**Universitat Autònoma de Barcelona**

**Escola d'Enginyeria**
**Departament d'Enginyeria Química**

# Degradation of pharmaceuticals in sewage sludge by *Trametes versicolor*

**PhD Thesis**

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**Bellaterra, July 2012**

**Title:** Degradation of pharmaceuticals in sewage sludge by *Trametes versicolor*

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**Public presentation:** July 13th, 2012

PhD Program in Biotechnology

Departament d'Enginyeria Química.

Escola d'Enginyeria.

Universitat Autònoma de Barcelona. Bellaterra. 2012.

This work was supported by the Spanish Ministries of MMAMRM (project 010/PC08/3-04) and MICINN (project CTQ2010-21776-C2-01). The author acknowledges the predoctoral fellowship from Universidad de Costa Rica and Consejo Superior de Investigaciones Cientificas (UCR-CSIC partnership).

Part of this work has been done in collaboration with the Institute of Environmental Assessment and Water Studies from the Department of Environmental Chemistry, CSIC, Barcelona, Spain.

Part of the work presented has been performed at the IBB-Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho in Braga, Portugal, under the supervision of M. Madalena Alves, M. Alcina Pereira and Diana Z. Sousa (project AIB2010PT-00169).

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

CERTIFIQUEM:

Que el graduat en Microbiologia i Química Clínica, Carlos E. Rodríguez Rodríguez ha realitzat sota la nostra direcció, en els laboratoris del Departament d'Enginyeria Química el treball que amb el títol "**Degradation of pharmaceuticals in sewage sludge by *Trametes versicolor***", es presenta en aquesta memòria, la qual constitueix la seva Tesi per optar al Grau de Doctor per la Universitat Autònoma de Barcelona.

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, Maig de 2012

Dra. Glòria Caminal Saperas Dra. Teresa Vicent i Huguet

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# List of Publications

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Rodríguez-Rodríguez, C.E., Marco-Urrea, E., Caminal, G.

***Degradation of naproxen and carbamazepine in spiked sludge by slurry and solid-phase* Trametes versicolor *systems***

Bioresour. Technol. 101 (2010), 2259-2266.

## Article 2:

Rodríguez-Rodríguez, C.E., Marco-Urrea, E., Caminal, G.

***Naproxen degradation test to monitor* Trametes versicolor *activity in solid-state bioremediation processes***

J. Hazard. Mater. 179 (2010), 1152-1155.

## Article 3:

Rodríguez-Rodríguez, C., Jelić, A., Llorca, M., Farré, M., Caminal, G., Petrović, M., Barceló, D., Vicent, T.

***Solid-phase treatment with the fungus* Trametes versicolor *substantially reduces pharmaceutical concentrations and toxicity from sewage sludge***

Bioresour. Technol. 102 (2011), 5602-5608.

## Article 4:

Rodríguez-Rodríguez, C.E., García-Galán, M.J., Blánquez, P., Díaz-Cruz, M.S., Barceló, D., Caminal, G., Vicent, T.

***Continuous degradation of a mixture of sulfonamides by* Trametes versicolor *and identification of metabolites from sulfapyridine and sulfathiazole***

J. Hazard. Mater. 213-214 (2012), 347-354.

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## Article A1:

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***Removal of pharmaceuticals, polybrominated flame retardants and UV filters from sludge by the fungus* Trametes versicolor *in bioslurry reactor***

J. Hazard. Mater. (2012) *Submitted*.

## Article A2:

Rodríguez-Rodríguez, C.E., Jelić, A., Pereira, M.A., Sousa, D.Z., Petrović, M., Alves, M.M., Barceló, D., Caminal, G., Vicent, T.

***Bioaugmentation of sewage sludge with* Trametes versicolor *in solid-phase biopiles produces degradation of pharmaceuticals and affects microbial communities***

Environ. Sci. Technol. (2012) *Submitted*.

# Summary

Given the the inefficiency of sewage treatment facilities to completely remove organic pollutants, the sludge produced in wastewater treatment plants constitutes an important source of contaminants. Subsequently, the increasingly popular practice of applying sludge in agricultural lands results in the spread of these micropollutants in the environment. The present work describes the development of a biotechnological approach based on the application of a white-rot fungus, *Trametes versicolor*, for the elimination of emerging contaminants, in particular pharmaceuticals, from sewage sludge. This fungus is known for its wide spectrum of degradation ability towards organopollutants.

The first stage in the research deals with the preliminary assessment of fungal colonization of sterile sludge systems (solid-phase and slurry) and the capacity of degradation of two model pharmaceuticals (carbamazepine and naproxen) in those systems. The development of a degradation test called ND24, designed to monitor the metabolic status of *T. versicolor* in solid-phase bioremediation process is also described.

Afterwards, in a second stage, the solid-phase and the biosurry treatments were employed for the degradation of pharmaceuticals at pre-existent concentrations from sterile sewage sludge. Work was undergone in sterile conditions in order to demonstrate that removal was due to the fungus and not to the microbial communities present in the sludge. In the solid-phase biopiles an important removal was observed for all the drugs detected, together with a remarkable reduction in the global toxicity. Similarly, elimination of pharmaceuticals and other groups of emerging pollutants was achieved in the slurry bioreactor. However, in terms of toxicological and removal values, the solid-

phase biopiles showed an enhanced performance compared to the slurry, reason why the current were further employed for the treatment of non-sterile sludge.

The third stage consisted in the application of *T. versicolor* in non-sterile biopiles for the elimination of pre-existent pharmaceuticals from sludge. This bioaugmentation process was compared with the action of solely the autochthonous microbiota. Molecular techniques permitted to monitor the colonization and survival of *T. versicolor* in the process and its effect over the microbial communities, parallel to the removal of the therapeutic drugs.

In an attempt to obtain further insights in the mechanism of degradation, the transformation of several sulfonamides, usually found in the sludge and removed by the fungus, was studied in detail. Results include the identification of transformation products and the participation of some enzymes. The use of a continuous fluidized bed reactor with fungal pellets for the simultaneous removal of three sulfonamides is also described.

# Resumen

Dada la ineficiencia de las plantas de tratamiento de aguas residuales para eliminar completamente los contaminantes orgánicos, los lodos en ellas producidos constituyen una importante fuente de agentes tóxicos. Consecuentemente, la cada vez más popular práctica de aplicar lodos in tierras de uso agrícola resulta en la diseminación de estos microcontaminantes en el ambiente. El presente trabajo describe el desarrollo de una estrategia biotecnológica para la eliminación de contaminantes emergentes en lodos de depuradora, con particular énfasis en compuestos farmacéuticos, y basado en la aplicación de un hongo de la podredumbre blanca (white-rot fungus), *Trametes versicolor*. Este hongo es conocido por su amplia capacidad degradativa de organocontaminantes.

La primera etapa de la investigación describe la evaluación preliminar de la colonización de sistemas de lodos estériles por parte del hongo (fase sólida y suspensión o "slurry") y la capacidad de degradación de dos drogas terapéuticas modelo (carbamazepina y naproxeno) en dichos sistemas. El desarrollo de una prueba de degradación llamada ND24, diseñada para el monitoreo del estatus metabólico de *T. versicolor* en procesos de biorremediación en fase sólida es también descrita.

A continuación, en una segunda etapa, los tratamientos en fase sólida y bioslurry fueron empleados para la degradación de fármacos, a concentraciones preexistentes, en lodos de depuradora estériles. Esta parte del trabajo fue llevada a cabo en condiciones estériles para demostrar que la eliminación se debe al hongo y no a las comunidades microbianas presentes en los lodos. En los sistemas de fase sólida (biopilas), una eliminación importante fue observada para todos los agentes terapéuticos detectados, así como una destacable reducción en la toxicidad. Similarmente, la eliminación de

fármacos y otros grupos de contaminantes emergentes fue también conseguida en los reactores en "slurry". Sin embargo, en términos de toxicidad y eliminación, el tratamiento en fase sólida mostró un mejor rendimiento comparado con el bioslurry, razón por la cual, las biopilas fueron posteriormente empleadas para el tratamiento de lodo no estéril.

La tercera etapa consistió en la aplicación de *T. versicolor* en biopilas no estériles para la eliminación de fármacos preexistentes en lodos. Este proceso de bioaumentación fue comparado con la acción de únicamente los microorganismos autóctonos. Mediante técnicas moleculares se pudo dar monitoreo a la colonización y supervivencia de *T. versicolor* en el proceso y su efecto sobre las comunidades microbianas, paralelo a la degradación de los agentes terapéuticos.

En un intento por obtener mayores detalles en el mecanismo de degradación, la transformación de varias sulfonamidas, usualmente encontradas en los lodos y eliminadas por el hongo, fue estudiada en profundidad. Los resultados incluyen la identificación de productos de transformación y la participación de algunas enzimas. El uso de un reactor de lecho fluidizado con pellets de *T. versicolor* para la eliminación simultánea de tres sulfonamidas es también descrito.

# Chapter 1
# BACKGROUND AND OBJECTIVES

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## 1. Emerging pollutants in sludge

Sludge is the residue generated during the biological treatment process of municipal wastewater, and its handling is one of the most important challenges in wastewater management (Fytili and Zabaniotou, 2008). Nowadays there are several ways by which the final sludge from wastewater treatment plants (WWTPs), referred to by the term biosolids, is disposed. In this sense, the fate of biosolids includes application in agricultural lands (37%), incineration (11%), landfilling (40%) and minor areas such as forestry and land reclamation (12%) (Fytili and Zabaniotou, 2008). Agricultural use is the internationally favored choice for sludge management, as it contributes to recycling nutrients and improving soil properties and fertility (Clarke and Smith, 2011). However, due to the incapability of conventional wastewater treatments to completely remove undesirable agents, sludge contains contaminants such as metals, pathogens and organic pollutants and though current regulations require pathogen reduction and monitoring of metals, the concern on organopollutants is only limited to halogenated compounds, alkylbenzene sulfonates, phthalates, nonylphenols, polychlorinated biphenyls, polycyclic aromatic hydrocarbons and dioxins , as shown in the EU's Working Document on Sludge (European Union, 2000). Consequently, an appropriate legislation for most of the groups of emerging pollutants, including pharmaceuticals, polybrominated diphenyl ethers (PBDEs) and UV filters, is still lacking.

In Europe, around 4000 pharmaceutical active compounds employed both for human or veterinary purposes are susceptible to reach the environment (Mompelat et al., 2009). The production and administration of pharmaceuticals varies between countries and over time, with fluctuations from one year to another (Alexy et al., 2006;

Goossens et al., 2007). Moreover, factors such as the continuous improving in the quality of life and the use of generic drugs, suggest that pharmaceuticals consumption is likely to increase in the years to come (Van der Aa et al., 2011). The annual consumption of the most common pharmaceuticals reaches hundreds tons in Europe; in particular, anti-inflammatory drugs like acetylsalicylic acid, paracetamol, ibuprofen and diclofenac were produced at amounts ranging from 836 to 86 t in Germany by 2001, coincident with the high concentrations detected in the influent of WWTPs for these drugs; meanwhile production of the antiepileptic carbamazepine reached 88 t (Fent et al., 2006). Similarly, annual antibiotic consumption reaches astonishing levels, more than 13 000 t in E.U. in 1999, especially due to their wide use in medicine, veterinary, farming and aquaculture (Kemper, 2008).

After administration, pharmaceuticals and their metabolites may reach the environment via WWTP discharges, manufacturing and hospital effluents, concentrated animal feeding operations, direct disposal and land application of biosolids (Daughton and Ternes, 1999). Parental compounds and metabolites can suffer additional transformations due to biological or chemical processes in the WWTPs and the receiving environmental compartments (Deblonde et al., 2011). However, the sewage treatment facilities are not designed to remove these micropollutants which are present at very low concentrations (usually ng $L^{-1}$), and therefore they fail to produce a contaminant-free sludge.

The scientific community widely agrees with the possibility that negative ecotoxicological effects may arise from the presence of pharmaceuticals in the environment (Santos et al., 2010). In particular, since antibiotics have the potential to affect microbial communities, the inhibition of natural occurring processes such as degradation of organic matter in water and sediments, anaerobic digestion or processes

related to the $N_2$ cycle may occur (Kümmerer, 2009); moreover, it is still unknown whether their presence in nature contributes to the spread of microbial antibiotic resistance (Kümmerer, 2009). Although chronic ecotoxicity data are scarce compared to acute studies, accumulative effects have been shown to damage some ecosystems (Daughton and Ternes, 1999). Acute and chronic ecotoxicology of different groups of pharmaceuticals are reviewed by Santos et al. (2010). Similarly, recent reports review and evaluate the potential risk of the presence of pharmaceuticals in different water compartments (de Jongh et al., 2012; Stuart et al., 2012)

## 2. White-rot fungi

White-rot fungi (WRF) comprise an ecophysiological group of fungi capable to biodegrade lignin. Taxonomically WRF include mostly basidiomycetes and some ascomycetes (Eaton and Hale, 1993). As the most significant lignin degraders among the wood inhabiting microorganisms, WRF present an extracellular oxidative system employed in the primary attack of lignin and its posterior mineralization (Martínez et al., 2005) in a non-specific and non-selective mechanism. This enzymatic system includes lignin-modifying enzymes (LMEs), which are extracellular and metal-containing oxidoreductases, especially peroxidases and laccases. The extracellular enzymes generate diffusible oxidizing agents that attack the molecules, many of these being activated oxygen species (Bumpus et al., 1985; Collins and Dobson, 1996). The reactions they catalyze include lignin polymerization and dimethoxylation, decarboxilation, hydroxylation and breakdown of aromatic rings.

WRF show several features which make them interesting agents for potential use in bioremediation: *i.)* their enzymatic system is highly non-specific, and therefore able to oxidize a wide range of pollutants; *ii).* expression of the enzymes involved in

degradation is not dependent of the presence of specific contaminants, thus reducing the need to adaptation at polluted sites or matrices; *iii.)* most oxidative enzymes are extracellular, which permits the degradation of low solubility contaminants; *iv.)* the wide distribution and hyphae-growth facilitate colonization and access to sequestered pollutants; *v.)* inexpensive lignocellulosic wastes can be employed as substrate/carrier for growth/inoculation of WRF, as they are also necessary as nutrient source during co-metabolic removal of organopollutans.

## 3. Enzymatic system of WRF

### 3.1. Lignin-modifying enzymes

Decomposition of lignin is possible thanks to the production of LMEs. WRF secrete mainly two different groups of LMEs, laccases and lignin-modifying peroxidases (LMPs), particualrly lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP), which act synergistically during lignin degradation (Lundell et al., 2010). The main difference is the electron acceptor, $O_2$ for laccases and $H_2O_2$ for peroxidases. Besides the fungal oxidative enzymes, the reactions of lignin biodegradation also involve secreted fungal metabolites like phenolic and other aromatic compounds, peptides, organic acids and lignocellulosic-derived compounds and metal ions (Guillén et al., 2000; Lundell et al., 2010).

The production of ligninolytic enzymes usually takes place during the secondary metabolism of several WRF. The lignin degrading system is induced when starvation of C or N occurs; moreover, agitation and temperature can significantly affect the levels of these enzymes; factors affecting LMEs are reviewed by Gao et al. (2010).

### 3.1.1. Laccase

Fungal laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) belong to the multicopper blue phenoloxidases. They comprise glycosylated proteins expressed in multiple forms and variable molecular weight, ranging from 59 to 110 kDa. Laccase is expressed as multiple constitutive and induced isoenzymes (Svobodová et al., 2008; Zhu et al., 2001). The enzyme contains four copper atoms (Cu), in different states of oxidation (I, II, III) (Thurston 1994) which play an important role in the catalytic mechanism. Laccase oxidizes different compounds while reducing $O_2$ to $H_2O$, a total reduction of four electrons. The catalytic cycle of laccase is shown in **Figure 1**.

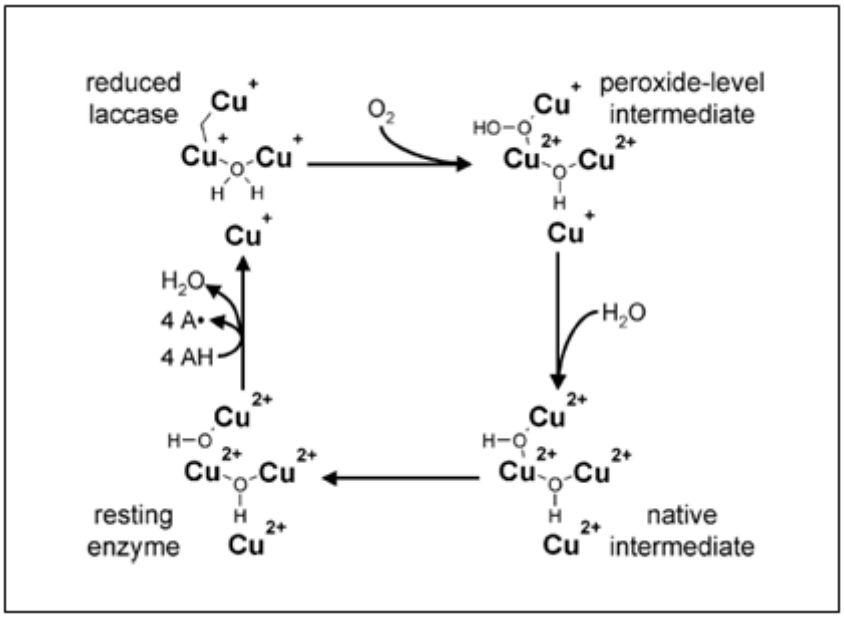


**Figure 1.** General catalytic cycle of laccase (Wesenberg et al., 2003).

The low specificity of electron-donating substrates is remarkable for laccases. These enzymes have high redox potential, making them able to oxidize a broad range of aromatic compounds (e.g. phenols, polyphenols, methoxy-substituted phenols, aromatic amines, benzenethiols) through the use of oxygen as electron acceptor. Other enzymatic reactions they catalyze include decarboxylations and demethylations (Nyanhongo et al., 2007).

Reported redox potentials of laccases are lower than those of non-phenolic compounds, and therefore these enzymes cannot oxidize such substances (Rodríguez-Couto and Toca-Herrera, 2006). However, it has been shown that in the presence of small molecules capable to act as electron transfer mediators, laccases are also able to oxidize non-phenolic structures (Bourbonnais and Paice, 1990; Call and Mücke, 1997). As part of their metabolism, WRF can produce several metabolites that play this role of laccase-mediators. They include compounds such as *N*-hydroxyacetanilide (NHA), *N*-(4-cyanophenyl)acetohydroxamic acid (NCPA), 3-hydroxyanthranilate, syringaldehyde, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,6-dimethoxyphenol (DMP), violuric acid, 1-hydroxybenzotriazole (HBT), 2,2,6,6-tetramethylpipperidin-*N*-oxide radical and acetovanillone, and by expanding the range of compounds that can be oxidized, their presence enhances the degradation of pollutants (Asgher et al., 2008; Camarero et al., 2005).

### 3.1.2. Peroxidases

The class II secreted fungal heme peroxidases include the LMPs LiP, MnP and VP (Hofrichter et al., 2010). All of these enzymes are extracellular and contain protoporphyrin IX (heme) as prosthetic group, and are secreted as glycosilated, 35-38 kDa size proteins. They use $H_2O_2$ or organic hydroperoxides as electron accepting cosubstrates during the oxidation of diverse compounds.

The classical peroxidative catalytic cycle (**Figure 2**) involves the formation of a so-called compound I intermediate, product of the binding of the hydrogen peroxide to the heme group of the enzyme and the subsequent release of a water molecule. The cycle operates through a second intermediate, compound II, to the resting state enzyme

by two individual, one electron withdrawals from the reducing substrates (Hofrichter et al., 2010).

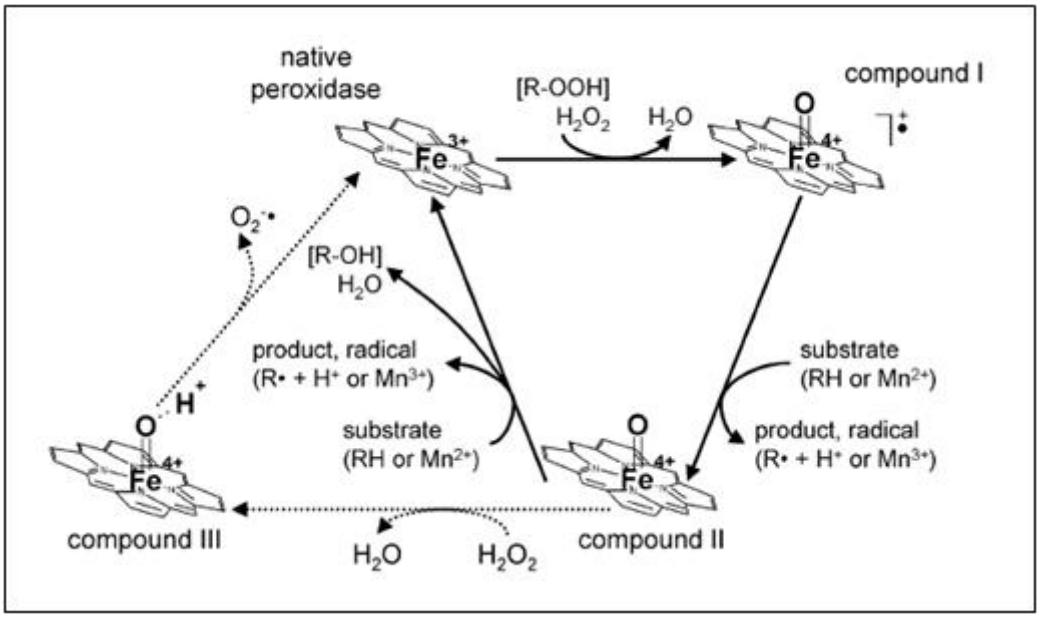


**Figure 2.** Generic catalytic cycle of peroxidases (Wesenberg et al., 2003).

LiP (EC 1.11.1.14) and MnP (EC 1.11.1.13) were first described in the WRF *P. chrysosporium* in the 80s (Cullen, 1997). MnP is the most commonly widespread of the class II peroxidases (Hofrichter 2002; Martínez et al., 2002). It catalyzes a $H_2O_2$-dependent oxidation of $Mn^{2+}$ to $Mn^{3+}$. The versatile oxidative capacity of MnP is apparently due to the production of chelated $Mn^{3+}$ ions, which act as diffusible redox-mediator and attacking, non-specifically, phenolic compounds such as biopolymers, milled wood, humic substances and several xenobiotics (Hatakka 2001; Hofrichter 2002; Husain et al., 2009).

LiPs are secreted as multiple isozymes and isoforms. They are catalytically the most powerful fungal peroxidase, and have the ability to directly oxidize dimeric lignin model compounds. In the presence of $H_2O_2$, LiP catalyzes oxidation of an endogenously generated low-molecular-mass redox mediator veratryl alcohol (the preferred aromatic

electron donor, (Reddy and D'Souza, 1994), which subsequently generates aryl cation radicals through one-electron oxidations of non-phenolic aromatic nuclei in lignin. These are then degraded to aromatic and aliphatic products, which are mineralized intracellularly. The produced radicals can participate in diverse reactions, including benzylic alcohol oxidation, carbon-carbon bond cleavage, hydroxylation, phenol dimerization/polymerization, and demethylation (Pointing, 2001). The substrate oxidation capacity of LiP includes the bleaching of colored compounds, depolymerization of synthetic lignin and transformation of organopollutants such as polycyclic aromatic hydrocarbons, chlorophenols and explosives (Bumpus 1989; Joshi and Gold 1993; Michels and Gottschalk 1994; Wesenberg et al., 2003).

VP (E.C. 1.11.1.46) was first described in liquid cultures of *Pleurotus eryngii* growing on peptone as nitrogen source (Camarero et al., 1999; Martínez et al., 1996) and *Bjerkandera* sp. (Mester and Field, 1998). VP is a heme containing structural hybrid between MnP and LiP, as it is able to oxidize $Mn^{2+}$, veratryl alcohol, simple amines, phenolic, nonphenolic and high molecular weight aromatic compounds and high-redox potential dyes in reactions which are of manganese-independent character (Heinfling et al., 1998). Therefore this enzyme has a wider catalytic versatility for electron donors as compared to LiP and MnP, and it has been postulated that its catalytic cycle is constituted by the sum of the catalytic cycles of those enzymes (Hofrichter et al., 2010).

### 3.2. Cytochrome P450 system

Ability of WRF to degrade pollutants was first ascribed to the LMEs in nitrogen limiting conditions, particularly LiP and MnP in *P. chrysosporium*, one of the first WRF employed as potential bioremediation agent. However, it was demonstrated afterwards

that some xenobiotics such as 2,4-dichlorophenoxiacetic acid and 2,4,5-trichlorophenoxacetic acid could be degraded in nitrogen-rich media, without the expression of LiP and MnP (Yadav and Reddy, 1992, 1993), thus suggesting the role of additional enzymatic systems. Posterior research demonstrated that the intracellular cytochrome P450 system exerts a leading role in the degradation of organic contaminants by WRF, as reviewed by Cerniglia (1997). Evidence is based on the induction pattern of cytochrome P450 codifying genes in response to exposure to organic pollutants (Doddapaneni and Yadav, 2004) and also on the important reduction in the extent of degradation in the presence of cytochrome P450 inhibitors, observed in the metabolism of PAHs by *P. ostreatus* (Bezalel et al., 1997), dibenzil sulfide (van Hamme et al, 2003), aromatic sulfur compounds such as dibenzothiophene (Ichinose et al., 1999), chlorinated hydrocarbons such as trichloro- and perchloroethylene by *T. versicolor* (Marco-Urrea et al., 2006, 2008). Moreover, the complete sequencing of *P. chrysosporium*'s genome revealed an estimated of 148 genes corresponding to P450 monooxygeneses, the highest number of sequenced fungi so far (Martínez et al., 2004; Yadav et al., 2006). The intracellular cytochrome P450 monooxygenases catalyze a broad range of reactions, which include hydroxylation, heteroatom oxygenation, dealkylation, epoxidation of C=C bonds, reduction and dehalogenation (Bernhardt 2006).

**4. Potential use of fungi in the degradation of pollutants**

The concept of developing a technology for the environmental application of fungi, particularly WRF appeared in the 80's (Gao et al., 2010). Since then, the development of biotechnologies using WRF has been studied to treat a wide variety of wastes and their role in the bioremediation of hazardous compounds in soils has been

established. Most studies on bioremediation have focused on bacteria as degraders because of their rapid growth, and their usual ability to employ the pollutants as only substrates. Bacterial degradation of xenobiotics differs from WRF-mediated degradation: while bacteria usually employ the pollutants as nutrient sources (C and N), in WRF oxidation by means of their LMEs yields no net energy, and the degradation becomes a co-metabolic process in which additional C and N sources are required (Pointing, 2001). This capacity represents an advantage respect bacteria as it prevents the need to internalize the pollutant into the cell, thus permitting to attack low-soluble compounds and avoiding toxicity problems.

The capacity of WRF to transform and mineralize a wide range of pollutants without a pre-conditioning period via co-metabolic pathways makes them interesting for the degradation of recalcitrant xenobiotics. The use of WRF and their LMEs for the removal of xenobiotics has been reviewed elsewhere (Asgher et al., 2008; Cabana et al., 2007a, 2007b; Gao et al., 2010; Majeau et al., 2010; Pointing, 2001; Rodríguez-Couto and Toca-Herrera, 2006).

Chemicals degraded by WRF include pesticides such as the organoclorines dichlorodiphenyltrichloroethane (DDT) and its very toxic metabolite dichlorodiphenyldichloroethylene (DDE) (Bumpus et al., 1987, 1993b) and organophosphate pesticides such as chlorpyrifos, fonofos and terbufos (Bumpus et al., 1993a); polychlorinated biphenyls (PCBs) of different degrees of chlorine substitution Novotný et al., 1997; Yadav et al., 1995; Zeddel et al., 1993), some even to mineralization (Beaudette et al, 2000; Dietrich et al., 1995); diverse polycyclic aromatic hydrocarbons (PAHs) in liquid media and from contaminated soils or in complex mixtures such as creosote (Byss et al., 2008; Field et al., 1992; Lamar et al., 2002); components of munition wastes including 2,4,6-trinitrotoluene (TNT) and its metabolite

2,4-dinitrotoluene (DNT) (Bumpus and Tatarko, 1994; Donelly et al., 1997; Hawari et al., 1999; Jackson et al., 1999; van Aken et al., 1997), nitroglycerin (Bhaumik et al., 1997) and cyclotrimethylenetrinitramine (RDX) (Bayman et al., 1995).

Specific effluents have also been subjected to WRF-mediated remediation studies. Decolorization, dechlorination and detoxification of highly toxic bleach plant effluents derived from the pulp and paper industry have been reported (Font et al., 2006; Selvam et al., 2002, 2006), while degradation and decolorization of synthetic dyes due to the non specificity of the LMEs have been widely documented (Blánquez et al., 2008; Svobodová et al., 2008). Likewise, treatment of the acidic, phenolic-rich olive oil mill wastewater has shown chemical oxygen demand reduction, decolorization and dephenolization (Blánquez et al., 2002; D'Annibale et al., 2006; Dhouib et al., 2006; Tsioulipas et al., 2002).

More recently, research moved towards the application of WRF to remove the so called emerging pollutants. From these organic contaminants, the endocrine disrupting compounds (EDC) comprise the most studied group. Removal of EDC has been demonstrated in aqueous phase and soil with WRF and LMEs (Cabana et al., 2007a; Soares et al., 2005; Tanaka et al., 2000), and even some work has been conducted at different reactor scale configurations (Auriol et al., 2007, 2008; Blánquez and Guieysse 2008; Tanaka et al., 2001). Contrary, approaches on the removal of pharmaceutical compounds are incipient, and most of the work has been performed in liquid media, aiming at demonstrating the ability of WRF to degrade the xenobiotics. Reports include the degradation of analgesics (Eibes et al., 2011; Marco-Urrea et al., 2009, 2010a, 2010b, 2010c; Tran and Urase Kusakabe, 2010), antibiotics (Accinelli et al., 2010; Rodarte-Morales et al., 2011; Schwarz et al., 2010), psychiatric drugs (Hata et al., 2010; Jelić et al., 2012; Marco-Urrea et al., 2010d) and lipid regulators (Marco-Urrea et al.,

2009; Tran and Urase Kusakabe 2010). Similar is the panorama of brominated flame retardants (BFR) (Uhnáková et al., 2009, 2011; Zhou et al., 2007) and UV filters (Gago-Ferrero et al., 2012), whose degradation by WRF has been barely studied.

## 5. Treatment of sludge by fungi

Sludge is an inevitable by-product of the wastewater treatment process. The terms sewage sludge and biosolids refer to the insoluble residue produced during biological wastewater treatment and subsequent sludge stabilization procedures (Arthurson, 2008). Usually sludge resulting from these treatment operations is mainly liquid, with a solid content ranging from 0.5% to 15%. In this respect, treatment processes are aimed at increasing the solid content in order to reduce the sludge volume, or decreasing volatile solids and stabilizing the fraction of organic matter. The increase in solids involves sludge thickening and dewatering, a requisite prior the application of subsequent processes such as composting, incineration and landfilling. Dewatering techniques include centrifugation, filtration, water evaporation and percolation (wetlands) (Uggetti et al., 2010). On the other hand, common stabilization approaches include biological anaerobic and aerobic digestion, lime stabilization, composting and heat drying, or new techniques such as radiation (Arthurson, 2008; Goldfarb et al., 1999; Wang and Wang, 2007). Nonetheless, recent research of the fate of emerging pollutants in WWTPs by environmental chemists has revealed that those treatments are not efficient enough in the removal of organic micropollutants (Carballa et al., 2007; Clara et al., 2004; Gerecke et al., 2006; Plagellat et al., 2006; Radjenović et al., 2009; Shin et al., 2010).

In this context, the search for alternative sustainable technologies approaches becomes an imperative task. Among the potential biotechnological possibilities, fungal

feasible bioremediation approaches for removal of organic pollutants from sludge are circumscribed to those in which the fungus is able to survive and colonize, and include slurry phase bioremediation and solid phase, compost-like biotreatment with dewatered sludge.

A bioslurry phase system consists of the suspension of a solid phase in water or other liquid medium to a concentration typically between 5% and 40% (w/v) and kept under agitation conditions to allow the microbial growth of the indigenous microbiota or a particular inoculated microorganism (Rubilar et al., 2007). Although bioslurry systems for bioremediation purposes have been mostly conducted with bacterial cultures (Robles-González et al., 2008; Wang et al., 2005), in the last few years fungal-mediated slurries have been applied (not for the treatment of sludge, though) for the ex situ bioremediation, at bench or reactor scale, of soils contaminated with PAHs (Garon et al., 2004; Valentín et al., 2007; Wang et al., 2008; Zhang et al., 2002), hexachlorocyclohexane (Quintero et al., 2007), pentachlorophenol (Rubilar et al., 2007) or dioxin-like compounds (Kamei et al., 2009).

On the other hand, the use of solid-phase techniques has especially increased in the last decades in the case of fungal-mediated bioprocesses, since they reproduce the natural habitat and growth conditions for filamentous fungi. Moreover, solid-phase bioreactors constitute an inexpensive treatment method for remediation of solids, since they require minimum maintenance and small amounts of energy for aeration and mixing, thus making these systems cost-effective when long treatments are required (Nano et al., 2003). This feature is especially important in the potential treatment of sludge, as the spectrum of pollutants with variable degrees of recalcitrance it contains is clearly wide. As good fungal colonization of the solid matrix (i.e. dry sludge) is necessary in bioremediation, the fungal inoculum should facilitate the delivery of the

biomass and provide a lignocellulosic nutrient source for growth (Schmidt et al., 2005). In this respect, WRF are produced on organic lignocellulosic wastes such as wood chips, wheat straw, corn cobs, grape stalks or barely bran, which in addition, promote the production of LMEs (Rodríguez-Couto and Sanromán, 2005), for the posterior application in the target matrix. Several reports deal with the application of fungal bioaugmentation for the treatment of PAH-, pentachlorophenol- and DDT-contaminated soil with dissimilar results in the efficiency of removal, in biopile-processes (Anastasi et al., 2009; Cea et al., 2010; Purnomo et al., 2011; Sayara et al. 2011; Yu et al., 2011; Zeng et al., 2009), including field-scale trials for the removal of pentachlorophenol with *T. versicolor* (Walter et al., 2005).

Despite the high potential described for *T. versicolor* and WRF in general in terms of degrading ability, most of the reports refer to liquid media and soils; however research concerning their application in complex matrixes such as sludge is limited. It should also be taken into account that in several studies of elimination of pollutants from solids by different means (not only by microorganisms), the contaminants were spiked in the matrix instead of being pre-existent; in this context the use of real contaminated samples can provide a better estimation of the potential environmental impact of the application of such approaches. Moreover, as degradation of many hazardous materials has been demonstrated mostly at laboratory scale, and especially under sterile conditions, the technical challenges including bacterial competition and the scale-up of the processes, remain before considering a real application.

## 6. Objectives

The main goal of the present thesis is to demonstrate the degradation of pharmaceuticals in sewage sludge by the WRF *T. versicolor* in order to set the first stages of a bioremediation process. To achieve the general objective, the work comprised in the thesis has been organized according to fit the following specific objectives:

- Evaluating the colonization of sewage sludge systems by *T. versicolor*.
- Assessing different strategies (bioslurry and solid-phase) for the fungal-mediated removal of emerging pollutants, particularly pharmaceuticals at pre-existent concentration from sewage sludge under sterile conditions.
- Applying the fungal treatment in a non-sterile sludge system to remove pharmaceuticals.
- Describing in liquid medium the degradation process of a group of pharmaceuticals removed in the sludge systems.

# Chapter 2:
# THESIS OUTLINE

## THESIS OUTLINE

The increasing use of sludge as amendment in agricultural lands makes it a potential source for the spread of micropollutants in the environment. As described above, the potential use of WRF for the degradation of organic pollutants is high; however in the particular case of emerging pollutants most of the studies so far conducted in this respect have been performed in liquid media and approaches in solid matrixes are scarce. Moreover, in the literature there is a lack in reports regarding fungal treatments in sewage sludge.

This work aimed at demonstrating the ability of the WRF *T. versicolor* to remove pharmaceuticals from sewage sludge. This fungus was selected based on its recently demonstrated capacity to degrade a wide range of emerging pollutants in defined liquid medium, by whole cells and enzymes (Auriol et al., 2008; Blánquez and Guieysse, 2008; Cabana et al., 2011; Hundt et al., 2000; Marco-Urrea et al., 2010a, 2010b, 2010c; Rode and Müller, 1998; Tran and Urase Kusakabe 2010). The results are grouped in a series of articles, as described below and chapter 3 presents a brief summary of the most remarkable results accomplished during the research period.

To accomplish the main thesis goal, it was first necessary to evaluate the colonization of the sewage sludge by *T. versicolor* and to demonstrate its degrading capacity in this complex matrix, under sterile conditions. **Articles 1 and 2** describe this stage of the research. In **article 1**, two different approaches for the colonization of sludge were studied: solid-phase and bioslurry systems. Several parameters were analyzed to monitor the colonization as well as the activity of the fungus. In addition, the degradation of two spiked model pharmaceuticals (carbamazepine and naproxen) was evaluated in the best performance conditions of both processes. The **article 2** describes the development of a metabolic-degrading test (based on the results of

naproxen degradation in article 1), intended to be applied as an indicator to monitor the degrading capacity of the fungus in solid-phase systems, and that was employed at several posterior stages of the research.

Once demonstrated the colonization/degradation ability in sludge-systems, the removal of pre-existent pharmaceuticals was the next challenge, for which modified versions of the previously mentioned approaches were employed. **Article 3** describes the fungal-mediated removal of pharmaceuticals at pre-existent concentrations from thermally dried sludge in sterile solid-phase biopile-like systems, including important considerations regarding the changes in the toxicity of the sludge due to the process. Similarly, **article A1** presents the elimination of pre-existent pharmaceuticals (as well as members from other groups of emerging pollutants, i.e. brominated flame retardants, UV filters and estrogens) from sludge in a bioslurry reactor with fungal pellets, together with toxicological data. A final section in article A1 compares both processes, revealing a better performance of the solid-phase treatments over the bioslurry in terms of removal of pollutants and reduction of toxicity.

Therefore, the solid-phase system was employed in the next stage of the research, which consisted in the application of the fungal treatment to non-sterile sludge. **Article A2** describes the use of the biopiles for the removal of pharmaceuticals from non-sterile sludge, including bioaugmented biopiles with *T. versicolor* and biopiles without the fungus. The colonization and survival of the fungus as well as the effect over the indigenous microbiota was monitored through the molecular analyses of the dynamics in the fungal and bacterial communities during the treatment process.

In an effort to obtain more insights on the removal process, the degradation of sulfonamides (detected and eliminated in the sludge during the treatments described in articles 3 and A1) was studied in depth. **Article 4** presents the study on the degradation

of sulfapyridine and sulfathizole by *T. versicolor*. Results include the role of different enzymatic complexes, the identification of transformation intermediates and the simultaneous elimination of several sulfonamides by means of a continuous fluidized bed reactor with fungal pellets.

At last, the detailed conclusions and suggestions for further research on fungal-mediated bioremediation of sludge are presented in Chapter 4.

# Chapter 3:
# SUMMARY OF RESULTS

## SUMMARY OF RESULTS

Most of the work developed during the research involved the use of real sludge. In this respect, batches of sludge were acquired from the WWTP of El Prat de Llobregat, located near Barcelona, Spain, a plant with a total treatment capacity of around two million equivalent inhabitants. This is a typical biological activated sludge plant, with anaerobic digestion and a final stage of thermal drying. A scheme of the wastewater treatment process of the plant, including the sludge sampling points is shown in **Figure 3**.

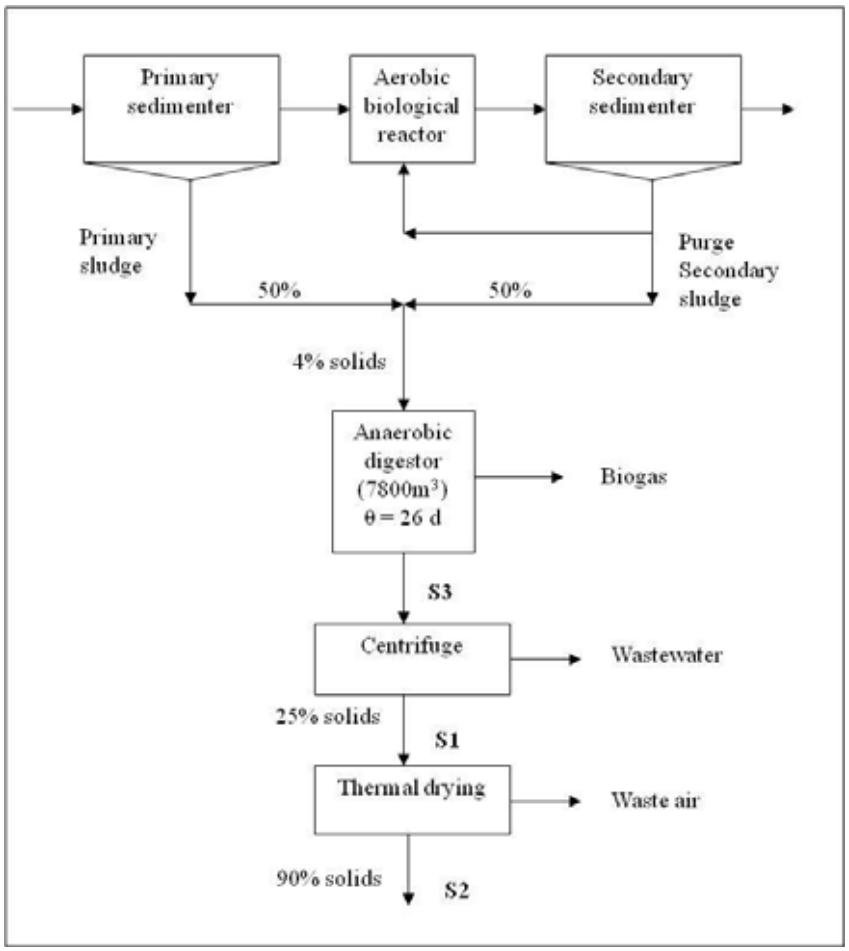


**Figure 3.** Wastewater treatment process of El Prat de Llobregat WWTP. S1, S2 and S3 indicate sludge sampling-points.

Sludge from sampling-point S1 and containing around 25% solids was first considered; however, preliminary experiments showed no evidences of colonization by *T. versicolor*, maybe due to poor oxygen diffusion (thick-gummy matrix). Then, sludge

from two other sampling-points were employed for the assessment of solid-phase systems (S2, mostly dry sludge with a solids content of around 90%) and slurry treatments (S3, around 4% in solids content). The intention here was to take advantage of the real physical characteristics of the sampled sludge (solid vs slurry) to evaluate the fungal treatments, with the minimum pre-conditioning.

