz, M., Kohler, H.-P.E., Ternes, T.A., 2010. Biotransformation of selected iodinated X-ray contrast media and characterization of microbial transformation pathways. Environ. Sci. Technol. 44, 4998–5007.

Kovalova, L., Siegrist, H., Singer, H., Wittmer, A., McArdell, C.S., 2012. Hospital wastewater treatment by membrane bioreactor: performance and efficiency for organic micropollutant elimination. Environ. Sci. Technol. 46, 1536–1545.

Kümmerer, K., 2009. The presence of pharmaceuticals in the environment due to human use – present knowledge and future challenges. J. Environ. Manage. 90, 2354–2366.

Kümmerer, K., Helmersb, E., 1997. Hospital effluents as a source for platinum in the environment. Sci. Total Environ. 193, 179–184.

Kunz, P.Y., Fent, K., 2006. Multiple hormonal activities of UV filters and comparison of in vivo and in vitro estrogenic activity of ethyl-4-aminobenzoate in fish. Aquat. Toxicol. 79, 305–324.

Kunz, P.Y., Fent, K., 2009. Estrogenic activity of ternary UV filter mixtures in fish ( Pimephales promelas ) — An analysis with nonlinear isobolograms. Toxicol. Appl. Pharmacol. 234, 77–88.

Kunz, P.Y., Galicia, H.F., Fent, K., 2004. Assessment of hormonal activity of UV filters in tadpoles of frog Xenopus laevis at environmental concentrations. Mar. Environ. Res. 58, 431–435.

Kunz, P.Y., Galicia, H.F., Fent, K., 2006a. Comparison of In Vitro and In Vivo Estrogenic Activity of UV Filters in Fish. Toxicol. Sci. 90, 349–361.

Kunz, P.Y., Gries, T., Fent, K., 2006b. The Ultraviolet Filter 3-Benzylidene Camphor Adversely Affects Reproduction in Fathead Minnow (Pimephales promelas). Toxicol. Sci. 93, 311–321.

Kupper, T., Plagellat, C., Brändli, R.C., de Alencastro, L.F., Grandjean, D., Tarradellas, J., 2006. Fate and removal of polycyclic musks , UV filters and biocides during wastewater treatment. Water Re 40, 2603 – 2612.

Lee, Y., von Gunten, U., 2010. Oxidative transformation of micropollutants during municipal wastewater treatment: comparison of kinetic aspects of selective (chlorine, chlorine dioxide, ferrate VI, and ozone) and non-selective oxidants (hydroxyl radical). Water Res. 44, 555–66.

Leisola, M., Pastinen, O., Axe, D.D., 2012. Lignin-Designed Randomness. BIOcomplexity. 2012, 1–11.

León, Z., Chisvert, A., Tarazona, I., 2010. Solid-phase extraction liquid chromatography – tandem mass spectrometry analytical method for the determination of 2-hydroxy-4-methoxybenzophenone and its metabolites in both human urine and semen. Anal. Bioanal. Chem. 831–843.

Li, W., Ma, Y., Guo, C., Hu, W., Liu, K., Wang, Y., Zhu, T., 2007. Occurrence and behavior of four of the most used sunscreen UV filters in a wastewater reclamation plant. Water Res. 41, 3506–12.

Li, W.C., 2014. Occurrence, sources, and fate of pharmaceuticals in aquatic environment and soil. Environ. Pollut. 187, 193–201.

Li, Y., Zhu, G., Ng, W.J., Tan, S.K., 2014. A review on removing pharmaceutical contaminants from wastewater by constructed wetlands: design, performance and mechanism. Sci. Total Environ. 468-469, 908–32.

Libra, J.A., Borchert, M., Banit, S., 2003. Competition strategies for the decolorization of a textile-reactive dye with the white-rot fungi Trametes versicolor under non-sterile conditions. Biotechnol. Bioeng. 82, 736–744.

Lishman, L., Smyth, S.A., Sarafin, K., Kleywegt, S., Toito, J., Peart, T., Lee, B., Servos, M., Beland, M., Seto, P., 2006. Occurrence and reductions of pharmaceuticals and personal care products and estrogens by municipal wastewater treatment plants in Ontario, Canada. Sci. Total Environ. 367, 544–58.

Liu, Y., Ying, G., Shareef, A., Kookana, R.S., 2011a. Biodegradation of three selected benzotriazoles under aerobic and anaerobic conditions. Water Res. 45, 5005–5014.

Liu, Y., Ying, G., Shareef, A., Kookana, R.S., 2011b. Simultaneous determination of benzotriazoles and ultraviolet filters in ground water , effluent and biosolid samples using gas chromatography – tandem mass spectrometry. J. Chromatogr. A 1218, 5328–5335.

Lloret, L., Eibes, G., Lú-Chau, T.A., Moreira, M.T., Feijoo, G., Lema, J.M., 2010. Laccase-catalyzed degradation of anti-inflammatories and estrogens. Biochem. Eng. J. 51, 124–131.

Loi, C.H., Busetti, F., Linge, K.L., Joll, C.A., 2013. Development of a solid-phase extraction liquid chromatography tandem mass spectrometry method for benzotriazoles and benzothiazoles in wastewater and recycled water. J. Chromatogr. A 1299, 48–57.

Loos, R., Carvalho, R., António, D.C., Comero, S., Locoro, G., Tavazzi, S., Paracchini, B., Ghiani, M., Lettieri, T., Blaha, L., Jarosova, B., Voorspoels, S., Servaes, K., Haglund, P., Fick, J., Lindberg, R.H., Schwesig, D., Gawlik, B.M., 2013. EU-wide monitoring survey on emerging polar organic contaminants in wastewater treatment plant effluents. Water Res. 47, 6475–87.

Lowe, N.J., Shaatg, N.A., Pathak, M.A., 1997. Sunscreens: development, evaluation and regulatory aspects. Marcel Dekker Inc., New York.

Lu, H., Chandran, K., 2010. Diagnosis and quantification of glycerol assimilating denitrifying bacteria in an integrated fixed-film activated sludge reactor via 13C DNA stable-isotope probing. Environ. Sci. Technol. 44, 8943–9.

Lu, Z., Sun, X., Yang, Q., Li, H., Li, C., 2009. Persistence and functions of a decolorizing fungal consortium in a non-sterile biofilm reactor. Biochem. Eng. J. 46, 73–78.

Machín-Ramírez, C., Morales, D., Martínez-Morales, F., Okoh, a. I., Trejo-Hernández, M.R., 2010. Benzo[a]pyrene removal by axenic- and co-cultures of some bacterial and fungal strains. Int. Biodeterior. Biodegradation 64, 538–544.

Marco-Urrea, E., Gabarrell, X., Sarrà, M., Caminal, G., Vicent, T., Reddy, C.A., 2006. Novel aerobic perchloroethylene degradation by the white-rot fungus Trametes versicolor. Environ. Sci. Technol. 40, 7796–802.

Marco-Urrea, E., Parella, T., Gabarrell, X., Caminal, G., Vicent, T., Reddy, C.A., 2008. Mechanistics of trichloroethylene mineralization by the white-rot fungus Trametes versicolor. Chemosphere 70, 404–10.

Marco-Urrea, E., Pérez-Trujillo, M., Blánquez, P., Vicent, T., Caminal, G., 2010a. Biodegradation of the analgesic naproxen by Trametes versicolor and identification of intermediates using HPLC-DAD-MS and NMR. Bioresour. Technol. 101, 2159–66.

Marco-Urrea, E., Pérez-Trujillo, M., Cruz-Morató, C., Caminal, G., Vicent, T., 2010b. Degradation of the drug sodium diclofenac by Trametes versicolor pellets and identification of some intermediates by NMR. J. Hazard. Mater. 176, 836–842.

Marco-Urrea, E., Pérez-Trujillo, M., Cruz-Morató, C., Caminal, G., Vicent, T., 2010c. White-rot fungus-mediated degradation of the analgesic ketoprofen and identification of intermediates by HPLC-DAD-MS and NMR. Chemosphere 78, 474–81.

Marco-Urrea, E., Pérez-Trujillo, M., Vicent, T., Caminal, G., 2009. Ability of white-rot fungi to remove selected pharmaceuticals and identification of degradation products of ibuprofen by Trametes versicolor. Chemosphere 74, 765–772.

Marco-Urrea, E., Seifert, J., von Bergen, M., Adrian, L., 2012. Stable isotope peptide mass spectrometry to decipher amino acid metabolism in Dehalococcoides strain CBDB1. J. Bacteriol. 194, 4169–77.

Margot, J., Maillard, J., Rossi, L., Barry, D.A., Holliger, C., 2013. Influence of treatment conditions on the oxidation of micropollutants by Trametes versicolor laccase. N. Biotechnol. 30, 803–13.

Martinez, D., Larrondo, L.F., Putnam, N., Gelpke, M.D.S., Huang, K., Chapman, J., Helfenbein, K.G., Ramaiya, P., Detter, J.C., Larimer, F., Coutinho, P.M., Henrissat, B., Berka, R., Cullen, D., Rokhsar, D., 2004. Genome sequence of the lignocellulose degrading fungus Phanerochaete chrysosporium strain RP78. Nat. Biotechnol. 22, 695–701.

Marvalin, C., Denoux, M., Pérard, S., Roy, S., Azerad, R., 2012. Microbial production of phase I and phase II metabolites of midazolam. Xenobiotica. 42, 285–93.

Matamoros, V., Bayona, J.M., 2006. Elimination of pharmaceuticals and personal care products in subsurface flow constructed wetlands. Environ. Sci. Technol. 40, 5811–6.

Matamoros, V., Jover, E., Bayona, J.M., 2010. Occurrence and fate of benzothiazoles and benzotriazoles in constructed wetlands. Water Sci. Technol. 61, 191–8.

Mcgrath, R., Singleton, I., 2000. Pentachlorophenol transformation in soil : a toxicological assessment. Soil Biol. Biochem. 32, 1311–1314.

Mester, T., Field, J.A., 1998. Characterization of a Novel Manganese Peroxidase-Lignin Peroxidase Hybrid Isozyme Produced by Bjerkandera Species Strain BOS55 in the Absence of Manganese. J. Biol. Chem. 273, 15412–15417.

Mikesková, H., Novotný, C., Svobodová, K., 2012. Interspecific interactions in mixed microbial cultures in a biodegradation perspective. Appl. Microbiol. Biotechnol. 95, 861–70.

Milagres, A.M.F., Arantes, V., Medeiros, C.L., Machuca, A., 2002. Production of metal chelating compounds by white and brown-rot fungi and their comparative abilities for pulp bleaching. Enzyme Microb. Technol. 30, 562–565.

Millán, B., Lucas, R., Robles, A., García, T., Alvarez de Cienfuegos, G., Gálvez, A., 2000. A study on the microbiota from olive-mill wastewater (OMW) disposal lagoons, with emphasis on filamentous fungi and their biodegradative potential. Microbiol. Res. 155, 143–147.

Miller, C.A., 1997. Expression of the Human Aryl Hydrocarbon Receptor Complex in Yeast. Activation of transcription by indole compounds. J. Biol. Chem. 272, 32824–32829.