**1. Colonization and degrading ability of *T. versicolor* in sewage sludge (articles 1 & 2).**

To determine the capacity of *T. versicolor* to remove pharmaceuticals from sludge, it was first necessary to study its ability to colonize such a complex matrix. In this respect, two different sludge-containing systems were employed under sterile conditions: bottle-scale bioslurries and solid-phase tube-scale biopiles. The degrading ability was also analyzed in these systems by means of spiking two model therapeutic drugs: carbamazepine (CBZ) and naproxen (NAP). To monitor the fungal growth, ergosterol, a specific sterol from fungal cell membranes, was analyzed. Similarly, as an indicator of the activity of the fungus, laccase was extracted and determined. Bioslurry cultures containing different loads of sludge (10%, 25% and 38% w/v) were performed. Despite a lag-phase, the 25%- and 38%-slurries yielded a higher biomass production than 10%-slurries over a period of 30 d, as reveled by ergosterol analyses. Important laccase levels were detected in the 10%- and 25%-slurries (up to 1308 and 2500 U $L^{-1}$), but negligible activity was found in the 38%-slurries. In the solid-phase systems (**Figure 4**) the sludge was amended with different concentrations of a lignocellulosic substrate (wheat straw pellets, WSP, 0%, 24% and 38% w/w) which acted as a bulking material and as a source of nutrients for the fungus. Over a 60 d period, data of ergosterol and laccase indicated an efficient colonization of the WSP-containing

systems, while colonization failed in the cultures lacking WSP. Considering the overall results, the 25%-slurry and the 38%-solid-phase culture were employed for the evaluation of degrading capacity. In the bioslurry, elimination reached a plateau at 47% and 57% within 24 h for NAP and CBZ. Meanwhile, complete removal of NAP and around 48% for CBZ was achieved in the solid-phase systems after 72 h.

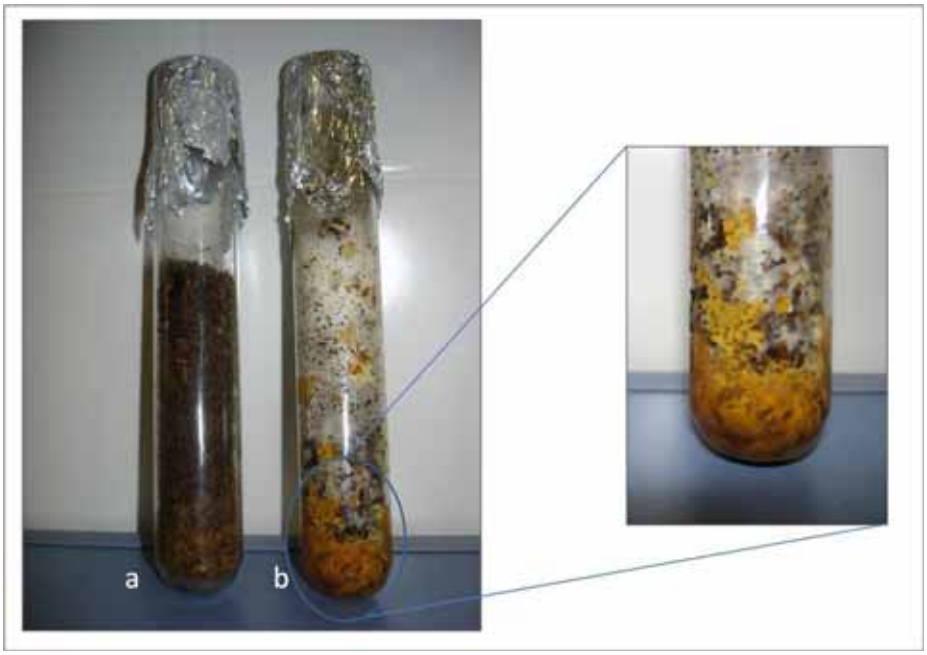


**Figure 4**. Colonization of solid-phase sludge systems by *T. versicolor*; (a) uninoculated and (b) inoculated sludge.

Given the results of NAP in solid-phase, a "degrading test", intended to monitor *T. versicolor* degrading ability in solid-state processes, was developed. The test was called ND24 (naproxen degradation in 24 h), and consisted of measuring the removal (%) of a determined concentration of NAP spiked on a solid-phase system after 24 h. The test was thought to be applied complementary to other analyses such as ergosterol, and laccase, as ND24 does not necessarily correlates those parameters. For samples collected over 45 d in sludge-systems, high ND24 values supported the feasibility of

applying *T. versicolor* as a bioremediation agent, indicated above in terms of colonization and removal of model drugs.

**2. Degradation of pre-existent pharmaceuticals in solid-phase treatment of sewage sludge (article 3).**

The capacity of *T. versicolor* to degrade pharmaceuticals at pre-existent concentrations was first assessed through solid-phase biopiles-like treatments. The same agricultural semi-processed residue, WSP, was employed as a component of the solid-phase system (Novotný et al., 1999), as *T. versicolor* grown on this substrate showed a better degrading performance according to ND24 results, though less biomass and laccase production, when compared to other wastes (Borràs et al., 2011). The biopiles consisted of sewage sludge (obtained after stabilization by thermal drying, ~90% solids, S2, **Figure 3**), supplemented with 38% (w/w, dry basis) *T. versicolor* inoculums previously grown on WSP.

Treatment time was set in 42 d, similar to that employed in typical sludge-composting processes. The determination of ergosterol content demonstrated fungal growth and colonization of the system, while important laccase activity was also achieved during the treatment process. Moreover, the results from the ND24 test suggested that the pollutant-removal potential of the fungus persisted in the sewage sludge biopiles. Overall biological parameters indicated that *T. versicolor* had been active throughout the treatment period.

**2.1. Removal of pharmaceuticals**

From 43 pharmaceuticals analyzed, 14 were detected in the sludge and exhibited some degree of removal; **Table 1** at the end of the chapter summarizes all

removal results from solid-phase systems. The most abundant pollutants belonged to the group of analgesic/anti-inflammatory compounds: ibuprofen and diclofenac, which were removed at around 75% and 64% respectively, after the fungal treatment. Mefenamic acid, another analgesic, was reduced in 72%, while complete removal of the analgesic/antipyretic phenazone was accomplished. Two psychiatric drugs were detected and partially degraded in the sludge: diazepam and carbamazepine (43% for both). The findings regarding the antiepileptic carbamazepine are highly remarkable, as its almost negligible removal has been widely documented in conventional activated sludge (AS) and membrane bioreactor (MBR) treatments (Carballa et al., 2007; Radjenović et al., 2007).

Antibiotics exhibited highly efficient elimination: clarithromycin and the sulfonamides (sulfamethazine, sulfapyridine and sulfathiazole) were completely removed. Reports of analogous treatments applied to solid wastes for the removal of antibiotics are scarce, with different levels of success depending on the sulfonamides (Radjenović et al., 2007; Wu et al., 2009).

Complete elimination was achieved for other pharmaceuticals, including atenolol (β-blocker), furosemide (diuretic), cimetidine and ranitidine (histamine H2-receptor antagonists), bezafibrate and fenofibrate (lipid regulators). On the other hand, the diuretic hydrochlorothiazide was eliminated with an efficiency of 52% and atorvastatin, a cholesterol lowering statin, decreased its concentration in 80%.

As reports of natural attenuation from biosolids suggest high half lives for several compounds (Walters et al., 2010), these findings highlight the potential use of *T. versicolor* to reduce the impact of biosolids once released to the environment, which could reduce the concentrations of pharmaceuticals in much shorter periods of treatment.

### 2.2. Removal of other emerging pollutants

At this point and taking advantage of the treatment applied to the sludge, the removal of other pre-existent emerging pollutants was also assessed, in order to obtain a wider vision of the potential of the process. This was also performed in the bioslurry treatment (section 3).

**Removal of deca-BDE-209:** Deca-BDE-209 (deca-bromodiphenyl ether) is considered as one of the major sources of pollution by polybrominated diphenyl ethers (PBDEs) (North 2004), and it is usually the one found at higher concentrations in sewage sludge samples. Characterization of the raw sludge showed an initial deca-BDE-209 concentration of 285 ng $g^{-1}$, which decreased, after the fungal treatment to 25.4 ng $g^{-1}$, equivalent to a reduction in 86%.

**Removal of UV filters:** The fungal treatment with *T. versicolor* resulted in an important reduction in the concentration of UV filters. 4DHB (4,4'-dihydroxybenzophenone) and BP3 (benzophenone-3) were completely removed, while the remaining compounds, i.e. OC (octocrylene), 4-MBC (3-(4-methylbenzylidene) camphor), and EHMC (ethylhexyl-methoxycinnamate), were depleted from 87% to 93%. The slightly lower removal efficiency of 4-MBC, OC and EHMC could be partially explained by the higher $\log K_{ow}$ of these compounds, which may translate in a reduced bioavailability for the fungus.

**Removal of estrogens:** Raw sludge presented concentrations of E1 (estrone), E2 (17β-estradiol) and E3 (estriol) ranging from 3.4 to 19.7 ng $g^{-1}$, E1 being the most abundant.

The fungal treatment showed a high efficiency, by completely removing all of the estrogens.

### 2.3. Reduction of sludge toxicity

A global estimation of toxicity was necessary to determine the reduction of toxic potential in the fungal-treated sludge. The standardized tests of acute immobilization of *Daphnia magna* and bioluminescence inhibition of *Vibrio fischeri* showed a strong decrease in toxicity when comparing the values obtained for the untreated sludge with those for the treated sludge. Considering that the primary use of biosolids is land application, and particularly on agricultural soils, a reduction in global phytotoxicity is desired and tests focused on seed germination were also performed. The results of the germination index and root elongation (Tiquia et al. 1996) clearly demonstrated that the fungal-treated sludge is more suitable for germination and growth of the tested vegetable-seeds.

## 3. Degradation of pre-existent pharmaceuticals from sewage sludge in bioslurry reactor (article A1).

Degradation of pharmaceuticals at pre-existent concentrations was then assessed in a lab scale slurry bioreactor. The bioslurry employed for the removal of emerging pollutants consisted of a stirred tank bioreactor containing sewage sludge obtained from the outlet of an anaerobic digestor (3.6% w/w in solid content, S3, **Figure 3**) from a WWTP, and inoculated with *T. versicolor* pellets (**Figure 5**). The system operated at 25 ºC in batch mode for 26 days, similar to the real residence time of the sludge in the anaerobic reactor of the WWTP. Ergosterol profiles revealed a continuous increase in fungal biomass, however laccase was detected in the reactor only between days 14 and

18. Although the pH was set at 4.5, near the optimum for maximum laccase production (Tavares et al., 2006), enzymatic activity was lower compared to higher solid-content sludge slurries without pH control, as described in article 1. Despite the absence of laccase activity in the last stage of the treatment, the steep increase in ergosterol during this period indicated the presence of metabolically active biomass.

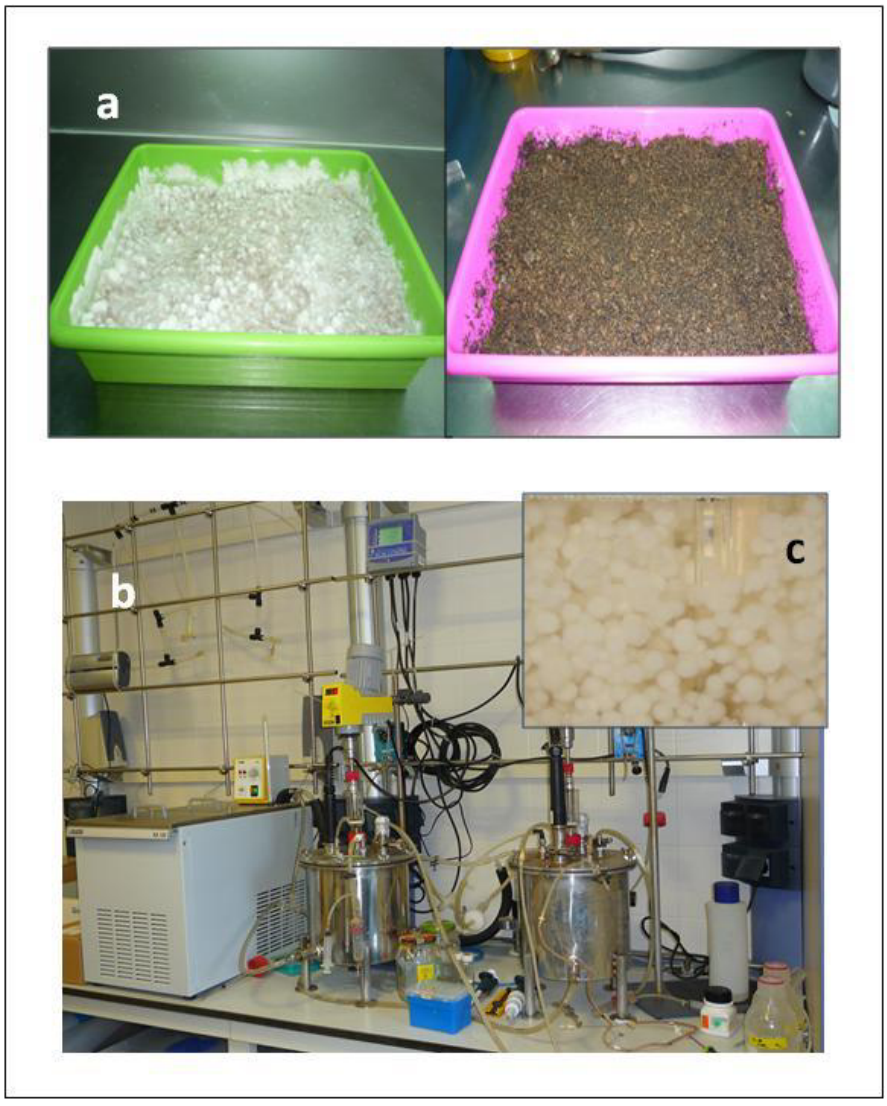


**Figure 5.** Different approaches employed for the treatment of sewage sludge: biopiles (a); bioslurry reactor (b); (c) shows the fungal pellets used to inoculate the slurry reactor.

### 3.1. Removal of pharmaceuticals

Twenty-four therapeutic drugs were detected in the sludge. Complete removal results from the bioslurry reactor are summarized in **Table 2** at the end of the chapter. The highest concentrations corresponded to the analgesics diclofenac and ibuprofen, and the sulfonamide sulfathiazole. Next in abundance were the diuretic compounds furosemide and hydrochlorothiazide, and the analgesic ketoprofen. Removal of analgesics varied from 40% to 92%, ibuprofen being the most efficiently removed.

The bioslurry treatment successfully removed several of the pharmaceuticals to non detectable levels after 26 d: three histamine H2-receptor antagonists (ranitidine, famotidine, cimetidine), two β-blockers (atenolol, sotalol), one barbiturate (butalbital) and one antidiabetic compound (glibenclamide). The elimination of the sulfonamide antibiotics sulfapyridine (100%), sulfamethazine (91.0%) and sulfathiazole (85.9%) and the diuretic drugs (furosemide and hydrochlorothiazide at 65-83%) was also high but incomplete.

Other groups of pharmaceuticals showed lower removal values, although some degree of elimination was achieved in every case. These groups included the phenazone type drugs (phenazone, 44.7%) and the lipid regulators and cholesterol lowering statin compounds (bezafibrate, gemfibrozil, atorvastatin, 41.1-49.8%). Similar to findings from solid-phase treatment, the psychiatric drug carbamazepine showed the lowest elimination value at 30.7% together with diazepam (26.1%).

### 3.2. Removal of other emerging pollutants

**Removal of BFR:** As usually reported, the most abundant BFR detected in the sludge was deca-BDE-209 at a concentration of 232 ng $g^{-1}$. The other congeners were present

at concentrations below 10 ng $g^{-1}$, except the so called "emerging BFR" decaBDEthane (deca-bromodiphenyl ethane), detected at 26.3 ng $g^{-1}$. Removal after the fungal slurry was rather low, and ranged from 16% to 53%; however only hepta-BDE-183 (2,2',3,4,4',5',6-hepta-bromodiphenyl ether) remained unchanged after the treatment. The most degradable congener was hexa-BDE-154 (53%, 2,2',4,4',5,6'-hexa-bromodiphenyl ether), while deca-BDE-209 was removed at only 38%, much less compared to the high removal obtained in solid-phase systems. Remarkably, no correlation in the elimination was observed according to the bromination degree of the detected congeners.

**Removal of UV filters:** OC, EHMC and 4-MBC were the most abundant UV filter detected in the raw sludge. The remaining sunscreens were found at concentrations below 0.1 μg $g^{-1}$. Removal values were higher than 58% for most of the compounds, including the complete elimination of BP1 (benzophenone-1). Only BP3 was poorly removed (22%) while 4DHB showed complete recalcitrance and could not be transformed in the process.

**Removal of estrogens:** E1, E2 and E3 were detected at values ranging from 10 to 21 ng $g^{-1}$. As in the case of the solid-phase systems, the fungal treatment was extremely efficient, with complete removal of the three chemical species.

### 3.3. Toxicity

Contrary to the solid-phase biopiles, results from toxicological assays (*D. magna* and *V. fischeri*) indicated an increase in the toxicity of residual sludge after the fungal treatment in the bioslurry. These findings suggest that micropollutants could be more

bioavailable in this system than in solid phase, and that aqueous media could favor abiotic reactions among the metabolic intermediates, not so common in solid phase. This is supported by the increase in the toxicity observed in the aqueous-phase degradation for some of the organic pollutants detected in the sludge, including ibuprofen (Marco-Urrea et al. 2009), carbamazepine (Jelić et al., 2012) and some BFR (Vilaplana et al., 2012).

Globally, degrading performance of the solid-phase biopiles was better in terms of pollutants removal (except for ibuprofen and hydrochlorothiazide) and reduction in sludge toxicity. Therefore, the biopile approach was chosen to assay the elimination of pharmaceuticals from non-sterile sludge.

**4. Degradation of pre-existent pharmaceuticals in solid-phase treatment of non-sterile sewage sludge (article A2).**

Degradation of pharmaceuticals was studied under non-sterile conditions in biopiles (**Figure 5**). In order to compare the performance of the fungal bioaugmentation vs the action of indigenous microflora, three sets of treatments were applied. The first set of biopiles contained sludge plus WSP but not fungus (non-bioaugmented, NB); the second set sludge plus *T. versicolor* pregrown on WSP as described in previous experiments (bioaugmented with *T. versicolor*, BTV) and the third was a "sterile control" with the same composition as the second set of biopiles (control with *T. versicolor*, CTV). One of the most important achievements from this stage of the research was obtaining successful fungal colonization of non-sterile systems with real sludge. Under non-sterile conditions, microbial community analysis by denaturing gradient gel electrophoresis (DGGE) revealed the prevalence of *T. versicolor* as the main organism in the system up to 21 d (half of the process), however by day 42, other

fungi took over as the most abundant taxons. Remarkably, comparison of fungal bioaugmented with non-bioaugmented sludge-biopiles showed a marked inhibitory effect of *T. versicolor* over bacterial populations, which is a promising finding, regarding the usual limitations and challenges pointed to the use of WRF in real applications (Gao et al., 2010). The most important fungal and bacterial taxons were identified in the systems by the cloning and sequencing approach.

Nine pharmaceuticals were detected. The elimination profiles revealed an enhanced removal of atorvastatin, diclofenac and hydrochlorothiazide (during the whole treatment) and ranitidine and fenofibrate (at short periods) in the BTV biopiles in respect to NB biopiles, coincident with the presence of the fungus. For ibuprofen, clarithromycin and furosemide, the elimination profiles were similar irrespective of the system, and with carbamazepine no significant degradation was obtained. These results suggest that fungal colonization is a promising process for pharmaceuticals remediation in complex matrices such as sludge.

**5. Degradation of selected pharmaceuticals: sulfonamides (article 4).**

The occurrence and elimination of several sulfonamides among the groups of pharmaceuticals detected in the sewage sludge treatments motivated further study on the degradation mechanism of these drugs by *T. versicolor*. The antibiotics chosen were sulfapyridine and sulfathiazole. Assays in defined liquid medium with fungal pellets revealed a complete degradation of both drugs at initial concentrations of around 10 mg $L^{-1}$, which was faster in the case of sulfapyridine (48 h vs >72 h). Experiments conducted with cytochrome P450 inhibitors indicated the partial participation of this enzymatic complex in the degradation of sulfathiazole, as its degradation was partially suppressed; on the contrary, this role was not clear in the case of sulfapyridine. When

purified laccase and laccase mediators were employed, removal was higher than 75%, supporting its role in the removal of sulfonamides.

Analyses with ultra-performance liquid chromatography-quadrupole time of flight mass spectrometry (UPLC-Qq-TOF-MS) permitted the identification of eight degradation metabolites from the fungal and enzymatic removal of sulfapyridine, among which, the desulfonated moiety was the only common intermediate. Similarly, five metabolites were identified in the case of sulfathiazole, in most cases analogous with those found for sulfapyridine and suggesting similar transformation pathways. Similar analyses permitted to describe the degradation of sulfamethazine (data not included in the present thesis, García-Galán et al., 2011).

The simultaneous removal of the three sulfonamides (sulfapyridine, sulfathiazole and sulfamethazine) was assayed in a fluidized-bed reactor (**Figure 6**), resulting in successful elimination (> 94% each) when operated at a hydraulic residence time (HRT) of 72 h.

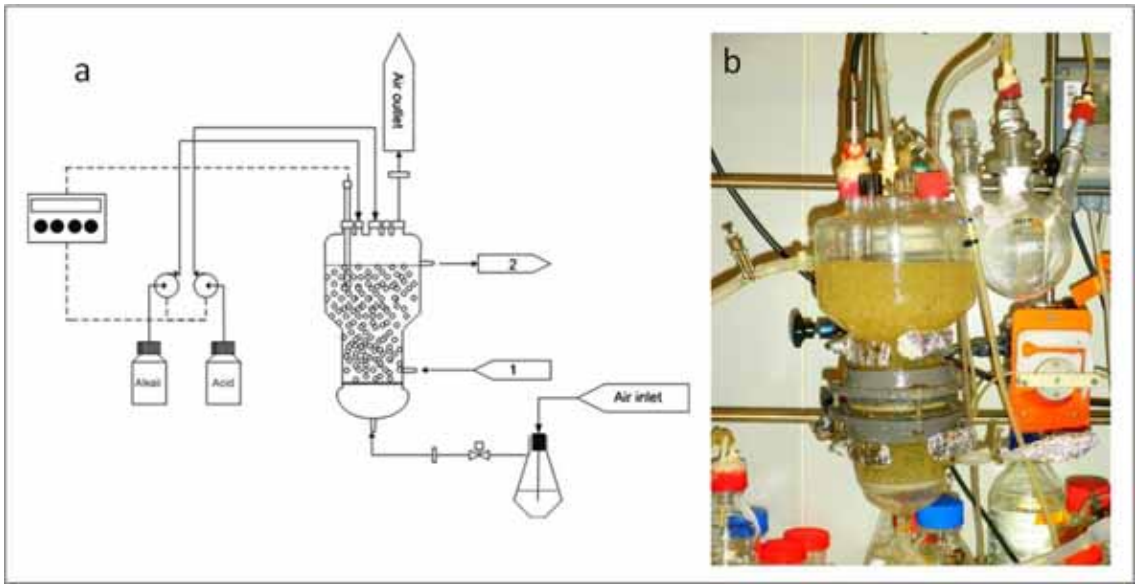


**Figure 6**. Fluidized-bed reactor employed for the continuous removal of sulfonamides. (a) Diagram, (1 inlet; 2 outlet) (Blánquez et al., 2007) and (b) reactor in operation.

**Table 1.** Occurrence and removal of emerging pollutants in thermally dried sewage sludge after solid-phase treatment with *T. versicolor*. Values corresponding to biopiles containing non-sterile sludge are presented in parenthesis. Removal values consider dilution effect by WSP; all concentrations are referred to a dw basis.

| Group of emerging contaminants | | Compounds | Raw sludge | | Treated sludge | | Removal (%) |
|---|---|---|---|---|---|---|---|
| | | | Concentration | RSD (%) | Concentration | RSD (%) | |
| | | | ($ng\ g^{-1}$) | | ($ng\ g^{-1}$) | | |
| Pharmaceuticals | Analgesics / anti-inflammatories | Ibuprofen | 85.9 (161.0) | 10.7 (13.3) | 13.1 (74.6) | 9.2 (8.6) | 75 (54) |
| | | Diclofenac | 60.3 (53.6) | 15.9 (9.0) | 13.6 (24.7) | 11.0 (6.1) | 64 (54) |
| | | Mefenamic acid | 17.9 | 11.7 | 3.1 | 16.1 | 72 |
| | Phenazone type drugs | Phenazone | 9.6 | 22.9 | n.d. | - | 100 |
| | Lipid regulators and cholesterol lowering statin drugs | Bezafibrate | 4.5 | 2.2 | n.d. | - | 100 |
| | | Fenofibrate | 4.2 (13.7) | 14.3 (13.1) | n.d. (n.d.) | - | 100 (100) |
| | | Atorvastatin | 37.8 (19.3) | 9.3 (13.5) | 4.7 (6.7) | 23.4 (23.9) | 80 (65) |
| | Psyquiatric drugs | Diazepam | 19.3 | 14.5 | 6.8 | 2.9 | 43 |
| | | Carbamazepine | 25.6 (10.5) | 19.5 (6.7) | 9.1 (11.1) | 1.1 (11.7) | 43 (-4.9) |
| | Histamine H2-receptor antagonists | Cimetidine | 11.4 | 5.3 | n.d. | - | 100 |
| | | Ranitidine | (17.5) | (7.4) | (n.d.) | - | (100) |
| | Diuretics | Hydrochlorothiazide | 26.7 (10.8) | 11.6 (15.7) | 8.0 (3.7) | 10.0 (8.1) | 52 (65) |
| | | Furosemide | (23.4) | (7.3) | (n.d.) | - | (100) |
| | β-blockers | Atenolol | 13.6 | 16.2 | n.d. | - | 100 |
| | Antibiotics | Clarithromycin | 21.0 (35.8) | 10.5 (19.0) | n.d. (6.5) | - (18.5) | 100 (82) |
| | | Sulfamethazine | 19.1 | 25.4 | n.d. | - | 100 |
| | | Sulfapyridine | 29.4 | 15.6 | n.d. | - | 100 |
| | | Sulfathiazole | 71.1 | 9.0 | n.d. | - | 100 |
| | | | ($ng\ g^{-1}$) | | ($ng\ g^{-1}$) | | |
| BFR | | Deca-BDE-209 | 285 | 5 | 41.0 | 14 | 86 |

**Table 1.** (continued)

| Group of emerging contaminants | Compounds | Raw sludge | | Treated sludge | | Removal (%) |
|---|---|---|---|---|---|---|
| | | Concentration | RSD (%) | Concentration | RSD (%) | |
| | | ($\mu g\ g^{-1}$) | | ($\mu g\ g^{-1}$) | | |
| **UV filters** | 4DHB | 0.07 | 6 | n.d | - | 100 |
| | BP3 | 0.06 | 11 | n.d | - | 100 |
| | 4-MBC | 3.10 | 9 | 0.40 | 11 | 87 |
| | OC | 8.00 | 11 | 0.92 | 10 | 89 |
| | EHMC | 2.20 | 5 | 0.16 | 5 | 93 |
| | | ($ng\ g^{-1}$) | | ($ng\ g^{-1}$) | | |
| **Estrogens** | E1 | 19.66 | - | n.d. | - | 100 |
| | E2 | 3.70 | 16.21 | n.d. | - | 100 |
| | E3 | 3.40 | 20.23 | n.d. | - | 100 |

**Table 2.** Occurrence and removal of emerging pollutants in sewage sludge after treatment in a bioslurry reactor with *T. versicolor*. Concentrations are referred to a dw basis.

| Group of emerging contaminants | | Compounds | Raw sludge | | Treated sludge | | Removal (%) |
|---|---|---|---|---|---|---|---|
| | | | Concentration | RSD (%) | Concentration | RSD (%) | |
| | | | ($ng\ g^{-1}$) | | ($ng\ g^{-1}$) | | |
| **Pharmaceuticals** | Analgesics / anti-inflammatories | Ketoprofen | 42.4 | 6.83 | 21.3 | 1.01 | 49.8 |
| | | Naproxen | 6.17 | 0.91 | 3.38 | 0.68 | 45.3 |
| | | Ibuprofen | 135 | 14.1 | 10.9 | 0.88 | 91.9 |
| | | Indomethacine | 9.60 | 1.00 | 3.65 | 0.29 | 61.9 |
| | | Diclofenac | 209 | 5.86 | 84.0 | 1.37 | 59.8 |
| | | Mefenamic acid | 14.2 | 3.05 | 8.37 | 0.20 | 41.1 |
| | Phenazone type drugs | Phenazone | 36.7 | 10.9 | 20.3 | 3.64 | 44.7 |
| | Lipid regulators and cholesterol lowering statin drugs | Bezafibrate | 11.8 | 1.21 | 6.43 | 0.26 | 45.3 |
| | | Gemfibrozil | 14.2 | 3.05 | 8.37 | 0.20 | 41.1 |
| | | Atorvastatin | 38.0 | 3.36 | 19.1 | 0.57 | 49.8 |
| | Psychiatric drugs | Diazepam | 7.71 | 0.49 | 5.70 | 0.72 | 26.1 |
| | | Carbamazepine | 29.2 | 2.57 | 20.2 | 3.49 | 30.7 |
| | Histamine H2-receptor antagonists | Ranitidine | 7.92 | 0.78 | n.d. | - | 100 |
| | | Famotidine | 12.0 | 0.78 | n.d. | - | 100 |
| | | Cimetidine | 10.4 | 1.31 | n.d. | - | 100 |
| | Sulfonamide antibiotics | Sulfamethazine | 6.1 | 22.0 | 0.5 | - | 91.0 |
| | | Sulfapyridine | 21.4 | 2.6 | n.d. | - | 100 |
| | | Sulfathiazole | 143.0 | | 20.1 | 26.4 | 85.9 |
| | ß-blockers | Atenolol | 1.70 | 0.17 | n.d. | - | 100 |
| | | Sotalol | 4.88 | 0.89 | n.d. | - | 100 |
| | Barbiturates | Butalbital | 16.3 | 2.30 | n.d. | - | 100 |

**Table 2.** (continued)

| Group of emerging contaminants | | Compounds | Raw sludge | | Treated sludge | | Removal (%) |
|---|---|---|---|---|---|---|---|
| | | | Concentration | RSD (%) | Concentration | RSD (%) | |
| | | | (ng $g^{-1}$) | | (ng $g^{-1}$) | | |
| **Pharmaceuticals** | Diuretics | Hydrochlorothiazide | 41.3 | 0.50 | 6.89 | 0.11 | 83.3 |
| | | Furosemide | 79.9 | 11.3 | 27.7 | 2.30 | 65.3 |
| | Antidiabetics | Glibenclamide | 17.4 | 1.94 | n.d. | - | 100 |
| | | | (ng $g^{-1}$) | | (ng $g^{-1}$) | | |
| **BFR** | PBDEs | Tetra-BDE-47 | 5.39 | 13 | 4.11 | 10 | 23.7 |
| | | Penta-BDE-99 | 6.05 | 23 | 3.24 | 25 | 46.4 |
| | | Hexa-BDE-154 | 5.48 | 35 | 2.56 | 15 | 53.3 |
| | | Hepta-BDE-183 | 0.61 | 13 | 0.62 | 12 | 0 |
| | | Deca-BDE-209 | 232 | 5.5 | 145 | 16 | 37.5 |
| | Emerging BFRs | DecaBDEthane | 26.3 | 22 | 22.1 | 18 | 16.0 |
| | | | (µg $g^{-1}$) | | (µg $g^{-1}$) | | |
| **UV filters** | | BP1 | 0.08 | 7 | n.d | - | 100 |
| | | 4DHB | 0.051 | 7 | 0.050 | 9 | 1 |
| | | BP3 | 0.034 | 10 | 0.019 | 7 | 22 |
| | | 4-MBC | 0.520 | 11 | 0.205 | 11 | 61 |
| | | OC | 7.71 | 11 | 3.214 | 8 | 58 |
| | | OD-PABA | 0.012 | 11 | 0.004 | 12 | 70 |
| | | EHMC | 1.031 | 7 | 0.211 | 8 | 79 |
| | | | (ng $g^{-1}$) | | (ng $g^{-1}$) | | |
| **Estrogens** | | E1 | 21.33 | | n.d. | - | 100 |
| | | E2 | 14.79 | 6.39 | n.d. | - | 100 |
| | | E3 | 10.15 | 11.07 | n.d. | - | 100 |

# Chapter 4:
# CONCLUDING REMARKS

## CONCLUDING REMARKS

The fungal-mediated degradation of emerging pollutants from sewage sludge by WRF was demonstrated for the first time through the application of solid-phase and bioslurry systems with *T. versicolor*. First, the colonization of the sludge by the fungus was assessed in depth in both systems under sterile conditions, followed by the demonstration of its removal ability in this matrix. In addition a degrading test, ND24, was developed to monitor solid-phase bioremediation treatments with the fungus. Solid-phase biopiles had in common the use of a lignocellulosic residue and thermally dried sludge, and included studies under sterile and non-sterile conditions. Slurry treatments, on the other hand, employed sludge from the effluent of an anaerobic digester and the reported removals refer only to sterile conditions.

Comparison of the removal efficiency under sterile conditions (which includes only the pollutants found in both kinds of sludge before fungal treatment), indicates that a solid-phase treatment is more efficient than the bioslurry process, as only two compounds (ibuprofen and hydrochlorothiazide) were removed at a higher extent in the latter system. In the case of a few compounds (cimetidine, atenolol and sulfapyridine), complete degradation was accomplished regardless of the treatment employed, but in most of the cases the elimination was significantly higher in solid-phase. That was especially highlighted in the case of several pharmaceuticals (phenazone and bezafibrate) and other emerging pollutants such as some UV filters (4DHB and BP3), and the deca-DBE-209, as the removal in solid-phase was more than twice the obtained in the bioslurry.

The remarkable difference in the final toxicity of the sludge obtained from both strategies, increased in the slurry and dramatically decreased in the biopiles, also

supports the possibility of employing a solid-phase treatment with WRF as an eco-friendly strategy to reduce the release of toxic contaminants into the environment.

Molecular analysis of microbial communities revealed the colonization and survival of *T. versicolor* in non-sterile biopiles, remarking the potential of the fungus for bioremediation process, sometimes underestimated due to reports of poor colonization capacity. After overcoming issues of matrix colonization, one of the biggest challenges in bioaugmentation, the process resulted in the removal of pharmaceuticals in non-sterile sludge, at efficiencies close to those under sterile conditions in most of the cases.

In addition, given the challenge inherent to structural elucidation studies in complex environmental matrices such as sludge, the identification of the transformation intermediates produced during the degradation of some sulfonamides by *T. versicolor* was performed in liquid media, as some of these therapeutic drugs were degraded in the sludge systems previously described.

Further research should focus on: *i.)* the description of the mechanisms involved in the degradation *in situ* and *ii.)* the identification of the transformation products released during the process to better estimate the toxicity of the residues; *iii.)* more insights in the relationship between the WRF and the microbiota, and their combined effects on the degrading capacity; and clearly, *iv.)* the optimization of the process and *v.)* the scale-up necessary to implement a possible real application.

# Chapter 5:
# REFERENCES

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# PUBLISHED ARTICLES

# Article 1:

Rodríguez-Rodríguez, C.E., Marco-Urrea, E., Caminal, G.

## Degradation of naproxen and carbamazepine in spiked sludge by slurry and solid-phase *Trametes versicolor* systems

Bioresour. Technol. 101 (2010), 2259-2266.

Bioresource Technology 101 (2010) 2259–2266

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

# Degradation of naproxen and carbamazepine in spiked sludge by slurry and solid-phase *Trametes versicolor* systems

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ARTICLE INFO

*Article history:*
Received 11 August 2009
Received in revised form 17 November 2009
Accepted 19 November 2009

*Keywords:*
*Trametes versicolor*
Sewage sludge
Bioslurry
Solid-phase fermentation
Emerging pollutants

ABSTRACT

Growth and activity of the white-rot fungus *Trametes versicolor* on sewage sludge were assessed in bioslurry and solid-phase systems. Bioslurry cultures with different loads of sludge (10%, 25% and 38%, w/v) were performed. A lag phase of at least 2 d appeared in the 25 and 38%-cultures, however, the total fungal biomass was higher for the latter and lower for the 10%-culture after 30 d, as revealed by ergosterol determination. Detectable laccase activity levels were found in the 10 and 25%-cultures (up to 1308 and 2588 AU $L^{-1}$, respectively) while it was negligible in the 38%-culture. Important levels of ergosterol and laccase were obtained over a 60 d period in sludge solid-phase cultures amended with different concentrations of wheat straw pellets as lignocellulosic bulking material. Degradation experiments in 25%-bioslurry cultures spiked with naproxene (NAP, analgesic) and carbamazepine (CBZ, antiepileptic) showed depletion of around 47% and 57% within 24 h, respectively. Complete depletion of NAP and around 48% for CBZ were achieved within 72 h in sludge solid cultures with 38% bulking material. CBZ degradation is especially remarkable due to its high persistence in wastewater treatment plants. Results showed that *T. versicolor* may be an interesting bioremediation agent for elimination of emerging pollutants in sewage sludge.



## 1. Introduction

In the last years, the presence of emerging micropollutants such as pharmaceuticals and personal care products (PPCP) and endocrine disruptors chemicals (EDC) in the environment has received much attention (Nakada et al., 2006; Radjenović et al., 2007, 2009a). After their consumption and excretion, these compounds and their metabolites reach sewage systems, where they are barely reduced. Consequently, they are released into the environment either by the receiving waters of the sewage treatment plants or adsorbed to solids in sludge disposal. Therefore, the development of new techniques for PPCP and EDC removal is intensively studied nowadays in wastewater, both by physicochemical or biologically-mediated techniques (Marco-Urrea et al., 2009; Quintana et al., 2005). However, it is noteworthy to mention the lack of studies regarding PPCP degradation in sewage sludge.

*Trametes versicolor* belongs to the category of white-rot fungi (WRF), a cosmopolitan group of microorganisms with a high capability to degrade a wide range of xenobiotics and recalcitrant pollutants due to the complexity of their enzymatic systems, able to act on diverse substrates through the attack of both extracellular (i.e., laccase, lignin peroxidase, manganese peroxidase, versatile peroxidase) and intracellular (i.e., cytochrome P450 system) enzymes (Asgher et al., 2008; Martínez et al., 1996). In the last few years, studies related to PPCP and EDC degradation by *T. versicolor* and other WRF in liquid media have revealed promising results (Blánquez and Guieysse, 2008; Cabana et al., 2007; Marco-Urrea et al., 2009).

Biopiles and bioslurry reactors are among the potential process options for sewage sludge bioremediation. A bioslurry phase system consists of a suspension of the solid phase in water or culture medium to a concentration typically between 5% and 40% (w/v), which is inoculated with the organism of interest and kept under agitation conditions to allow microbial growth (Rubilar et al., 2007). Bioslurry systems for bioremediation purposes have been mostly conducted by bacterial cultures (Robles-González et al., 2008; Wang et al., 2005), although in the last few years WRF were also successfully applied to soil bioremediation of polycyclic aromatic hydrocarbons (PAHs), hexachlorocyclohexane and pentachlorophenol (Quintero et al., 2007; Rubilar et al., 2007; Valentín et al., 2007).

On the other hand, solid-phase remediation systems include bioaugmentation in piles, usually referred to as biopiles or tray bioreactors, depending on the scale of the process. They have been largely described as an efficient mean to remove many pollutants

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0960-8524/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2009.11.089

by bacteria, but reports employing fungi are markedly less frequent and refer to pentachlorophenol (Walter et al., 2005), oil, petroleum hydrocarbons and di(2-ethylhexyl) phthalate (Chiu et al., 2009). Biopiles may be static with forced aeration or they may be turned or mixed by special devices, while moisture is provided by spraying the solid matrix with water until it is wet but avoiding puddles. Additionally, they may be amended with a bulking agent, usually with straw, saw dust, bark or wood chips or some other organic material.

The application of the above mentioned processes for sewage sludge bioremediation with *T. versicolor* required a preliminary study of the viability and biological activity of the microorganism under different operation conditions in bioslurry and solid-phase systems. After growth conditions were established, degradation tests with sewage sludge spiked with the antiepileptic carbamazepine (CBZ) and analgesic naproxen (NAP) were performed, based on previous evidence of *T. versicolor* ability to degrade them in a defined liquid medium (Marco-Urrea et al., 2009,2010). These pharmaceuticals were selected according the reported non-biodegradability of CBZ in sewage treatment plants and the extensive use of NAP as non-prescription drug (Quintana et al., 2005; Radjenović et al., 2007).

The present work evaluates for the first time the ability of WRF to colonize and degrade PPCP in spiked sewage sludge via bioaugmentation.

## 2. Methods

### 2.1. Chemicals and fungal strain

CBZ (5H-dibenzo[*b*,*f*]azepine-5-carboxamide), NAP ((S)-(+)-methoxy-α-methyl-2-naphthalene acetic acid, 98%) and ergosterol (ergosta-5,7,22-trien-3β-ol, >95%) were obtained from Sigma–Aldrich Co. (St. Louis, MO).

The strain *T. versicolor* ATCC 42530 was acquired from the American Type Culture Collection, and maintained by subculturing every 30 d on 2% malt extract agar slants (pH 4.5) at 23 °C.

### 2.2. Sewage sludge

Dry sewage sludge (17.7% humidity; water holding capacity 0.47 g $g^{-1}$ dry weight, DW) was obtained from the wastewater treatment plant of El Prat de Llobregat, The plant is located near Barcelona, Spain and it has a total treatment capacity of two million equivalent inhabitants. Wastewater treated is a mixture of municipal and industrial wastewater.

### 2.3. Media and cultures

Two different liquid media were employed in this study. Defined medium contained per liter: 10 g glucose, 2.1 g $NH_4Cl$, 10 mg thiamine, 10 mL micronutrient solution (per liter: nitrilotriacetic acid 1.5 g, $MgSO_4{\cdot}7H_2O$ 3.0 g, $MnSO_4{\cdot}H_2O$ 0.5 g, NaCl 1.0 g, $FeSO_4{\cdot}7H_2O$ 0.1 g, $CoSO_4$ 0.1 g, $ZnSO_4{\cdot}7H_2O$ 0.1 g, $CaCl_2{\cdot}2H_2O$ 0.1 g, $CuSO_4{\cdot}5H_2O$ 0.01 g, $AlK(SO_4)_2{\cdot}12H_2O$ 0.01 g, $H_3BO_3$ 0.01 g, $NaMoO_4$ 0.01 g) and 100 mL macronutrient solution (per liter: $KH_2PO_4$ 20 g, $MgSO_4{\cdot}7H_2O$ 5 g, $CaCl_2$ 1 g). Malt extract medium contained 20 g $L^{-1}$ malt extract (Scharlau, Barcelona, Spain). All media were adjusted to a final pH of 4.5. *T. versicolor* blended mycelial suspension and pellets were prepared according to Marco-Urrea et al. (2008). The wheat straw pellets (ATEA Praha s r.o., Czech Republic) used as bulking material in solid-phase cultures were kindly provided by Č. Novotný and V. Šašek from the Academy of Science of the Czech Republic.

### 2.4. Experimental procedures

#### 2.4.1. Growth in bottle-scale bioslurry systems

*T. versicolor* growth studies in bioslurry cultures were performed in 600 mL cotton-stoppered bottles. Each bottle contained 50 mL defined medium, 2.5 mL blended mycelium suspension and 5, 15 or 30 g of autoclaved dry sewage sludge, to give approximate concentrations of 10%, 25% and 38% (w/v). The bottles were incubated on an orbital shaker (135 rpm) at 25 °C. In time-course experiments, triplicate bottles of each solid concentration were sacrificed and their content was fractioned by filtration to analyze glucose, laccase activity, dissolved organic carbon (DOC) and pH from the liquid phase and ergosterol from the solid phase. During the incubation period, glucose was periodically added to maintain a final concentration of 4–8 g $L^{-1}$ (except from 10 to 19 d).

#### 2.4.2. T. versicolor colonization in solid-phase cultures

Solid state cultures were performed in flasks containing dry sewage sludge and the corresponding concentration of wheat straw pellets as lignocellulosic bulking material. Each flask contained 8 g dry sewage sludge and 0, 2.5 or 5 g of wheat straw pellets to obtain concentrations of 0%, 24% and 38% (w/w, dry basis), respectively. Wheat straw pellets were previously hydrated in a 1:2 ratio (w/v). Each culture was inoculated with 0.25 mL blended mycelium suspension per gram of solid dry weight. Solid content of the cultures was sterilized before mycelium addition. All the flasks were incubated at 25 °C, periodically homogenized and sterile distilled water was often added to the cultures to provide moisture. For each time-point, triplicate flasks were sacrificed to analyze ergosterol and laccase activity.

#### 2.4.3. Degradation of pharmaceuticals in bioslurry systems

Two sets of 14 d-old triplicate cultures (volume 120 mL) were prepared as described before containing 25% sludge (w/v). One set was sterilized at 120 °C for 30 min to act as a heat-killed control. All the cultures were then spiked with a non-sterile stock solution of the corresponding pharmaceutical (CBZ or NAP) in ethanol, to give a final concentration of ~0.067 mg $g^{-1}$ dry solid each. For each time point analysis, 5 mL samples were taken from each culture and centrifuged at 8000 rpm for 5 min, the supernatant was decanted and the pellet was washed with 4 mL water:methanol (2:1) and centrifuged as mentioned. Both liquid phases were mixed and the volume was adjusted to 10 mL in a volumetric flask for subsequent HPLC analyses. The solid phase was lyophilized (Virtis Sentry freeze-drying equipment, Gardiner, NY) and then subjected to pressurized liquid extraction (PLE) in a PSE One extractor (Applied Separations, Allentown, PA), as previously described (Radjenović et al., 2009a). The volume of the extract was adjusted to 25 mL in a volumetric flask; a 1.5 mL aliquot was centrifuged at 10,000 rpm for 5 min and the supernatant transferred to amber HPLC vials for subsequent analysis. The total mass of each pharmaceutical remaining after fungal treatment was calculated considering both solid and liquid phases of the bioslurry. The recovery yields of these pharmaceuticals were higher than 95% by this procedure.

#### 2.4.4. Degradation of pharmaceuticals in solid-phase cultures

Solid-phase cultures with a total dry solid weight content of 2.6 g were performed in 40 mL screw cap vials (28 × 95 mm, Grace, Deerfield, IL), containing 38% bulking material, as previously described. After fungal growth the cultures were spiked with the CBZ or NAP stock solution to give a final concentration of ~0.077 and 0.096 mg g $DW^{-1}$, respectively. Triplicate cultures previously autoclaved were employed as heat-killed control. At each time point, triplicate culture flasks were sacrificed, lyophilized and all

the content was subjected to PLE. The volume of extraction was adjusted to 50 mL and processed as previously described.

### 2.5. Analytical methods

#### 2.5.1. Ergosterol quantification

Ergosterol was analyzed in homogeneously-mixed samples of solid-phase cultures and solid fraction of bioslurries employing a modified method described by Novotný et al. (1999). Briefly, 0.5–0.8 g from each sample were weighed and placed in a test tube to be extracted with a mixture of 1 mL cyclohexane and 3 mL KOH–methanol solution (10% w/v) for 90 min at 70 °C (sonicating for the first 15 min, J.P. Selecta, Barcelona, Spain). Then 1 mL distilled water and 2 mL cyclohexane were added; the tube was vortexed for 30 s and centrifuged at 3500 rpm for 5 min. The organic phase was recovered and the aqueous phase was washed twice with 2 mL cyclohexane. The organic phases were pooled and evaporated to dryness with nitrogen. The residue was dissolved in 1 mL methanol for 15 min at 40 °C, vortexed for 30 s and centrifuged in Eppendorf vials at 6000 rpm for 3 min. Finally the resultant solution was transferred to amber vials and analyzed in a Dionex 3000 Ultimate HPLC (Sunnyvale, CA) equipped with an UV detector at 282 nm, using a reverse phase Grace Smart RP18 column (250 mm × 4 mm, particle size 5 μm, Deerfield, IL). Methanol was isocratically supplied at 1 mL $min^{-1}$ as eluent and retention time was ~6.8 min. Ergosterol content was expressed as milligrams per gram of solid dry weight (mg g $DW^{-1}$), unless otherwise stated. In order to correlate ergosterol content and fungal biomass, a calibration curve was obtained from the ergosterol extraction of different weights of *T. versicolor* pelleted-form. Results were expressed in terms of fungal dry weight.

#### 2.5.2. Laccase activity

Laccase determination was performed in centrifuged (15,000*g*, 15 min) samples from the liquid phase of bioslurries. In solid-phase cultures, the enzyme was previously extracted according to a modified method by Lang et al. (1998): 30 mL sodium acetate buffer (0.16 M, pH 5) were added to 3 g of homogenized sample and shaken for 30 min at 4 °C; 1.5 mL from the extracts were transferred to Eppendorf vials and centrifuged at 15,000*g* for 15 min; the supernatant was then analyzed. In both cases, laccase activity was measured using the first step of the method for determination of manganese peroxidase (Wariishi et al., 1992) and 2,6-dimethoxyphenol as the substrate. Results were expressed as activity units (AU) per liter or gram.

#### 2.5.3. Pharmaceuticals quantification

CBZ and NAP analyses were performed using a Dionex 3000 Ultimate HPLC equipped with a UV detector at 230 nm. Chromatographic separation was achieved by injection of 20 μL samples on a Grace Smart RP18 column (250 mm × 4 mm, particle size 5 μm) and a mobile phase of 65% 6.9 mmol $L^{-1}$ acetic acid (pH 4.0) plus 35% acetonitrile, added isocratically at 1 mL $min^{-1}$ (Stafiej et al., 2007). Retention times were ~8.4 min for CBZ and ~14.1 min for NAP.

#### 2.5.4. Other analyses

Glucose was analyzed by the glucose oxidase method in an YSI 2700 analyzer (Yellow Springs, OH). DOC was measured in a 1020A Total Carbon Analyzer (O I Analytical, College Station, TX). pH determinations were conducted with a Basic20 pH meter (Crison, Barcelona, Spain).

## 3. Results and discussion

### 3.1. Growth and activity of *T. versicolor* in sewage sludge bioslurry systems

To asses the feasibility of employing *T. versicolor* as a bioremediation agent for sewage sludge treatment, it is first necessary to demonstrate the growth and biological activity of the fungus within the sewage sludge systems. Ergosterol, main sterol in fungal cell membranes, was employed as fungal biomass indicator, given the inability of its determination by direct means in the bioremediation systems (Barajas-Aceves et al., 2002; D'Annibale et al., 2005; Niemenmaa et al., 2006; Pant and Adholeya, 2007). In order to monitor *T. versicolor* growth in sewage sludge systems, a previous calibration curve correlating ergosterol and pelleted-biomass was obtained. The slope in the straight line ($r = 0.9974$) indicated that ergosterol content of *T. versicolor* ATCC 42530 corresponds to 6.61 mg g $DW^{-1}$ of fungal biomass. Although few reports are available in the literature, this value is similar to the contents of 4.78 or 6.95 mg g $DW^{-1}$ reported by Barajas-Aceves et al. (2002) for different strains of *T. versicolor*.

The growth and activity patterns of *T. versicolor* were analyzed over a 30 d period in bioslurry cultures containing different concentrations of sewage sludge. Visual examination of cultures indicated that inoculated mycelium grew in the form of pellets, due to the effect of orbital shaking. As it is shown in Fig. 1A, a noticeable increase of ergosterol concentration was observed on the second day of incubation in the 10%-bioslurry, whereas 5 d

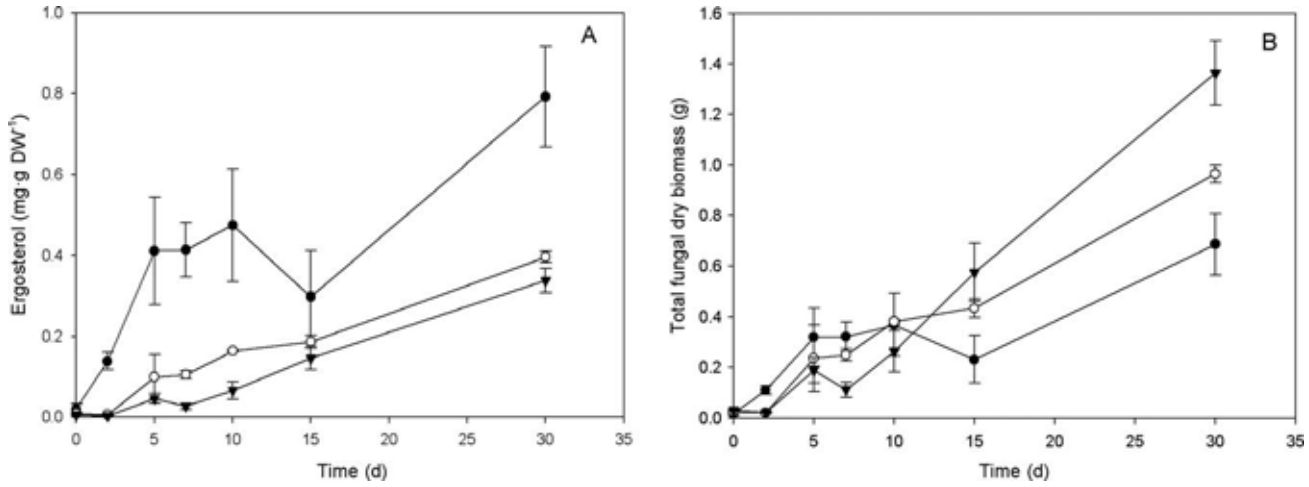


**Fig. 1.** Time courses of *T. versicolor* growth in the presence of 10% (●), 25% (○) and 38% (▼) sewage sludge (w/v). (A) Relative growth expressed as ergosterol content per unit of solid dry weight; (B) absolute growth expressed as total fungal dry biomass per bottle. Values plotted are means ± standard deviations for triplicate cultures.

were necessary to detect an increment in 25- and 38%-bioslurries. Thereafter, ergosterol concentration increased over time in all cultures, up to the end of the experiment (30 d), where it was higher (0.79 mg g $DW^{-1}$) for the 10%-bioslurry and lower (0.34 mg g $DW^{-1}$) for the 38%-bioslurry. However, as these results are relative to the sludge mass, the time-course of total fungal biomass production allows a better comparison of sludge concentration influence. As shown in Fig. 1B, there was a lag phase of at least 2 d for 25- and 38%-bioslurries. The appearance of a lag phase due to increasing solid concentrations in bioslurry systems has been also reported by Quintero et al. (2007). Possible explanations for this phenomenon are: (i) the shear stress exerted by solid sludge particles on mycelia biomass, (ii) the critical stressful conditions due to limitations in oxygen transfer at solid concentrations over 30%, as was reported by Woo et al. (1997), and (iii) the presence of some unidentified sludge components showing inhibitory effect on fungal growth. Inhibitory effect seemed to be higher for the 38%-bioslurry at culture onset, since the amounts of biomass tended to be lower in the early stages of the incubation. However, by the end of the experiment this culture produced the highest amount of biomass, followed by the 25%-bioslurry. Although *T. versicolor* presented no lag phase in the 10%-bioslurry, this culture yielded the lower biomass level.

The initial inhibitory effect of sludge concentration was also reflected in glucose consumption by *T. versicolor* (Fig. 2A). Glucose depletion occurred by day 2 for 10%-bioslurry whereas it occurred by day 5 in 25%-bioslurry, and at this time 38%-bioslurry still contained 1.27 g $L^{-1}$ of glucose. Nonetheless, after day 5, all bioslurries presented very similar glucose consumption rates, thus indicating the end of lag phase and continuous growth. Previous reports demonstrate prolonged lag phases in 10%-soil bioslurry reactors with other WRF. For instance Valentín et al. (2007) obtained a 6 d lag period inoculating the WRF *Bjerkandera* sp. at even higher inoculums concentrations than those tested here. Similarly, Quintero et al. (2007) did not observe complete depletion of glucose until 5–6 d in 10%-bioslurries containing *B. adusta*. Although shear effects produced by sandy soils might be higher than those due to sludge, the growth profiles here reported suggest that *T. versicolor* is a promising microorganism for sludge slurry applications.

In order to determine whether *T. versicolor* was able to grow solely at the expenses of organic compounds released from the sludge, DOC was monitored during incubation. Glucose addition was stopped from days 10 to 19, but DOC, used as a possible indicator of fungal activity, did not decrease over this period in any of the bioslurrry treatments (data not shown). Nonetheless, stable or higher levels of ergosterol were found in all bioslurry treatments (Fig. 1A). No clear conclusions were extracted from these results, however, they suggest that the failure to detect a decrease in DOC when glucose addition was stopped might be ascribed to two non-mutually exclusive possibilities: (i) an adsorption–desorption equilibrium favored the release of C containing compounds as they were depleted in the liquid medium, or (ii) consumption of sludge occurred in a slow rate, not detectable in comparison to DOC amounts, which were in the range of 5000–20,000 mg C $L^{-1}$ depending on the treatment.

*T. versicolor* produces both constitutive and inducible laccases and therefore its extracellular activity was used as indicator of the oxidative potential of the cultures. Moreover, to monitor laccase activity is interesting since this enzyme is involved in the biodegradation of different environmental pollutants (Asgher et al., 2008; Blánquez and Guieysse, 2008). Laccase activity results are presented in Fig. 2C. Activity onset was at day 5, and the profiles show a peak of 2588 AU $L^{-1}$ at day 10 for the 25%-bioslurry and 1308 AU $L^{-1}$ at day 7 for the 10%-bioslurry. In both cases, those peaks were followed by a continuous decay in activity, more abrupt in the former case. This decrease can be ascribed to the high

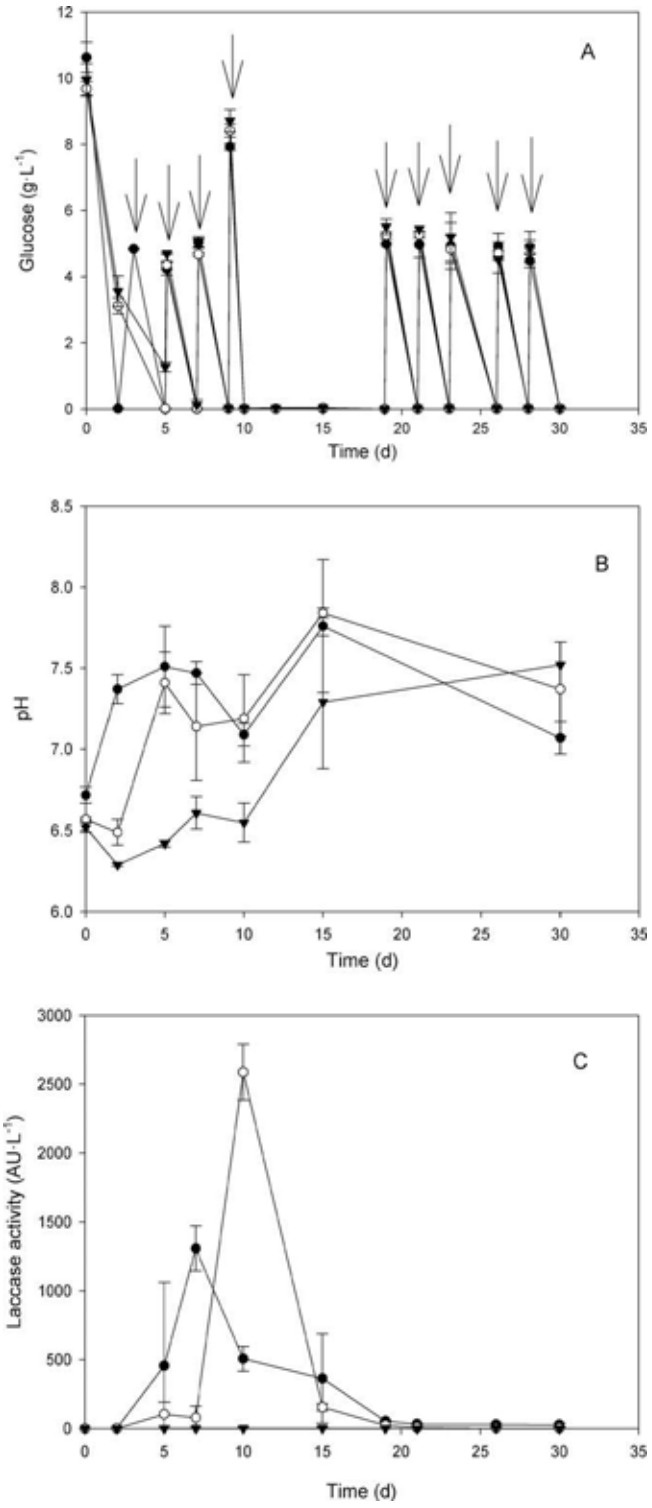


**Fig. 2.** (A) Glucose consumption, (B) pH and (C) laccase activity profiles in *T. versicolor* bioslurry cultures. (●) 10%, (○) 25% and (▼) 38% sewage sludge (w/v). Glucose addition was intentionally stopped from days 10 to 19 of incubation. Values plotted are means ± standard deviations for triplicate cultures. Vertical arrows represent times of glucose addition.

values of pH obtained from day 15 (Fig. 2B) since maximum laccase activity occurs at pH around 5 (Tavares et al., 2006) and values over 7.4 have shown inactivation in WRF enzymes (Lu-Chau et al., 2004). Although pH 4.5 of the medium used in this study was the optimal for *T. versicolor*, after the mixture with sewage sludge it rose up to 6.5–6.7 for all the cultures and at the end of the

incubation period it was between 7.1 and 7.5 (Fig. 2B). The increase of pH during incubation period might be associated to glucose depletion, as it has been reported for soil bioslurry cultures of other WRF (Lu-Chau et al., 2004; Rubilar et al., 2007; Valentín et al., 2007), given that assimilation of other carbon sources from the sludge may lead to the release of amine-like products. In fact, pH peaks for 10 and 25%-bioslurries were coincident with the glucose-free period (10–19 d). However, as we stated in Section 3.1, our results regarding consumption of C compounds from sludge by *T. versicolor* are not conclusive. In any case, to keep more stable laccase activity profiles, future approaches should emphasize on pH control as is reported for continuous liquid-phase reactors (Blánquez et al., 2006).

On the basis of the high laccase activity and continuous fungal growth observed, the 25%-bioslurry treatment was selected for further studies. This is in contrast with the small loads ranging from 5% to 10% solids commonly used for degradation processes, which obviously favors microbial activity and pollutant removal efficiency (Quintero et al., 2007; Rubilar et al., 2007; Valentín et al., 2007). Therefore, degradation of xenobiotics in the aforementioned conditions provides a promising option for simultaneous treatment of higher sewage sludge amounts.

### 3.2. T. versicolor colonization of sewage sludge solid-phase cultures

WRF are a heterogeneous group of organisms that have in common the capacity to degrade lignin as well as other wood components in the environment. Degradation of lignin present in wood is carried out by a battery of oxidative enzymatic mechanisms that enable WRF to gain access to the holocellulose, which is their carbon and energy source in nature. Therefore, to ensure the growth of *T. versicolor* as well as the production of its ligninolytic enzymes, commonly involved in xenobiotic degradation, it is recommended to amend soils with ligninolytic substrates such as corn cobs or wood chips. In this regard, the use of lignocellulosic materials as potential bulking agents has shown to stimulate laccase activity and provide some necessary nutrients to WRF (Rodríguez-Couto et al., 2001). In the present study, wheat straw pellets were assessed not only as a bulking material, but also to act as a substrate for *T. versicolor* growth to allow efficient colonization of sludge.

Colonization of solid-phase cultures consisting of mixtures of sewage sludge and wheat straw pellets were monitored by quantification of ergosterol and laccase production for a 60 d period. Two different concentrations of bulking material and a culture lacking it were used. The latter presented slight growth, limited to one or two points of colonization, not enough to promote an increase in ergosterol content (Fig. 3A), demonstrating that unamended sewage sludge is not an appropriate substrate for *T. versicolor* development. Meanwhile, visual examination showed a clear and complete colonization in the cultures with 24% and more especially with 38% bulking material after 10 d. However, the difference was not that clear in the ergosterol amounts, since the highest peak appeared first for the 24%-culture (0.136 mg g $DW^{-1}$) at day 7 and then at day 22 for the 38%-culture (0.129 mg g $DW^{-1}$). Ergosterol contents ranging from 0.084 to 0.464 mg g $DW^{-1}$ have been obtained for *Pleurotus ostreatus* in wheat straw and corncob cultures moistened with nutrient-rich agents (Pant and Adholeya, 2007), while very low levels (up to 0.002 mg g $DW^{-1}$) were achieved in soil colonized by *T. versicolor* exploratory mycelium (Novotný et al., 1999). After the maximum ergosterol levels were reached, the 24%-culture presented a continuous decay in the ergosterol content, indicating lysis of the fungal cell walls (Niemenmaa et al., 2006). Contrary, the 38%-culture showed a first decay after day 22, but a second continuous increase in ergosterol content was achieved after day 43. Fluctuations on ergosterol content through the incubation period were also observed in subsequent experiments (see Section 3.3.2).

Time-course of ergosterol content, which has not been previously reported in white-rot fungi, demonstrated unequivocal growth of *T. versicolor* throughout the studied period.

Laccase activity profiles are shown in Fig. 3B. Activity peaks appeared after 22 d for both 24- and 38%-cultures, in the order of 7.28 and 4.11 AU g $DW^{-1}$, respectively. After decay, however, there was a continuous level of around 2 AU g $DW^{-1}$ that kept until the end of the experiment by day 60. Laccase peaks are coincident in magnitude and time of appearance with those previously reported by Novotný et al. (1999) in straw cultures of *T. versicolor*. The results can be considered as high (more than 40-fold) if compared to production in solid-phase soil cultures of various fungi, including some WRF (D'Annibale et al., 2005).

Since substantial levels on ergosterol and laccase production were continuously observed in both treatments, 38%-cultures were chosen for further studies.

### 3.3. Degradation of CBZ and NAP by T. versicolor

#### 3.3.1. Degradation in bioslurry systems

The degradation of the pharmaceuticals was tested adding CBZ and NAP to 14-d-old bioslurry cultures containing 25% of sewage sludge, as previously mentioned.

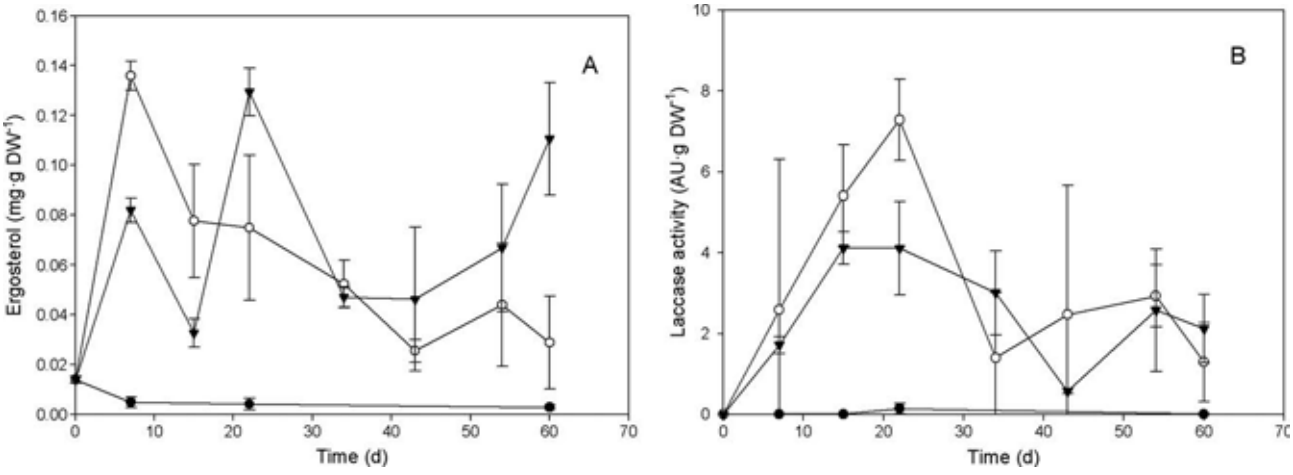


**Fig. 3.** Colonization of sewage sludge solid-phase cultures amended with different amounts of wheat straw pellets as bulking material: (■) 0%, negative control (○) 24% and (▼) 38% (w/w, dry basis). (A) Growth expressed as ergosterol content per unit of dry weight and (B) laccase activity expressed as AU per unit of dry weight. Values plotted are means ± standard deviations for triplicate cultures.

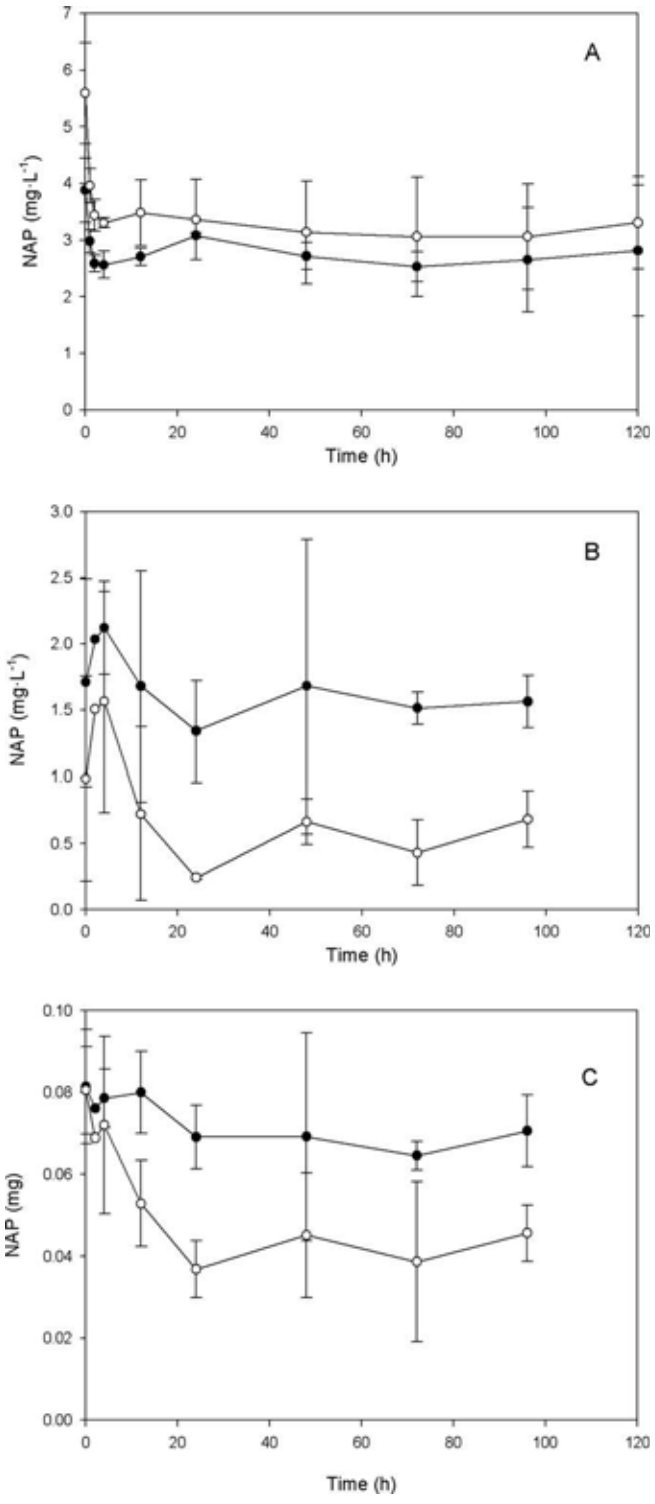


**Fig. 4.** Profiles of NAP degradation by *T. versicolor* in 14 d bioslurry cultures containing 25% of sewage sludge. Concentration in the liquid phase (A), the solid phase (B) and the total amount of NAP recovered (C) are shown. Treatments: (●) heat-killed controls and (○) active cultures. Details regarding conditions of the experiment and NAP extraction are found in Section 2. Values plotted are means ± standard deviations for triplicate cultures.

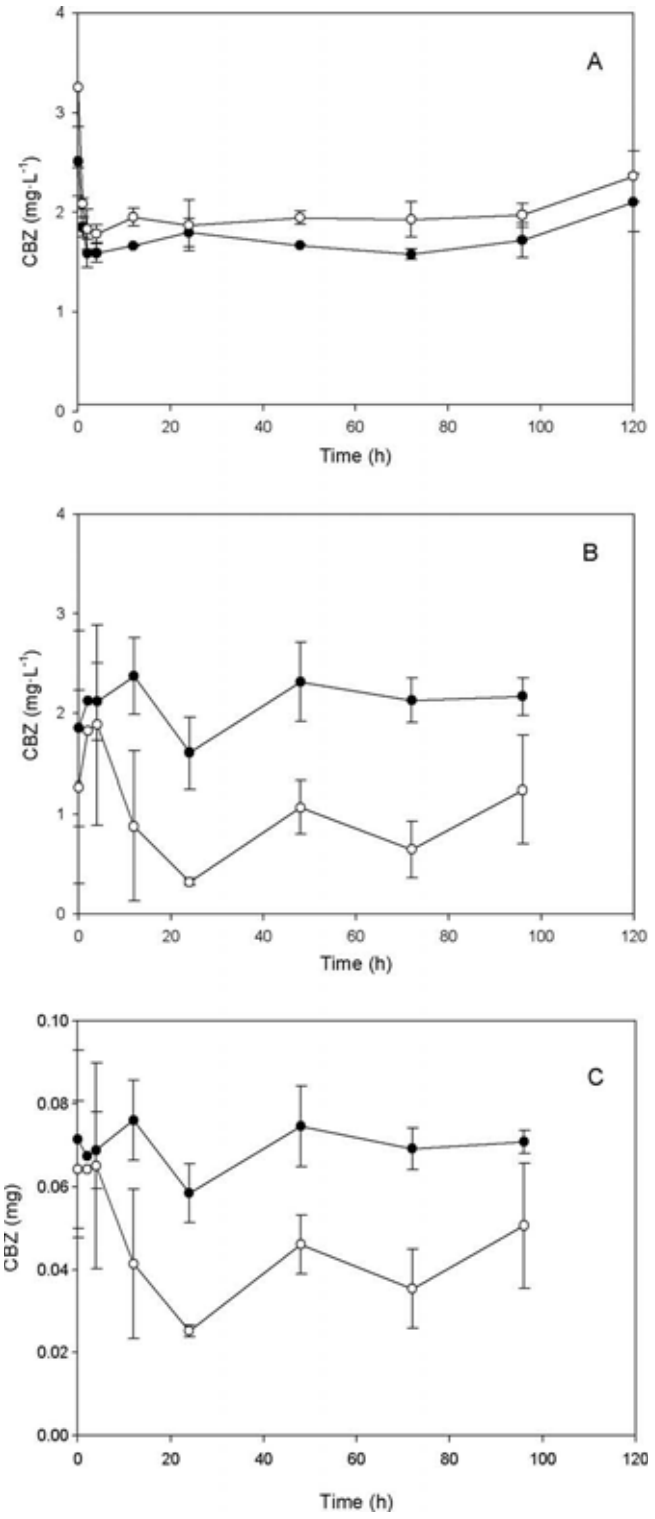


**Fig. 5.** Profiles of CBZ degradation by *T. versicolor* in 14 d bioslurry cultures containing 25% of sewage sludge. Concentration in the liquid phase (A), the solid phase (B) and the total amount of CBZ recovered (C) are shown. Treatments: (●) heat-killed controls and (○) active cultures. Details regarding conditions of the experiment and CBZ extraction are found in Section 2. Values plotted are means ± standard deviations for triplicate cultures.

Fig. 4A shows the concentration of NAP in the liquid phase of the slurry. It is remarkable that approximately 45% of the pharmaceutical was adsorbed to the sludge in the first 2 h, reaching an equilibrium that remained constant throughout the rest of the experiment. Lower levels of NAP were observed in heat-killed controls, which might be ascribed to the greater bioadsorptive capacities of fungal cell walls after heat treatments (Arıca et al., 2003).

Regarding solid phase of bioslurries, a first increase of NAP corresponding to the sorption process and a subsequent stabilization

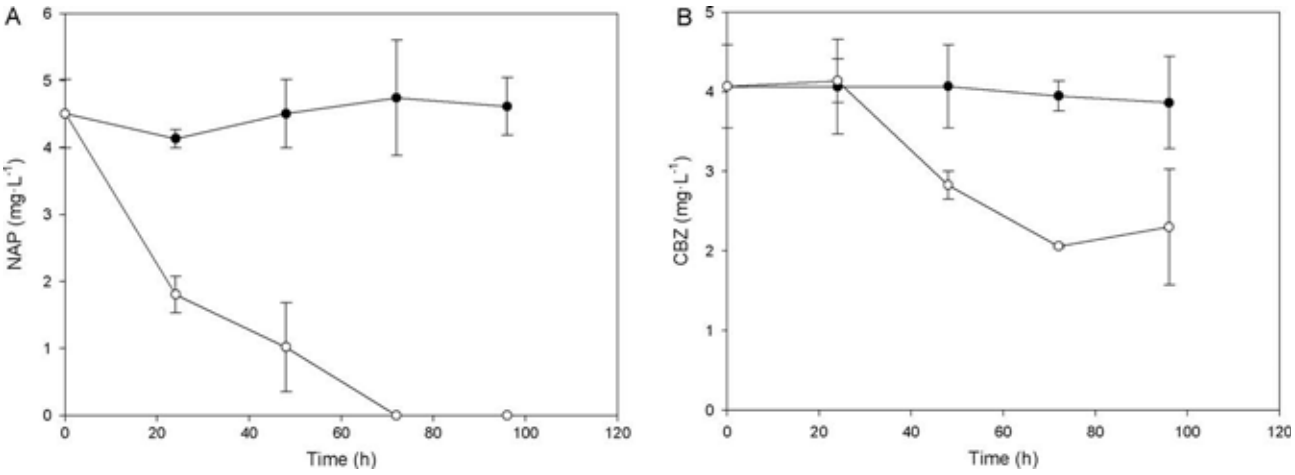


**Fig. 6.** Degradation profiles in 17 d-old sewage sludge solid-phase cultures containing 38% of bulking material. (A) NAP and (B) CBZ. Treatments: (●) heat-killed controls and (○) active cultures. Values plotted are means ± standard deviations for triplicate cultures.

for the heat-killed control can be observed in Fig. 4B. It is in the live culture plot of the solid phase where NAP degradation can be detected, as a constant decrease in concentration during the first 24 h. The total amount of remaining NAP in the slurry, considering liquid and solid phases is presented in Fig. 4C. The process of NAP elimination stopped at 24 h, time after which no further degradation was achieved, reaching a maximum degradation of around 47%. These results suggest that a sorption–desorption equilibrium restores NAP concentration in the liquid phase as it is depleted by *T. versicolor* activity. Previous research demonstrated that complete NAP degradation at low (50 μg $L^{-1}$) and higher concentration (10 mg $L^{-1}$) can be accomplished in 5 h by *T. versicolor* in liquid defined medium catalyzed by laccase and cytochrome P450 (Marco-Urrea et al., 2010). Thus, reduction in laccase levels due to increase in pH may explain the depletion stop in NAP degradation profiles, as observed in Fig. 2. Reports of NAP removal in activated sludge treatment vary from 50% to 65% in periods usually of months, while complete removal has been achieved in treatments involving disinfection (Carballa et al., 2004; Quintana et al., 2005).

CBZ degradation took place in the solid phase (57% in 24 h), analogously to NAP (Fig. 5). CBZ degradation is highly remarkable since it has been considered a nondegradable compound in wastewater treatment plants and anaerobic digestion processes of sewage sludge (Carballa et al., 2007; Radjenović et al., 2009b). *T. versicolor* was previously demonstrated to degrade CBZ (58%) after 7 d in a defined liquid medium (Marco-Urrea et al., 2009) and cytochrome P450 system appeared to be involved.

### 3.3.2. Solid-phase cultures

According to the aforementioned results for solid-phase profiles, 17 d cultures containing 38% of bulking material were employed to determine pharmaceuticals degradation during a 96 h period. Fig. 6A and B shows the results for NAP and CBZ, respectively. NAP degradation reached a 56% after 24 h of treatment with *T. versicolor*, a similar result in comparison to bioslurry experiments. Thereafter, degradation proceeded and complete removal was achieved in 72 h, contrary to bioslurry systems, where depletion stopped after 24 h. On the other hand, CBZ elimination was not observed in the first day of treatment, although ~48% degradation was obtained after 72 h.

A problem sometimes referred to bioprocesses carried out by fungi is the slow rate of growth and activity, if compared to bacterial systems. However, our results indicate that short treatment periods, in the order of few days, are enough for *T. versicolor* to accomplish degradation of some emerging pollutants such as the pharmaceuticals evaluated.

## 4. Conclusions

*T. versicolor* is suitable as a bioremediation agent for sewage sludge treatment. Efficient colonization and important enzymatic activity were demonstrated in both bioslurry and solid-phase sludge cultures by ergosterol and laccase determinations, respectively. Degradation of NAP and CBZ, two pharmaceuticals considered emerging pollutants, was accomplished. NAP removal was around 50% in 25%-bioslurry whereas complete depletion was achieved in solid-phase. CBZ removal was around 50% in both systems. CBZ elimination is noteworthy, since this compound is highly persistent in wastewater treatment plants. As far as the authors know, this is the first report regarding pharmaceutical degradation on sewage sludge by WRF.

## Acknowledgements

This work was supported by MMAMRM (Project 010/PC08/3-04). The Department of Chemical Engineering of the Universitat Autònoma de Barcelona is the Unit of Biochemical Engineering of the Centre de Referència en Biotecnologia de la Generalitat de Catalunya. The authors are members of a Consolidated Research Group of Catalonia (2009-SGR-656). C. Rodríguez-Rodríguez received a predoctoral fellowship from Universidad de Costa Rica and Consejo Superior de Investigaciones Científicas (UCR-CSIC collaboration).

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# Article 2:

Rodríguez-Rodríguez, C.E., Marco-Urrea, E., Caminal, G.