Miura, Y., Hiraiwa, M.N., Ito, T., Itonaga, T., Watanabe, Y., Okabe, S., 2007. Bacterial community structures in MBRs treating municipal wastewater: relationship between community stability and reactor performance. Water Res. 41, 627–37.

Moore, D., Robson, G.D., Trinci, A.P.J., 2011. 21st century guidebook to fungi. Cabridge University Press, Cambridge, UK.

Morasch, B., 2013. Occurrence and dynamics of micropollutants in a karst aquifer. Environ. Pollut. 173, 133–7.

Morasch, B., Höhener, P., Hunkeler, D., 2007. Evidence for in situ degradation of mono-and polyaromatic hydrocarbons in alluvial sediments based on microcosm experiments with 13C-labeled contaminants. Environ. Pollut. 148, 739–48.

More, T.T., Yan, S., Tyagi, R.D., Surampalli, R.Y., 2010. Potential use of filamentous fungi for wastewater sludge treatment. Bioresour. Technol. 101, 7691–7700.

Moreira, M.. T., Palma, C., Feijoo, G., Lema, J.M., 1998. Strategies for the continuous production of ligninolytic enzymes in fixed and fluidised bed bioreactors. J. Biotechnol. 66, 27 – 39.

Morozova, O. V., Shumakovich, G.P., Shleev, S. V., Yaropolov, Y.I., 2007. Laccase-mediator systems and their applications: A review. Appl. Biochem. Microbiol. 43, 523–535.

Müller, A., Weiss, S.C., Beißwenger, J., Leukhardt, H.G., Schulz, W., Seitz, W., Ruck, W.K.L., Weber, W.H., 2012. Identification of ozonation by-products of 4- and 5-methyl- 1H-benzotriazole during the treatment of surface water to drinking water. Water Res. 46, 679–690.

Murray, K.E., Thomas, S.M., Bodour, A.A., 2010. Prioritizing research for trace pollutants and emerging contaminants in the freshwater environment. Environ. Pollut. 158, 3462–3471.

Negreira, N., Rodríguez, I., Ramil, M., Rubí, E., Cela, R., 2009. Sensitive determination of salicylate and benzophenone type UV filters in water samples using solid-phase microextraction , derivatization and gas chromatography tandem mass spectrometry. Anal. Chim. Acta 638, 36–44.

Nguyen, L.N., Hai, F.I., Yang, S., Kang, J., Leusch, F.D.L., Roddick, F., Price, W.E., Nghiem, L.D., 2013. Removal of trace organic contaminants by an MBR comprising a mixed culture of bacteria and white-rot fungi. Bioresour. Technol. 148, 234–41.

Nieto, A., Borrull, F., Marcé, R.M., Pocurull, E., 2009. Determination of personal care products in sewage sludge by pressurized liquid extraction and ultra high performance liquid chromatography-tandem mass spectrometry. J. Chromatogr. A 1216, 5619–25.

Noguerol, T., Boronat, S., Casado, M., Raldúa, D., Barceló, D., 2006. Evaluating the interactions of vertebrate receptors with persistent pollutants and antifouling pesticides using recombinant yeast assays. Anal. Bioanal. Chem. 385, 1012–1019.

Nübel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R.I., Ludwig, W., Backhaus, H., 1996. Sequence heterogeneities of genes encoding 16S rRNAs in Paenibacillus polymyxa detected by temperature gradient gel electrophoresis. J. Bacteriol. 178, 5636–43.

Oaks, J.L., Gilbert, M., Virani, M.Z., Watson, R.T., Meteyer, C.U., Rideout, B.A., Shivaprasad, H.L., Ahmed, S., Chaudhry, M.J.I., Arshad, M., Mahmood, S., Ali, A., Khan, A.A., 2004. Diclofenac residues as the cause of vulture population decline in Pakistan. Nature 427, 630–3.

Odjadjare, E.E.O., Okoh, A.I., 2010. Physicochemical quality of an urban municipal wastewater effluent and its impact on the receiving environment. Environ. Monit. Assess. 170, 383–94.

Oller, I., Malato, S., Sánchez-Pérez, J.A., 2011. Combination of Advanced Oxidation Processes and biological treatments for wastewater decontamination-A review. Sci. Total Environ. 409, 4141–4166.

Ortega-Clemente, A., Caffarel-Méndez, S., Ponce-Noyola, M.T., Barrera-Córtes, J., Poggi-Varaldo, H.M., 2009. Fungal post-treatment of pulp mill effluents for the removal of recalcitrant pollutants. Bioresour. Technol. 100, 1885–1894.

Osorio, V., Marcé, R., Pérez, S., Ginebreda, A., Cortina, J.L., Barceló, D., 2012. Occurrence and modeling of pharmaceuticals on a sewage-impacted Mediterranean river and their dynamics under different hydrological conditions. Sci. Total Environ. 440, 3–13.

Parkinson, A., 2001. Biotransformation of xenobiotics, in: Klaassen, C.D., Thomas, M.J., Hazucha, M.J. (Eds.), Casarett and Doull's Toxicology: The Basic Science of Poisons. McGraw-Hill Medical Publishing Division, New York, pp. 113–186.

Pedrouzo, M., Borrull, F., Marcé, R.M., Pocurull, E., 2010. Stir-bar-sorptive extraction and ultra-high-performance liquid chromatography – tandem mass spectrometry for simultaneous analysis of UV filters and antimicrobial agents in water samples. Anal. Bioanal. Chem. 2833–2839.

Pérez, G., Fernández-Alba, A.R., Urtiaga, A.M., Ortiz, I., 2010. Electro-oxidation of reverse osmosis concentrates generated in tertiary water treatment. Water Res. 44, 2763–72.