## Naproxen degradation test to monitor *Trametes versicolor* activity in solid-state bioremediation processes

J. Hazard. Mater. 179 (2010), 1152-1155.

Journal of Hazardous Materials 179 (2010) 1152–1155

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Short communication

# Naproxen degradation test to monitor *Trametes versicolor* activity in solid-state bioremediation processes

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ARTICLE INFO

*Article history:*
Received 29 November 2009
Received in revised form 23 February 2010
Accepted 28 February 2010
Available online 4 March 2010

*Keywords:*
*Trametes versicolor*
Biodegradation test
Bioremediation
Naproxen
Sewage sludge

ABSTRACT

The white-rot fungus *Trametes versicolor* has been studied as a potential agent for the removal of environmental pollutants. For long-time solid-phase bioremediation systems a test is required to monitor the metabolic status of *T. versicolor* and its degradation capability at different stages. A biodegradation test based on the percentage of degradation of a spiked model pharmaceutical (anti-inflammatory naproxen) in 24 h (ND24) is proposed to monitor the removal of pharmaceuticals and personal care products in sewage sludge. ND24 is intended to act as a test complementary to ergosterol quantification as specific fungal biomarker, and laccase activity as extracellular oxidative capacity of *T. versicolor*. For samples collected over 45 d, ND24 values did not necessarily correlate with ergosterol or laccase amounts but in most cases, they were over 30% degradation, indicating that *T. versicolor* may be suitable for bioremediation of sewage sludge in the studied period.



## 1. Introduction

*Trametes versicolor* is a white-rot fungus (WRF) capable to biodegrade a wide range of organic pollutants due to its capacity to attack substrates through the action of nonspecific intracellular (i.e. cytochrome P450 system) and extracellular enzymes (i.e. laccases and peroxidases). Given its degrading versatility, the potential use of *T. versicolor* has been recently studied for the removal of pharmaceuticals and personal care products (PPCP), a group of xenobiotics widespread distributed in the environment at very low concentrations [1,2]. A more recent demonstration of the ability of *T. versicolor* to colonize sewage sludge in both solid-phase and bioslurry systems and the subsequent degradation of spiked pharmaceuticals in such matrixes open an optimistic horizon for a possible real scale application [3].

Monitoring solid-state bioremediation processes is a very complex task. Time-demanding extraction-based methodologies are required to analyze activity indicators throughout the process, which, in addition, usually provide only indirect proofs of biological activity that do not coincide with the removal of pollutants in every case. Therefore the development of tests which allow a more complete knowledge of the physiological status of the fungus in such systems is of great interest.

The present research was aimed at defining an activity test to be employed as a complement to laccase activity and ergosterol determinations, to provide wider information on the metabolic status of *T. versicolor* at different stages through long incubation periods in sewage sludge for PPCP bioremediation purposes.

Determination of ergosterol, a specific component of fungal cell membranes, can be a reliable indicator of fungal growth in solid matrixes, however it does not necessarily correlate enzymatic activity or degradation patterns. On the other hand, laccase, an extracellular enzyme produced constitutively in *T. versicolor*, has been directly associated with the oxidative potential of WRF cultures. Moreover, laccase has been involved in the conversion of several PPCP such as natural and synthetic hormones and anti-inflammatory drugs [2,4] and therefore its activity level can provide an indication of the potential transformation of some PPCP in sewage sludge. However, extracellular enzymes may become stabilized on clay or humic substances within the sludge, thus resulting in loss of some activity or extended viability even when the fungal cells have decayed [5].

The test reported here partially overcomes the limitations of the aforementioned indicators by yielding complementary information. It is based on the addition of an external pharmaceutical in a sample and the estimation of its removal after 24 h, which

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doi:10.1016/j.jhazmat.2010.02.091

can determine the specific degradation activity of *T. versicolor* in the sewage sludge. In this case, the pharmaceutical selected was naproxen, an anti-inflammatory drug being extensively used, whose degradation can be catalyzed by laccase and cytochrome P450 in *T. versicolor* [6].

The naproxen degradation test was applied together with ergosterol and laccase determinations to monitor the activity of *T. versicolor* in sewage sludge solid-phase cultures.

## 2. Materials and methods

### 2.1. Chemicals and fungal strain

Naproxen ((S)-(+)-methoxy-α-methyl-2-naphthalene acetic acid, 98%) and ergosterol (ergosta-5,7,22-trien-3β-ol, >95%) were obtained from Sigma–Aldrich Co. (St. Louis, MO).

The strain *T. versicolor* ATCC 42530 was acquired from the American Type Culture Collection, and maintained by subculturing every 30 d on 2% malt extract agar slants (pH 4.5) at 23 °C. *T. versicolor* blended mycelial suspension was prepared according to Marco-Urrea et al. [7].

### 2.2. Sewage sludge and bulking material

Dry sewage sludge (17.7% humidity; water holding capacity 0.47 g $g^{-1}$ dry weight, DW) was obtained from the wastewater treatment plant of El Prat de Llobregat. The plant is located near Barcelona, Spain and it has a total treatment capacity of two million equivalent inhabitants. It is a typical biological activated sludge plant with anaerobic digestion and thermal dehydration. The wheat-straw pellets (ATEA Praha s.r.o., Czech Republic) used as bulking material in solid-phase cultures were kindly provided by Č. Novotný and V. Šašek from the Academy of Science of the Czech Republic.

### 2.3. Solid-phase cultures

Solid-phase cultures with a total dry solid weight content of 2.6 g were performed in 40 mL screw cap vials (28 mm × 95 mm, Grace, Deerfield, IL), containing sewage sludge and 38% bulking material (w/w, dry basis). Wheat-straw pellets were previously hydrated in a 1:2 ratio (material weight:water volume). Each culture was inoculated with 0.25 mL blended mycelium suspension per gram of solid dry weight. Solid content of the cultures was sterilized before mycelium addition. All the cultures were incubated at 25 °C, periodically homogenized and often sprinkled with sterile distilled water to provide moisture. A total of 54 cultures were prepared in parallel to act as unitary samples throughout the experiments.

### 2.4. Degradation studies

Naproxen degradation was evaluated for cultures of different age (10, 17, 25, 31, 38, 45 d). At each time point, triplicate cultures were spiked with a naproxen stock solution to give a final concentration of ~0.096 mg g $DW^{-1}$. Additional triplicate cultures previously autoclaved were employed as heat-killed controls at every time point. After 24 h, the complete content of the cultures was lyophilized (Virtis Sentry freeze-drying equipment, Gardiner, NY) and then subjected to pressurized liquid extraction (PLE) in a PSE-One extractor (Applied Separations, Allentown, PA), as previously described [8]. The volume of extraction was adjusted to 50 mL; a 1.5 mL aliquot was centrifuged at 10,000 rpm for 5 min and the supernatant transferred to amber HPLC vials for subsequent analysis. Quantitative extraction of naproxen was previously verified by this methodology (>95%) [3]. Results were expressed as the percentage of naproxen degradation in 24 h by comparing naproxen concentration in the cultures with those in the heat-killed controls. Similarly, the time-course degradation within a period of 24 h was determined for 10 d-old cultures. In parallel, triplicate flasks were sacrificed to analyze ergosterol and laccase activity.

### 2.5. Analytical methods

Ergosterol was measured in homogeneously-mixed samples of solid-phase cultures and extraction was performed as previously described [3]. Analysis was carried out in a Dionex 3000 Ultimate HPLC (Sunnyvale, CA) equipped with an UV detector at 282 nm, using a reverse phase Grace Smart RP18 column (250 mm × 4 mm, particle size 5 μm, Deerfield, IL). Methanol was isocratically supplied at 1 mL $min^{-1}$ as eluent and retention time was ~6.8 min. Ergosterol content was expressed as milligrams per gram of solid dry weight of culture material (mg g $DW^{-1}$).

Laccase was first extracted according to a modified method by Lang et al. [9]: 30 mL sodium acetate buffer (0.16 M, pH 5) were added to 3 g of homogenized sample and shaken for 30 min at 4 °C; extracts of 1.5 mL were centrifuged at 15,000 × *g* for 15 min and the supernatant was then analyzed. Enzymatic activity was measured using a modified version of the method for manganese peroxidase determination [10]: the reaction mixture consisted of 200 μL sodium malonate (250 mM, pH 4.5), 50 μL 2,6-dimethoxyphenol (DMP, 20 mM) and 600 μL sample. DMP is oxidized by laccase even in the absence of a cofactor. Changes in the absorbance at 468 nm were monitored for 2 min at 30 °C. Results were expressed as activity units (AU) per gram of solid dry weight. One AU was defined as the number of micromoles of DMP oxidized per min. The DMP extinction coefficient was 24,800 $M^{-1}$ $cm^{-1}$.

Naproxen analyses were performed using a Dionex 3000 Ultimate HPLC equipped with a UV detector at 230 nm. Chromatographic separation was achieved by injection of 20 μL samples on a Grace Smart RP18 column (250 mm × 4 mm, particle size 5 μm) and a mobile phase of 65% 6.9 mmol $L^{-1}$ acetic acid (pH 4.0) plus 35% acetonitrile, added isocratically at 1 mL $min^{-1}$ [11]. Retention time was ~14.1 min.

Dry weight of solid-phase culture material was determined by drying samples to constant weight in a 100 °C incubator.

## 3. Results and discussion

Studies with *T. versicolor* have demonstrated promising results for the removal of emerging pollutants in sewage sludge. These potential treatments are thought to be applied for long periods of 30 d or more, given the wide spectrum of contaminants with different degrees of biodegradability present in the sludge. Therefore, the degradation ability of the fungus needs to be assured throughout the whole treatment period. Thus, and based on previous demonstration of depletion mediated by several enzymatic complexes [6], we decided to employ naproxen degradation as a parameter to evaluate metabolic fungal activity during colonization of sewage sludge. Naproxen is a non-prescription analgesic of extensive use, being considered as an emerging pollutant.

In the first experiment with 10 d-old culture samples, 31% degradation of spiked naproxen was accomplished within 24 h in sewage sludge solid-phase systems (Fig. 1). Previous studies by our group showed that complete depletion of similar naproxen amounts occurs after 72 h of treatment in 17 d cultures in the same matrix [3], thus indicating that 24 h is an appropriate treatment time for the degradation test, since it assures that not all the pharmaceutical has been removed. This allows, therefore, the comparison of fungal activity in time-course bioremediation experiments.

Usually, a test of enzymatic activity determines initial reaction rates which are directly correlated with enzyme concentration. However, in this case degradation may be due to more than one

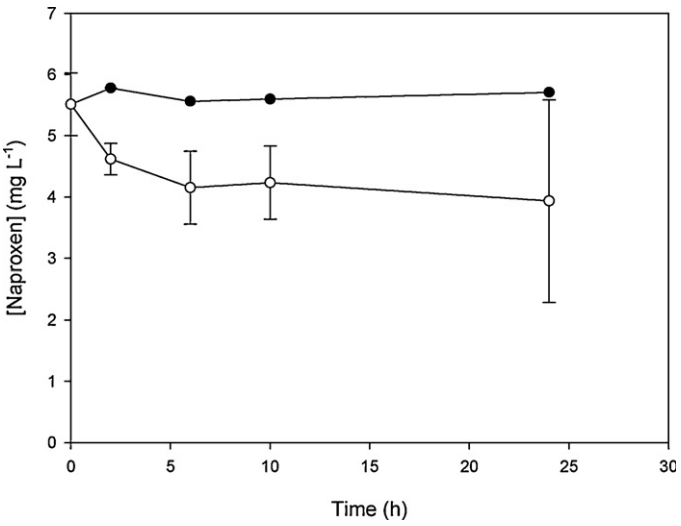


**Fig. 1.** Naproxen degradation profiles by *T. versicolor* in 10 d-old sewage sludge solid-phase cultures containing 38% of bulking material. Treatments: (●) heat-killed controls and (○) live cultures. Values plotted are means ± standard deviations for triplicate cultures.

enzyme and initial degradation rate may be masked by adsorption/diffusion processes mediated by the presence of the fungus. Therefore, and taking advantage of the aforementioned results, the degradation ability of *T. versicolor* was used to perform the degradation test to monitor the global metabolic status of the fungus. The test was defined as the percentage of naproxen degradation in 24 h under the stated conditions, and was called ND24.

To evaluate *T. versicolor* activity in sewage sludge solid-phase cultures two additional indicators were employed: ergosterol quantification as a component of viable mycelium [12,13] and laccase activity as constitutive enzyme involved in degradation machinery [4]. Results showing the follow up of ergosterol, laccase and the ND24 test are presented in Fig. 2. As it can be seen, important naproxen degradation was achieved along the whole experiment. A minimum value of ND24 appeared at 25 d, time which corresponds to the end of an ergosterol-decrease period. Moreover, a posterior ergosterol increase correlated with a new ND24 increase. It must be pointed out that similar tendencies have been observed in ergosterol content profiles [3]. Important ergosterol amounts were observed during 45 d, indicating the presence of physiologically active *T. versicolor* biomass. Laccase activity showed a maximum peak and a posterior slowly decay, though notable activity levels were maintained during all the experiment.

Determination of laccase is an indicator of extracellular oxidative capacity in *T. versicolor*. However this activity may last even after metabolic inactivation of the fungus. Contrary, laccase may be produced but inactivated by environment conditions such as high pH [14], making it undetectable in activity tests even if the fungus is still active. In both cases correlation between enzyme and degradation ability may not be achieved. On the other hand, ergostrol quantification, besides being highly laborious, gives information about viability of the fungus; however production of enzymes may depend on nutritional conditions of the substrate or culture medium. The test proposed measures in a global way the degrading-metabolic activity of *T. versicolor*. Although ND24 does not evaluate the whole range of metabolic pathways, but the ones implicated in the degradation of the selected pollutant, its objective is minimizing the limitations listed above and being complementary to laccase and ergosterol determinations.

Results of ND24 showed that all the cultures tested were capable to degrade naproxen at an important extent, over 31% (except the 25 d culture), with a maximum degradation of 56% in the 17 d

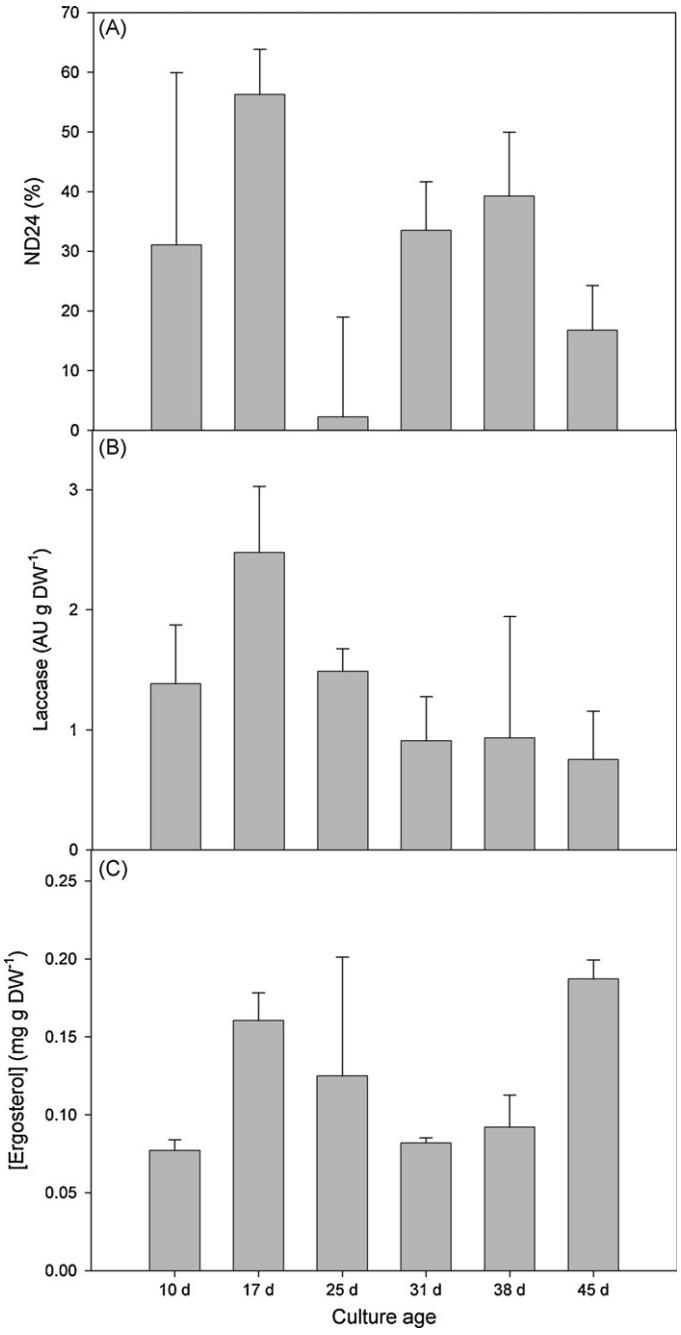


**Fig. 2.** Evolution of ND24 (A), laccase activity (B) and ergosterol content (C) by *T. versicolor* in solid-phase cultures of different age. Values plotted are means ± standard deviations for triplicate cultures.

cultures, thus demonstrating biodegradation ability in *T. versicolor* for the whole study period. ND24 indicated that in spite of laccase decay, notable degradation can be accomplished. Fig. 3 presents the profiles of laccase and ergosterol in terms of ND24 values. As we mentioned previously, naproxen is known to be degraded by the intracellular enzyme cytochrome P450 and laccase and therefore a highest correlation between the increase of ND24 and the increase of both laccase and ergosterol content would be expected. However, factors such as the bioavailability of naproxen due to sorption into sludge, for example, could explain low ND24 values at high ergosterol contents or the fact that similar laccase activities resulted in different degradation values. Additionally, it should be taken into account that relatively high variations within the triplicates were obtained in some cases (Fig. 2), which resulted in

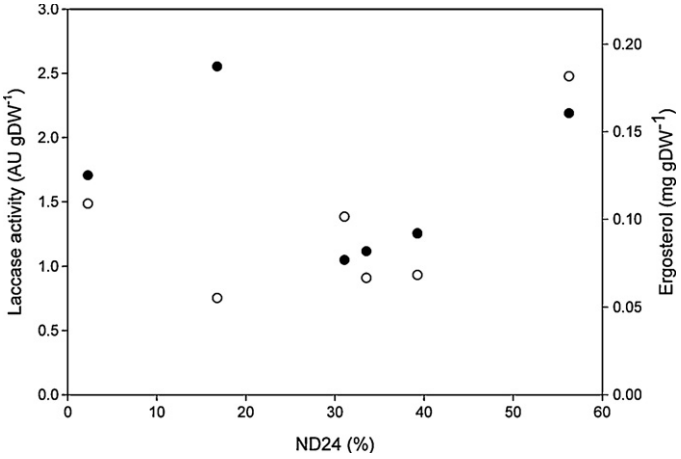


**Fig. 3.** Variation of usual indicators of WRF activity as function of ND24 values in sewage sludge solid-phase cultures of *T. versicolor*. Laccase activity (○) and ergosterol (●).

difficulty to categorically establish direct correlations between the parameters.

Since different but complementary information is obtained from the biodegradation test, ND24 may be a useful assay in the follow up of treatments applied in other solid-state matrixes (i.e. soil), whose control is difficult and usually little information of the process is available. Naproxen was chosen for the ND24 assay based on its fast removal by *T. versicolor* in solid-state, however this kind of test could be performed with other degradable pollutants.

Although the ND24 test is based on the profiles of only one pharmaceutical, the results indicate that *T. versicolor* is suitable for long-time treatments in sewage sludge bioremediation, since depletion ability remains for periods over 1 month.

## 4. Conclusions

*T. versicolor*'s ability to deplete spiked naproxen in sewage sludge solid-phase cultures, led to the definition of a complementary biodegradation test. The test corresponds to the percentage of naproxen degradation in 24 h, and it was aimed to be employed as an indicator of fungal activity in solid-phase systems. Application in sewage sludge cultures revealed that ND24 values do not necessarily correlate with ergosterol or laccase amounts. However, notable degradation results for periods over 1 month indicate that *T. versicolor* may be a suitable agent for bioremediation of sewage sludge and could be able to remove pollutants that require long treatments. ND24 is useful as a complementary test to monitor bioremediation processes in solid-state matrixes.

## Acknowledgments

This work was supported by MMAMRM (project 010/PC08/3-04). The Department of Chemical Engineering of the Universitat Autònoma de Barcelona is the Unit of Biochemical Engineering of the Centre de Referència en Biotecnologia de la Generalitat de Catalunya. The authors are members of a Consolidated Research Group of Catalonia (2009-SGR-656). C. Rodríguez-Rodríguez received a predoctoral fellowship from Universidad de Costa Rica and Consejo Superior de Investigaciones Científicas (UCR-CSIC collaboration).

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# Article 3:

Rodríguez-Rodríguez, C., Jelić, A., Llorca, M., Farré, M., Caminal, G., Petrović, M., Barceló, D., Vicent, T.

## Solid-phase treatment with the fungus *Trametes versicolor* substantially reduces pharmaceutical concentrations and toxicity from sewage sludge

Bioresour. Technol. 102 (2011), 5602-5608.

Bioresource Technology 102 (2011) 5602–5608

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

# Solid-phase treatment with the fungus *Trametes versicolor* substantially reduces pharmaceutical concentrations and toxicity from sewage sludge

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ARTICLE INFO

*Article history:*
Received 26 November 2010
Received in revised form 4 February 2011
Accepted 5 February 2011
Available online 4 March 2011

*Keywords:*
Sewage sludge
*Trametes versicolor*
Pharmaceuticals
Solid-phase
Biodegradation

ABSTRACT

For safe biosolid-land-applying, sludge should be contaminant-free. However, it may contain important amounts of micropollutants, not removed in the wastewater-treatment-processes. An alternative treatment with the fungus *Trametes versicolor* was applied in sterile solid-phase systems consisting of sludge and a lignocellulosic substrate. Fungal colonization and activity were demonstrated during the process, according to monitoring of ergosterol, laccase activity and the naproxen-degradation test (ND24). Fourteen out of 43 analyzed pharmaceuticals were found in the raw sludge. After treatment, phenazone, bezafibrate, fenofibrate, cimetidine, clarithromycin, sulfamethazine and atenolol were completely removed, while removals between 42% and 80% were obtained for the remaining pharmaceuticals. Toxicological analyses (*Daphnia magna*, *Vibrio fischeri* and seed germination) showed an important reduction in sludge toxicity after treatment. Results suggest that a solid-phase treatment with *T. versicolor* may reduce the ecotoxicological impact of micropollutants present in sewage sludge. This is the first report of a fungal-approach for elimination of emerging pollutants from biosolids.



## 1. Introduction

Sewage sludge is a by-product generated during the wastewater treatment process. Treated sewage sludge meeting specific regulations for microbial pathogens, nutrients and metal concentrations is known in the EU and the US by the term biosolids (Wu et al., 2009). Biosolids are used as soil amendment; however, scarce jurisdiction exists for the regulation of concentrations of organic pollutants. Regulation initiatives on biosolids in EU, US and Canada include the control of heavy metals and microbial pathogens, while the more recent concern in organic pollutants focuses on halogenated compounds, alkylbenzene sulphonates, phthalates, nonylphenols, polychlorinated biphenyls (PCB), polycyclic aromatic hydrocarbons (PAH) and dioxins (European Union, 2000; Hébert, 2008). However, legislation regarding the limits of emerging organic micropollutants such as pharmaceuticals is not included. Therefore, municipal biosolids may contain important amounts of contaminants, previously sequestered by suspended solids in the wastewater (Kinney et al., 2006). If high enough concentrations are repeatedly applied on soil, these micropollutants might reduce or counteract the benefits of land applying biosolids, resulting in contamination of arable soils. Results of the present work expect to contribute, among others in the field, to provide the information required to include the control of emerging pollutant concentrations in the future legislation.

In Europe, around 4000 pharmaceutical active compounds employed both for human or veterinary purposes are susceptible to reach the environment (Mompelat et al., 2009). The annual consumption of the most common pharmaceuticals reaches hundreds tons in Europe; in particular, anti-inflammatory drugs like aspirin, paracetamol, ibuprofen and diclofenac were produced in Germany (by 2001) at amounts ranging from 836 to 86 t, meanwhile the antiepileptic carbamazepine was produced at 88 t (Fent et al., 2006). On the other hand, annual antibiotic consumption may reach thousands tons, more than 13 000 t in EU in 1999, especially due to their wide use in medicine, veterinary, farming and aquaculture (Kemper, 2008). Pharmaceuticals and their metabolites may

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doi:10.1016/j.biortech.2011.02.029

reach the environment via WWTP discharges, manufacturing and hospital effluents, concentrated animal feeding operations, direct disposal and land application of biosolids (Daughton and Ternes, 1999).

The scientific community widely agrees with the possibility that negative ecotoxicological effects may arise from the presence of pharmaceuticals in the environment (Santos et al., 2010). In particular, since antibiotics have the potential to affect microbial communities, the inhibition of natural occurring processes such as degradation of organic matter in water and sediments, anaerobic digestion or processes related to the $N_2$ cycle may occur (Kümmerer 2009); moreover, it is still unknown whether their presence in nature contributes to the spread of microbial antibiotic resistance (Kümmerer, 2009). Although chronic ecotoxicity data are scarce if compared to acute studies, accumulative effects have been shown to damage some ecosystems (Daughton and Ternes, 1999). Acute and chronic ecotoxicology of different groups of pharmaceuticals are reviewed by Santos et al. (2010).

A promising approach to reduce organic pollution is the application of natural-degrading microorganisms, since bioremediation techniques are increasing attention as more environmentally friendly alternatives than conventional physicochemical treatments used for cleaning up of contaminated sludge. White-rot fungi (WRF) are considered as an interesting group of microorganisms from the biodegradation point of view, due to their non-specific extracellular ligninolytic enzymatic system, which includes laccases and high redox potential peroxidases such as lignin peroxidase, manganese peroxidase and versatile peroxidase (Martínez et al., 2005). Additionally, some xenobiotics can be potentially metabolized by WRF by means of the intracellular cytochrome P450 complex, which acts in a similar way in mammals (Doddapaneni and Yadav, 2004). In this respect, efficient colonization and degradation of spiked pharmaceuticals in sludge has been recently demonstrated by the fungus *Trametes versicolor* (Rodríguez-Rodríguez et al., 2010a), thus opening a whole new spectrum of interesting and promissory environmental-friendly decontamination processes.

This work aimed to demonstrate the biodegradation of pharmaceuticals in sterilized sewage sludge under solid-phase conditions with *T. versicolor*, as a first approach for the removal of emerging pollutants from biosolids.

## 2. Methods

### 2.1. Chemicals

Ergosterol (ergosta-5,7,22-trien-3β-ol, >95%) was obtained from Sigma–Aldrich Co. (St. Louis, MO). The pharmaceutical standards used for analysis were of high purity grade (>90%). Ibuprofen, Naproxen, Ketoprofen, Diclofenac and Gemfibrozil were supplied by Jescuder (Rubí, Spain). Acetaminophen, Indometacin, Mefenamic acid, Phenazone, Bezafibrate, Mevastatin, Fenofibrate, Pravastatin (as sodium salt), Carbamazepine, Famotidine, Ranitidine (as hydrochloride), Cimetidine (as hydrochloride), Erithromycin (as hydrate), Azithromycin (as dehydrate), Roxitromycin, Clarithromycin, Josamycin, Tylosin A, Sulfamethazine, Trimethoprim, Chloramphenicol, Atenolol, Sotalol, Metoprolol (as tartrate), Timolol, Pindolol, Nadolol, Salbutamol, Clenbuterol (as hydrochloride), Enalapril (as maleate), Glibenclamide, Furosemide, Hydrochlorothiazide and Metronidazole were purchased from Sigma–Aldrich (Steinheim, Germany). Standard Atorvastatin (as calcium salt) was provided by LGC Promochem (London, UK), while Diazepam, Lorazepam and Butalbital were from Cerilliant (Texas, USA).

The deuterated or $^{13}C$-labeled compounds, used as internal standards, were Sulfathiazole-$d_4$, Famotidine-$^{13}C_3$, rac-Timolol-$d_5$ maleate, Clarithromycin-*N*-methyl-$d_3$, Atorvastatin-$d_5$ sodium salt, Azithromycin-$d_3$, Fenofibrate-$d_6$, Metronidazole hydroxyl-$d_2$, Phenacetine-$^{13}C$, Ketoprofen-$^{13}C,d_3$, Indomethazine-$d_4$, rac-Naproxen-$d_3$, Mefenamic acid-$d_3$, Gemfibrozil-$d_6$ and Bezafibrate-$d_4$ from Toronto Research Chemicals; Diazepam-$d_5$ and Phenobarbital-$d_3$ from Cerilliant (Texas, USA); Atenolol-$d_7$, Carbamazepine-$d_{10}$, Ibuprofen-$d_3$, Enalapril–$d_5$, Glyburide-$d_3$, Albuterol-$d_3$, Cimetidine-$d_3$, Antipyrine-$d_3$, Diclofenac-$d_4$, Hydrochlorothiazide-3, 3-$d_2$ from CDN Isotopes (Quebec, Canada); Sotalol hydrochloride-$d_6$ from Dr. Ehrenstorfer (Augsburg, Germany) and Erythromycin-$^{13}C,d_3$ (*N*-Methyl-$^{13}C,d_3$) from Isotec (Ohio, USA).

The individual standard solutions as well as isotopically labeled internal standard solutions were prepared according to Jelić et al. (2009).

### 2.2. Fungal strain

The strain *T. versicolor* ATCC 42530 was acquired from the American Type Culture Collection, and maintained by subculturing every 30 days on 2% malt extract agar slants (pH 4.5) at 23 °C. *T. versicolor* blended mycelial suspension was prepared according to Font Segura et al. (1993).

### 2.3. Sewage sludge and bulking material

Dry sewage sludge was obtained from the wastewater treatment plant of El Prat de Llobregat. The plant is located near Barcelona, Spain and it has a total treatment capacity of two million equivalent inhabitants. It is a typical biological activated sludge plant with sludge anaerobic digestion and thermal dehydration. Sludge employed in the experiments was obtained from the final stage of processing, i.e., after thermal dehydratation (~10% water content). The wheat-straw pellets (WSP, ATEA Praha s.r.o., Czech Republic) used as bulking material and substrate in solid-phase cultures were kindly provided by Č. Novotný.

### 2.4. Solid-phase treatment

Solid-phase systems with a total dry solid weight content of 6.5 g were performed in 24 × 150 mm tubes (Barloworld Scientific Ltd., Staffordshire, UK), containing sterile-sewage sludge and 38% (w/w, dry basis) *T. versicolor* inoculum. Inocula were prepared by adding blended mycelium suspension to sterile WSP, 0.65 mL per gram of dry WSP and pre-growing for 7 d at 25 °C. The WSP were hydrated in a 1:2 ratio (w/v) prior mycelium inoculation. Sterilization process consisted of autoclaving at 121 °C for 30 min in every case. The solid-phase systems were incubated for up to 42 d at 25 °C, periodically homogenized and often sprinkled with sterile distilled water to provide moisture. Triplicate cultures were sacrificed for time-course and final-point analytical determinations. Uninoculated cultures consisting of sterile-sludge amended with 38% WSP were used as controls, and are referred to as "untreated sludge", unless otherwise stated. Unamended sterile sludge (lacking WSP) is referred to as "raw sludge".

### 2.5. Analytical methods

#### 2.5.1. Sample preparation and analysis of pharmaceuticals

The samples were prepared according to a previously developed multi-residue method for analysis of 43 pharmaceuticals in sludge (Jelić et al., 2009). The pharmaceuticals were isolated from solid samples by pressurized solvent extraction using Dionex ASE 200 (Dionex; Sunnyvale, CA), followed by a solid-phase extraction clean-up step onto a lipophilic–hydrophilic balanced Oasis HLB (60 mg, 3 mL) cartridge.

Instrumental analysis was performed by high-performance liquid chromatography (HPLC) coupled to tandem mass spectrometry (MS/MS) according to the method developed by Gros et al. (2009). The analysis was performed using a Symbiosis™ Pico (SP104.002, Spark, Holland), equipped with an autosampler, followed by a 4000 QTRAP Hybrid Triple Quadrupole-Linear Ion Trap mass spectrometer equipped with a Turbo Ion Spray source (Applied Biosystems-Sciex, Foster City, CA, USA). Chromatographic separation was achieved with a Purospher Star RP-18 end capped column (125 mm × 2.0 mm, particle size 5 μm) preceded by a C18 guard column (4 × 4.5 μm), both supplied by Merck (Darmstadt, Germany).

*2.5.2. Analysis of PCB and PAH*

PCB and PAH concentrations were analyzed after 42 d, according to methods EPA-8082 and EPA-8270C.

*2.5.3. Ergosterol quantification*

Ergosterol was measured in homogeneously-mixed samples of solid-phase cultures. Extraction and quantification were performed as previously described (Rodríguez-Rodríguez et al., 2010a). Ergosterol content was expressed as milligrams per gram of solid dry weight (mg $g^{-1}$ DW).

*2.5.4. Laccase activity*

Laccase was extracted from solid samples and measured as described by Rodríguez-Rodríguez et al. (2010a), using 2,6-dimethoxyphenol (DMP) as the substrate. Results were expressed as activity units (U) per gram of solid dry weight. One U was defined as the number of micromoles of DMP oxidized per min.

*2.5.5. ND24 test*

A test based on naproxen degradation, previously defined by Rodríguez-Rodríguez et al. (2010b) was employed to monitor the degrading ability of *T. versicolor* throughout the treatment. Briefly, triplicate cultures (total dry solid weight content of 2.6 g each) were spiked with a naproxen stock solution to give a final concentration of ~0.096 mg g $DW^{-1}$. Additional triplicate cultures previously autoclaved were employed as heat-killed controls at every time point. After 24 h, the complete content of the cultures was lyophilized (Virtis Sentry freeze-drying equipment, Gardiner, NY) and then subjected to pressurized liquid extraction (PSE-One extractor, Applied Separations, Allentown, PA). Naproxen was analyzed by HPLC in the processed extracts. Results were expressed as the percentage of naproxen degradation in 24 h by comparing the remaining naproxen concentrations in active cultures with those in triplicate heat-killed controls.

*2.5.6. Toxicological analyses*

*2.5.6.1. Daphnia magna immobilization test.* Static acute 24–48 h assays were conducted according to the internationally accepted Standard Method ISO 6341 (ISO 6341, 1996). The tests were performed using *D. magna* neonates which were hatched in about 3 days from the eggs at 20–22 °C, under continuous illumination of 6000 lux. Immobility at 24 h and 48 h was the bioassay endpoint, assumed as equivalent to mortality. A bench of dilutions of each sample (0%, 10%, 25%, 50%, 75% and 90%) was tested using two replicates for each dilution with 10 neonates per replicate. *D. magna* neonates exposed to the blanks and to the different dilutions were incubated in darkness at 20 °C. After 24 h and 48 h of exposure the number of immobilized organisms was determined and the percentage of immobilization was plotted against the logarithm concentration. A four parameters equation was fitted and the $EC_{50}$ was determined as the concentrations producing the 50% of immobilization. In addition, toxicity units of the different samples were calculated using Ramsay and Sprague expression: $TU = (EC_{50})^{-1} \times 100$ (Sprague and Ramsay, 1965).

*2.5.6.2. Vibrio fischeri bioluminescence inhibition test.* The experimental procedure for conducting the bacterial bioluminescence assay was based on the ISO 11348-3 protocol (ISO 11348-3, 1998). Osmolality was adjusted in order to obtain a 2% of saline in each solution or sample. In all measures, the percent of inhibition (% I) was determined by comparing the response given by a saline control solution to that corresponding to the diluted sample. The concentration of toxicants in the test which caused a 50% reduction in light (Inhibition = 50%) after exposure for 15 or 30 min was designed as the 15 or 30 min $EC_{50}$ value. Tests were performed at 15 °C.

*2.5.6.3. Seed germination test.* The phytotoxicity of the treated/untreated sludge was evaluated by the seed germination technique. The seed germination percentage and root length were determined for 10 seeds of lettuce (*Lactuca sativa*), tomato (*Solanum lycopersicum*), cucumber (*Cucumis sativus*) and pepper (*Capsicum annuum*) after 7–14 d of incubation at 25 °C with sludge extracts (1:4, material weight: distilled water). The same parameters were measured in distilled water to be used as germination controls. Tests were done in quadruple. The percentages of relative root elongation (RE) and germination index (GI) were calculated according to standard methods using equations (1–3) (US Department of Agriculture and US Composting Council, 2001).

$$RE = \frac{\text{mean root length}}{\text{mean root length in control}} \times 100 \quad (1)$$

$$GI = \frac{(SG) \times (RE)}{100} \quad (2)$$

where SG is the relative seed germination, defined as:

$$SG = \frac{\text{seeds germinated}}{\text{seeds germinated in control}} \times 100 \quad (3)$$

## 3. Results and discussion

*3.1. Growth and activity of T. versicolor in the solid-phase sludge system*

A solid-phase, composting-like system was chosen to determine the feasibility of using the fungus *T. versicolor* for the removal of several pharmaceuticals from sewage sludge. The use of solid-phase techniques has especially increased in the last decades in the case of fungal-mediated bioprocesses, since they reproduce the natural habitat and growth conditions for filamentous fungi. Moreover, solid-phase bioreactors constitute an inexpensive treatment method for remediation of solids, since they require minimum maintenance and small amounts of energy for aeration and mixing, thus making these systems cost-effective when long treatments are required (Nano et al., 2003). This is especially important in the removal of pharmaceuticals, since they comprise a wide spectrum of components with different degree of persistence.

In the present work, WSP were employed with a dual function: as a bulking material for the solid-phase treatment, and as a lignocellulosic substrate to enhance the growth of *T. versicolor* and the subsequent colonization of the sludge. Treatment time was set in 42 d, similar to that employed in typical sludge-composting systems.

The results of *T. versicolor* growth and activity are shown in Fig. 1. The initial time denotes the moment of mixing the sludge with the fungal inoculum. The ergosterol content, a specific com-

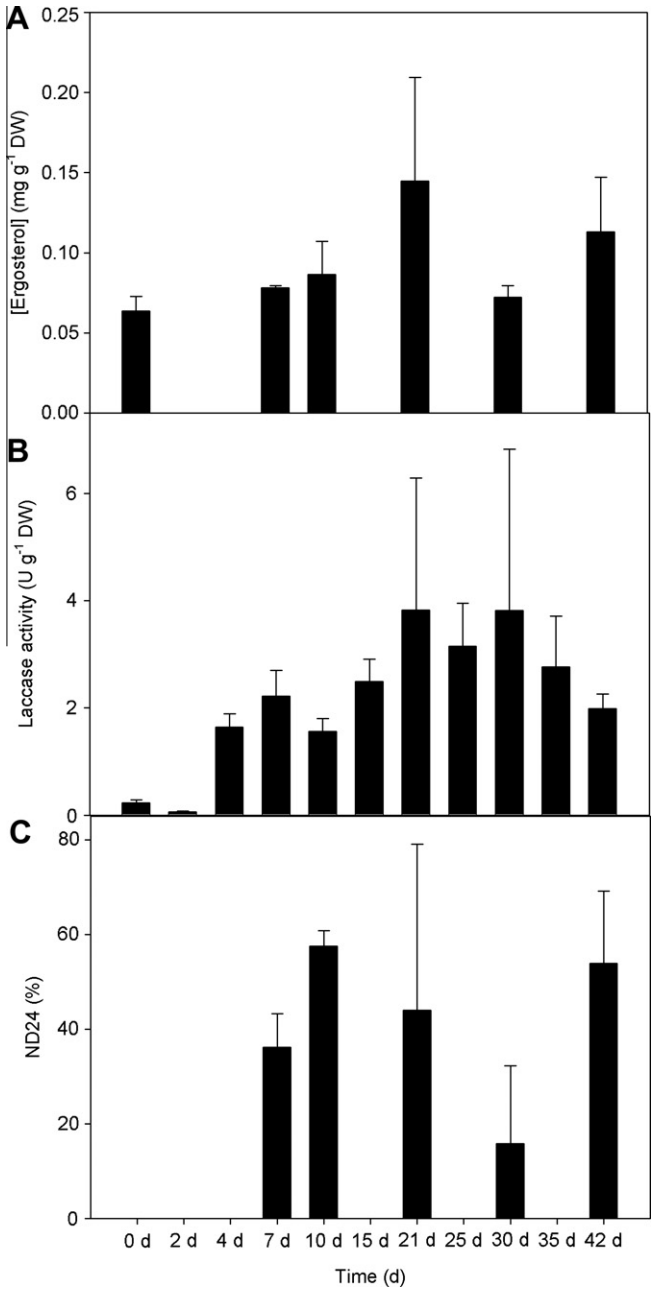


**Fig. 1.** Growth and activity profiles of *T. versicolor* during solid-phase treatment of sewage sludge. Growth expressed as ergosterol content (A), oxidative potential expressed as laccase activity (B) and metabolic degrading ability measured as ND24 (naproxen-degradation test) (C). The cultures contained 38% wheat straw pellets as bulking material/substrate for fungal growth. Values plotted are means ± standard deviations for triplicate cultures. Ergosterol content and ND24 were not determined in days 2, 4, 15, 25 and 35.

ponent of fungal cell membranes, was determined as a biomarker of viable mycelium, and therefore of fungal growth (Barajas-Aceves et al., 2002). Ergosterol profiles in Fig. 1A demonstrated fungal growth and colonization, and therefore supported the visual examination of the cultures. A peak value of 0.145 mg $g^{-1}$ DW (±0.065) was reached after 21 d of sludge colonization, followed by an apparent decrease, probably due to partial fungal cell wall lysis. However, due to the relatively high standard deviations innate to quantitative analyses in this kind of matrixes, it seems that a constant level of ergosterol was achieved up to the end of the treatment. Similar fluctuations have been observed in cultures of *T. versicolor* when growing in sludge (Rodríguez-Rodríguez et al., 2010a,b).

On the other hand, laccase activity is directly associated with the oxidative potential of WRF cultures and has been identified as a key factor in the degradation of several xenobiotics (Marco-Urrea et al., 2009). Results in Fig. 1B revealed important activity from day 4, which maintained high levels up to the end of the process. Maximum values ranging from 3 to 4 U $g^{-1}$ DW were obtained between days 20 and 30.

The ND24 test was also applied to monitor the metabolic status during the treatment. It employs naproxen as a model spiked xenobiotic of fast removal, whose degradation is evaluated after 24 h. Since both laccase and cytochrome P450 are known to be involved in the degradation of this compound (Marco-Urrea et al., 2010a), the test provides important complementary data on the actual ability of *T. versicolor* to remove some pollutants when growing in such matrixes. Remarkable ND24 values were obtained during the process (Fig. 1C). Moreover, values as high as 57.5% (±3.3) and 53.9% (±15.3) after 10 d and 42 d of treatment, respectively, clearly suggest that the degrading potential of the fungus persisted in the sewage sludge system. The peaks in ND24 might be linked to the periods of increase in ergosterol content, though both parameters are not necessarily correlated (Rodríguez-Rodríguez et al., 2010b).

Overall results demonstrate that *T. versicolor* had been active throughout the treatment period.

### 3.2. Removal of pharmaceuticals and PCBs from the sludge

Pharmaceuticals comprise one of the most common groups of organic microcontaminants present in biosolids, given that conventional biological wastewater treatment processes are inadequate for their complete removal (Kinney et al., 2006).

From 43 pharmaceuticals analyzed, 14 were detected in the thermally dehydrated sludge (Table 1). Considering that the biosolids employed in this study had previously received conventional activated sludge (AS) and anaerobic digestion treatments, the occurrence of pharmaceuticals indicates that many removal values described in the literature for WWTPs refer to elimination more than biodegradation or transformation, since they do not take into account the amounts sorbed to biosolids.

The most abundant pollutants belonged to the group of analgesic/anti-inflammatory compounds: ibuprofen (85.9 ng $g^{-1}$, ±9.2) and diclofenac (60.3 ng $g^{-1}$, ±9.6). Important removal of both compounds occurred after the fungal treatment, around 75% and 64%, respectively (Table 1). A removal of 41% was reported for ibuprofen in a study which considered both the dissolved and the adsorbed fraction during anaerobic digestion of sewage sludge (Carballa et al., 2007). On the other hand, dissimilar results from no elimination to removal values of up to 69% have been described for diclofenac in anaerobic digestion processes (Carballa et al., 2007; Radjenović et al., 2009). Marco-Urrea et al. (2009, 2010b) described the ability of *T. versicolor* to degrade both pharmaceuticals in defined liquid medium. 2-hydroxy ibuprofen and 1-hydroxy ibuprofen were identified as the main metabolites after 3 h, when complete depletion of the parent compound was achieved; nonetheless, after 7 d, 1,2-dihydroxy ibuprofen, a more toxic compound, remained as the final product. From the aforementioned products, the isomers of hydroxyl ibuprofen have been identified only in raw wastewater (Quintana et al., 2005). In the case of diclofenac, though laccase is able to transform the parent compound, cytochrome P450 seems to play the key role in the degradation by *T. versicolor* and a reduction in toxicity was reported after 24 h (Marco-Urrea et al., 2010b). Mefenamic acid, another analgesic, was also detected at a concentration of 17.9 ng $g^{-1}$ (±2.1) and subse-

**Table 1**
Detected pharmaceuticals and their residual concentration after solid-phase treatment of thermally dehydrated sewage sludge with *T. versicolor*.

| Compounds | CAS number | Raw sludge | | | Treated sludge | | | Removal[e] (±SD[d]) (%) |
|---|---|---|---|---|---|---|---|---|
| | | LOQ[a] (ng $g^{-1}$) | R[b] (%) | Concentration[c] (±SD[d]) (ng $g^{-1}$) | LOQ[a] (ng $g^{-1}$) | R[b] (%) | Concentration[c] (±SD[d]) (ng $g^{-1}$) | |
| Ibuprofen | 15687-27-1 | 0.7 | 64 | 85.9 (±9.2) | 0.8 | 65 | 13.1 (±1.2) | 75 (±0.4) |
| Diclofenac | 15307-86-5 | 2.1 | 109 | 60.3 (±9.6) | 3.2 | 83 | 13.6 (±1.5) | 64 (±1.8) |
| Mefenamic acid | 61-68-7 | 0.7 | 94 | 17.9 (±2.1) | 1.1 | 82 | 3.1 (±0.5) | 72 (±1.2) |
| Phenazone | 60-80-0 | 3.2 | 108 | 9.6 (±2.2) | 2.9 | 93 | n.d. | 100 |
| Bezafibrate | 41859-67-0 | 0.5 | 104 | 4.5 (±0.1) | 0.8 | 83 | n.d. | 100 |
| Fenofibrate | 49562-28-9 | 2.5 | 147 | 4.2 (±0.6) | 2.7 | 151 | n.d. | 100 |
| Atorvastatin | 134523-00-5 | 3.3 | 78 | 37.8 (±3.5) | 3.3 | 84 | 4.7 (±1.1) | 80 (±2.9) |
| Diazepam | 439-14-5 | 5.7 | 97 | 19.3 (±2.8) | 5.8 | 95 | 6.8 (±0.2) | 43 (±6.9) |
| Carbamazepine | 298-46-4 | 1.1 | 129 | 25.6 (±5.0) | 0.9 | 139 | 9.1 (±0.1) | 43 (±10.8) |
| Cimetidine | 51481-61-9 | 0.9 | 87 | 11.4 (±0.6) | 1.3 | 80 | n.d. | 100 |
| Clarithromycin | 81103-11-9 | 7.8 | 83 | 21.0 (±2.2) | 9.8 | 64 | n.d. | 100 |
| Sulfamethazine | 57-68-1 | 1.2 | 101 | 20.4 (±1.4) | 0.8 | 86 | n.d. | 100 |
| Atenolol | 29122-68-7 | 0.8 | 84 | 13.6 (±2.2) | 0.8 | 95 | n.d. | 100 |
| Hydrochlorothiazide | 58-93-5 | 1.2 | 59 | 26.7 (±3.1) | 0.9 | 73 | 8.0 (±0.8) | 52 (±0.8) |

[a] LOQ: limit of quantification.
[b] R: recovery ($n = 3$).
[c] Mean detected concentration ($n = 3$).
[d] Standard deviation of mean concentration ($n = 3$).
[e] Removal values were calculated considering that the treated sludge contained 62% sludge and 38% WSP (w/w).

quently reduced in 72% after treatment; meanwhile complete removal of the analgesic/antipyretic phenazone was accomplished.

Two psychiatric drugs were detected in the sludge. The findings regarding the antiepileptic carbamazepine are highly remarkable. Its poor, almost negligible removal has been widely reported in conventional AS and membrane bioreactor (MBR) treatments (Carballa et al., 2007; Radjenović et al., 2009). Furthermore, given its persistence, carbamazepine has been proposed as a molecular marker of sewage (Clara et al., 2004). In the present work, removal of 43% was achieved after applying the proposed fungal treatment. Previously, elimination values ranging from 48% to 57% were only reported for high concentrations of carbamazepine spiked in bioslurry and solid phase sludge treatments with *T. versicolor* (Rodríguez-Rodríguez et al., 2010a). Likewise, the anxiolytic diazepam, found at 19.3 ng $g^{-1}$ (±2.8), presented a removal of 43%, similar to the performance showed by anaerobic digestion processes (Carballa et al., 2007).

The lipid regulator drugs bezafibrate and fenofibrate, at initial concentrations of 4.5 ng $g^{-1}$ (±0.1) and 4.2 ng $g^{-1}$ (±0.6), were completely removed after the fungal treatment. Meanwhile, the most abundant atorvastatin (37.8 ng $g^{-1}$, ±3.5), a cholesterol lowering statin, decreased its concentration in 80%. So far, none of these compounds has been proved to be degradable by WRF.

Antibiotics exhibited a highly efficient elimination. The macrolide clarithromycin and the sulfonamide sulfamethazine (initial concentrations of approximately 20 ng $g^{-1}$) were completely removed. The fungal-mediated treatment seems to be promising, since no elimination was reported for sulfamethazine in anaerobic fermentation of swine manure (Mohring et al., 2009) and only ~40% in natural attenuation of biosolids (Wu et al., 2009).

Other pharmaceuticals were successfully removed. These included the β-blocker atenolol and the histamine H2-receptor antagonist cimetidine, both completely eliminated. On the other hand, the diuretic hydrochlorothiazide was eliminated with an efficiency of 52%.

Additional to pharmaceuticals, some congeners of PCBs (Table 2) and PAHs were analyzed, considering their inclusion in the EU's third draft of the Working Document on Sludge. Concentrations of the latter were below the detection limit, when analyzed on the range of μg $g^{-1}$ both in the untreated and treated sludge. Although PCB concentrations were below the limit value suggested in the draft (800 ng $g^{-1}$), a reduction of 28% was observed in the treated sludge for the sum of compounds (from 75.3 to 53.9 ng $g^{-1}$). Degradation of the less chlorinated congeners (less than 6 Cl substitutions) has been demonstrated for aerobic bacteria while more highly chlorinated congeners are barely transformed by anaerobic bacteria, meanwhile numerous studies have shown the degradation of PCBs by WRF (Pointing, 2001). Removal of around 50% has been achieved for some PCB commercial mixtures in liquid media and solid-phase with *T. versicolor*, though in some cases Cb-153 could not be degraded at all (Novotný et al., 1997). Comparable elimination values were reported by Yadav et al. (1995) with the WRF *Phanerochaete chrysosporium* in mixtures, though higher elimination was obtained for some congeners in the present study.

To the best of the authors' knowledge, this is the first report of removal of the highly recalcitrant carbamazepine and other pharmaceuticals at environmentally-relevant, real concentrations from sludge.

Given that in some opportunities degradation-intermediates can be even more toxic than their parent compounds, a global

**Table 2**
Removal of PCBs during solid-phase treatment of thermally dehydrated sewage sludge with *T. versicolor*.

| PCB | Common name | Untreated sludge (ng $g^{-1}$) | Treated sludge (ng $g^{-1}$) | Removal (%) |
|---|---|---|---|---|
| 2,4,4′-Trichlorobiphenyl | Cb-28 | 7.60 | <0.01 | 99.9 |
| 2,2′,5,5′-Tetrachlorobiphenyl | Cb-52 | 12.25 | 4.91 | 59.9 |
| 2,2′,4,5,5′-Pentachlorobiphenyl | Cb-101 | 11.60 | 9.69 | 16.5 |
| 2,3′,4,4′,5-Pentachlorobiphenyl | Cb-118 | 9.70 | 9.33 | 3.8 |
| 2,2′,4,4′,5,5′-Hexachlorobiphenyl | Cb-153 | 13.69 | 12.18 | 11.0 |
| 2,2′,3,4,4′,5′-Hexachlorobiphenyl | Cb-138 | 12.13 | 10.56 | 12.9 |
| 2,2′,3,4,4′,5,5′-Heptachlorobiphenyl | Cb-180 | 8.34 | 7.20 | 13.7 |
| Total | | 75.30 | 53.90 | 28.2 |

**Table 3**
Toxicity parameters obtained from sewage sludge using *V. fischeri* and *D. magna*, expressed as 50% effective concentration ($EC_{50}$) and toxicity units (TU).

| | | $EC_{50}$ (g sludge mL solution$^{-1}$) | | TU | |
|---|---|---|---|---|---|
| *D. magna* 24–48 h acute immobilization test | Time of exposure | 24 h | 48 h | 24 h | 48 h |
| | Treated sludge | 0.2293 | 0.1386 | 436 | 722 |
| | Untreated sludge | 0.1072 | 0.06045 | 933 | 1654 |
| | Raw sludge | 0.03512 | 0.02819 | 2847 | 3547 |
| Bioluminiscence inhibition of *V. fischeri* | Time of exposure | 15 min | 30 min | 15 min | 30 min |
| | Treated sludge | Non toxic solutions | Non toxic solutions | 0 | 0 |
| | Untreated sludge | 0.1286 | 0.1686 | 778 | 593 |
| | Raw sludge | 0.005811 | 0.006000 | 17209 | 16667 |

estimation of toxicity was performed to determine the global reduction of toxic potential in the treated sludge.

### 3.3. Reduction of sludge toxicity after treatment with T. versicolor

To assess ecotoxicity changes after treatment with *T. versicolor* two standardized toxicity tests were performed. The first one, using the micro-crustaceous *D. magna,* and the second using the marine bacteria *V. fischeri*. As it can be seen in Table 3, the raw sludge presented high toxicity values in both cases, but especially for the marine bacteria test. Untreated sludge was less toxic than raw sludge samples, but this effect can be ascribed merely to the dilution of the sludge when mixed with non toxic substrates (e.g. WSP). However, comparing the values obtained for the untreated sludge with those for the treated sludge, a strong decrement in toxicity was observed. In the case of *D. magna* a 56% of toxicity was removed. In the case of the *V. fischeri* based test, the sludge preparation presented a strong decrease in toxicity, much higher than the value expected according to a simple additive model, possibly because synergistic effects between toxics in the sample at concentrations higher to a critical value. Therefore, below this relative concentrations value synergistic effects disappeared. In spite of this initial effect, at time zero the untreated sludge presented a high toxicity value (593 TU), whereas after the treatment with *T. versicolor* none of the tested concentrations presented any inhibition, therefore it should be considered as a non toxic sludge sample to *V. fischeri*.

Since the primary use of biosolids is land application, and particularly on agricultural soils, a reduction in global phytotoxicity is desired. GI has been extensively used as an indicator of phytotoxicity in soils (Tiquia et al., 1996). This parameter combines germination and root growth, therefore reflecting a more complete estimation of toxicity. The results of the seed germination tests shown in Table 4 clearly demonstrate that the fungal-treated sludge is more suitable for germination and growth of the tested vegetable-seeds if compared to the untreated sludge, in which germination of lettuce, pepper and tomato was completely suppressed. On the other hand, the RE can be an indicator of stress or non-acute toxicological effects in the plant evolution; hence, RE may be more sensitive than GI when it comes to toxicity directly affecting root development. This parameter is also evidently reduced if compared to the treated sludge in the case of cucumber, the only seed that was able to germinate in the untreated sludge.

It is quite evident that the next step of research should be focused on the identification of the metabolites released during the degradation process, in those cases where mineralization cannot be accomplished, in order to obtain a more accurate estimation of their individual toxicity. Nonetheless, these results suggest that, in a global way, an important reduction of toxicity is achieved with the fungal treatment of the sludge.

Additionally, work is now underway in order to accomplish the colonization and degradation of organic micropollutants in non-sterile sewage sludge, which could lead to a real practical application. In non-sterile sludge systems, interactions of *T. versicolor* with microbiota may counteract fungal growth or its degrading capacity, or on the other hand may produce a synergic effect, contrary to sterile conditions where fungal colonization is enhanced due to lack of competition. In this regard, the biggest initial challenge is the fungal colonization of this complex matrix; however, the final thermal step of the sewage treatment and its consequent reduction of the microbial load in the sludge may favor the colonization of *T. versicolor*. Moreover, a posterior optimization should be conducted in order to scale-up the process. In this respect, achieving a reduction in the amount of inoculum is a key factor to make the process economically feasible, though it needs to assure the good colonization of the sludge.

Since traditional composting methods have failed to significantly reduce concentrations of many emerging contaminants (Kinney et al., 2006), a static biopile-like treatment for sludge bioaugmentation with *T. versicolor*, as presented in this study, may reduce the ecotoxicological impact of organic pollutants on the environment.

**Table 4**
Seed germination tests as indicators of phythotoxicity in treated/untreated sewage sludge. Values reported are means ± standard deviation for quadruple tests.

| Seed | Relative seed germination (%) | | Relative root elongation (%) | | Germination index | |
|---|---|---|---|---|---|---|
| | TS[a] | US[a] | TS | US | TS | US |
| Lettuce (7 d) | 93 ± 10 | 0 | 62 ± 39 | 0 | 58 ± 39 | 0 |
| Pepper (14 d) | 72 ± 6 | 0 | 93 ± 34 | 0 | 65 ± 20 | 0 |
| Cucumber (7 d) | 94 ± 36 | 50 ± 33 | 103 ± 31 | 40 ± 29 | 92 ± 27 | 25 ± 22 |
| Tomato (7 d) | 95 ± 14 | 0 | 44 ± 14 | 0 | 43 ± 20 | 0 |

[a] TS: treated sludge; US: untreated sludge.

## 4. Conclusions

The use of *T. versicolor* as a potential agent for the degradation of pharmaceuticals at environmentally relevant concentrations in sewage sludge was demonstrated for the first time. Proper growth and activity of the fungus during the process were achieved, as revealed by analyses of ergosterol, laccase and the ND24 test. Complete removal was obtained for half of the detected pharmaceuticals, while elimination values over 40% were accomplished for the remaining ones. The globally less toxic sludge produced after the treatment suggests that this fungal process may be a suitable eco-friendly strategy to reduce the release of toxic contaminants into the environment.

## Acknowledgements

This work was supported by the Spanish Ministry of MMAMRM (Project 010/PC08/3-04). The Department of Chemical Engineering of the Universitat Autònoma de Barcelona is the Unit of Biochemical Engineering of the Centre de Referència en Biotecnologia de la

Generalitat de Catalunya. The authors are members of a Consolidated Research Group of Catalonia (2009-SGR-656). C. Rodríguez-Rodríguez received a predoctoral fellowship from Universidad de Costa Rica and Consejo Superior de Investigaciones Científicas (UCR-CSIC collaboration).

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# Article 4:

Rodríguez-Rodríguez, C.E., García-Galán, M.J., Blánquez, P., Díaz-Cruz, M.S., Barceló, D., Caminal, G., Vicent, T.

## Continuous degradation of a mixture of sulfonamides by *Trametes versicolor* and identification of metabolites from sulfapyridine and sulfathiazole

J. Hazard. Mater. 213-214 (2012), 347-354.

Journal of Hazardous Materials 213–214 (2012) 347–354

Contents lists available at SciVerse ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

# Continuous degradation of a mixture of sulfonamides by *Trametes versicolor* and identification of metabolites from sulfapyridine and sulfathiazole

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ARTICLE INFO

*Article history:*
Received 15 November 2011
Received in revised form 1 February 2012
Accepted 3 February 2012
Available online 11 February 2012

*Keywords:*
*Trametes versicolor*
Sulfonamides
Degradation metabolites
Continuous degradation
Fluidized bed reactor

ABSTRACT

In this study, we assessed the degradation of the sulfonamides sulfapyridine (SPY) and sulfathiazole (STZ) by the white-rot fungus *Trametes versicolor*. Complete degradation was accomplished in fungal cultures at initial pollutant concentrations of approximately 10 mg $L^{-1}$, although a longer period of time was needed to completely remove STZ in comparison to SPY. When cytochrome P450 inhibitors were added to the fungal cultures, STZ degradation was partially suppressed, while no additional effect was observed for SPY. Experiments with purified laccase and laccase mediators caused the removal of greater than 75% of each antibiotic. Ultra-performance liquid chromatography-quadupole time of flight mass spectrometry (UPLC-QqTOF-MS) analyses allowed the identification of a total of eight degradation intermediates of SPY in both the *in vivo* and the laccase experiments, being its desulfonated moiety the commonly detected product. For STZ, a total of five products were identified. A fluidized bed reactor with *T. versicolor* pellets degraded a mixture of sulfonamides (SPY, STZ and sulfamethazine, SMZ) by greater than 94% each at a hydraulic residence time of 72 h. Because wastewater contains many diverse pollutants, these results highlight the potential of *T. versicolor* as a bioremediation agent not only for the removal of antibiotics but also for the elimination of a wide range of contaminants.



## 1. Introduction

Because of their low cost and relative efficiency in combating many common bacterial infections, sulfonamides (SAs) are some of the most widely used antibiotics [1]. In the EU, sulfonamides are the second most widely used class of veterinary antibiotics after tetracyclines [2]. SAs are sometimes used to treat human diseases, but they are more commonly used in veterinary medicine, especially on animal and fish farms. However, the spread of microbial resistance has raised concerns about the prevalence of SAs in the environment [3,4]. The excretion of SAs in the feces and urine of medicated animals and the subsequent application of the contaminated manure as fertilizer onto agricultural land are among the major routes through which SAs enter the environment. Previous studies showed that livestock excrete up to 50–90% of the administered dose, the parent drug making up ~9–50% of the excreted products [5,6]. SAs are highly soluble and weakly acidic, which allows them to be leached from the soil and run off into ground and surface waters [7,8]. Other environmental sources of SAs include aquaculture, hospital effluents, and the disposal of unused drugs from WWTPs, where SAs elimination is often incomplete [9,10].

Alternative eco-friendly treatments to remove organic pollutants such as SAs are of great interest. The white rot fungus (WRF) *Trametes versicolor* has the potential to remove a diverse range of xenobiotics [11], even from complex matrices such as sludge [12], which due to its extracellular and non-specific lignin-mineralizing enzymes (*i.e.*, laccases and peroxidases) and intracellular enzymatic complexes (*e.g.*, cytochrome P450) [13]. Previous studies have demonstrated the laccase-mediated processes of transformation of SAs in liquid medium and their coupling to organic matter [14–16]. However, bioreactor-scale approaches for the application of WRF in the bioremediation of emerging pollutants are still scarce [17].

This work aimed at demonstrating the ability of *T. versicolor* to degrade two SAs, sulfapyridine (SPY) and sulfathiazole (STZ),

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doi:10.1016/j.jhazmat.2012.02.008

through the identification of their metabolites and the determination of the role of specific enzymatic systems in the transformation process. Once described the degradation, the feasibility of the simultaneous elimination of a mixture of three SAs (SPY, STZ and sulfamethazine, SMZ) was also evaluated in a continuous fluidized bed reactor with fungal pellets.

## 2. Materials and methods

### 2.1. Fungal strain

The strain *T. versicolor* (ATCC 42530) was acquired from the American Type Culture Collection and maintained by subculturing every 30 days on 2% malt extract agar slants (pH 4.5) at 25 °C. *T. versicolor* pellets were produced as described by Font Segura et al. [18].

### 2.2. Chemicals and reagents

SPY (4-amino-*N*-(2-pyridinyl)benzenesulfonamide, 99%), STZ (4-amino-*N*-(2-thiazolyl)benzenesulfonamide, Vetranal®, 99.9%), SMZ (4-amino-*N*-(4,6-dimethylpyrimidin-2-yl)benzenesulfonamide, 99%), piperonyl butoxide (PB, ≥90%), 1-aminobenzotriazole (ABT), 3,5-dimethoxy-4-hydroxyacetophenon (DMHAP, 97%), 2,2′-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS, ∼98%), violuric acid (VA, ≥97%) and purified laccase from *T. versicolor* were obtained from Sigma-Aldrich (St. Louis, MO, USA). The internal standard $d_4$-sulfathiazole (99.9%) was purchased from Toronto Research Chemicals (Ontario, Canada). High performance liquid chromatography (HPLC)-grade acetonitrile, water and formic acid (98%) were purchased from Merck (Darmstadt, Germany). Nylon filters (0.45 μm) were purchased from Whatman (Maidstone, UK). The Hydromatrix dispersing agent was purchased from Agilent (Santa Clara, CA, USA).

### 2.3. Experimental procedures

#### 2.3.1. In vivo degradation experiments

The degradation experiments were performed in 250 mL Erlenmeyer flasks containing 10 g of fungal pellets (wet weight) in a total volume of 50 mL of a chemically defined medium at pH 4.5 (composition per liter: 8 g glucose, 498 mg nitrogen as ammonium tartrate, 10 and 100 mL micro- and macronutrient solutions [19], respectively, and 1.168 g 2,2-dimethylsuccinate). Uninoculated flasks containing 50 mL of defined medium and autoclaved cultures were employed as abiotic and heat-killed controls, respectively. All of the conditions were tested in triplicate. SPY or STZ was added from a stock solution in methanol to give the desired concentration (approximately 9–11 mg $L^{-1}$). The flasks were incubated in the dark on an orbital shaker (135 rpm) at 25 °C. In the time course experiments, 1 mL samples were periodically withdrawn, filtered (0.22 μm Millex-GV filters, Millipore, Billerica, MA) and subsequently analyzed by HPLC. Adsorption was estimated by comparing the concentration of the SAs in the heat-killed controls with the concentration in the abiotic controls. Degradation percentages were determined using the concentration values in the heat-killed controls as a baseline.

#### 2.3.2. Experiments with cytochrome P450 inhibitors and enzymatic degradation with laccase

To determine the effect of cytochrome P450 inhibitors, PB or ABT was added to a final concentration of 5 mM in the experiments performed as described in Section 2.3.1. The assays were performed in triplicate. Laccase-mediated degradation experiments were performed in Erlenmeyer flasks containing 50 mL of a purified laccase solution (pH 4.5) at an initial activity concentration of 50.4 ± 8.2 activity units (U) $L^{-1}$ for SPY and 55.4 ± 9.3 U $L^{-1}$ for STZ. The effect of laccase mediators was evaluated by adding VA, DMHAP or ABTS (0.8 mM each) to the reaction mixture. Controls containing milli-Q water at pH 4.5 were included in the analysis. SPY was added at a concentration of 20 mg $L^{-1}$, and STZ was added at a concentration of 16 mg $L^{-1}$. The flasks were incubated in the dark on an orbital shaker (135 rpm) at 25 °C. At designated time points, 1 mL samples were withdrawn, and 100 μL of acetic acid was added to each flask to stop the reactions prior HPLC analysis.

#### 2.3.3. Continuous degradation of SAs in a fluidized bed reactor

A fungal pellet fluidized bed bioreactor [20] was employed to degrade a mixture of SAs. The working volume was set at 1500 mL. Fluidized conditions were maintained by air pulses generated by an electrovalve that was alternately open for 1 s and a shut for 4 s. A pH controller was used to maintain the pH at 4.5 ± 0.2, and the system was kept at 25 °C. The bioreactor was initially loaded with 1.5 L of defined medium as described in Section 2.3.1, glucose (10 g $L^{-1}$) and SAs (SMZ, SPY and STZ, 5 mg $L^{-1}$ each). The reactor was inoculated with fungal pellets at 2.3 g $L^{-1}$ (dry cell weight, DCW) and operated in batch mode. Once the glucose concentration reached 1 g $L^{-1}$, the continuous stage was switched on with an initial hydraulic residence time (HRT) of 48 h; the HRT was changed according to the system's performance during the experiment. The feeding solution consisted of defined medium without glucose and SAs at 5 mg $L^{-1}$ each. Glucose was supplied separately at the consumption rate (approximately 2 g $L^{-1}$ $d^{-1}$, [20]). Throughout the experiment, the biomass was contained inside the reactor with a metal mesh in the outlet.

### 2.4. Analytical procedures

#### 2.4.1. Analyses of SAs

SAs were quantified using a Dionex 3000 Ultimate HPLC (Sunnyvale, CA) equipped with a UV detector at 264 nm. Chromatographic separation was achieved at 30 °C by injecting 20 μL samples on a Grace Smart RP18 column (250 mm × 4 mm, 5 μm particle size); the mobile phase consisted of 40 mM ammonium acetate buffer (A, pH 7) and methanol (B). For the analysis of individual SAs, the eluents were added isocratically (65% A, 35% B) at 1 mL $min^{-1}$ [21]. The retention times were 4.3 min and 3.9 min for SPY and STZ, respectively. The elution of the mixture of SAs in the reactor experiments was accomplished with a linear increase from 0% B to 35% B over 10 min, isocratic elution for 2 min, and then a return to the initial conditions in 2 min. The retention times in this case were 9.9 min (STZ), 10.6 min (SPY) and 12.1 min (SMZ).

#### 2.4.2. Identification of degradation products

MS and tandem MS (MS/MS) analyses of SPY and STZ and its degradation products were performed using a Waters/Micromass QqTOF-Micro system coupled to a waters acquity ultra performance liquid chromatography (UPLC) system (Micromass, Manchester, UK). A Waters Acquity BEH C18 column (10 mm × 2.1 mm, 1.7 μm particle size) was employed. The flow rate was set up at 0.3 mL $min^{-1}$. Eluent A (HPLC-grade water) and eluent B (acetonitrile) were both acidified with 10 mM of formic acid. The elution was performed with a linear increase of B from 5% to 60% over 7 min, a further increase of B to 95% over the following 2 min, and then a return to the initial conditions in 2 min. The injection volume of the sample was 5 μL. The analyses were performed in the positive ionization (PI) mode. The operating conditions are described in a previous publication by the authors of this work [22]. For continuous internal mass calibration, an independent reference (valine–tyrosine–valine) was used as a lock mass, with $m/z$ 380.2185, and was acquired in all of the measurements. For the MS

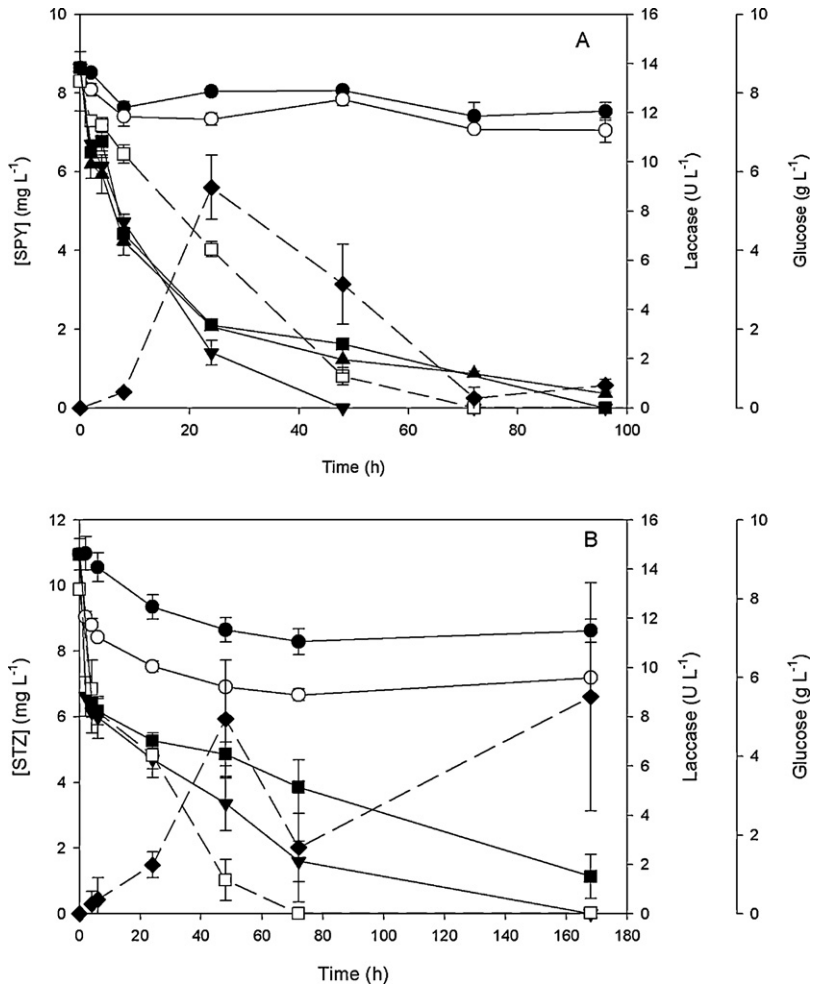


**Fig. 1.** Degradation profiles of SPY (A) and STZ (B) by *T. versicolor* at the bench scale. Symbols: uninoculated controls (●), heat-killed controls (○), reaction cultures (▼), cultures containing cytochrome P450 inhibitors PB (■) and ABT (▲), glucose consumption (□) and laccase activity (♦). Values plotted are the means ± standard deviation (SD) for triplicate cultures. The initial pellet biomass (dry weight) was 282.7 ± 5.2 mg and 353.7 ± 10.8 mg for SPY and STZ, respectively.

analyses on the QqTOF instrument, the MS data were collected by scanning from *m*/*z* 50 to *m*/*z* 500.

### 2.4.3. Other analyses

Laccase activity was measured as described by Wariishi et al. [23]; 2,6-dimethoxyphenol (DMP) was used as the substrate. The results are expressed as U $L^{-1}$. One activity unit is defined as the number of micromoles of DMP oxidized per min. The glucose levels were analyzed by the glucose oxidase method in a YSI 2700 analyzer (Yellow Springs, OH). The mycelia dry weight was determined by vacuum filtering the cultures through preweighed glass filters (Whatman GF/C). The filters containing the biomass were dried at 100 °C to constant weight.

## 3. Results and discussion

### 3.1. Degradation of SPY and STZ by T. versicolor

The fungal transformation of the SAs SPY and STZ was assayed and the degradation profiles are shown in Fig. 1. Complete removal of SPY was achieved after 48 h (Fig. 1A), although most of the degradation occurred during the first 24 h (81 ± 5%). The experimental degradation rate was 86.5 ± 5.5 ng $h^{-1}$ $mg^{-1}$ DCW (0.347 ± 0.022 nmol $h^{-1}$ $mg^{-1}$ DCW). Adsorption, which was determined by comparing the concentrations in the heat-killed controls and the uninoculated flasks, was negligible. STZ (Fig. 1B) removal was slightly slower, and more than 20% was still detected after 72 h, although the initial elimination rate was higher than with SPY (117.3 ± 19.2 ng $h^{-1}$ $mg^{-1}$ DCW, 0.459 ± 0.075 nmol $h^{-1}$ $mg^{-1}$ DCW). The adsorption to heat-killed biomass was 17 ± 4%. A slight reduction in the concentration of both SAs was observed in the uninoculated flasks, which can be ascribed to the production of $N^4$-glycosyl-SPY and $N^4$-glucosyl-STZ as by-products released due to the presence of glucose in the defined medium. These compounds were detected and confirmed by MS and tandem MS/MS analyses; an analogous phenomenon has been described for SMZ [22]. The confirmation of degradation metabolites in the reaction cultures, discussed in Section 3.3, indicates that the elimination of SPY and STZ, as demonstrated for the elimination of SMZ [22], is due to a degradation process and cannot be ascribed solely to sorption phenomena.

### 3.2. Effect of cytochrome P450 inhibitors and the role of laccase in the degradation of SAs

Cytochrome P450, an intracellular enzymatic complex, has been implicated in the degradation of several organic pollutants by WRF [24,25]. To evaluate its role in the transformation of the SAs, the cytochrome P450 inhibitors PB and ABT were employed [26]. When comparing the inhibitor-containing cultures and the reaction cultures in Fig. 1, no difference was observed in the first hours, possibly corresponding to the period of active transport of SAs into the

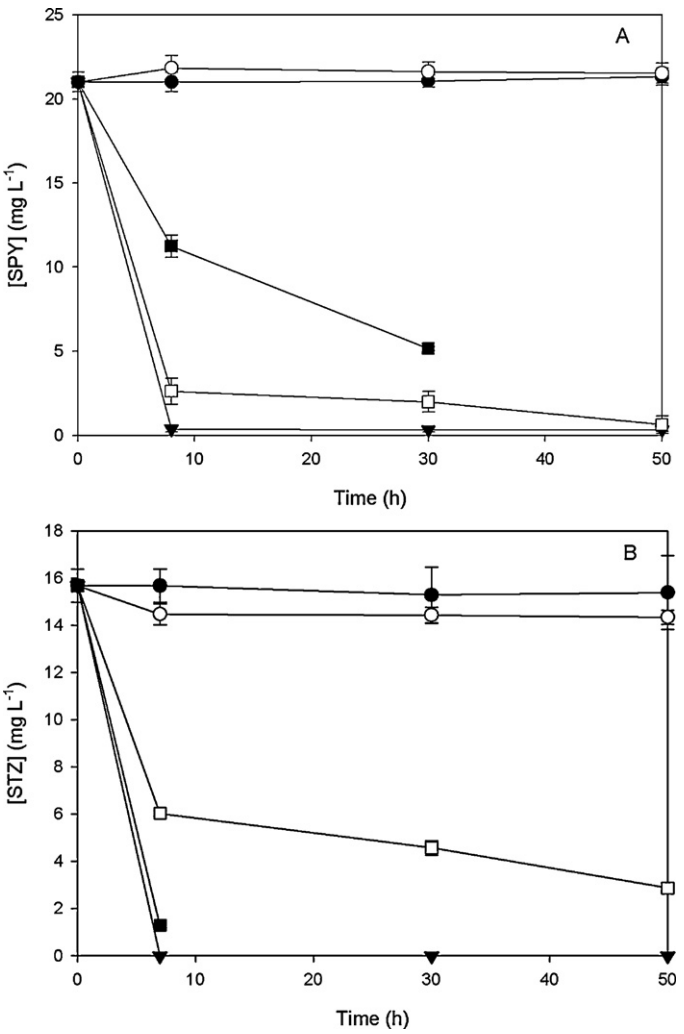


**Fig. 2.** Degradation profiles of SPY (A) and STZ (B) by purified laccase. Symbols: laccase-free controls (●), laccase without mediators (○), laccase with VA (▼), laccase with DMHAP (□) and laccase with ABTS (■). Values plotted are the means ± SD for triplicate cultures.

fungal cells. There was a slight delay in the degradation of SPY (24 h) in the inhibitor-containing cultures, but almost complete removal (>95%) was obtained after 96 h. On the other hand, a decelerated decline in STZ concentration was observed in the cultures with PB, if compared to inhibitor-free cultures. These results suggest that the cytochrome P450 complex may be involved in the removal of STZ, whereas there is no clear experimental evidence that cytochrome P450 plays a role in the degradation of SPY.

Similar to cytochrome P450, the participation of laccase in the removal of diverse groups of environmental contaminants has been widely investigated [27]. Because laccase activity levels up to 10 U $L^{-1}$ were obtained in the degradation assays (Fig. 1), further experiments that focused on determining the role of this enzyme were performed with purified laccase and laccase mediator systems [13]. Enzymatic degradation with solely commercial laccase (Fig. 2) caused minimal SA degradation in 50 h. However, when VA, DMHAP and ABTS—mediators in laccase reactions—were applied, the elimination of the antibiotics ranged from 75% to 98% (±4%) for SPY and 82% to 100% (±3%) for STZ by the end of the experiment. Laccase mediators favor the oxidation of non-phenolic compounds [28], and although they are not produced by *T. versicolor* itself, molecules with analogous functions may be released during active growth. This, together with the combined activity of other enzymatic systems (*e.g.* cytochrome P450), results in shorter treatment periods with the use of whole fungal cells, compared to those reported by purified laccase, in which significant transformation of SAs has been achieved only with longer incubation times (9–16 d) [16]. Laccase-mediated degradation has been demonstrated for some pharmaceuticals, including naproxen and other SAs [16,22,29].

### 3.3. Identification of degradation products from sulfapyridine

#### 3.3.1. In vivo experiments

SPY was fully degraded after 48 h in the fungal cultures, and the presence of 7 different transformation products confirmed this finding (Fig. 3). Although the relative mass errors of the degradation products were sufficiently accurate for identification, confirmation by MS/MS spectra was not always achieved, as sometimes no more than a single fragment could be obtained for each of them. In these cases, the predicted structures were confirmed with the double bond equivalent (DBE) values and the sodium adduct signals from the chromatograms (see Supplementary information, SI).

Upon chromatographic separation with the UPLC, SPY was detected at a retention time (RT) of 2.95 min. At reaction time 0, a peak at an RT of 2.20 min was already present in both the abiotic and heat-killed control samples and the reaction samples, with a base peak *m*/*z* of 412.1177 (average value, $n = 4$) in the corresponding mass spectra. The elemental composition for this mass was $C_{17}H_{22}N_3O_7S$ (SI, Table S1), corresponding to the by-product $N^4$-glycosyl-SPY (P6 in Fig. 3), which is formed due to the presence of glucose in the defined culture medium described in Section 2.3.1. To confirm this finding, a CE of 30 eV was applied, and stable fragment ions at *m*/*z* 255.1083 and *m*/*z* 108.0961 were obtained (SI, Fig. S1). In the reaction samples, this adduct was also present at a concentration profile similar to that of SPY, indicating that both were consumed by the fungi to a similar extent (Fig. 4A). The glycosylated adduct was subjected to transformation itself, and a second glycosylated product was detected after 8 h in the reaction samples, with a RT of 1.52 min and a base peak of *m*/*z* 348.1550 (mean value, $n = 3$) in the corresponding mass spectra. It was identified as the desulfonated product of $N^4$-glycosyl-SPY (P2).

A new peak appeared at a reaction time of 8 h and at a RT of 1.9 min, with an observed *m*/*z* of 186.1039 ($n = 4$). Its elemental composition was elucidated as $C_{11}H_{12}N_3$ and corresponded to the desulfonated product of SPY, reaching a maximum relative intensity of 80% after 24 h (Fig. 4, P4). The product ion spectrum (CE of 30 eV) yielded peaks at *m*/*z* of 108.0698 and 93.0574 (SI, Fig. S1). This desulfonated product of SPY was the only common metabolite present also in the enzymatic degradation experiments. Previous studies have demonstrated that desulfonated products are frequently detected after the enzymatic degradation, physicochemical oxidation or photodegradation of SAs [16,22,30–32].