Pérez, S., Barceló, D., 2007. Application of advanced MS techniques to analysis and identification of human and microbial metabolites of pharmaceuticals in the aquatic environment. TrAC Trends Anal. Chem. 26, 494–514.

Pérez-González, A., Urtiaga, A.M., Ibáñez, R., Ortiz, I., 2012. State of the art and review on the treatment technologies of water reverse osmosis concentrates. Water Res. 46, 267–283.

Pillard, D.A., Cornell, J.S., Dufresne, D.L., Hernandez, M.T., 2001. Toxicity of benzotriazole and benzotriazole derivatives to three aquatic species. Water Res. 35, 557–560.

Pinedo-Rivilla, C., Aleu, J., Collado, I., 2009. Pollutants Biodegradation by Fungi. Curr. Org. Chem. 13, 1194–1214.

Plagellat, C., Kupper, T., Furrer, R., de Alencastro, L.F., Grandjean, D., Tarradellas, J., 2006. Concentrations and specific loads of UV filters in sewage sludge originating from a monitoring network in Switzerland. Chemosphere 62, 915–25.

Plósz, B.G.Y., Leknes, H., Thomas, K. V, 2010. Impacts of competitive inhibition, parent compound formation and partitioning behavior on the removal of antibiotics in municipal wastewater treatment. Environ. Sci. Technol. 44, 734–42.

Pointing, S.B., 2001. Feasibility of bioremediation by white-rot fungi. Appl. Microbiol. Biotechnol. 57, 20–33.

Prenafeta-Boldú, F.X., Luykx, D.M.A.M., Vervoort, J., de Bont, J.A.M., 2001. Fungal Metabolism of Toluene : Monitoring of Fluorinated Analogs by 19 F Nuclear Magnetic Resonance Spectroscopy. Appl. Environ. Microbiol. 67, 1030–1034.

Prieto, A., Möder, M., Rodil, R., Adrian, L., Marco-urrea, E., 2011. Degradation of the antibiotics norfloxacin and ciprofloxacin by a white-rot fungus and identification of degradation products. Bioresour. Technol. 102, 10987–10995.

Purdom, C.E., Hardiman, P. a., Bye, V.V.J., Eno, N.C., Tyler, C.R., Sumpter, J.P., 1994. Estrogenic Effects of Effluents from Sewage Treatment Works. Chem. Ecol. 8, 275–285.

Rabinovich, M.L., Bolobova, A. V, Vasil'chenko, L.G., 2004. Fungal Decomposition of Natural Aromatic Structures and Xenobiotics : A Review. Appl. Biochem. Microbiol. 40, 1–17.

Radjenovic, J., Bagastyo, A., Rozendal, R. a, Mu, Y., Keller, J., Rabaey, K., 2011. Electrochemical oxidation of trace organic contaminants in reverse osmosis concentrate using RuO2/IrO2-coated titanium anodes. Water Res. 45, 1579–86.

Radjenovic, J., Petrovic, M., Barceló, D., 2007. Analysis of pharmaceuticals in wastewater and removal using a membrane bioreactor. Anal. Bioanal. Chem. 387, 1365–77.

Radjenović, J., Petrović, M., Barceló, D., 2009. Fate and distribution of pharmaceuticals in wastewater and sewage sludge of the conventional activated sludge (CAS) and advanced membrane bioreactor (MBR) treatment. Water Res. 43, 831–41.

Radjenović, J., Petrović, M., Ventura, F., Barceló, D., 2008. Rejection of pharmaceuticals in nanofiltration and reverse osmosis membrane drinking water treatment. Water Res. 42, 3601–10.

Rafin, C., Potin, O., Veignie, E., Lounes-Hadj Sahraoui, A., Sancholle, M., 2000. Degradation of benzo[a]pyrene as sole carbon source by a non white rot fungus, Fusarium solani. Polycycl. Aromat. Compd. 21, 311–329.

Rajala, T., Velmala, S.M., Tuomivirta, T., Haapanen, M., Müller, M., Pennanen, T., 2013. Endophyte communities vary in the needles of Norway spruce clones. Fungal Biol. 117, 182–190.

Reemtsma, T., Miehe, U., Duennbier, U., Jekel, M., 2010. Polar pollutants in municipal wastewater and the water cycle : Occurrence and removal of benzotriazoles. Water Res. 44, 596–604.

Reungoat, J., Macova, M., Escher, B.I., Carswell, S., Mueller, J.F., Keller, J., 2010. Removal of micropollutants and reduction of biological activity in a full scale reclamation plant using ozonation and activated carbon filtration. Water Res. 44, 625–637.

Rodarte-Morales, A.I., Feijoo, G., Moreira, M.T., Lema, J.M., 2011. Degradation of selected pharmaceutical and personal care products (PPCPs) by white-rot fungi. World J. Microbiol. Biotechnol. 27, 1839–1846.

Rodarte-Morales, A.I., Feijoo, G., Moreira, M.T., Lema, J.M., 2012a. Operation of stirred tank reactors (STRs) and fixed-bed reactors (FBRs) with free and immobilized Phanerochaete chrysosporium for the continuous removal of pharmaceutical compounds. Biochem. Eng. J. 66, 38–45.

Rodarte-Morales, A.I., Feijoo, G., Moreira, M.T., Lema, J.M., 2012b. Biotransformation of three pharmaceutical active compounds by the fungus Phanerochaete chrysosporium in a fed batch stirred reactor under air and oxygen supply. Biodegradation 23, 145–56.

Rodil, R., Moeder, M., Altenburger, R., Schmitt-Jansen, M., 2009a. Photostability and phytotoxicity of selected sunscreen agents and their degradation mixtures in water. Anal. Bioanal. Chem. 395, 1513–24.

Rodil, R., Quintana, B., Lo, P., 2008. Multiclass Determination of Sunscreen Chemicals in Water Samples by Liquid Chromatography-Tandem Mass Spectrometry. Database 80, 1307–1315.

Rodil, R., Schrader, S., Moeder, M., 2009b. Pressurised membrane-assisted liquid extraction of UV filters from sludge. J. Chromatogr. A 1216, 8851–8.

Rodríguez, A., Falcón, M.A., Carnicero, A., Perestelo, F., De la Fuente, G., Trojanowski, J., 1996. Laccase activities of Penicillium chrysogenum in relation to lignin degradation. Appl. Microbiol. Biotechnol. 45, 399–403.

Rodríguez-Rodríguez, C.E., Barón, E., Gago-Ferrero, P., Jelić, A., Llorca, M., Farré, M., Díaz-Cruz, M.S., Eljarrat, E., Petrović, M., Caminal, G., Barceló, D., Vicent, T., 2012a. Removal of pharmaceuticals, polybrominated flame retardants and UV-filters from sludge by the fungus Trametes versicolor in bioslurry reactor. J. Hazard. Mater. 233-234, 235–43.

Rodríguez-Rodríguez, C.E., Jelić, A., Llorca, M., Farré, M., Caminal, G., Petrović, M., Barceló, D., Vicent, T., 2011. Solid-phase treatment with the fungus Trametes versicolor substantially reduces pharmaceutical concentrations and toxicity from sewage sludge. Bioresour. Technol. 102, 5602–8.

Rodríguez-Rodríguez, C.E., Jelic, A., Pereira, M.A., Sousa, D.Z., Petrovic, M., Alves, M.M., Barcelo, D., Caminal, G., Vicent, T., 2012b. Bioaugmentation of Sewage Sludge with Trametes versicolor in Solid- Phase Biopiles Produces Degradation of Pharmaceuticals and Affects Microbial Communities. Environ. Sci. Technol. 46, 12012–12020.

Rosal, R., Rodríguez, A., Perdigón-Melón, J.A., Petre, A., García-Calvo, E., Gómez, M.J., Agüera, A., Fernández-Alba, A.R., 2010. Occurrence of emerging pollutants in urban wastewater and their removal through biological treatment followed by ozonation. Water Res. 44, 578–88.

Rousk, J., Bååth, E., 2007. Fungal and bacterial growth in soil with plant materials of different C/N ratios. FEMS Microbiol. Ecol. 62, 258–67.

Ruiz-Dueñas, F.J., Martínez, M.J., 1996. Enzymatic Activities of Trametes versicolor and Pleurotus eryngii Implicated in Biocontrol of Fusarium oxysporum f. sp. lycopersici. Curr. Microbiol. 32, 151–155.

Sagarkar, S., Mukherjee, S., Nousiainen, A., Björklöf, K., Purohit, H.J., Jørgensen, K.S., Kapley, A., 2013. Monitoring bioremediation of atrazine in soil microcosms using molecular tools. Environ. Pollut. 172, 108–15.

Sakkas, V. a., Calza, P., Islam, M.A., Medana, C., Baiocchi, C., Panagiotou, K., Albanis, T., 2009. TiO2/H2O2 mediated photocatalytic transformation of UV filter 4-methylbenzylidene camphor (4-MBC) in aqueous phase: Statistical optimization and photoproduct analysis. Appl. Catal. B Environ. 90, 526–534.

Salvador, A., Chisvert, A., 2005. An environmentally friendly (“green”) reversed-phase liquid chromatography method for UV filters determination in cosmetics. Anal. Chim. Acta 537, 15–24.

Sander, R., 1999. Compilation of Henry’s Law Constants for Inorganic and Organic Species of Potential Importance in Environmental Chemistry (Version 3) [WWW Document]. URL http://www.henrys-law.org/henry.pdf (accessed 5.4.13).

Santos, L.H.M.L.M., Gros, M., Rodriguez-Mozaz, S., Delerue-Matos, C., Pena, A., Barceló, D., Montenegro, M.C.B.S.M., 2013. Contribution of hospital effluents to the load of pharmaceuticals in urban wastewaters: identification of ecologically relevant pharmaceuticals. Sci. Total Environ. 461-462, 302–16.

Saratale, R.G., Saratale, G.D., Chang, J.S., Govindwar, S.P., 2009. Decolorization and biodegradation of textile dye Navy blue HER by Trichosporon beigelii NCIM-3326. J. Hazard. Mater. 166, 1421–8.

Sauzeau, V., Carvajal-González, J.M., Riolobos, A.S., Sevilla, M.A., Menacho-Márquez, M., Román, A.C., Abad, A., Montero, M.J., Fernández-Salguero, P., Bustelo, X.R., 2011. Transcriptional factor aryl hydrocarbon receptor (Ahr) controls cardiovascular and respiratory functions by regulating the expression of the Vav3 proto-oncogene. J. Biol. Chem. 286, 2896–909.

Schlumpf, M., Cotton, B., Conscience, M., Haller, V., Steinmann, B., Lichtensteiger, W., 2001. In vitro and in vivo estrogenicity of UV screens. Environ. Health Perspect. 109, 239–44.

Schlumpf, M., Jarry, H., Wuttke, W., Ma, R., 2004a. Estrogenic activity and estrogen receptor β binding of the UV filter 3-benzylidene camphor. Comparison with 4-methylbenzylidene camphor. Toxicology 199, 109–120.

Schlumpf, M., Kypke, K., Vökt, C.C., Birchler, M., Durrer, S., Faass, O., Ehnes, C., Fuetsch, M., Gaille, C., Henseler, M., Hofkamp, L., Maerkel, K., Reolon, S., Zenker, A., Timms, B., Tresguerres, J. a. F., Lichtensteigera, W., 2008. Endocrine Active UV Filters: Developmental Toxicity and Exposure Through Breast Milk. Chim. Int. J. Chem. 62, 345–351.