Two different signals corresponded to the same mass and yielded the same mass spectra: P5 and P5′. Both stereoisomers appeared in the chromatogram at RTs of 1.69 and 2.01 min, respectively, with a base peak *m*/*z* of 214.0984 (average value, $n = 10$), corresponding to the addition of a formyl group to the aforementioned desulfonated moiety. An elemental composition of $C_{12}H_{12}N_3O$ was assigned to this product. The presence of formic acid in the UPLC eluents used may have led to the formation of this molecule as a by-product in the samples. However, it was not detected in either the abiotic or the heat-killed control samples, which were analyzed with the same UPLC eluents and gradient. At a RT of 2.98 min, a different peak with *m*/*z* of 278 was recorded, corresponding to formyl-SPY (P7), but its relative peak intensity remained below 5%. Similar formyl metabolites were predicted to be produced during the photodegradation of sulfadiazine in water,

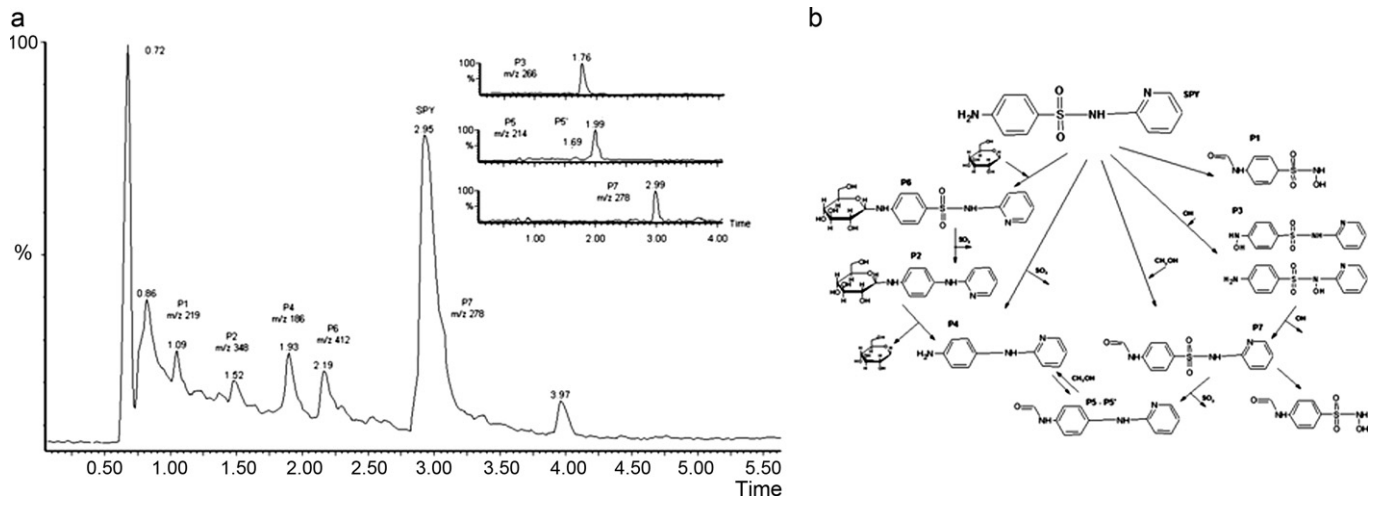


**Fig. 3.** Total ion chromatogram (TIC) of SPY degradation after 8 h in *in vivo* experiments (a) and proposed transformation products (b).

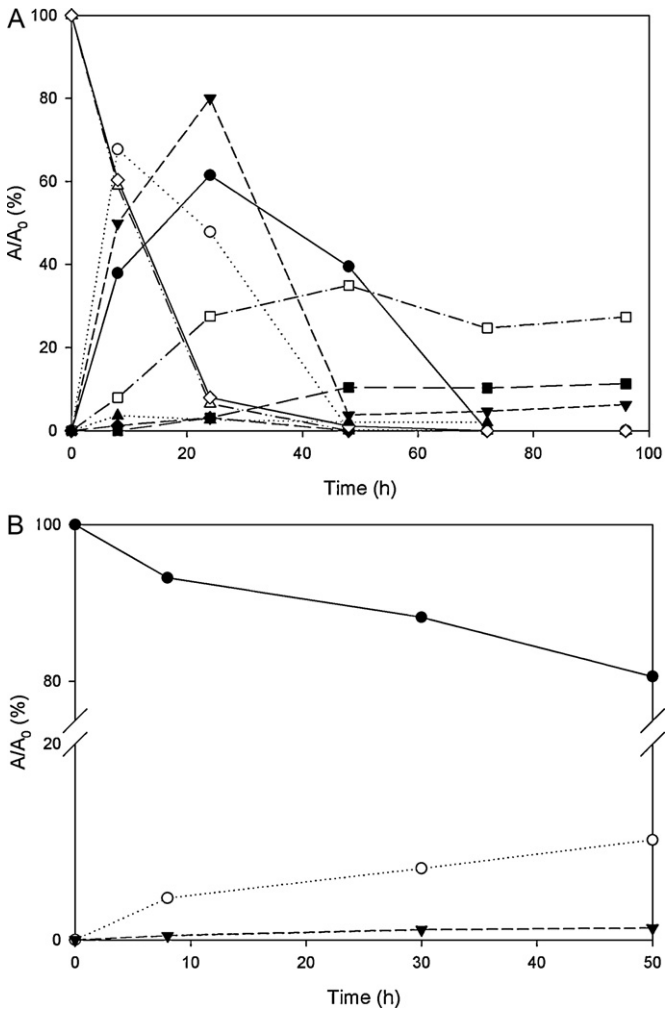


**Fig. 4.** Degradation of SPY over time and its major transformation products in *in vivo* experiments (A) and laccase experiments (B). Symbols (A): SPY (◊), *m/z* 219 (●), *m/z* 348 (○), *m/z* 266 (♦), *m/z* 186 (▼), *m/z* 214-1 (□), *m/z* 214-2 (■), *m/z* 412 (△), and *m/z* 278 (▲). Symbols (B): SPY (●), *m/z* 186 (○), and *m/z* 200 (▼).

the laccase-mediated degradation of SPY and during the degradation of SMZ by *T. versicolor* [16,22,33].

The loss of the pyridine ring and the addition of a hydroxyl group and a formyl group yielded a new product of *m/z* 219.0449 ($n = 3$) with an assigned elemental composition of $C_7H_{11}N_2O_4S$ (P1), which reached its maximum relative intensity after 24 h (61%). The hydroxylated moiety of SPY was also detected at a RT of 1.78 min with an observed mass of 266.0603 and an elemental composition of $C_{11}H_{12}N_3O_3S$ (P3). It was identified only in the samples taken after 8 h and 24 h and always at relative peak intensities <5%. Similar to SMZ degradation by *T. versicolor* [22], the OH group of the hydroxylated product was lost during MS/MS fragmentation.

### 3.3.2. Enzymatic degradation experiments

Although phenol-like compounds are the typical substrates of laccase-mediated oxidation, aromatic amines can also act as substrates [34]. As shown in Fig. 4, the decrease of SPY was significantly slower in laccase-mediated cultures than in the *in vivo* experiments, with a relative peak intensity of only 20% after 50 h. Two chromatographic peaks could be identified in the enzymatic degradation assays. The first peak appeared in the chromatogram at a RT of 1.16 min, with a base peak of *m/z* 200.0266 (average value, $n = 3$). The proposed elemental composition was $C_7H_8N_2O_3S$, which could correspond to a formyl intermediate (SI, Table S1). At a RT of 1.9 min, the desulfonated metabolite of SPY previously identified in the *in vivo* assays (*m/z* of 186.1052, $n = 3$; see Section 3.3.1) was also present. Although the relative peak intensity was lower in this case (10% maximum value), the presence of a common intermediate metabolite in the *in vivo* experiments supports the role of laccase in SPY transformation by *T. versicolor*. Mass spectral information could not be obtained for either of these degradation products primarily because they were present at low concentrations. As in Section 3.3.1, some of the predicted chemical structures were confirmed by obtaining the DBE values and detecting the sodium adducts.

## 3.4. Identification of degradation products from sulfathiazole

### 3.4.1. In vivo experiments

STZ was not fully degraded after 96 h of culture with the fungus. The glycosylated adduct and two transformation products were detected (Fig. 5).

The first signal in the chromatogram corresponded to $N^4$-glycosyl-STZ (P1), with an observed *m/z* of 418.0764 and an RT of 2.2 min. In contrast to $N^4$-glycosyl-SPY, which decreased during the reaction at rate similar to that of SPY, $N^4$-glycosyl-STZ decreased faster than STZ (Fig. 6). The STZ concentration started to decrease after 24 h, when 88% of the adduct had been used up by the fungus. This lag phase required by *T. versicolor* to start degrading STZ was not observed for SPY or SMZ [22], SAs that have 6-membered

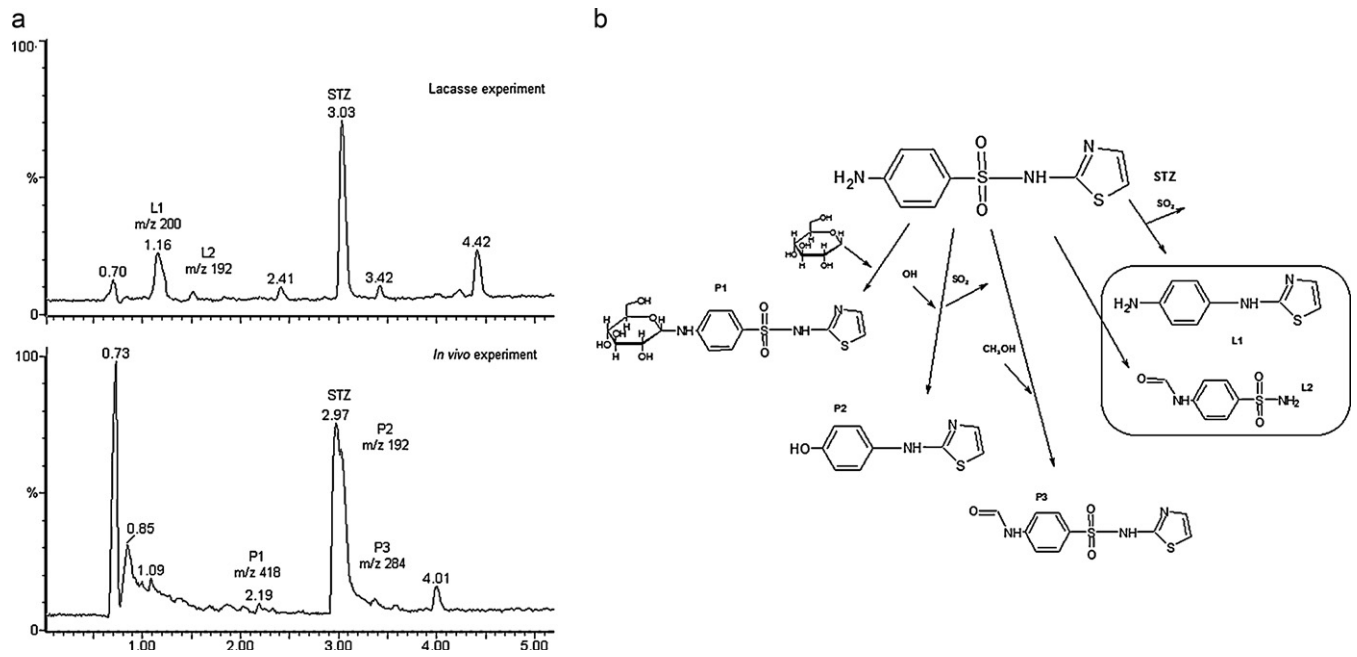


**Fig. 5.** Total ion chromatogram (TIC) of STZ degradation after 8 h in *in vivo* experiments (a) and proposed transformation products (b).

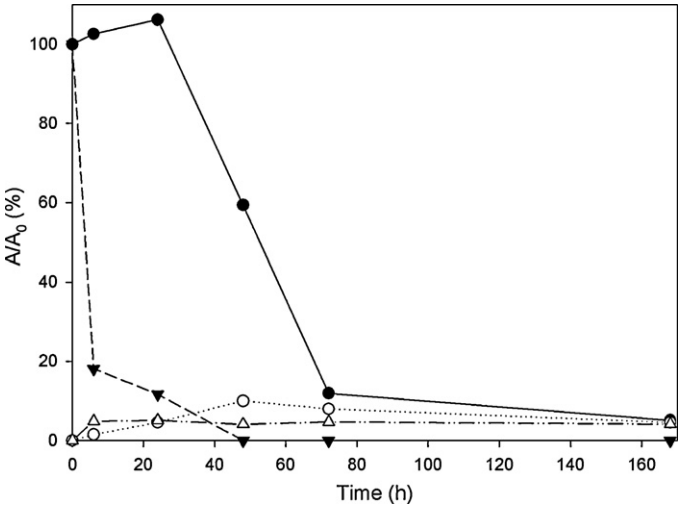


**Fig. 6.** Degradation of STZ over time and its major transformation products in *in vivo* experiments. Symbols: STZ (●), *m*/*z* 418 (▼), *m*/*z* 192 (△), and *m*/*z* 284 (○).

heterocyclic rings (pyridine and pyrimidine rings, respectively), suggesting that the substituent group of the SA secondary amine, in this case the 1,3-thiazole ring of STZ, determines the ability of the SA to be assimilated and/or degraded. The desulfonated product of the glycosylated adduct was not present for STZ.

At a RT of 2.99 min, a new peak was present in the chromatogram (Fig. 5a), with a base peak of *m*/*z* 192.0359 (average value, $n = 3$) and an elemental composition of $C_9H_8N_2OS$ (P2), which could correspond to the desulfonated and hydroxylated moiety of STZ, although the hydroxylated moiety of STZ was not detected in either the *in vivo* or laccase assays. The predicted molecular structure is shown in SI, Table S2. The normalized area was never higher than 5%. At a RT of 3.23 min, an additional signal with a base peak of *m*/*z* 284.0172 was identified as formyl-STZ (P3), which was at its highest concentration after 48 h of incubation. Upon applying a CE of 25, the product ion spectrum obtained showed two main stable fragment ions of *m*/*z* 120.0450 and *m*/*z* 100.0101 (SI, Fig. S1).

### 3.4.2. Enzymatic degradation experiments

We further investigated only two samples from the enzymatic degradation experiments, taken after 30 h and 50 h. Therefore, the trends over time for the intermediate products could not be determined. The desulfonated moiety of STZ was only detected in the laccase experiments. This peak appeared at a RT of 1.61 min, with an observed mass of 192.0584 and a corresponding elemental composition of $C_9H_{10}N_3S$. The signal intensity for this metabolite was similar for both samples (30 h and 50 h). As with SPY, the enzymatic degradation of STZ generated a compound with an observed *m*/*z* of 200.0257 and a proposed elemental composition of $C_7H_8N_2O_3S$ (L2, Fig. 5). The intensity of the signal seemed to decrease slightly between 30 h and 50 h.

## 3.5. Continuous degradation of SAs in a fluidized bed reactor

The promising results that we obtained at flask-scale, combined with previous studies that demonstrated the ability of *T. versicolor* to degrade SMZ [22], encouraged us to assess the simultaneous removal of the three antibiotics (SMZ, SPY and STZ) at the reactor scale. The performance of the reactor is shown in Fig. 7. The first stage (I) consisted of a batch setup reaction, during which complete removal of the SAs was accomplished. In this case, STZ was the last antibiotic to be fully depleted. To reduce the time of treatment, the continuous stage (II) was switched on with a HRT of 48 h when the glucose levels reached 1 g $L^{-1}$ (at day 4). From this time on, glucose was supplied at the consumption rate to maintain a concentration of approximately zero. After four HRTs (a steady state was assumed after three HRTs), the degradation stabilized at 35% to 55% for the three SAs and SPY exhibited the highest removal. By the 12th day of operation, an excess in the growth of the biomass required us to purge the equivalent to nearly a third of the previous working volume so that the system could regain proper fluidization. Strategies such as purging and partial biomass renovation have been successfully applied to overcome operational issues and to achieve long-term continuous degradation in similar reactors such as those that use *T. versicolor* for decolorization [35]. The period of time during which the system was refilled to the original volume level and then stabilized at a HRT of 48 h is indicated in Fig. 7 as the

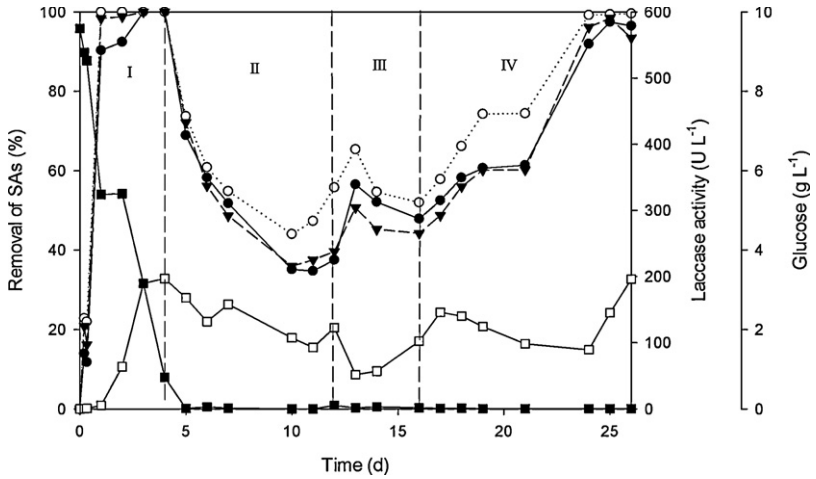


**Fig. 7.** Elimination of STZ (●), SPY (○) and SMZ (▼) (5 mg $L^{-1}$ each) during treatment with *T. versicolor* pellets in a fluidized bed reactor. Stages of treatment: initial batch (I); continuous treatment, HRT 48 h (II); transition period (III); and continuous treatment, HRT 72 h (IV). Laccase activity (□) and glucose (■).

transition period (III). In an attempt to increase the removal efficiency, the HRT was changed to 72 h (IV). The elimination amount was significantly improved, reaching values of greater than 94% for all of the SAs after three HRTs. The laccase activity was monitored, and the activity values ranged from 100 U $L^{-1}$ to 200 U $L^{-1}$ during the continuous stages (II and IV). The simultaneous transformation of diclofenac, ibuprofen and naproxen has been accomplished in a fed-batch reactor containing *Phanerochaete chrysosporium* pellets [36]; however, reports of continuous depletion are uncommon and include the removal of textile dyes by *T. versicolor* [20,37] and endocrine-disrupting compounds by immobilized laccase [38].

## 4. Conclusions

The degradation of SPY and SMZ by *T. versicolor* has been demonstrated in this work. Despite the high concentrations used in the assays, SPY was fully degraded after the experimental time in the fungal cultures, and 7 different degradation products were elucidated, including formylated and hydroxylated products. STZ was more resilient toward degradation, and 12% of the initial concentration was still present after 96 h of incubation with the fungus in the *in vivo* assays, in which three different intermediate products were present. The respective desulfonated moieties and a common degradation product of *m*/*z* 200 were detected in the purified laccase experiments for both SAs. A continuous fluidized bed reactor with fungal pellets successfully eliminated a mixture of three SAs at a HRT of 72 h. Although degradation was accomplished and many metabolites disappeared or remained at very low concentrations at the end of the batch experiments, a toxicity assessment should be performed to determine any potential increase in the toxicity before a real application of *T. versicolor* for the removal of SAs.

## Acknowledgments

This work has been supported by the Spanish Ministries of MMAMRM (project 010/PC08/3-04), and MEC (Project CEMAGUA, CGL2007-64551), and the Generalitat de Catalunya (projects SGR 2009-SGR00796 and 2009-SGR00656). The Department of Chemical Engineering (UAB) is the Unit of Biochemical Engineering of the XRB de la Generalitat de Catalunya. Prof. D. Barcelò acknowledges King Saud University (Riyadh, Saudi Arabia) for his contract position as Visiting Professor. C.E. Rodríguez-Rodríguez received a pre-doctoral grant from the Universidad de Costa Rica and Consejo Superior de Investigaciones Científicas (UCR-CSIC collaboration). M.J. García acknowledges AGAUR (Generalitat de Catalunya, Spain) for economic support through an FI pre-doctoral grant.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2012.02.008.

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**SUPPLEMENTARY INFORMATION**

**Table S1**. Accurate mass measurement of sulfapyridine biodegradation products as determined by UPLC/ESI-QqToF in $MS^2$ mode and optimized cone voltage (25 V) and collision energies (15-25 eV).

**Table S2**. Accurate mass measurement of sulfathiazole biodegradation products as determined by UPLC/ESI-QqToF in $MS^2$ mode and optimized cone voltage (25 V) and collision energies (15-25 eV).

**Figure S1.** Product ion spectra obtained from ESI (+)MS/MS experiments with UPLC-QqTOF for the enzymatic degradation assays after 48 h of reaction: a) $N^4$-glycosyl-SPY ( CE 25 eV); B) Desulfonated-SPY (CE 20 eV) ; C) $N^4$-formyl-STZ (CE 20 eV) (Cone voltage: 30V for all the experiments).

Table S1.

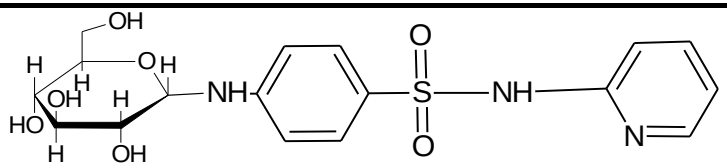
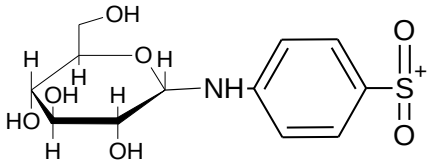
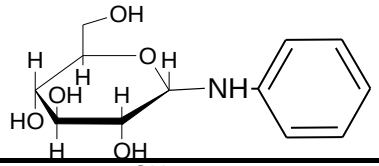
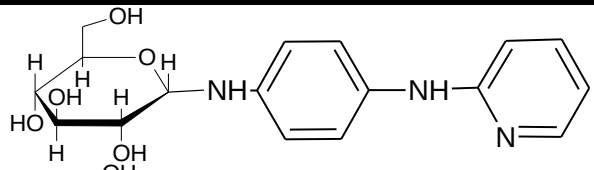
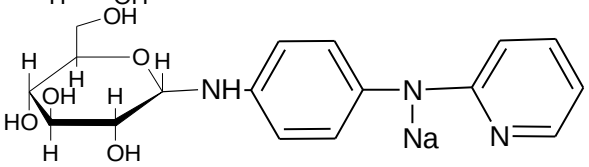
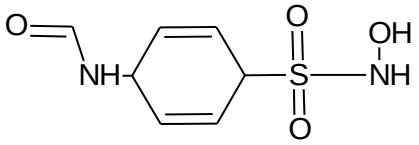
| OBSERVED MASS (m/z) | RT / FRAGMENTS | ELEMENTAL COMPOSITION | CALCULATED MASS | ERROR (mDA) | ERROR (ppm) | DBE | STRUCTURE |
|---|---|---|---|---|---|---|---|
| | | | ***FUNGAL CULTURES*** | | | | |
| **412.1168** | 2.2 | $C_{17}H_{22}N_3O_7S$ | 412.1178 | 1 | 2.4 | 8.5 |  |
| | **318.0665** | $C_{12}H_{16}NO_7S$ | 318.0647 | 1.8 | 5.2 | 5 |  |
| | **255.1083** | $C_{12}H_{17}NO_5$ | 255.1107 | 2.4 | 9.5 | 5 |  |
| **348.1539** | 1.5 | $C_{17}H_{22}N_3O_5$ | 348.1559 | 2 | 5.7 | 8.5 |  |
| | **370.1371** | $C_{17}H_{21}N_3O_5Na$ | 370.1379 | 0.8 | 2.2 | 8.5 |  |
| **219.0443** | 1.1 | $C_7H_{11}N_2O_4S$ | 219.0.440 | 0.3 | 1.4 | 3.5 |  |

Table S1 (cont).

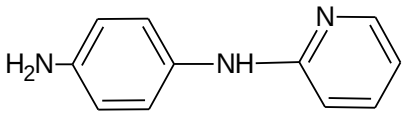
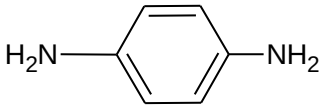
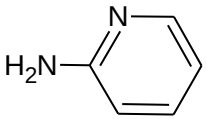
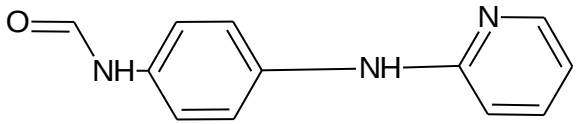
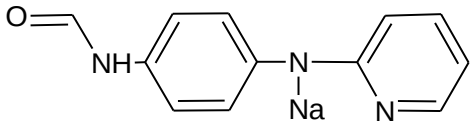
| OBSERVED MASS (m/z) | RT / FRAGMENTS | ELEMENTAL COMPOSITION | CALCULATED MASS | ERROR (mDA) | ERROR (ppm) | DBE | STRUCTURE |
|---|---|---|---|---|---|---|---|
| **186.1035** | 1.93 | $C_{11}H_{12}N_3$ | 186.1031 | 0.3 | 1.8 | 7.5 |  |
| | **108.0698** | $C_6H_8N_2$ | 108.0687 | 1.1 | 9.7 | 4 |  |
| | **93.0574** | $C_6H_7N$ | 93.0578 | 0.4 | 4.8 | 4 |  |
| **214.0964** | 2 | $C_{12}H_{12}N_3O$ | 214.0980 | 1.6 | 7.5 | 8.5 |  |
| | **236.0831** | $C_{12}H_{11}N_3ONa$ | 3.1 | 13.1 | 13.1 | 8.5 |  |

Table S1 (cont).

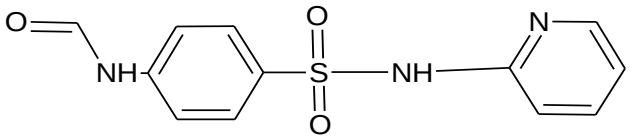
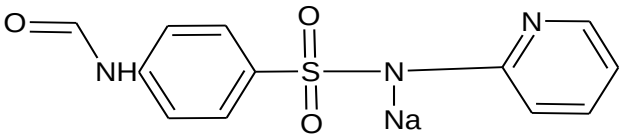
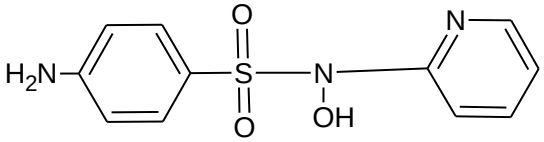
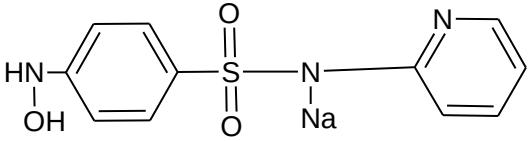
| OBSERVED MASS (m/z) | RT / FRAGMENTS | ELEMENTAL COMPOSITION | CALCULATED MASS | ERROR (mDA) | ERROR (ppm) | DBE | STRUCTURE |
|---|---|---|---|---|---|---|---|
| **278.0617** | 2.98 | $C_{12}H_{12}N_3O_3S$ | 278.0599 | 1.8 | 6.5 | 8.5 |  |
| | **300.0432** | $C_{12}H_{11}N_3ONaS$ | 300.0419 | 3.4 | 11.3 | 8.5 |  |
| **266.0603** | 1.78 | $C_{11}H_{12}N_3O_3S$ | 266.0599 | 0.4 | 1.5 | 7.5 |  |
| | **288.0418** | $C_{11}H_{11}N_3O_3NaS$ | 288.0419 | 0.1 | 0.3 | 7.5 |  |

Table S1 (cont).

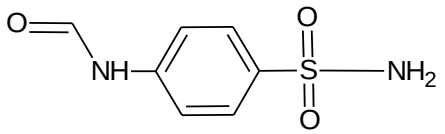
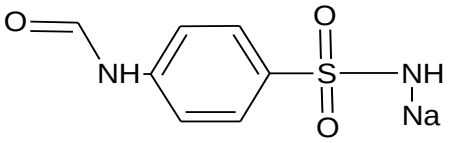
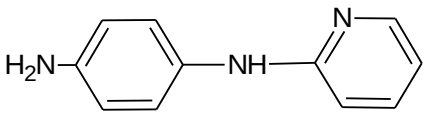
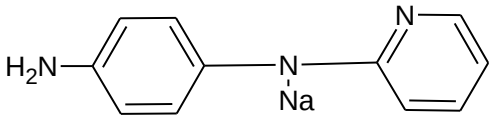
| OBSERVED MASS (m/z) | RT / FRAGMENTS | ELEMENTAL COMPOSITION | CALCULATED MASS | ERROR (mDA) | ERROR (ppm) | DBE | STRUCTURE |
|---|---|---|---|---|---|---|---|
| ***PURIFIED LACASSE EXPERIMENTS*** | | | | | | | |
| **200.0267** | 1.16 | $C_7H_8N_2O_3S$ | 200.0256 | 1.2 | 5.8 | 5 |  |
| | **222.0095** | $C_7H_7N_2O_3SNa$ | 222.0075 | 2 | 8.9 | 5 |  |
| **186.1059** | 1.87 | $C_{11}H_{12}N_3$ | 186.1031 | 2.8 | 15.1 | 7.5 |  |
| | **208.0862** | $C_{11}H_{11}N_3Na$ | 208.0851 | 1.1 | 5.3 | 7.5 |  |

Table S2.

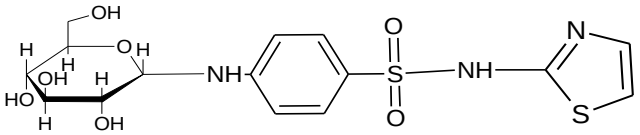
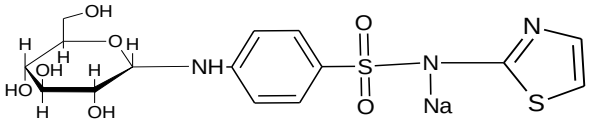
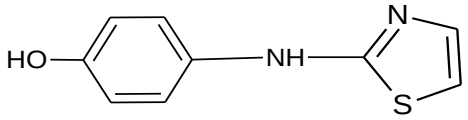
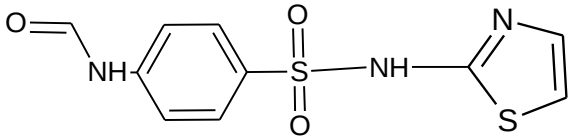
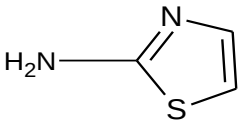
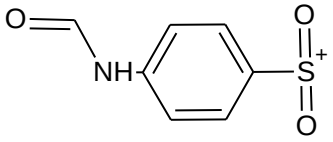
| OBSERVED MASS (m/z) | RT / FRAGMENTS | ELEMENTAL COMPOSITION | CALCULATED MASS (m/z) | ERROR (mDA) | ERROR (ppm) | DBE | STRUCTURE |
|---|---|---|---|---|---|---|---|
| | | | ***FUNGAL CULTURES*** | | | | |
| **418.0764** | 2.2 | $C_{15}H_{20}N_3O_7S_2$ | 418.0743 | 2.1 | 5.1 | 7.5 |  |
| | **440.0544** | $C_{15}H_{19}N_3O_7NaS_2$ | 440.0562 | 1.8 | 4.2 | 7.5 |  |
| **192.0367** | 2.99 | $C_9H_8N_2OS$ | 192.0357 | 1 | 5.2 | 7 |  |
| **284.0172** | 3.23 | $C_{10}H_{10}N_3O_3S_2$ | 284.0164 | 0.8 | 2.8 | 7.5 |  |
| | **100.0101** | $C_3H_4N_2S$ | 100.0095 | 0.6 | 5.8 | 3 |  |
| | **184.0072** | $C_7H_6NO_3S$ | 184.0068 | 0.4 | 2 | 5.5 |  |

Table S2 (cont).

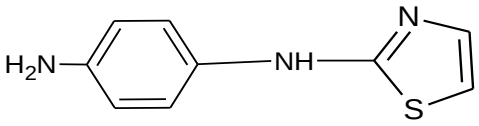
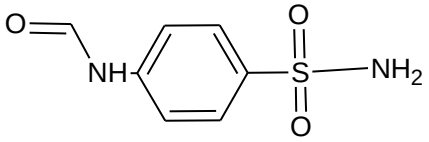
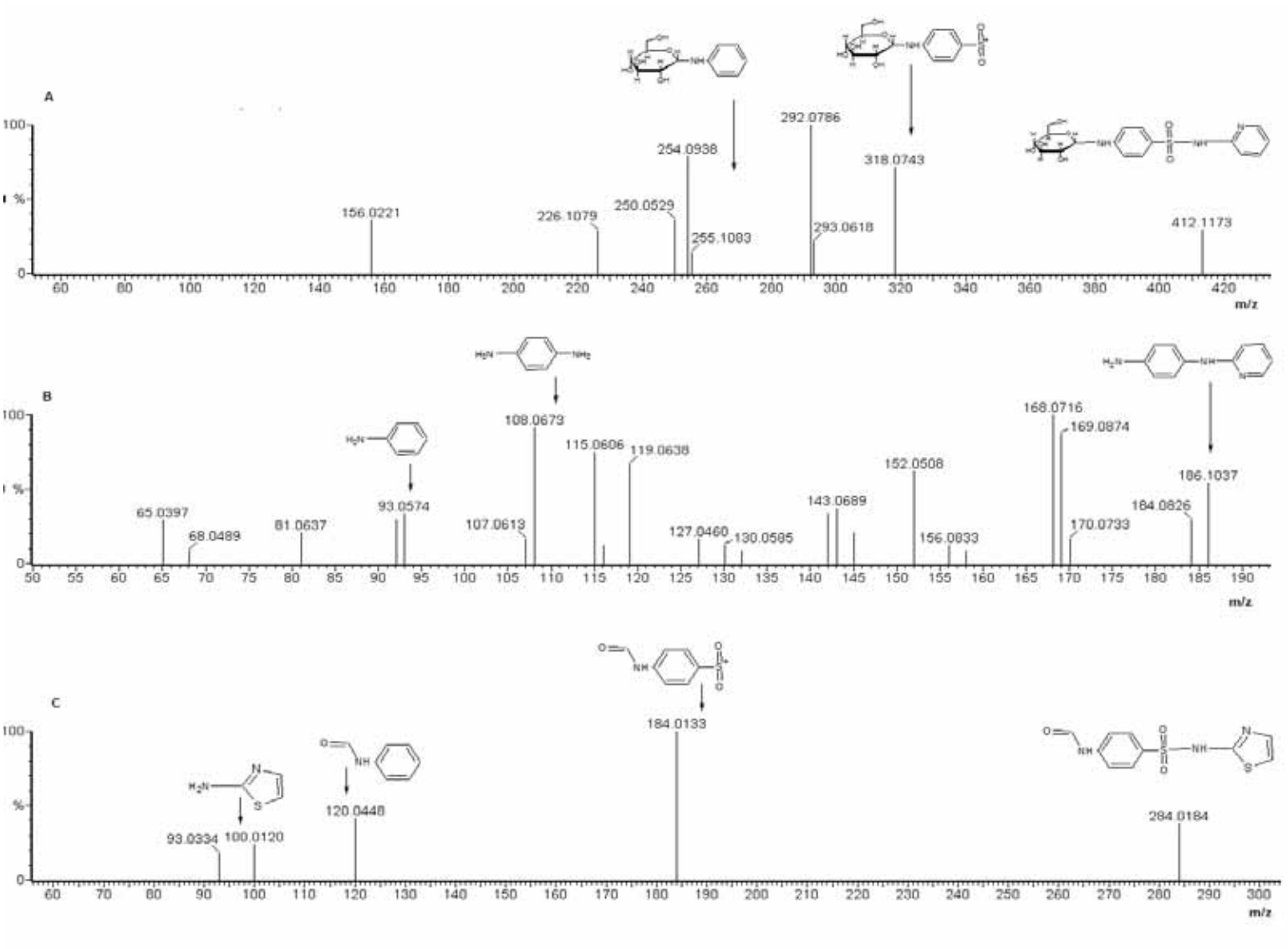
| OBSERVED MASS (m/z) | RT / FRAGMENTS | ELEMENTAL COMPOSITION | CALCULATED MASS (m/z) | ERROR (mDA) | ERROR (ppm) | DBE | STRUCTURE |
|---|---|---|---|---|---|---|---|
| ***PURIFIED LACASSE EXPERIMENTS*** | | | | | | | |
| **192.0584** | 1.61 | $C_9H_{10}N_3S$ | 192.0595 | 1.1 | 5.9 | 6.5 |  |
| **200.0257** | 1.16 | $C_7H_8N_2O_3S$ | 200.0256 | 0.1 | 0.7 | 5 |  |

Figure S1.


A
100
%
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156.0221
226.1079
250.0529
254.0938
255.1083
292.0786
293.0618
318.0743
412.1173
60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420
m/z
B
100
%
0
65.0397
68.0489
81.0637
93.0574
107.0613
108.0679
115.0606
119.0638
127.0460
130.0595
143.0689
152.0508
156.0933
168.0716
169.0874
170.0793
184.0826
186.1037
50 55 60 65 70 75 80 85 90 95 100 105 110 115 120 125 130 135 140 145 150 155 160 165 170 175 180 185 190
m/z
C
100
%
0
93.0334
100.0120
120.0448
184.0133
284.0184
60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300
m/z

# Appendix I
# SUBMITTED ARTICLES

# Article A1:

Rodríguez-Rodríguez, C.E., Barón, E., Gago-Ferrero, P., Jelić, A., Llorca, M., Farré, M., Díaz-Cruz, S., Eljarrat, E., Petrović M., Caminal, G., Barceló, D., Vicent, T.

## Removal of pharmaceuticals, polybrominated flame retardants and UV filters from sludge by the fungus *Trametes versicolor* in bioslurry reactor

J. Hazard. Mater. (2012) *Submitted*.

Elsevier Editorial System(tm) for Journal of Hazardous Materials
Manuscript Draft

Manuscript Number: HAZMAT-D-12-00489

Title: Removal of pharmaceuticals, polybrominated flame retardants and UV-filters from sludge by the fungus Trametes versicolor in bioslurry reactor

Article Type: Research Paper

Keywords: sewage sludge; Trametes versicolor; degradation; emerging pollutants; bioslurry

Corresponding Author: gloria caminal, Dr

Corresponding Author's Institution:

First Author: Carlos Rodriguez

Order of Authors: Carlos Rodriguez; Enrique Barón; Pablo Gago-Ferrero; Aleksandra Jelic; Marta Llorca; Marinella Farré; M. Silvia Díaz-Cruz; Ethel Eljarrat; Mira Petrovic; Gloria Caminal; Damià Barceló; Teresa Vicent

Abstract: Conventional wastewater treatments are inefficient in the removal of many organic pollutants. The presence of these contaminants in the final sludge represents a source of environmental pollution due to the increasing use of biosolids in land application. A biotechnological approach which employed the fungus Trametes versicolor in a sludge-bioslurry reactor was assessed in order to remove several groups of emerging pollutants. Biological fungal activity was monitored by means of ergosterol and laccase determinations. Fifteen out of 24 detected pharmaceuticals were removed at efficiencies over 50% after the treatment, including eight completely degraded. Removal ranged between 16%-53% and 22%-100% for the brominated flame retardants and the UV filters, respectively. Only two of all the detected compounds remained unchanged after the treatment. Despite the good elimination results, the toxicity of the final sludge increased, contrary to similar treatments of sludge with T. versicolor in solid-phase.

Barcelona, January 23rd , 2012.

Dear Editor,

I am herewith enclosing the manuscript "**Removal of pharmaceuticals, polybrominated flame retardants and UV-filters from sludge by the fungus *Trametes versicolor* in bioslurry reactor"** to be considered for publication in the *Journal of Hazardous Materials.* This manuscript has not been previously published, in whole or in part, it is not under consideration by any other journal, and it has not been sent to HAZMAT previously. All authors are aware of, and accept responsibility for the manuscript; additionally, the authors mutually agreed the submission to HAZMAT.

The **word count** of the manuscript is **4701** (excluding references).

Our manuscript describes a biotechnological approach for the removal of several emerging pollutants from real sewage sludge, which employs a lab-scale bioreactor with *Trametes versicolor*. Promising results included the removal of more than 20 pharmaceuticals and more than 10 UV-filters and brominated flame retardants, all of them at the pre-existent concentrations in the sludge. Based on the results, the authors consider that this manuscript could fit to the interests of HAZMAT readers.

Thank you for your time and consideration.

Sincerely yours,

Glòria Caminal

***Novelty Statement**

Since current treatment techniques do not efficiently remove micropollutants from sludge in WWTP, they can enter the environment. Published reports usually focus on the occurrence/fate of micropollutants but little is known about alternative strategies for their removal. In this manuscript we demonstrated for the first time the simultaneous biodegradation of several groups of emerging-pollutants (i.e. pharmaceuticals, UV-filters and brominated-flame-retardants; more than 30 compounds in total) naturally-occurring at pre-existent concentrations in sewage sludge by a white-rot fungus (*Trametes versicolor*). The configuration of bioslurry reactor was employed. The results could be a reference for possible applications of fungi in wastewater treatment.

***Highlights**

- Sludge from a WWTP was treated in a fungal slurry reactor with *Trametes versicolor*.
- Twenty-four pharmaceuticals were removed at important extents.
- UV-filters and brominated flame retardants were also degraded.
- Overall toxicity of sludge increased despite the pollutant removal.

# Removal of pharmaceuticals, polybrominated flame retardants and UV-filters from sludge by the fungus *Trametes versicolor* in bioslurry reactor

Carlos E. Rodríguez-Rodríguez[1,2], Enrique Barón[3], Pablo Gago-Ferrero[3], Aleksandra Jelić[3], Marta Llorca[3], Marinella Farré[3], M. Silvia Díaz-Cruz[3], Ethel Eljarrat[3], Mira Petrović[4,5], Glòria Caminal[1,*], Damià Barceló[3,5,6], Teresa Vicent[7]

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[3] Department of Environmental Chemistry, Institute of Environmental Assessment an Water Research (IDAEA), Spanish Council for Scientific Research (CSIC), Jordi Girona 18-26, 08034 Barcelona, Spain

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Tel: +34 93 581 2144; email: Gloria.Caminal@uab.cat

**Abstract**

Conventional wastewater treatments are inefficient in the removal of many organic pollutants. The presence of these contaminants in the final sludge represents a source of environmental pollution due to the increasing use of biosolids in land application. A biotechnological approach which employed the fungus *Trametes versicolor* in a sludge-bioslurry reactor was assessed in order to remove several groups of emerging pollutants. Biological fungal activity was monitored by means of ergosterol and laccase determinations. Fifteen out of 24 detected pharmaceuticals were removed at efficiencies over 50% after the treatment, including eight completely degraded. Removal ranged between 16%-53% and 22%-100% for the brominated flame retardants and the UV filters, respectively. Only two of all the detected compounds remained unchanged after the treatment. Despite the good elimination results, the toxicity of the final sludge increased, contrary to similar treatments of sludge with *T. versicolor* in solid-phase.