Schlumpf, M., Kypke, K., Wittassek, M., Angerer, J., Mascher, H., Mascher, D., Vökt, C., Birchler, M., Lichtensteiger, W., 2010. Exposure patterns of UV filters, fragrances, parabens, phthalates, organochlor pesticides, PBDEs, and PCBs in human milk: correlation of UV filters with use of cosmetics. Chemosphere 81, 1171–83.

Schlumpf, M., Schmid, P., Durrer, S., Conscience, M., Maerkel, K., Henseler, M., Gruetter, M., Herzog, I., Reolon, S., Ceccatelli, R., Faass, O., Stutz, E., Jarry, H., Wuttke, W., Lichtensteiger, W., 2004b. Endocrine activity and developmental toxicity of cosmetic UV filters — an update. Toxicology 205, 113–122.

Schmitt, C., Oetken, M., Dittberner, O., Wagner, M., Oehlmann, J., 2008. Endocrine modulation and toxic effects of two commonly used UV screens on the aquatic invertebrates Potamopyrgus antipodarum and Lumbriculus variegatus. Environ. Pollut. 152, 322–329.

Schreiner, K.M., Filley, T.R., Blanchette, R. a, Bowen, B.B., Bolskar, R.D., Hockaday, W.C., Masiello, C. a, Raebiger, J.W., 2009. White-rot basidiomycete-mediated decomposition of C60 fullerol. Environ. Sci. Technol. 43, 3162–8.

Schreurs, R., Lanser, P., Seinen, W., van der Burg, B., 2002. Estrogenic activity of UV filters determined by an in vitro reporter gene assay and an in vivo transgenic zebrafish assay. Arch. Toxicol. 76, 257–61.

Schreurs, R., Sonneveld, E., Jansen, J.H.J., Seinen, W., van der Burg, B., 2005. Interaction of polycyclic musks and UV filters with the estrogen receptor (ER), androgen receptor (AR), and progesterone receptor (PR) in reporter gene bioassays. Toxicol. Sci. 83, 264–72.

Schriks, M., Heringa, M.B., Kooi, M.M.E. Van Der, Voogt, P. De, Wezel, A.P. Van, 2010. Toxicological relevance of emerging contaminants for drinking water quality. Water Res. 44, 461–476.

Sigoillot, C., Camarero, S., Vidal, T., Record, E., Asther, M., Pérez-Boada, M., Martínez, M.J., Sigoillot, J.-C., Asther, M., Colom, J.F., Martínez, A.T., 2005. Comparison of different fungal enzymes for bleaching high-quality paper pulps. J. Biotechnol. 115, 333–43.

Silfer, J.A., Engel, M.H., Limited, V.G.I., Way, A., Oht, C.C., 1991. Stable Carbon Isotope Analysis of Amino Acid Enantiomers by Conventional Isotope Ratio Mass Spectrometry and Combined Gas Chromatography/Isotope Ratio Mass Spectrometry. Alytical Chem. 63, 370–374.

Sim, W.-J., Lee, J.-W., Lee, E.-S., Shin, S.-K., Hwang, S.-R., Oh, J.-E., 2011. Occurrence and distribution of pharmaceuticals in wastewater from households, livestock farms, hospitals and pharmaceutical manufactures. Chemosphere 82, 179–86.

Sirés, I., Arias, C., Cabot, P.L., Centellas, F., Garrido, J.A., Rodríguez, R.M., Brillas, E., 2007. Degradation of clofibric acid in acidic aqueous medium by electro-Fenton and photoelectro-Fenton. Chemosphere 66, 1660–9.

Smith, A.E., Hristova, K., Wood, I., Mackay, D.M., Lory, E., Lorenzana, D., Scow, K.M., 2004. Comparison of Biostimulation versus Bioaugmentation with Bacterial Strain PM1 for Treatment of Groundwater Contaminated with Methyl Tertiary Butyl Ether (MTBE). Environ. Health Perspect. 113, 317–322.

Snyder, S.A., Adham, S., Redding, A.M., Cannon, F.S., Decarolis, J., Oppenheimer, J., Wert, E.C., Yoon, Y., 2007. Role of membranes and activated carbon in the removal of endocrine disruptors and pharmaceuticals. Sci. Technol. 202, 156–181.

Snyder, S.A., Wert, E.C., Rexing, D.J., Zegers, R.E., Drury, D.D., 2006. Ozone Oxidation of Endocrine Disruptors and Pharmaceuticals in Surface Water and Wastewater. Ozone Sci. Eng. 28, 445–460.

Soares, A., Guieysse, B., Jefferson, B., Cartmell, E., Lester, J.N., 2008. Nonylphenol in the environment: a critical review on occurrence, fate, toxicity and treatment in wastewaters. Environ. Int. 34, 1033–49.

Song, H., 1999. Comparison of pyrene biodegradation by white rot fungi. World J. Microbiol. Biotechnol. 15, 669–672.

Stasinakis, A.S., Thomaidis, N.S., Arvaniti, O.S., Asimakopoulos, A.G., Samaras, V.G., Ajibola, A., Mamais, D., Lekkas, T.D., 2013. Contribution of primary and secondary treatment on the removal of benzothiazoles, benzotriazoles, endocrine disruptors, pharmaceuticals and perfluorinated compounds in a sewage treatment plant. Sci. Total Environ. 463-464, 1067–75.

Subramanian, V., Yadav, J.S., 2008. Regulation and heterologous expression of P450 enzyme system components of the white rot fungus Phanerochaete chrysosporium. Enzyme Microb. Technol. 43, 205–213.

Suda, T., Hata, T., Kawai, S., Okamura, H., Nishida, T., 2012. Treatment of tetracycline antibiotics by laccase in the presence of 1-hydroxybenzotriazole. Bioresour. Technol. 103, 498–501.

Takatori, S., Kitagawa, Y., Oda, H., Miwa, G., Nishikawa, J., Nishihara, T., Nakazawa, H., Hori, S., 2003. Estrogenicity of Metabolites of Benzophenone Derivatives Examined by a Yeast Two-Hybrid Assay. J. Heal. Sci. 49, 91–98.

Tangtian, H., Bo, L., Wenhua, L., Shin, P.K.S., Wu, R.S.S., 2012. Estrogenic potential of benzotriazole on marine medaka (Oryzias melastigma ). Ecotoxicol. Environ. Saf. 80, 327–332.

Tarazona, I., Chisvert, A., León, Z., Salvador, A., 2010. Determination of hydroxylated benzophenone UV filters in sea water samples by dispersive liquid – liquid microextraction followed by gas chromatography – mass spectrometry. J. Chromatogr. A 1217, 4771–4778.

Tavares, A.P.M., Coelho, M.A.Z., Agapito, M.S.M., Coutinho, J.A.P., Xavier, A.M.R.B., 2006. Optimization and Modeling of Laccase Production by Trametes versicolor in a Bioreactor Using Statistical Experimental Design. Appl. Biochem. Biotechnol. 134, 233–248.

Temp, U., Eggert, C., 1999. Novel Interaction between Laccase and Cellobiose Dehydrogenase during Pigment Synthesis in the White Rot Fungus Pycnoporus cinnabarinus. Appl. Environ. Microbiol. 65, 389–395.

Ternes, T.A., 1998. Occurrence of drugs in german sewage treatment plants and rivers. Water Res. 32, 3245–3260.

Tien, M., Kirk, T.K., 1983. Lignin-Degrading Enzyme from the Hymenomycete Phanerochaete chrysosporium Burds. Science (80-. ). 221, 661–663.

Tomsovský, M., Homolka, L., 2003. Laccase and other ligninolytic enzyme activities of selected strains of Trametes spp . from different localities and substrates. Folia Microbiol. (Praha). 48, 413–6.

Tran, N.H., Urase, T., Kusakabe, O., 2010. Biodegradation Characteristics of Pharmaceutical Substances by Whole Fungal Culture Trametes versicolor and its Laccase. J. Water Environ. Technol. 8, 125–140.

Tuomela, M., Lyytika, M., Oivanen, P., Hatakka, A., 1999. Mineralization and conversion of pentachlorophenol (PCP) in soil inoculated with the white-rot fungus Trametes versicolor. Soil Biol. Biochem. 31, 65–74.

Urtiaga, A.M., Pérez, G., Ibáñez, R., Ortiz, I., 2013. Removal of pharmaceuticals from a WWTP secondary effluent by ultrafiltration/reverse osmosis followed by electrochemical oxidation of the RO concentrate. Desalination 331, 26–34.

Vanquerp, V., Rodriguez, C., Coiffard, C., Coiffard, L.J.M., de Roeck-Holtzhauer, Y., 1999. Technical note High-performance liquid chromatographic method for the comparison of the photostability of five sunscreen agents. J. Chromatogr. A 832, 273–277.

Vasilyeva, E., Forgac, M., 1998. Interaction of the clathrin-coated vesicle V-ATPase with ADP and sodium azide. J. Biol. Chem. 273, 23823–9.

Verdin, A., Lounès-Hadj Sahraoui, A., Newsam, R., Robinson, G., Durand, R., 2005. Polycyclic aromatic hydrocarbons storage by Fusarium solani in intracellular lipid vesicles. Environ. Pollut. 133, 283–91.

Verlicchi, P., Al Aukidy, M., Zambello, E., 2012. Occurrence of pharmaceutical compounds in urban wastewater: removal, mass load and environmental risk after a secondary treatment--a review. Sci. Total Environ. 429, 123–55.

Verlicchi, P., Galletti, A., Petrovic, M., Barceló, D., 2010. Hospital effluents as a source of emerging pollutants: An overview of micropollutants and sustainable treatment options. J. Hydrol. 389, 416–428.

Verlicchi, P., Zambello, E., 2014. How efficient are constructed wetlands in removing pharmaceuticals from untreated and treated urban wastewaters? A review. Sci. Total Environ. 470-471, 1281–306.

Vila, J., María Nieto, J., Mertens, J., Springael, D., Grifoll, M., 2010. Microbial community structure of a heavy fuel oil-degrading marine consortium: linking microbial dynamics with polycyclic aromatic hydrocarbon utilization. FEMS Microbiol. Ecol. 73, 349–62.

Vilchez-Vargas, R., Junca, H., Pieper, D.H., 2010. Metabolic networks, microbial ecology and “omics” technologies: towards understanding in situ biodegradation processes. Environ. Microbiol. 12, 3089–104.

Vogna, D., Marotta, R., Andreozzi, R., Napolitano, A., d’Ischia, M., 2004. Kinetic and chemical assessment of the UV/H2O2 treatment of antiepileptic drug carbamazepine. Chemosphere 54, 497–505.

Völkel, W., Colnot, T., Schauer, U.M.D., Broschard, T.H., Dekant, W., 2006. Toxicokinetics and biotransformation of 3-(4-methylbenzylidene)camphor in rats after oral administration. Toxicol. Appl. Pharmacol. 216, 331–8.