**Key words:** sewage sludge, *Trametes versicolor*, degradation, emerging pollutants, bioslurry

## 1. Introduction

Sludge is the residue generated during the biological treatment process of municipal wastewater [1]. Nowadays the fate of final sludge from wastewater treatment plants (WWTPs), also known as biosolids, includes cement production, and composting or thermal dewatering with subsequent land application. The latter option is internationally favoured for sludge management, as it contributes to recycling nutrients and improving soil properties and fertility [2]. However, due to the incapability of conventional wastewater treatments to completely remove undesirable agents, sludge contains contaminants such as metals, pathogens and organic pollutants and though current regulations require pathogen reduction and monitoring of metals, the concern on organopollutants is only limited to some compounds, as shown in the EU's Working Document on Sludge [3]. Hence, there is a lack of legislation for most of the groups of emerging pollutants, including pharmaceuticals, polybrominated diphenyl ethers (PBDEs) and UV-filters.

Pharmaceutically active agents represent an important fraction of the organic contaminants present in urban aquatic environments, where they reach after human or veterinary consumption or discharge. Moreover, the main transfer pathway for pharmaceuticals of human use to enter the environment is via WWTPs [2].

Polybrominated diphenyl ethers (PBDEs) comprise a group of brominated flame retardants, extensively employed in plastics, textiles and other materials [2]. Due to the potential threat to health and environment, PBDEs were listed as Persistent Organic Pollutants (POPs) in 2008 by the United Nations Environment Programme (UNEP) [4]. These compounds are routinely detected in sewage sludge in the range of a few mg $kg^{-1}$ (on a dry basis), while trace amounts (ng $L^{-1}$) have been also found in treated effluents [5], making them a source of PBDE contamination for the environment [6]. BDE 47, BDE 99 (pentaBDE)

and BDE209 (decaBDE) are the congeners that comprise the majority of PBDEs in sludge [2].

UV-filters are added to sunscreens and some other cosmetics. In sunscreens, the concentration of a specific UV-filter varies from 0.5% to 10%, but may be as high as 25% [7]. Their accumulation in the environment has been demonstrated, due both to direct input in water sources from swimming or bathing activities or industrial wastewater discharges, and indirect input, especially related to discharge from WWTPs [8], as they have been detected in treated wastewater and digested sewage sludge [9,10].

Potential effects of emerging pollutants in the environment range from the increase of antibiotic resistance in the case of antimicrobial agents, thyroidal disruption, neuro-developmental defects and cancer for PBDEs [2], to the possible endocrine-disrupting effects of UV-filters [11].

In this context, the search of green technologies to mitigate the pollution derived from emerging pollutants is of high interest. Bioremediation is a popular alternative to conventional methods for the treatment of wastes; in particular, white-rot fungi (WRF) have been successfully employed for the removal of a wide range of xenobiotics. They have been applied in the removal of textile dyes, PAHs, PCBs and other compounds, as reviewed elsewhere [12]. However, reports of WRF application on sludge are still scarce, and include the demonstration of sludge colonization by *Trametes versicolor* [13] and its use for the elimination of pharmaceuticals with concomitant reduction in toxicity in solid-phase sludge systems [14].

The present work aimed to evaluate the ability of *T. versicolor* to remove several groups of organic micropollutants from sterile sludge in a bioslurry system. The use of a bioslurry approach represents an advantage over solid-phase treatments, as the sludge can be employed directly from the effluent of the anaerobic digester. More precisely, some biological

parameters of the process were determined together with the fate of pharmaceuticals, PBDEs and UV-filters, as well as the changes in toxicity after the fungal treatment.

## 2. Materials and methods

### *2.1 Chemicals and reagents*

All the pharmaceutical standards for target compounds were of high purity grade (>90%). Naproxen, ketoprofen, diclofenac and gemfibrozil were supplied by Jescuder (Rubí, Spain). Indomethacin, mefenamic acid, phenazone, bezifibrate, mevastatin, fenofibrate, pravastatin (as sodium salt), carbamazepine, famotidine, ranitidine (as hydrochloride), cimetidine (as hydrochloride), erythromycin (as hydrate), roxitromycin, clarithromycin, josamycin, tylosin a, sulfamethazine, trimethoprim, chloramphenicol, atenolol, sotalol, metoprolol (as tartrate), timolol, pindolol, nadolol, salbutamol, clenbuterol (as hydrochloride), enalapril (as maleate), glibenclamide, furosemide, hydrochlorothiazide and metronidazole were purchased from Sigma-Aldrich (Steinheim, Germany). Standard atorvastatin (as calcium salt) was provided by LGC Promochem (London, UK), while diazepam, lorazepam and butalbital were from Cerilliant (Texas, USA).

The isotopically labelled compounds, used as internal standards, were sulfamethazine-$d_4$, famotidine-$^{13}C_3$, rac-timolol-$d_5$ maleate, clarithromycin-n-methyl-$d_3$, atorvastatin-$d_5$ sodium salt, fenofibrate-$d_6$, metoprolol-$d_7$, metronidazole hydroxyl-$d_2$, pravastatin-$d_3$, ketoprofen-$^{13}C$,$d_3$, indomethazine-$d_4$, rac-naproxen-$d_3$, mefenamic acid-$d_3$, gemfibrozil-$d_6$, bezafibrate-$d_4$ and furosemide-$d_5$ from Toronto Research Chemicals; diazepam-$d_5$ and phenobarbital-$d_3$ from Cerilliant (Texas, USA); atenolol-$d_7$, carbamazepine-$d_{10}$, ibuprofen-$d_3$, clotrimazole-$d_5$, enalapril–$d_5$, hydrochlorothiazide-$d_2$, glyburide-$d_3$, albuterol-$d_3$, cimetidine-$d_3$, antipyrine-$d_3$, acetaminophen-$d_4$, diclofenac-$d_4$, clofibric-$d_4$ acid, hydrochlorothiazide-3,3-

$d_2$ from CDN Isotopes (Quebec, Canada); sotalol hydrochloride $d_6$ from Dr. Ehrenstorfer (Augsburg, Germany) and erythromycin-$^{13}C,d_3$ (N-methyl-$^{13}C,d_3$) from Isotec (Ohio, USA).

The individual standard solutions as well as isotopically labelled internal standard solutions were prepared according to Jelić et al. [15].

The PBDE native compounds stock solution BFR-PAR, BDE-77, BDE-181 and $^{13}C_{12}$-BDE-209 were purchased from Wellington Laboratories (Guelph, Ontario, Canada). The components of BFR-PAR solution were: 3 di-BDEs (7, 10, 15), 3 tri-BDEs (17, 28, 30), 5 tetra-BDEs (47, 49, 66, 71, 77), 5 penta-BDEs (85, 99, 100, 119, 126), 7 hexa-BDEs (138, 139, 140, 153, 154, 156, 169), 5 hepta-BDEs (171, 180, 183, 184, 191), 6 octa-BDEs (196, 197, 201, 203, 204, 205), 3 nona-BDEs (206, 207, 208), deca-BDE (209), pentabromoethylbenzene (PBEB), hexabromobenzene (HBB) and decabromodiphenylethane (DecaBDEthane).

Analytical standards of benzophenone-3 (BP3), octocrylene (OC), ethylhexyldimethyl PABA (OD-PABA), 2,4-dihydroxybenzophenone (BP1), 4-hydroxybenzophenone (4HB), 4,4'-dihydroxybenzophenone (4DHB) and the isotopically labelled compound benzophenone-$d_{10}$, used as internal standard, were of the highest purity and were obtained from Sigma-Aldrich (Steinheim, Germany); 4-methylbenzylidenecamphor (4MBC) was supplied by Dr Ehrenstorfer (Augsburg, Germany); and ethylhexylmethoxycinnamate (EHMC) by Merck (Darmstadt, Germany). Standards solutions for the analysis of UV filters in sewage sludge are described in Gago-Ferrero et al. [16].

Malt extract was obtained from Scharlau (Barcelona, Spain). Glucose, $NH_4Cl$ and thiamine (<99%) were purchased from Sigma-Aldrich (Barcelona-Spain).

*2.2 Fungal strain*

The strain *T. versicolor* ATCC 42530 was acquired from the American Type Culture Collection, and maintained by subculturing every 30 days on 2% malt extract agar slants (pH 4.5) at 23 °C. *T. versicolor* pellets were produced by inoculating a 1 L Erlenmeyer flask containing 250 mL malt extract medium with 1 mL blended mycelium suspension and shaking (135 rpm) at 25 °C for 5 d [17].

*2.3 Sewage sludge*

Sewage sludge was obtained from the wastewater treatment plant of El Prat de Llobregat. The plant, located near Barcelona, Spain, has a total treatment capacity of two million equivalent inhabitants and it is a typical biological activated sludge (AS) plant with sludge anaerobic digestion and thermal dehydration. Sludge employed in the fungal slurry bioreactor was obtained from the outlet of the anaerobic digestor (3.6% w/w in solid content).

*2.4 Bioslurry reactor and operation conditions*

An 8 L steel stirred tank bioreactor with a working volume of 6 L was used. The reactor was equipped with a pH controller (InPro 325X probe, M300 controller, Mettler Toledo, Urdof, Switzerland) and an oxygen probe (InPro 6800, Mettler Toledo, Urdof, Switzerland). The reactor was filled with 5.34 L thermally sterilized (15 min at 121 ºC, twice) sewage sludge, supplemented with 600 mL macronutrient solution, 60 mL micronutrient solution [18] and 5 g $L^{-1}$ glucose, 2.1 g $L^{-1}$ $NH_4Cl$ and 10 mg $L^{-1}$ thiamine. Concentrated HCl was added (~45 mL) in order to adjust the pH to the set point, which was 4.5 ± 0.3. Agitation with an anchor stirrer was set at 115 rpm, which supported the establishment of the homogeneous slurry. Aeration was supplied in order to keep the dissolved oxygen level over 40%. Temperature was maintained at 25 °C by a continuous flux of water from a RA120 cooling thermostat (Lauda, Koenigshofen, Germany) to the reactor jacket. The bioreactor was

inoculated with an amount of *T. versicolor* pellets equivalent to 1.8 g biomass $L^{-1}$ (measured as dry weight, dw), and operated in batch mode for 26 d. Glucose was frequently measured (YSI 2700 Analyzer, Yellow Springs, OH) and supplied at a concentration of approximately 5 g $L^{-1}$ when completely depleted.

### *2.5 Analytical methods*

#### *2.5.1 Sample preparation and analysis of pharmaceuticals*

After the treatment with *T. versicolor*, the whole content of the reactor was lyophilized (Virtis Sentry freeze-drying equipment, Gardiner, NY). An equal amount of untreated sewage sludge was also lyophilized and analyzed to provide the reference values. The two types of samples, i.e. treated and untreated sludge, were grounded and extracted using an accelerated solvent extraction (ASE) system as described in detail by Jelić et al. [15]. Concentrated extracts were diluted in water and purified/preconcentrated by solid phase extraction and analyzed by high performance liquid chromatography coupled to a hybrid triple quadrupole – linear ion trap mass spectrometer (HPLC-QLIT-MS/MS) according to the previously developed multi-residual methodology for analysis of pharmaceuticals in wastewater [19].

#### *2.5.2 Sample preparation and analysis of BFRs*

One gram (dw) of sample was spiked with internal standards (BDE-77, BDE-181 and $^{13}C_{12}$-BDE-209), and kept overnight to equilibrate. Pressurized liquid extraction (PLE) was carried out using a fully automated ASE 200 system (Dionex, Sunnyvale, CA, USA). Extraction cell was heated to 100ºC and extraction was carried out using a mixture of hexane:dichloromethane (1:1). Extracts were subjected to an acid treatment with concentrated sulphuric acid, followed by a cleanup with a solid phase extraction (SPE) cartridge of alumina.

Analysis were performed by gas chromatography coupled to mass spectrometry working with negative ion chemical ionization (GC-NCI–MS) on a trace GC ultra gas chromatograph connected to a dual stage quadrupole mass spectrometer (Thermo Electron, Texas, USA). A DB-5ms capillary column (15 m, 0.25 mm i.d., 0.1 μm film thickness, Agilent, California, USA) was used with ammonia as the carrier gas. Quantification of di- to penta-BDEs, PBEB and HBB was carried out by internal standard procedure using BDE-77, whereas for hexa- to hepta-BDEs, BDE-181 standard was used. In the case of octa- to deca-BDEs and DecaBDEthane, the quantification was carried out using $^{13}C_{12}$-BDE-209 as internal standard.

Quality parameters of applied methodology include: recoveries ranging from 56% to 110%, relative standard deviation (RSD) from 0.6% to 8%, and limit of detection (LOD) between 1 and 86 pg $g^{-1}$ dw [20,21].

*2.5.3 Sample preparation and analysis of UV filters*

Samples were extracted by pressurized liquid extraction (PLE) using an automatic extractor ASE 200 (Dionex Corporation, Sunnyvale, CA, USA). Aliquots of freeze-dried and grinded sludge (1 g) were mixed in the extraction cells with aluminium oxide in order to perform the purification step within the cell. The PLE extract (c.a. 20 mL) was brought to 25 mL with methanol. A 2 mL aliquot of this solution was passed through 0.45 μm filter to a Lc-vial and finally the extract was reconstituted in 250 μL of acetonitrile.

Analyses were performed by ultra high resolution liquid chromatography-tandem mass spectrometry, using an Acquity UPLC chromatograph attached to a TQD mass spectrometer (Waters) according to the previously developed multi-residual methodology for analysis of UV filters in sewage sludge [16].

*2.5.4 Ergosterol quantification*

Ergosterol was measured in homogeneously-mixed lyophilized samples. Extraction and quantification were performed as previously described [13]. Ergosterol content was expressed as milligrams per gram of solid dry weight (mg $g^{-1}$ dw).

*2.5.5 Laccase activity*

Laccase determination was performed in centrifuged (15 000 *g*, 15 min) samples from the liquid phase of the bioslurry. Laccase activity was measured using a modified version of the method for the determination of manganese peroxidase [22], which employs 2,6-dimethoxyphenol as the substrate (extinction coefficient: 24 800 $M^{-1}cm^{-1}$, [23]). Results were expressed as activity units (U) per milliliter. One U was defined as the number of micromoles of DMP oxidized per min.

*2.5.6 Ecotoxicological studies*

Two standard toxicity assays were applied for the toxicity studies: *Daphnia magna* [24] and *Vibrio fischeri* [25]. Toxicity of each sample was evaluated in duplicate with the test based on immobilization of *Daphnia magna*, and the samples were run in triplicate with the tests based on the bioluminescence inhibition of *V. fischeri*. Data treatment was performed using GrapPad Prism 4.00. In addition toxicity units for each sample were calculated according to the Sprague equation [26]: $TU_{ij}= (EC_{50})^{-1}{}_{ij}$ x 100, where $(EC_{50})_{ij}$ is the 50% effective concentration.

*2.5.6.1 D. magna 24–48 h acute immobilization tests*

The tests were performed using neonates which were hatched in about 3 d from the eggs at 20–22 °C, under continuous illumination of 6000 lux. Immobility at 24 h and 48 h is

the bioassay endpoint, assumed to be equivalent to mortality. For each test a control solution was included. A bench of dilutions of each sample was tested using two replicates for each dilution with 10 neonates per replicate. *D. magna* neonates exposed to the blanks and to the different dilutions were incubated in darkness at 20 °C. After 24 h and 48 h of exposure the number of immobilized organisms was determined.

*2.5.6.2 Bioluminescence inhibition of Vibrio fischeri*

Bacterial reagents were reconstituted just prior to the analysis and the pre-incubation times followed standard protocols. Osmolality was adjusted in order to obtain a 2% of saline in each sample dilution. In all measures, the percentage of inhibition (% I) was determined by comparing the response given by a saline control solution to that corresponding to the diluted sample. Each dilution was tested in triplicate. The concentration of a sample which causes a 50% of bioluminescence inhibition after exposure for 15 or 30 min is assigned as the $EC_{50}$ value, for that time of exposure. Tests were performed at 15 °C.

## 3. Results and discussion

*3.1 Growth and activity of T. versicolor in the bioslurry reactor*

A bioslurry system was employed to study the ability of *T. versicolor* to biodegrade several groups of emerging pollutants from sewage sludge. Although the solid content in slurry reactors employed for ex-situ remediation of soils is usually over 5% [27], in the present study the content was approximately 3.6%, the concentration in the outlet of the anaerobic digester of the WWTP. In this regard, to mimic real conditions, the reactor operated for 26 d, which corresponded to the residence time of the sludge in the digestor.

The results of growth and activity of *T. versicolor* are shown in Figure 1. Ergosterol, main sterol of fungal cell membranes, was employed as an indicator of fungal growth [28],

given the impossibility to apply direct techniques for biomass quantification in the slurry. Time course results revealed a continuous increase in fungal biomass, reaching a maximum value of 2.05 mg $g^{-1}$ dw. Glucose consumption profile supports that fungal growth during the treatment.

Production of laccase was monitored during the process as an indicator of the oxidative potential of the system. Moreover, this extracellular activity is of great interest, since it is involved in the removal of diverse organic contaminants [12]. Laccase activity was detected in the reactor only between days 14 and 18, with a peak of 28.5 U $L^{-1}$ at day 18. Although the pH was set at 4.5, near the optimum for maximum laccase production [29], enzymatic activity was lower if compared to previous reports with higher sludge contents, up to 25% solids [13], nonetheless, in that case slurries were at bottle-scale and with orbital agitation. In the present study shear stress exerted by the stirrer may have contributed to the reduced laccase production, as it has been previously described for ligninolytic enzymes in other WRF [27,30]. Despite the absence of laccase activity in the last stage of the treatment, the steep increase in ergosterol during this period indicated the presence of metabolically active biomass.

### *3.2 Removal of emerging pollutants from sludge by T. versicolor in bioslurry reactor*

#### *3.2.1 Pharmaceuticals*

Among pharmaceuticals, the highest concentrations in the raw sludge were found for the analgesic drugs diclofenac (209 ng $g^{-1}$) and ibuprofen (135 ng $g^{-1}$) and the sulfonamide antibiotic sulfathiazole (143 ng $g^{-1}$). They were followed by the diuretic compounds hydrochlorothiazide (41.3 ng $g^{-1}$), furosemide (79.9 ng $g^{-1}$) and the analgesic ketoprofen (42.4 ng $g^{-1}$). The remaining pharmaceuticals were found at concentrations below 40 ng $g^{-1}$, as it is shown in the table 1.

The fungal treatment resulted in the highly efficient removal of histamine H2 receptor antagonists (three drugs), β-blockers (two drugs), barbiturates (one drug) and antidiabetic compounds (one drug), with a complete elimination within 26 d. Also high, though not complete, was the removal of sulfonamide antibiotics: sulfapyridine (100%), sulfamethazine (91%) and sulfathiazole (86%), followed by the diuretic drugs with removals ranging from 65% to 83%.

Some therapeutic groups were more resistant to degradation/transformation by fungi, as the removal efficiency of the treatment was low to moderate (20-50%). These groups included the phenazone type drugs (phenazone, 44.7%), lipid regulators and cholesterol lowering statin compounds (three drugs, 41.1-49.8%) and the psychiatric drugs carbamazepine and diazepam with the lowest removal values of 30.7% and 26.1%, respectively. Regarding the removal of carbamazepine by *T. versicolor*, a similar sludge slurry system achieved an efficiency of 57% [13]. The result for carbamazepine is remarkable though, since this compound is usually recognized as non-degradable in conventional AS and membrane bioreactor treatments for wastewater [31-33]. This result confirms the recently demonstrated degradation of carbamazepine by *T. versicolor* in aqueous phase [34].

In the case of analgesics, removal values varied from very high for ibuprofen (91.9%), over medium for indomethacine and diclofenac (~60%), to medium-low values observed for ketoprofen, naproxen and mefenamic acid (~40-50%). Similar removal of naproxen, i.e. 47%, was previously obtained in a smaller scale spiked slurry system containing *T. versicolor* and 25% solid sludge [13].

The degradation of some of these compounds has been demonstrated in liquid medium with *T. versicolor*. Moreover, cytochrome P450 and/or laccase seem to be involved in the fungal-mediated transformation of sulfamethazine, ketoprofen, naproxen, diclofenac and carbamazepine [34-39], and other ligninolytic enzymes for other fungi [40,41].

*3.2.2 BFRs*

Regarding the group of BFRs, its most abundant congener was, by far, deca-BDE-209 at a concentration of 232 ng $g^{-1}$. The remaining most common congeners were present at concentrations below 10 ng $g^{-1}$ (Table 2). Only decaBDEthane, a considered "emerging BFR" was detected at 26.3 ng $g^{-1}$. Though tri-BDE-28, penta-BDE-100 and hexa-BDE-153 are also considered among the most abundant in municipal sewage sludge, none of them was detected in the raw sludge. Removal after the slurry ranged from 16% to 53%, and only hepta-BDE-183 showed total recalcitrance to the fungal treatment. The most degradable was hexa-BDE-154 at 53%, while deca-BDE-209 was removed at 38%; however, no pattern in the removal was observed according to the bromination degree of the molecules. Biotransformation of PBEs has been recently demonstrated by bacterial action in both aerobic and anaerobic environments, including soil, sediments and anaerobic degradation in sewage sludge [42-45]; however, degradation with WRF is more scarce [46,47]. The high $\log K_{ow}$ values of BFR, which lead to very low aqueous concentrations, have been considered as a limiting factor for biologically mediated transformation processes.

*3.2.3 UV filters*

The most abundant UV filters in the raw sludge were OC and OMC, found at 7.71 µg $g^{-1}$ and 1.03 µg $g^{-1}$, respectively. 4-MBC was detected at a lower concentration (0.520 µg $g^{-1}$) and the remaining at concentrations below 0.1 µg $g^{-1}$ (Table 2). It is well known that UV filters tend to adsorb on the solid phase fraction of the sludge, thus it was expected that removal would be higher for the least hydrophobic compounds (such as 4DHB and BP3, $\log K_{ow}$ 2.55 and 3.79) and lower for the most hydrophobic ($\log K_{ow}$: 5.8, 6.15 and 7.35 for OMC, OD-PABA and OC, respectively). However, that pattern was not followed in the

removal profiles. This might suggest one or more of the following possibilities: i. bioavailability is not a limitant factor for degradation ii. the fungus was able to properly colonize the solid particles in the slurry or iii. desorption to reestablish equilibrium occurred during degradation in aqueous phase. Removal values ranged from 22% to 100%, and just 4DHB remained unchanged after the fungal treatment (which also occurred only to the aforementioned BFR hepta-BDE-183). The degradation of 4-MBC and BP3 by *T. versicolor* in liquid medium has been described, with participation of cytochrome P450 in the former and laccase in the latter [48,49].

### *3.3 Toxicity*

Contrary as expected, the results of both standardized toxicity tests with *D. magna* and *V. fischeri* showed an acute increase on toxicity of the treated sludge. Figure 2 shows toxicity values before and after the treatment in the bioslurry reactor. Previous sludge treatments using *T. versicolor* in biopile systems proved to be an efficient approach to remove several organic pollutants and reducing toxicity of final sludge [14]. Different factors could have affected the results in the bioslurry, which seems to produce more toxic metabolic intermediates after 26 d. First, in the case of bioslurries a reduction of pH of the sludge was done with hydrochloric acid in order to enhance the *T. versicolor* growth rate. This acidic treatment could also lead to the formation of degradation intermediates with higher polarity and higher toxicity than the parent compounds. In addition, in order to perform the ecotoxicity assays, the pH of the sludge samples was readjust to the neutral range, and the samples were treated again with sodium hydroxide, producing a new disturbance on the initial nature of the samples. Therefore, these toxicity values cannot be considered as conclusive and it should be taken into account the good results obtained by using *T. versicolor* in previous studies, when its growth was carried out under neutral conditions. In future works it should be consider to avoid drastic

pH changes, and maybe increase the time of residence in case on bioslurry to achieve better toxic removals. Results are not completely surprising, as an increase in the toxicity has been shown in aqueous-phase degradation studies for some of the micropollutants here detected, including ibuprofen [50], carbamazepine [34] and BFR [46]. These observations may suggest that contaminants are more bioavailable in the slurry than in solid phase. In addition, aqueous media could favor abiotic reactions of the intermediates which are less likely to occur in solid phase.

### *3.4 Comparative performance of bioslurry vs solid-state T. versicolor systems in the removal of emerging pollutants*

The degradation of emerging pollutants from sludge by *T. versicolor* has been reported in some recent works through the application of solid-phase systems [14,35,46,48]. These reports have in common the use of a lignocellulosic residue (WSP) which acts both as a bulking agent and as a substrate/carrier for the fungus, and the fact that the sludge employed comes from the final stage (thermal dehydration) of the same WWTP. Therefore, the initial concentrations (before the fungal treatment) vary if compared to the sludge employed in the bioslurry, given that in the dried sludge the most hydrophobic compounds would have been removed during the mechanical drying (centrifugation). The present section aims to compare the performance of both fungal systems: bioslurry and solid-phase, when it comes to the removal of emerging pollutants. Table 3 shows the occurrence of emerging pollutants in sewage sludge and the removal after solid-phase treatment with *T. versicolor*.

Comparison of the efficiency removal is shown in Figure 3, which includes only the pollutants found in both kinds of sludge before fungal treatment. Global results indicate that a solid-phase treatment is more efficient than the bioslurry process. Only two compounds were better removed by the bioslurry, ibuprofen and hydrochlorothiazide. This finding could be

ascribed to the higher hydrophilic nature of these pollutants, which may be translated into a higher bioavailability for the fungus in the liquid phase. In the case of a few compounds (cimetidine, atenolol and sulfapyridine), complete degradation was accomplished regardless of the treatment employed, but in most of the cases the elimination was significantly higher in solid-phase. That was especially highlighted in the case of some UV filters (4DHB and BP3), several pharmaceuticals (phenazone and bezafibrate) and the deca-DBE-209, as the removal in solid-phase was more than twice the obtained in bioslurry.

Highly remarkable is the difference in the remaining toxicity after both treatments, as it has been discussed in section 3.3.

## 4. Conclusions

The potential of *T. versicolor* as a bioremediation agent for the simultaneous degradation of different groups of pollutants was demonstrated in sewage sludge. The removal of pre-existent micropollutants from this complex matrix was assessed for the first time in a fungal bioslurry reactor, resulting in the elimination of several pharmaceuticals, UV filters and BFR. Despite the versatile degradation showed by *T. versicolor* in the biolurry reactor, the elimination performance of the fungus is higher in solid-phase systems, where in addition, a significant reduction in the toxicity of the residual sludge is obtained. Eventhough we clearly recommend fungal biopiles over bioslurries to remove micropollutants, a proper risk assessment should be performed before the application of the final sludge in agricultural lands.

## Acknowledgements

This work was supported by the Spanish Ministries of MMAMRM (project 010/PC08/3-04) and MICIN (project CTQ2010-21776-C2-01). The Department of Chemical Engineering of

the Universitat Autònoma de Barcelona is the Unit of Biochemical Engineering of the Xarxa de Referència en Biotecnologia de la Generalitat de Catalunya. The authors are members of a Consolidated Research Group of Catalonia (2009-SGR-656). C. Rodríguez-Rodríguez is recepient of a predoctoral fellowship from Universidad de Costa Rica and Consejo Superior de Investigaciones Científicas (UCR-CSIC collaboration).

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**Figure captions**

**Fig. 1.** Time course of ergosterol content (□), laccase activity (▼) and glucose concentration (●) in a bioslurry reactor for the treatment of sewage sludge with *T. versicolor*.

**Fig. 2.** Toxicity of the sewage sludge before and after the fungal treatment in the bioslurry reactor. Assays: *V. fischeri* ( ■ ) and *D. magna* ( ■ ).

**Fig. 3.** Comparison of removal efficiencies for several emerging pollutants during different strategies of sewage sludge treatment with *T. versicolor*. Treatment strategies: biopile like system ( ■ ) and bioslurry reactor ( □ ). Only compounds found in both kinds of sludge are included.

**Table legends:**

**Table 1.** Occurrence of pharmaceuticals in sewage sludge and residual concentration after treatment in a bioslurry reactor with *T. versicolor*. Concentrations are referred to a dw basis.

**Table 2.** Occurrence of emerging pollutants in sewage sludge and residual concentration after treatment in a bioslurry reactor with *T. versicolor*. Concentrations are referred to a dw basis.

**Table 3**. Occurrence of emerging pollutants in thermally dehydrated sewage sludge and their residual concentration after solid-phase treatment with *T. versicolor.*

Table 1

**Table 1.** Occurrence of pharmaceuticals in sewage sludge and residual concentration after treatment in a bioslurry reactor with *T. versicolor*. Concentrations are referred to a dw basis.

| Therapeutic groups | Compounds | CAS Number | Internal standards | Raw sludge | | Treated sludge | | Removal (%) |
|---|---|---|---|---|---|---|---|---|
| | | | | Concentration[a] (ng $g^{-1}$) | RSD[b] (%) | Concentration[a] (ng $g^{-1}$) | RSD[b] (%) | |
| Analgesics / anti-inflammatories | Ketoprofen | 22071-15-4 | Ketoprofen $d_3$ | 42.4 | 6.83 | 21.3 | 1.01 | 49.8 |
| | Naproxen | 22204-53-1 | Naproxen $d_3$ | 6.17 | 0.91 | 3.38 | 0.68 | 45.3 |
| | Ibuprofen | 15687-27-1 | Ibuprofen $d_3$ | 135 | 14.1 | 10.9 | 0.88 | 91.9 |
| | Indomethacine | 53-86-1 | Indomethacine $d_4$ | 9.60 | 1.00 | 3.65 | 0.29 | 61.9 |
| | Diclofenac | 15307-86-5 | Diclofenac $d_4$ | 209 | 5.86 | 84.0 | 1.37 | 59.8 |
| | Mefenamic acid | 61-68-7 | Mefenamic acid $d_3$ | 14.2 | 3.05 | 8.37 | 0.20 | 41.1 |
| Phenazone type drugs | Phenazone | 60-80-0 | Antypyrine $d_3$ | 36.7 | 10.9 | 20.3 | 3.64 | 44.7 |
| Lipid regulators and cholesterol lowering statin drugs | Bezafibrate | 41859-67-0 | Bezafibrate $d_4$ | 11.8 | 1.21 | 6.43 | 0.26 | 45.3 |
| | Gemfibrozil | 25812-30-0 | Gemfibrozil $d_6$ | 14.2 | 3.05 | 8.37 | 0.20 | 41.1 |
| | Atorvastatin | 134523-00-5 | Atorvastatin $d_5$ | 38.0 | 3.36 | 19.1 | 0.57 | 49.8 |
| Psychiatric drugs | Diazepam | 439-14-5 | Diazepam $d_5$ | 7.71 | 0.49 | 5.70 | 0.72 | 26.1 |
| | Carbamazepine | 298-46-4 | Carbamazepine $d_{10}$ | 29.2 | 2.57 | 20.2 | 3.49 | 30.7 |
| Histamine H2 receptor antagonists | Ranitidine | 66357-35-5 | Famotidine $^{13}C$ | 7.92 | 0.78 | n.d.[c] | - | 100 |
| | Famotidine | 76824-35-6 | Famotidine $^{13}C$ | 12.0 | 0.78 | n.d. | - | 100 |
| | Cimetidine | 51481-61-9 | Cimetidine $d_3$ | 10.4 | 1.31 | n.d. | - | 100 |
| Sulfonamide antibiotics | Sulfamethazine | 57-68-1 | Sulfathiazole $d_4$ | 6.1 | 22.0 | 0.5 | - | 91.0 |
| | Sulfapyridine | 144-83-2 | Sulfathiazole $d_4$ | 21.4 | 2.6 | n.d. | - | 100 |
| | Sulfathiazole | 72-14-0 | Sulfathiazole $d_4$ | 143.0 | | 20.1 | 26.4 | 85.9 |
| ß-blockers | Atenolol | 29122-68-7 | Atenolol $d_7$ | 1.70 | 0.17 | n.d. | 0.00 | 100 |
| | Sotalol | 3930-20-9 | Sotalol $d_6$ | 4.88 | 0.89 | n.d. | 0.00 | 100 |
| Barbiturates | Butalbital | 77-26-9 | Phenobarbital $d_5$ | 16.3 | 2.30 | n.d. | 0.00 | 100 |
| Diuretics | Hydrochlorothiazide | 58-93-5 | Hydrochlorthiazide $d_2$ | 41.3 | 0.50 | 6.89 | 0.11 | 83.3 |
| | Furosemide | 54-31-9 | Ibuprofen $d_3$ | 79.9 | 11.3 | 27.7 | 2.30 | 65.3 |
| Antidiabetics | Glibenclamide | 10238-21-8 | Glibenclamide $d_3$ | 17.4 | 1.94 | n.d. | 0.00 | 100 |

[a]Mean detected concentration (n=3); [b]Relative standard deviation of mean concentration (n=3); [c] n.d.: not detected

**Table 2.** Occurrence of emerging pollutants in sewage sludge and residual concentration after treatment in a bioslurry reactor with *T. versicolor*. Concentrations are referred to a dw basis.

| Group of emerging contaminants | | Compounds | Raw sludge | | Treated sludge | | Removal (%) |
|---|---|---|---|---|---|---|---|
| | | | Concentration[a] (ng $g^{-1}$) | RSD[b] (%) | Concentration[a] (ng $g^{-1}$) | RSD[b] (%) | |
| **Brominated flame retardants** | **PBDEs** | Tri-BDE-28 | n.d.[c] | - | n.d. | - | |
| | | Tetra-BDE-47 | 5.39 | 13 | 4.11 | 10 | 23.7 |
| | | Penta-BDE-99 | 6.05 | 23 | 3.24 | 25 | 46.4 |
| | | Penta-BDE-100 | n.d. | - | n.d. | - | |
| | | Hexa-BDE-153 | n.d. | - | n.d. | - | |
| | | Hexa-BDE-154 | 5.48 | 35 | 2.56 | 15 | 53.3 |
| | | Hepta-BDE-183 | 0.61 | 13 | 0.62 | 12 | 0 |
| | | Deca-BDE-209 | 232 | 5.5 | 145 | 16 | 37.5 |
| | **Emerging BFRs** | HBB | n.d. | - | n.d. | - | |
| | | PBEB | n.d. | - | n.d. | - | |
| | | DecaBDEthane | 26.3 | 22 | 22.1 | 18 | 16.0 |
| | | | Concentration (µg $g^{-1}$) | RSD (%) | Concentration (µg $g^{-1}$) | RSD (%) | |
| **UV filters** | | BP1 | 0.08 | 7 | n.d | - | 100 |
| | | 4DHB | 0.051 | 7 | 0.050 | 9 | 1 |
| | | BP3 | 0.034 | 10 | 0.019 | 7 | 22 |
| | | 4-MBC | 0.520 | 11 | 0.205 | 11 | 61 |
| | | OC | 7.71 | 11 | 3.214 | 8 | 58 |
| | | OD-PABA | 0.012 | 11 | 0.004 | 12 | 70 |
| | | OMC | 1.031 | 7 | 0.211 | 8 | 79 |

[a]Mean detected concentration (n=3); [b]Relative standard deviation of mean concentration (n=3); [c] n.d.: not detected

**Table 3**

**Table 3**. Occurrence of emerging pollutants in thermally dehydrated sewage sludge and their residual concentration after solid-phase treatment with *T. versicolor.*

| Group of emerging contaminants | Compounds | Raw sludge | | Treated sludge | | Removal[c] (%) | Reference |
|---|---|---|---|---|---|---|---|
| | | Concentration[a] (ng $g^{-1}$) | RSD[b] (%) | Concentration[a] (ng $g^{-1}$) | RSD[b] (%) | | |
| **Pharmaceuticals** | Ibuprofen | 85.9 | 10.7 | 13.1 | 9.2 | 75 | [14,35] |
| | Diclofenac | 60.3 | 15.9 | 13.6 | 11.0 | 64 | |
| | Mefenamic acid | 17.9 | 11.7 | 3.1 | 16.1 | 72 | |
| | Phenazone | 9.6 | 22.9 | n.d. | - | 100 | |
| | Bezafibrate | 4.5 | 2.2 | n.d. | - | 100 | |
| | Fenofibrate | 4.2 | 14.3 | n.d. | - | 100 | |
| | Atorvastatin | 37.8 | 9.3 | 4.7 | 23.4 | 80 | |
| | Diazepam | 19.3 | 14.5 | 6.8 | 2.9 | 43 | |
| | Carbamazepine | 25.6 | 19.5 | 9.1 | 1.1 | 43 | |
| | Cimetidine | 11.4 | 5.3 | n.d. | - | 100 | |
| | Clarithromycin | 21.0 | 10.5 | n.d. | - | 100 | |
| | Atenolol | 13.6 | 16.2 | n.d. | - | 100 | |
| | Hydrochlorothiazide | 26.7 | 11.6 | 8.0 | 10.0 | 52 | |
| | Sulfamethazine | 19.1 | 25.4 | n.d. | - | 100 | |
| | Sulfapyridine | 29.4 | 15.6 | n.d. | - | 100 | |
| | Sulfathiazole | 71.1 | 9.0 | n.d. | - | 100 | |
| **Brominated flame retardants (BFRs)** | Deca-BDE-209 | 285 | 5 | 41.0 | 14 | 86 | [46] |
| | | Concentration (µg $g^{-1}$) | RSD (%) | Concentration (µg $g^{-1}$) | RSD (%) | | |
| **UV filters** | 4DHB | 0.07 | 6 | n.d | - | 100 | [48] |
| | BP3 | 0.06 | 11 | n.d | - | 100 | |
| | 4-MBC | 3.10 | 9 | 0.40 | 11 | 87 | |
| | OC | 8.00 | 11 | 0.92 | 10 | 89 | |
| | OMC | 2.20 | 5 | 0.16 | 5 | 93 | |

[a]Mean detected concentration (n=3); [b]Relative standard deviation of mean concentration (n=3)

[c] Removal values were calculated considering that the treated sludge contained 62% sludge and 38% WSP (w/w).