Vom Saal, F.S., Hughes, C., 2005. An Extensive New Literature Concerning Low-Dose Effects of Bisphenol A Shows the Need for a New Risk Assessment. Environ. Health Perspect. 113, 926–933.

Wariishi, H., Valli, K., Gold, M.H., 1992. Manganese(II) oxidation by manganese peroxidase from the basidiomycete Phanerochaete chrysosporium. Kinetic mechanism and role of chelators. J. Biol. Chem. 267, 23688–95.

Weber, S.D., Ludwig, W., Schleifer, K.-H., Fried, J., 2007. Microbial composition and structure of aerobic granular sewage biofilms. Appl. Environ. Microbiol. 73, 6233–40.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173, 697–703.

Weiss, S., Jakobs, J., Reemtsma, T., 2006. Discharge of three benzotriazole corrosion inhibitors with municipal wastewater and improvements by membrane bioreactor treatment and ozonation. Environ. Sci. Technol. 40, 7193–9.

Wells, T., Ragauskas, A.J., 2012. Biotechnological opportunities with the β-ketoadipate pathway. Trends Biotechnol. 30, 627–37.

Wetzstein, H.-G., Stadler, M., Tichy, H.-V., Dalhoff, A., Karl, W., 1999. Degradation of Ciprofloxacin by Basidiomycetes and Identification of Metabolites Generated by the Brown Rot Fungus Gloeophyllum striatum. Appl. Environ. Microbiol. 65, 1556–1563.

White, D.C., Davis, W.M., Nickels, J.S., King, J.D., Bobbie, R.J., 1979. Determination of the sedimentary microbial biomass by extractible lipid phosphate. Oecologia 40, 51–62.

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, in: Innins, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols. Academic Press, San Diego, p. 315.

Widsten, P., Kandelbauer, A., 2008. Laccase applications in the forest products industry: A review. Enzyme Microb. Technol. 42, 293–307.

Winklert, R., Sandermann, H., 1992. N-Glucosyl Conjugates of Chlorinated Anilines: Spontaneous Formation and Cleavage. J. Agric. Food Chem. 40, 2008–2012.

Witorsch, R.J., Thomas, J.A., 2010. Personal care products and endocrine disruption : A critical review of the literature. Physiology 40, 1–30.

Wong, D.W.S., 2009. Structure and action mechanism of ligninolytic enzymes. Appl. Biochem. Biotechnol. 157, 174–209.

Wu, X., Chou, N., Lupher, D., Davis, L.C., 1998. Benzotriazoles: toxicity and degradation, in: Proceedings of the 1998 Conference on Hazardous Waste Research. pp. 374–382.

Wymelenberg, A. Vanden, Gaskell, J., Mozuch, M., Kersten, P., Sabat, G., Martinez, D., Cullen, D., 2009. Transcriptome and Secretome Analyses of Phanerochaete chrysosporium Reveal Complex Patterns of Gene Expression. Appl. Environ. Microbiol. 75, 4058–4068.

Wymore, A.S., Compson, Z.G., Liu, C.M., Price, L.B., Whitham, T.G., Keim, P., Marks, J.C., 2013. Contrasting rRNA gene abundance patterns for aquatic fungi and bacteria in response to leaf-litter chemistry. Freshw. Sci. 32, 663–672.

Yang, Q., Angly, F.E., Wang, Z., Zhang, H., 2011. Wastewater treatment systems harbor specific and diverse yeast communities. Biochem. Eng. J. 58-59, 168–176.

Yang, S., Hai, F.I., Nghiem, L.D., Nguyen, L.N., Roddick, F., Price, W.E., 2013a. Removal of bisphenol A and diclofenac by a novel fungal membrane bioreactor operated under non-sterile conditions. Int. Biodeterior. Biodegradation 85, 483–490.

Yang, S., Hai, F.I., Nghiem, L.D., Price, W.E., Roddick, F., Moreira, M.T., Magram, S.F., 2013b. Understanding the factors controlling the removal of trace organic contaminants by white-rot fungi and their lignin modifying enzymes: a critical review. Bioresour. Technol. 141, 97–108.

Yoshida, H., 1883. LXIII. Chemistry of lacquer (Urushi). Part I. Communication from the Chemical Society of Tokio. J. Chem. Soc. Trans. 43, 472.

Zenker, A., Cicero, M.R., Prestinaci, F., Bottoni, P., Carere, M., 2014. Bioaccumulation and biomagnification potential of pharmaceuticals with a focus to the aquatic environment. J. Environ. Manage. 133, 378–87.

Zhang, D., Yang, Y., Leakey, J.E.A., Cerniglia, C.E., 1996. Phase I and phase II enzymes produced by Cunninghamella : elegans for the metabolism of xenobiotics. FEMS Microbiol. Lett. 138, 221–226.

Zhang, F., Knapp, J.S., Tapley, K.N., 1999. Decolourisation of cotton bleaching effluent with wook rotting fungus. Water Res. 33, 919–928.

Zhang, Y., Geissen, S.U., 2010. In vitro degradation of carbamazepine and diclofenac by crude lignin peroxidase. J. Hazard. Mater. 176, 1089–92.

Zhang, Y., Geissen, S.U., 2012. Elimination of carbamazepine in a non-sterile fungal bioreactor. Bioresour. Technol. 112, 221–7.

Zhang, Y., Geissen, S.U., Gal, C., 2008. Carbamazepine and diclofenac: removal in wastewater treatment plants and occurrence in water bodies. Chemosphere 73, 1151–61.

Zhou, T., Lim, T.-T., Chin, S.-S., Fane, A.G., 2011. Treatment of organics in reverse osmosis concentrate from a municipal wastewater reclamation plant : Feasibility test of advanced oxidation processes with / without pretreatment. Chem. Eng. J. 166, 932–939.