Figure 1

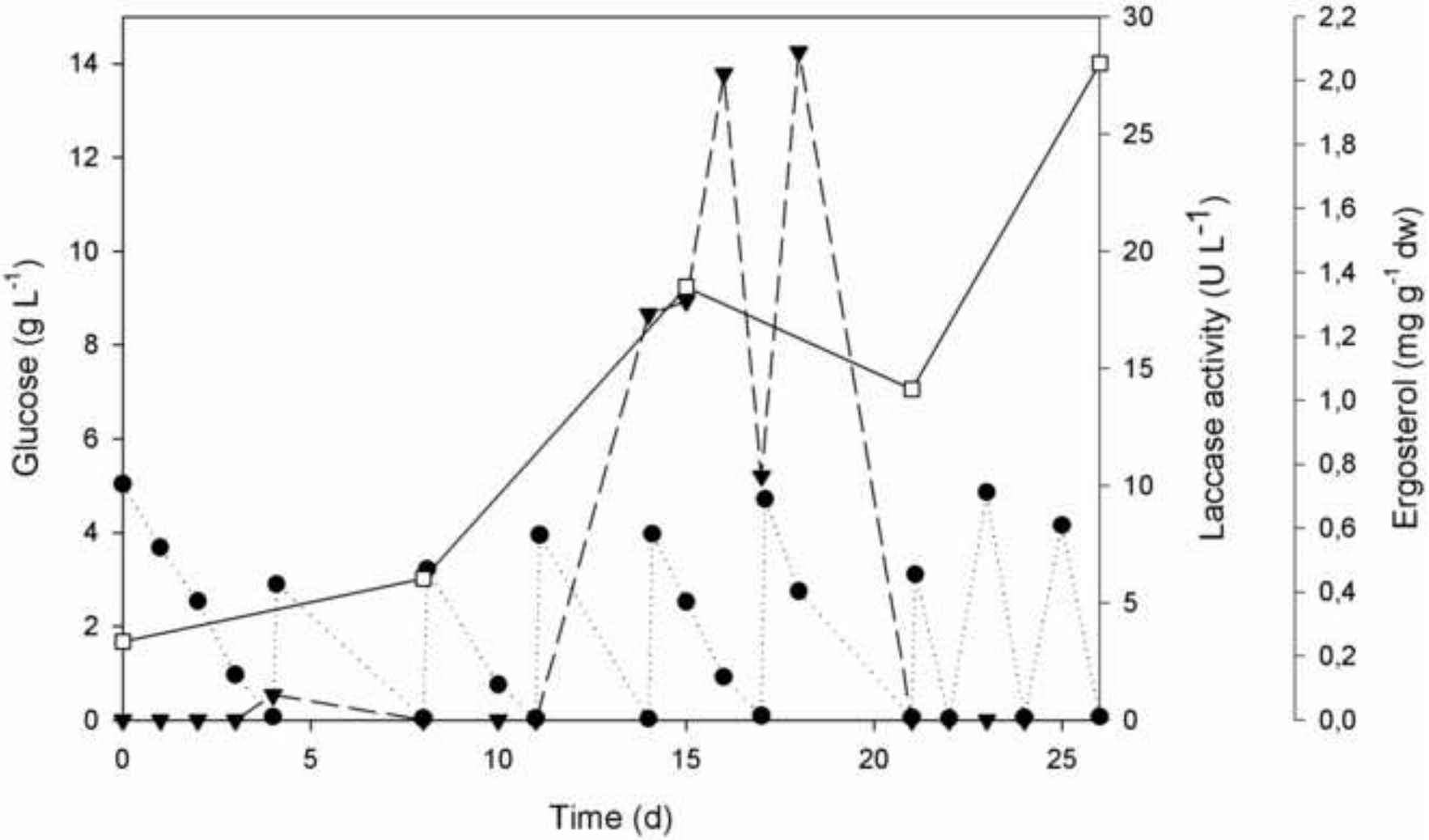

Glucose (g L-1)
Laccase activity (U L-1)
Ergosterol (mg g-1 dw)
Time (d)
0
2
4
6
8
10
12
14
0
5
10
15
20
25
30
0,0
0,2
0,4
0,6
0,8
1,0
1,2
1,4
1,6
1,8
2,0
2,2

**Figure 2**

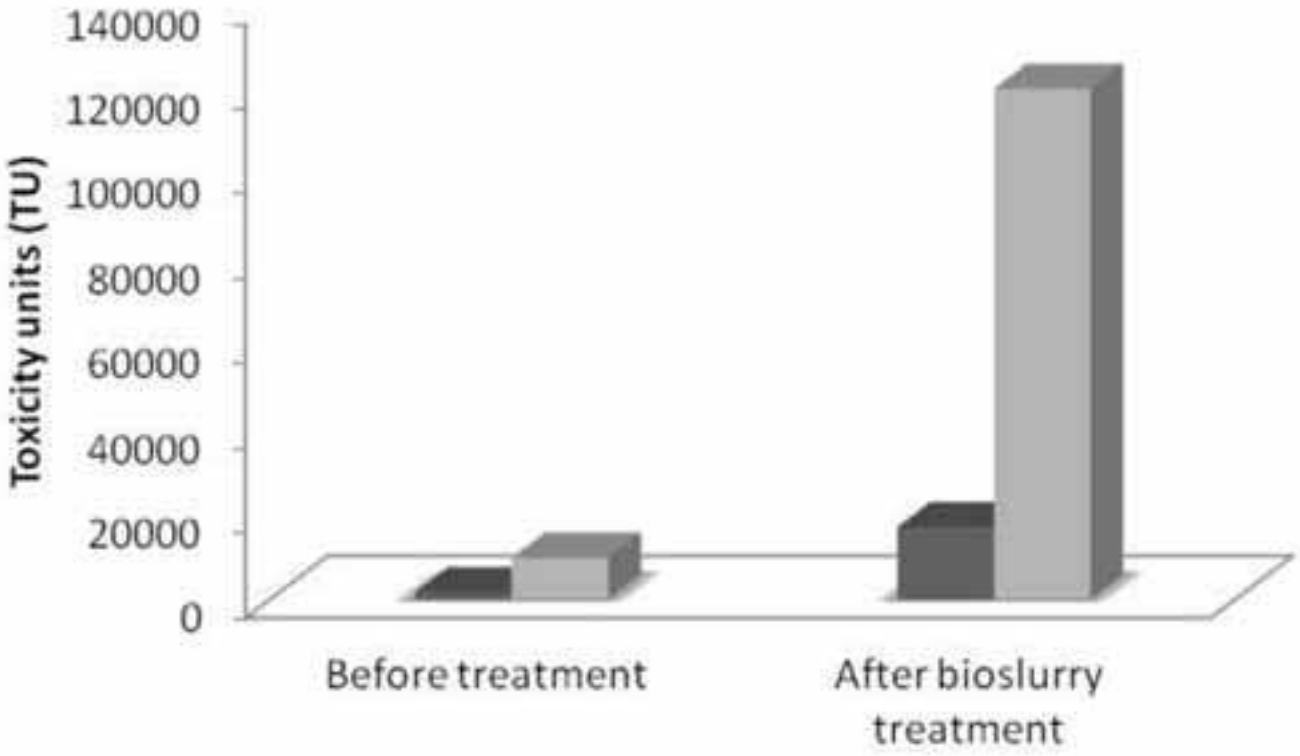
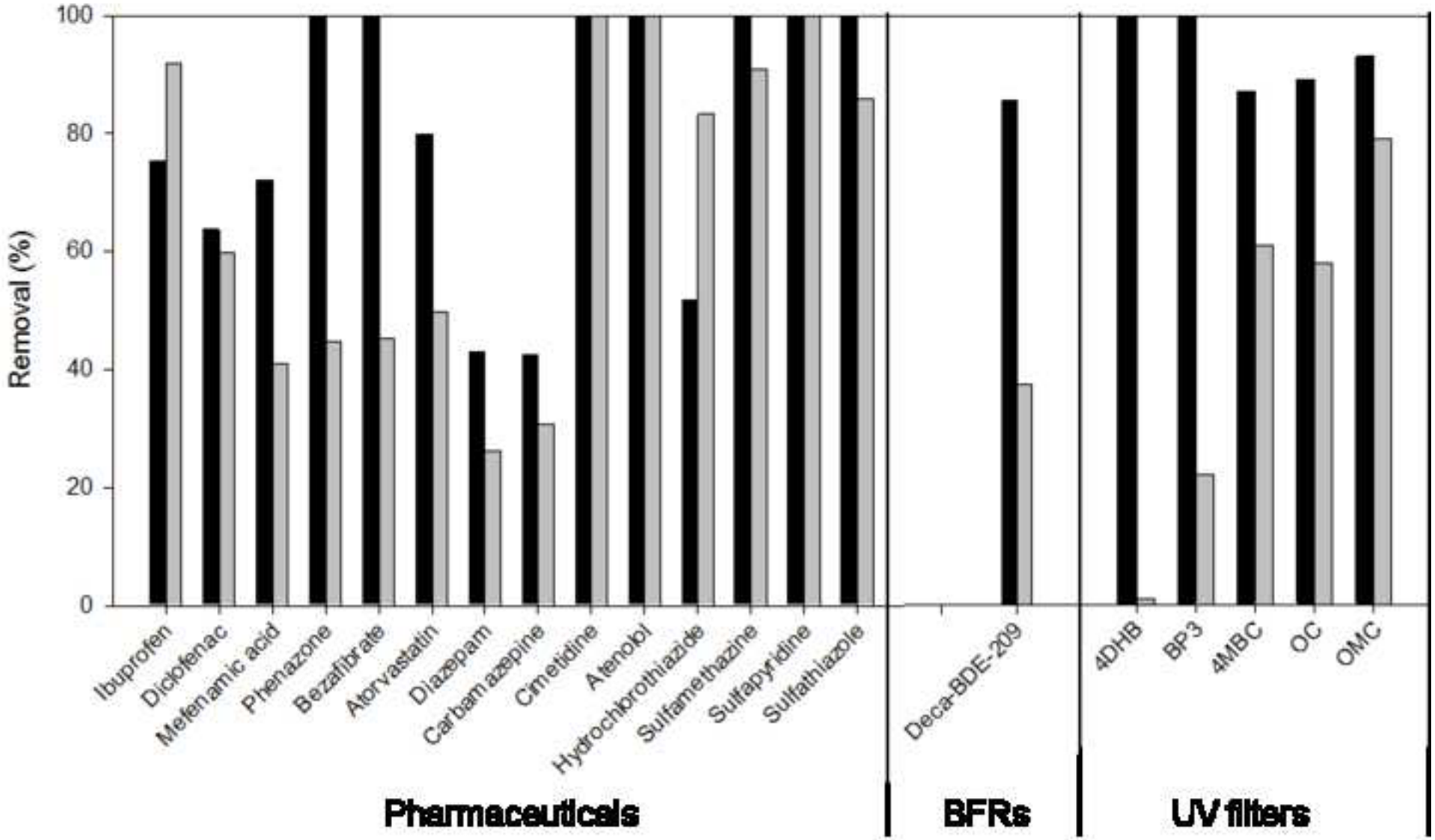

140000
120000
100000
80000
60000
40000
20000
0
Toxicity units (TU)
Before treatment
After bioslurry treatment

Figure 3


Removal (%)
100
80
60
40
20
0
Ibuprofen
Diclofenac
Mefenamic acid
Phenazone
Bezafibrate
Atorvastatin
Diazepam
Carbamazepine
Cimetidine
Atenolol
Hydrochlorothiazide
Sulfamethazine
Sulfapyridine
Sulfathiazole
Deca-BDE-209
4DHB
BP3
4MBC
OC
OMC
Pharmaceuticals
BFRs
UV filters

Dear editor:

Changes in the format of the manuscript were performed according to the indications from the technical check, as follows:

Comments:

1) Novelty statement provided should not exceed the maximum length of 100 words.

Novelty statement was reduced to less than 100 words.

2) References in reference list should be numbered sequentially with each number within square brackets.

Square brackets were added to the reference list.

3) Table 2 provided in the manuscript is not cited in the text.

Table 2 was cited in the text accordingly (sections 3.2.2 and 3.2.3)

The authors expect that after these corrections, the manuscript can undergo the reviewing procedure.

Regards,

Carlos E. Rodríguez-Rodríguez

Universitat Autònoma de Barcelona

# Article A2:

Rodríguez-Rodríguez, C.E., Jelić, A., Pereira, M.A., Sousa, D.Z., Petrović, M., Alves, M.M., Barceló, D., Caminal, G., Vicent, T.

## Bioaugmentation of sewage sludge with *Trametes versicolor* in solid-phase biopiles produces degradation of pharmaceuticals and affects microbial communities

Environ. Sci. Technol. (2012) *Submitted* .

# Bioaugmentation of sewage sludge with Trametes versicolor in solid-phase biopiles produces degradation of pharmaceuticals and affects microbial communities



| | |
|---|---|
| Journal: | *Environmental Science & Technology* |
| Manuscript ID: | es-2012-01788n |
| Manuscript Type: | Article |
| Date Submitted by the Author: | 04-May-2012 |
| Complete List of Authors: | Rodríguez, Carlos; Universitat Autònoma de Barcelona, Chemical Engineering<br>Jelic, Aleksandra; Environmental Assessment and Water Research (IDAEA), Environmental Chemistry<br>Pereira, Alcina; IBB-Institute for Biotechnology and Bioengineering, Center of Biological Engineering,<br>Sousa, Diana; IBB-Institute for Biotechnology and Bioengineering, Center of Biological Engineering,<br>Petrovic, Mira; Institució Catalana de Reserca i Estudis Avançats (ICREA),<br>Alves, Madalena; Universidade do Minho, Departamento de Engenharia Biológica<br>Barcelo, Damia; IDAEA-CSIC, Environmental Chemistry Department<br>caminal, Gloria; Universitat Autònoma de Barcelona, Enginyeria Quimica<br>Vicent, Teresa; Universitat Autònoma de Barcelona, Enginyeria Quimica |
| | |

# Bioaugmentation of sewage sludge with *Trametes versicolor* in solid-phase biopiles produces degradation of pharmaceuticals and affects microbial communities

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**Abstract**

The use of sludge (biosolids) in land application may contribute to the spread of organic micropollutants, as wastewater treatments do not completely remove these compounds. Therefore, the development of alternative strategies for sludge treatment is a matter of recent concern. The elimination of pharmaceuticals at pre-existent concentrations from sewage sludge was assessed, for the first time, in non-sterile biopiles by means of fungal bioaugmentation with *Trametes versicolor* (BTV-systems) and compared with the effect of autochthonous microflora (NB-systems). The competition between the autochthonous fungal/bacterial communities and *T. versicolor* was studied, using denaturing gradient gel electrophoresis (DGGE) and the cloning/sequencing approach. An inhibitory effect exerted by *T. versicolor* over bacterial populations was suggested. However, after 21 days, *T. versicolor* was no longer the main taxon in the fungal communities. The elimination profiles revealed an enhanced removal of atorvastatin-diclofenac-hydrochlorothiazide (during the whole treatment) and ranitidine-fenofibrate (at short periods) in the BTV biopiles in respect to NB biopiles, coincident with the presence of the fungus. For ibuprofen-clarithromycin-furosemide, the elimination profiles were similar irrespective of the system, and with carbamazepine no significant degradation was obtained. The results suggest that fungal colonization is a promising process for pharmaceuticals remediation in complex matrices such as biosolids.

## INTRODUCTION

Pharmaceuticals comprise a diverse group of therapeutic compounds intended to improve health. The expanding world population, discovery of new drugs and the use of cheaper generic agents are some of the reasons of the increasing use of pharmaceuticals.[1] After human or veterinary consumption, pharmaceuticals reach urban aquatic environments, ending up in the wastewater treatment plants (WWTPs), which are usually regarded as the main points of discharge of pharmaceuticals into ecosystems.[2] The recent concern on the occurrence and fate of pharmaceuticals in WWTPs and receiving surface waters and its subsequent monitoring, have revealed the incapability of conventional wastewater treatments to completely remove, and thus prevent the entrance of these emerging pollutants into the environment.[3,4] Although a fraction of the pharmaceuticals is removed from the aqueous phase of wastewater, important amounts remain adsorbed in the solid sludge, which for some compounds represents the main removal route during wastewater treatment.[5] The sewage sludge constitutes a final residue in the WWTPs. The application of sludge (biosolids) in agriculture as soil amendment or in landfills, is one of the most applied solutions for their disposal.[6,7] However, legislation on biosolids only focuses on heavy metal and bacterial pathogens, and more recently in some organic pollutants such as polychlorinated biphenyls, polycyclic aromatic hydrocarbons, dioxins and halogenated compounds,[8] without considering pharmaceuticals and other groups of micropollutants. Therefore, biosolids become another important input of pharmaceuticals to the environment.[9]

Nowadays, although the potential impact of pharmaceuticals in the environment is little understood, studies have emerged showing their potential deleterious impact on

wildlife and possible effects on ecosystems.[3,10-13] Moreover, the potential risk of toxicity due to mixtures, as those found in sludge, has gained more attention.[3]

In this context, the search of alternative approaches, especially eco-friendly treatments for the elimination of organic pollutants from sludge, is a matter of great interest. Bioremediation has appeared as an advantageous clean-up technique of xenobiotics from the environment, and bioaugmentation, the addition of pre-grown microorganisms to enhance the degradation of unwanted compounds, is a promising approach of bioremediation.[14] White-rot fungi (WRF) comprise a group of extensively studied organisms capable to degrade a wide range of pollutants by means of their extracellular ligninolytic enzymes and intracellular enzymatic complexes. The degrading versatility of WRF has been reviewed elsewhere,[15-17] however their use in bioaugmentation processes has been reduced due to their limited colonization potential.[18] Most of the research on degradation by WRF has been performed in defined media or sterile matrices and bioaugmentation in solid phase is reduced to few reports in soil for the elimination of PAHs[19-22] or pentachlorophenol.[23-25] Moreover, bioaugmentation of sewage sludge with single microorganisms has only been tested with bacteria,[26] and as far as the authors know, never for the treatment of emerging pollutants.

In previous works, we demonstrated the proper colonization of sewage sludge by the WRF *Trametes versicolor* and its ability to remove pharmaceuticals (both spiked and pre-existent) from this complex matrix in sterile conditions.[27,28] The present work focuses in the next step, which deals with the challenge of applying the fungus in non-sterile sludge, where the indigenous microbiota may counteract the fungal growth and its degrading ability.

This paper describes a bioremediation process which employs *T. versicolor* as a bioaugmentation agent for the removal of pre-existent pharmaceuticals from sewage sludge. The dynamics of the fungal and bacterial communities during the process was also monitored to understand how indigenous microflora is affected by the inoculation of an external fungus.

## MATERIALS AND METHODS

### Pharmaceutical standards

All the pharmaceutical and the corresponding isotopically labelled standards were of high purity grade (>90%). Detailed information on the providers of the analytical standards can be found elsewhere.[29] The individual standard solutions as well as isotopically labelled standard solutions were prepared according to Gros et al.[30] The solvents, HPLC grade methanol, acetonitrile, water (Lichrosolv) and formic acid (98%) were provided by Merck (Darmstadt, Germany).

### Sewage sludge and bulking material

Dry sewage sludge was obtained from the wastewater treatment plant of El Prat de Llobregat. The plant is located near Barcelona, Spain and it has a total treatment capacity of two million equivalent inhabitants. It is a typical biological activated sludge plant with sludge anaerobic digestion and thermal drying. Sludge employed in the experiments was obtained from the final stage of processing, i.e., after thermal drying (~10% water content). The wheat-straw pellets (WSP, ATEA Praha s.r.o., Czech Republic) used both as bulking material and lignocellulosic substrate for fungal growth in bioaugmentation experiments were kindly provided by Č. Novotný.

**Solid-phase bioremediation treatments**

Two treatments were carried out to evaluate the efficiency of pharmaceuticals degradation in sewage sludge and the dynamics of microbial communities: i.) *non-bioaugmented treatment* (addition of WSP, moisture and regulated temperature, NB) and ii.) *bioaugmentation* with the fungus *T. versicolor* (BTV). To compare the removal performance of *T. versicolor* alone, a sterilized system inoculated with the fungus was included (CTV).

**Microcosm description**

Sludge biopile systems with a total solid dry weight (DW) of approximately 368 g were prepared in plastic trays with a 20 x 27 cm surface and a height of 6 cm. The non-bioaugmented sludge contained 38% WSP as amendment (w/w, dry basis), which were hydrated (1:2 ratio, w/v) prior mixing with the sludge. The bioaugmented sludge contained 38% (w/w, dry basis) *T. versicolor* inoculum grown on WSP. The strain *T. versicolor* ATCC 42530 was acquired from the American Type Culture Collection. Inocula were prepared by the addition of a blended mycelium suspension[31] to WSP (0.65 mL per gram of dry WSP) and pre-growing for 7 d at 25 ºC. WSP were hydrated (1:2 ratio, w/v) prior mycelium addition. Both conditions were done in triplicate biopiles. Duplicate sterilized biopiles inoculated with *T. versicolor* were prepared with autoclaved sludge (121 ºC for 30 min) as described for bioaugmented sludge. In every case, biopiles were incubated for up to 42 d at 25 ºC, periodically homogenized and moisturized, and sampled for different analyses on times 0, 3, 6, 10, 14, 21, 28, 35 and 42 d; time-points are indicated on the text as tX, where X corresponds to the day of sampling.

## Analytical procedures

### Sample preparation and analysis of pharmaceuticals

The preparation of the solid samples for the analysis of selected pharmaceuticals was performed according to Jelić et al.[29] In brief, the samples were freeze dried and ground, and extracted using an accelerated solvent extraction system (ASE) (Dionex ASE 200, Dionex; Sunnyvale, CA). The concentrated extracts were diluted in HPLC water (methanol content < 5%), and processed by solid phase extraction using Oasis HLB (200 mg, 6 ml) cartridges (Waters, Milford, MA) at neutral pH. Instrumental analysis of the samples was performed by high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). HPLC analysis was performed using Symbiosis™ Pico (SP104.002, Spark, Holland), equipped with an autosampler and connected in series with a 4000 QTRAP Hybrid Triple Quadrupole - Linear Ion Trap mass spectrometer equipped with a Turbo Ion Spray source (Applied Biosystems-Sciex, Foster City, CA, USA). Chromatographic separation was achieved with a Purospher Star RP-18 endcapped column (125 mm × 2.0 mm, particle size 5 μm) preceded by a $C_{18}$ guard column (4 mm × 4mm, particle size 5 μm), both supplied by Merck (Darmstadt, Germany). The concentrations of pharmaceuticals were determined using a previously developed multi-residual methodology for analysis of pharmaceuticals.[30] The recoveries calculated for triplicate samples spiked with the target compounds at 50 ng/g DW ranged from 32 to 136%, with RSD <15%. The method quantification limits (MQL), calculated as ten times signal-to-noise, were in the range from 0.5 – 14 ng/g DW.

### Ergosterol quantification

Ergosterol was measured in homogeneously-mixed samples of solid-phase cultures. Extraction and quantification were performed as previously described.[27] Ergosterol content was expressed as milligrams per gram of solid DW.

**Laccase activity**

Laccase was extracted from solid samples according to Rodríguez-Rodríguez et al.[27] Activity was measured using a modified version of the method for the determination of manganese peroxidase,[32] using 2,6-dimethoxyphenol (DMP) as the substrate (extinction coefficient: 24 800 $M^{-1}cm^{-1}$ ).[33] Results were expressed as activity units (U) per gram of solid DW. One U was defined as the number of micromoles of DMP oxidized per min.

**ND24 test**

A test based on naproxen degradation, previously defined by Rodríguez-Rodríguez et al.[34] was employed to monitor the degrading ability of *T. versicolor* throughout the treatment. The test indicates the percentage of degradation of spiked naproxen in 24 h by comparing the remaining naproxen concentrations in active cultures to those in heat-killed controls.

**Microbial community analysis**

**Sampling and DNA extraction**

Homogenized samples were withdrawn from the biopiles over a period of 42 d and stored at -20 ºC. Total genomic DNA was extracted from approximately 0.4 g samples using the Fast DNA® Spin kit for soil (MP Biomedicals, USA), according to

the manufacturer's instructions. Extracted DNA was maintained at -20 ºC and used as a template for PCR amplification.

**PCR amplification**

Bacterial 16S rRNA genes and internal transcribed spacer (ITS) regions of fungal rRNA genes were amplified from total genomic DNA using a *Taq* polymerase kit (Invitrogen, Carlsbad, CA, USA). PCR amplification was performed in reaction mixture (50 µL) containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 200 µM each of the four deoxynucleoside triphosphates (dNTP), 1.25 U *Taq* polymerase, 200 nM each primer and 1 µL of properly diluted template DNA. All primers employed were synthesized by Invitrogen. Complete bacterial 16S rRNA genes were amplified for cloning and sequencing by using the forward primer Bact27-f (5'-GTT TGA TCC TGG CTC AG-3') and the universal reverse primer Uni1492-r (5'-CGG CTA CCT TGT TAC GAC-3').[35] 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 (25 cycles), and 72 ºC for 5 min. In the case of fungal ITS regions the forward primer ITS1F (5'-CTT GGT CAT TTA GAG GAA GTA A-3')[36] and the reverse primer ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3')[37] were employed. The thermocycling program was as described above but with an annealing temperature of 55 ºC. For DGGE analysis, PCR products were generated using bacterial 16S rRNA gene primers U968-f (5'-ACC GCG AAG AAC CTT AC-3') and L1401-r (5'-CGG TGT GTA CAA GAC CC-3')[38] for amplification of the V6-V8 bacterial region, and the fungal rRNA primers ITS1F and ITS2 (5'-GCT GCG TTC TTC ATC GAT GC-3').[37] A 40-base GC clamp (CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G) was attached to the primers U968-f and ITS1F at the 5' end. The thermocycling program was as described above but with 35 cycles, an

annealing temperature of 56 ºC for bacterial primers and elongation time of 1 min for fungal primers. The size and amount of PCR products was estimated by electrophoresis in 1% agarose gels (w/v) using a DNA marker and ethidium bromide staining.

**DGGE analysis**

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 20-60% 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 60 ºC. Gels were then stained with silver nitrate and scanned in an Epson Perfection V750 PRO (Epson, USA).

**Cloning and sequencing**

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 *E. cloni*® 10G Electrocompetent Cells (Lucigen® Corporation) according to the manufacturer's instructions. Positive transformants were selected after growth in LB medium supplemented with ampicillin. 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 sludge. 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 (5'-GAT TTA GGT GAC ACT ATA G-3') and T7 (5'-TAA TAC GAC TCA CTA TAG GG-3') (Promega), 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).

**Phylogenetic analysis**

Partial sequences were assembled using the CAP application included in the BioEdit v7.0.9 software package.[39] Similarity searches for the assembled sequences were performed using the NCBI Blast search program within the GenBank database (http://www.ncbi.nlm.nih.gov/blast/).[40]

## RESULTS

**Parameters of growth and activity in bioaugmentation with *T. versicolor***

Solid-phase biopile systems were employed to study the bioaugmentation of sewage sludge with *T. versicolor* for the removal of pharmaceuticals. The lignocellulosic WSP employed in the biopiles acted both as a substrate (C and N source) for fungal growth and a carrier of *T. versicolor*. Preliminary studies revealed that the preparation of the biopiles as described by Rodríguez-Rodríguez et al. in sterile conditions could not support the colonization of the non-sterile sludge by the fungus (data not shown); therefore, a gradual reduction in the moisture of the system was used as a strategy to favor the visual colonization of the matrix in the BTV biopiles. Figure 1 shows the results of ergosterol, employed as an indicator of fungal biomass,[41] and laccase, an extracellular enzyme of *T. versicolor*, directly related to the oxidative potential of the culture and involved in the degradation of several polutants.[15] BTV and

CTV presented similar ergosterol profiles (Figure 1A), with maximum values (0.091 mg $g^{-1}$ DW and 0.092 mg $g^{-1}$ DW, respectively) in the first days of the treatment. The profile of NB biopiles was more oscillatory, though values were usually higher than those shown by BTV and CTV biopiles after 6 d. However, by the end of the process, ergosterol in the NB biopiles matched the final value of the BTV biopiles. Laccase activity profile of BTV systems revealed high levels in the first 10 d (maximum 4.58 U $g^{-1}$ DW at day 3), with a posterior decrease and a new raise between t28 and t35 (Figure 1B). Results were similar, though higher, for the replicates of the CTV, with peak values of around 7.66 U $g^{-1}$ DW, but the expression of the enzymatic activity started later in this case. No activity was detected in the NB biopiles, except at t35 (0.08 U $g^{-1}$ DW), at a negligible value compared to the other systems. The ND24 test, employed as an indicator of degrading ability of *T. versicolor*, presented only significant degradation values at t6 (> 10%) in the BTV biopiles.

**Removal of pharmaceuticals from sludge**

From 43 pharmaceuticals analyzed, 9 were detected in the sewage sludge; their elimination profiles are shown in Figure 2. The most abundant therapeutic agents were the analgesic/anti-inflammatory compounds ibuprofen and diclofenac (Table 1). Removal of ibuprofen after 42 d was similar in all the bioremediation treatments, ranging from 55.9% to 61.0% (Table 1). Contrary, diclofenac was better degraded in the BTV (54.0%) and CTV (60.5%) biopiles, against only 22.0% in the NB systems.

Two lipid regulators, fenofibrate and atorvastatin, were detected. The former was easily removed from the sludge, as it completely disappeared at the end of the process, with or without bioaugmentation. On the other hand, atorvastatin was more

efficiently removed in the BTV (65.4%) and CTV (70.4%) biopiles, while elimination exclusively due to indigenous microflora reached 48.4%.

In the cases of the histamine H2-receptor antagonist ranitidine and the diuretic furosemide, the degradation was complete in all the systems, by the end of the treatment for the former, and by t10 for the latter. Similarly, for the antibiotic clarithromycin the removal was the same irrespective of the process, between 81.7% and 84.5% after 42 d. The other diuretic, hydrochlorothiazide showed a slightly better removal in the biopiles with *T. versicolor* (65.4% in BTV and 62.7% in CTV) than the NB biopiles (55.3%). Finally, carbamazepine, detected at an initial concentration of 10.5 ng $g^{-1}$ (± 0.7) was the only drug that remained unchanged regardless of the applied treatment.

**Dynamics and characterization of fungal and bacterial communities**

DGGE profiles were obtained for samples of the triplicate BTV and NB biopiles. Profiles in the replicates were highly similar for bacterial and fungal communities, and thus only the results of one of the replicates are included. A more detailed analysis of the structure of the microbial communities was also performed by cloning and sequencing analyses of the 16S rRNA gene and ITS regions of fungal rRNA gene Figure 3.

DGGE profiles of fungal communities are shown in Figure 3A and 3B. In the NB biopiles, bands were absent at t0 and only one band weakly appeared at t6; after t10 a higher fungal diversity was observed, with several bands that were present up to t42 (Figure 3A). Sample TV corresponds to the product amplified from the genomic DNA of a pure culture of *T. versicolor*. In the BTV biopiles this band prevailed alone until t10, and shared predominance in the community on t21; however, it disappeared by the end of the treatment at t42, when the predominant bands included those also found in

the NB biopiles (Figure 3B). Fungal sequences corresponding to bands F1, F5 and F8 showed high similarity to *Scopulariopsis brevicaulis*, a common soil saprophyte.[42] These bands developed with higher relative intensity in the samples from NB biopiles. Bands F2, F6 and F9, whose sequences matched with isolates of *Gymnascella dankaliensis* (99-100% identity) were predominant in BTV systems. The sequences corresponding to the bands F3 and F7 were 91% identical to the sequence of the cephalosporin-producer *Acremonium chrysogenum*,[43] which appeared by t42 in both systems. At t42 but only in samples from BTV biopiles a band (F4) appeared, whose sequence has a 99% identity to an isolate of *Gymnoascus* sp. Both *G. dankaliensis* and *Gymnoascus* sp. are gymnoascaceous keratinophilic fungi usually found in soils.[44] An additional clone not assigned in the DGGEs, with a gene sequence related to *Cephaliophora tropica* (98% identity), a soil fungus,[45] was obtained from the sample collected at t21 from the BTV biopiles. Overall results indicate that, with exception of *T. versicolor*, the fungal communities were similar in the NB and BTV treatments.

The DGGE profiles corresponding to the bacterial communities are presented in Figure 3C and 3D. The NB biopiles showed higher diversity (in terms of number of bands) than the BTV biopiles. Several bands appeared after t0, and although weak, some prevailed along the process. However, in the BTV biopiles the diversity observed at t0 disappeared during the treatment to produce the same pattern up to t42, which consisted of a predominant band that co-existed with the fungal population. The 16S rRNA gene sequence corresponding to band B1 showed high identity (99%) to an isolate of *Staphylococcus equorum*. This band was present in the NB biopiles but absent in the presence of *T. versicolor*. The two bands present throughout each of the processes, e.g. B2 and B3, have sequences that matched isolates belonging to the family Bacillaceae, *Oceanobacillus* sp. (99% identity) and *Bacillus* sp. (99% identity), which

are genera that normally occur in wastewater treatment systems.[46] An additional clone obtained from the sample collected at t10 from BTV biopiles, whose sequence was 100% identical to the 16S rRNA gene sequence of *Bacillus cereus*, could not be assigned to a band in the DGGE profiles.

## DISCUSSION

The present work addresses a fungal approach for the elimination of pharmaceuticals, a group of emerging pollutants, from thermally dried sewage sludge. The bioaugmentation with *T. versicolor* (BTV) was compared with the effect achieved with solely the indigenous microbiota of the sludge, which given its proliferation due to the use of WSP (observed in community analyses), could be considered as a biostimulation process (NB).

Although ergosterol has been previously applied to monitor fungal biomass production, results from NB biopiles (usually higher than BTV and CTV, contrary to what was expected given the size of the inoculum) suggest that it is not a suitable parameter to follow up the specific bioaugmentation with *T. versicolor*. This can be ascribed to the fact that ergosterol production (respect to total fungal biomass) is species-specific,[41] and growth of indigenous fungi in NB biopiles with higher ergosterol production yields could surpass the amount due only to *T. versicolor*. On the other hand, laccase determination seemed to be a good parameter to monitor activity of *T. versicolor* in the bioaugmented systems, as production was negligible in NB biopiles.

Fungal DGGE profiles of BTV biopiles demonstrated that *T. versicolor* was able to survive in the sludge system (at least during half of the process), i.e. the fungus was not inhibited by autochthonous microflora. Moreover, comparison of DGGE profiles of bacterial communities, on the contrary, revealed an inhibitory effect of *T. versicolor*

over bacteria, as diversity shown in NB biopiles was reduced to one predominant band in the profile of BTV biopiles. This finding corresponds to a usually challenging key factor to overcome in bioaugmentation with fungi, since these microorganisms are commonly surpassed by bacteria in terms of colonization ability. The success of fungi in situations of competition with indigenous microbiota widely differs,[20,47,48] in particular with respect to colonization of soil by *T. versicolor*.[18,49,50] In the present case, the colonization could have been enhanced by the reduction of microflora by the thermal treatment of the sludge in the WWTP and the application of low humidity conditions, together with the use of the WSP as lignocellulosic substrate that favors the activity of WRF. The sporulated nature of most of the bacterial taxa identified is not strange given the origin of the sludge (thermally dried), and might correspond to survivors of that thermal process. Similarly, *T. versicolor* seemed to delay the colonization of the system by other fungi, which appeared as intense bands at t10 and t21 in DGGE profiles from NB and BTV biopiles, respectively. Those fungi can also correspond to survivors of the stabilization treatment or to recontamination organisms.

Degradation of all the pharmaceuticals found in this work has been reported from sterile sludge systems with *T. versicolor* in solid-phase (except ranitidine and furosemide),[28] and in bioslurry reactors (except fenofibrate and clarithromycin).[51] The persistence of carbamazepine (widely known for its recalcitrance), which remained unchanged after the treatment, is remarkable under non-sterile conditions, as degradation values of 31% and 43% were obtained in sterile sludge systems with this fungus,[28,51] which could suggest a counteract effect of the indigenous flora on the degrading ability of the fungus towards this drug. Moreover, the degradation of this therapeutic drug has been evidenced by *T. versicolor* in liquid medium.[52] Nonetheless, several analytical issues which point the appearance of a carbamazepine metabolite at

the same retention time as the parent compound (during liquid phase degradation by *T. versicolor*), could indicate an underestimation in the degradation obtained in the biopiles (unpublished results).

For the removal of some pharmaceuticals no significant difference was obtained between BTV and NB biopiles, which indicates that the addition of the WSP may stimulate also the growth of indigenous microorganisms, as shown in NB-DGGE profiles. Likewise, degrading profiles of BTV and CTV systems are highly similar in most cases, which suggest that microbial interactions between the sludge microbiota and *T. versicolor* did not counteract the degrading ability of the fungus, a common issue when bioaugmentation agents are employed. In this respect, cooperation and antagonism phenomena have been described in microflora-fungi interactions in pollutant degradation processes.[47,48,53]

In general, the fungal-mediated treatments (BTV and CTV) tended to result in higher degradation in shorter periods (by t10 or t21) than non-bioaugmented systems. Nonetheless, by the end of the process the NB biopiles equated the degradation accomplished in the former systems for many pharmaceuticals. That accelerated removal effect due to the presence of *T. versicolor* was coincident with its predominance in the systems during the first half of the treatment, as demonstrated in the DGGE profiles. When comparing the removal of pharmaceuticals in NB and BTV biopiles, three kinds of profiles were observed: *i.)* compounds with higher removal in BTV biopiles; *ii.)* pharmaceuticals with similar removal regardless of the process; and *iii.)* drugs with negligible removal. The group *i.)* profiles include atorvastatin, diclofenac and hydrochlorothiazole, in which the action of *T. versicolor* resulted in enhanced removal values throughout the process. In the case of fenofibrate and ranitidine an improved removal in the BTV biopioles was obtained during the first 21 d

(complete elimination in the former), coincident with the period in which *T. versicolor* is the predominant taxon of the system, according to the results of the DGGEs of fungal communities, and more active according to the results from the ND24 test. However, a complete removal was achieved with both BTV and NB systems at the end of the process, indicating a slower but efficient degrading ability by the indigenous microbiota. Ibuprofen, clarithromycin and furosemide comprise the group *ii.)* degrading profiles, in which no clear difference between the BTV and NB systems was found. Complete elimination of furosemide was fast (by t10), while in the case of ibuprofen and clarithromycin a steep elimination occurred in the first 10 d followed by a plateau (~56-60% and 82-85%, respectively). These results suggest that the fungal bioaugmentation process is not necessary for the removal of such pharmaceuticals, yet the presence of the fungus does not negatively affect the elimination of the drugs. Group *iii.)* profiles includes only carbamazepine, which in this case was not significantly removed as indicated above. Though this therapeutic drug has been widely considered as a non-biodegradable compound in anaerobic digestion processes and WWTPs.[2,54], its depletion has been demonstrated in sterile conditions with *T. vesicolor* in liquid phase and sludge.[28,51,52]

Summarizing, important potential in bioremediation of sludge was demonstrated by *T. versicolor* for the elimination of pharmaceuticals. Molecular and analytical results suggest a reduction in the activity of *T. versicolor* after 21 d of treatment. Therefore, additional strategies such as fungal reinoculation should be conducted in order to improve and optimize a potential bioremediation process. Similarly, the degrading range of the fungus for the elimination of other emerging as well as traditional contaminants must be evaluated in these real conditions to estimate the reach of such process. Likewise, important, although usually slower removal of pharmaceuticals by

autochthonous microbiota should be further studied to consider the application of biostimulation or natural attenuation techniques as it has been described with partial success for some traditional environmental pollutants. The proposed treatment could be interesting from the applicability point of view, as the use of these composting-like technologies is widely extended in WWTPs.

**ACKNOWLEDGEMENTS**

This work was supported by the Spanish Ministries of MMAMRM (project 010/PC08/3-04) and MICIN (projects CTQ2010-21776-C2-01 and AIB2010PT-00169). The co-funds from CRUP – Rectors Council of Portuguese Universities, through the integrated Portuguese-Spanish action E51/11 are gratefully acknowledged. The Department of Chemical Engineering of the UAB is the Unit of Biochemical Engineering of the Xarxa de Referència en Biotecnologia de la Generalitat de Catalunya. C. Rodríguez-Rodríguez acknowledges UCR-CSIC for a predoctoral fellowship.

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## Tables

**Table 1.** Initial concentration of pharmaceuticals detected in the sewage sludge and degradation in different bioremediation strategies.

| Therapeutic agent | Initial concentration ± SD (ng $g^{-1}$ DW) | Degradation (%) | | |
|---|---|---|---|---|
| | | NB t42 | BTV t42 | CTV t42 |
| Fenofibrate | 13.7 ± 1.8 | 100,0 | 100,0 | 100,0 |
| Atorvastatin | 19.3 ± 2.6 | 48,4 | 65,3 | 70,4 |
| Hydrochlorothiazide | 10.8 ± 1.7 | 55,3 | 65,4 | 62,7 |
| Diclofenac | 53.6 ± 4.8 | 22,0 | 54,0 | 60,5 |
| Clarithromycin | 35.8 ± 6.8 | 84,5 | 81,7 | 83,5 |
| Ranitidine | 17.5 ± 1.3 | 100,0 | 100,0 | 100,0 |
| Carbamazepine | 10.5 ± 0.7 | 5,7 | -4,9 | 9,2 |
| Ibuprofen | 161.0 ± 21.4 | 61,0 | 55,9 | 60,5 |
| Furosemide | 23.4 ± 1.7 | 100,0 | 100,0 | 100,0 |

## Figure captions

**Figure 1.** Profiles of ergosterol (A) and laccase activity (B) over time in the bioremediation treatments of sewage sludge: NB (○), BTV (●), CTV (□). Values plotted are the means ± standard deviation (SD) for triplicate biopiles, except for TVE in B (two replicates individually plotted).

**Figure 2.** Time-course removal of detected pharmaceuticals in the sludge biopiles during a 42-d bioremediation treatment: NB (●), BTV (○) and CTV (▼).Values plotted are the means ± SD for triplicate biopiles.

**Figure 3.** DGGE profiles of fungal PCR-amplified ITS fragments and bacterial PCR-amplified 16S rRNA gene fragments from sludge microcosms over a period of 42 d. Fungal profiles: (A) non bioaugmented, NB and (B) bioaugmented, BTV; bacterial profiles: (C) non bioaugmented, NB and (B) bioaugmented, BTV. TV corresponds to the amplified product from genomic DNA of a *T. versicolor* pure culture. Phylogenetic affiliation of the retrieved ITS or 16S rRNA sequences and their corresponding band position in the DGGE profiles are shown on the right.

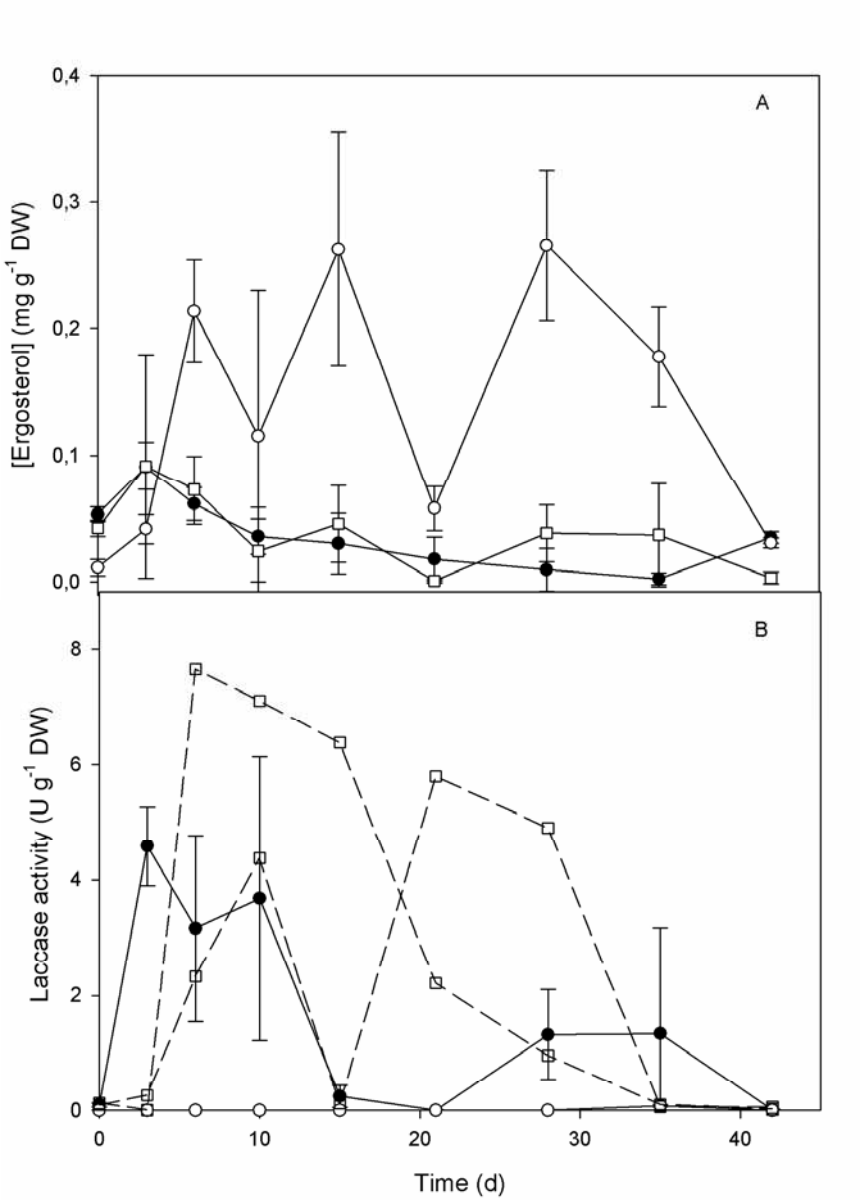


Figure 1
148x207mm (300 x 300 DPI)

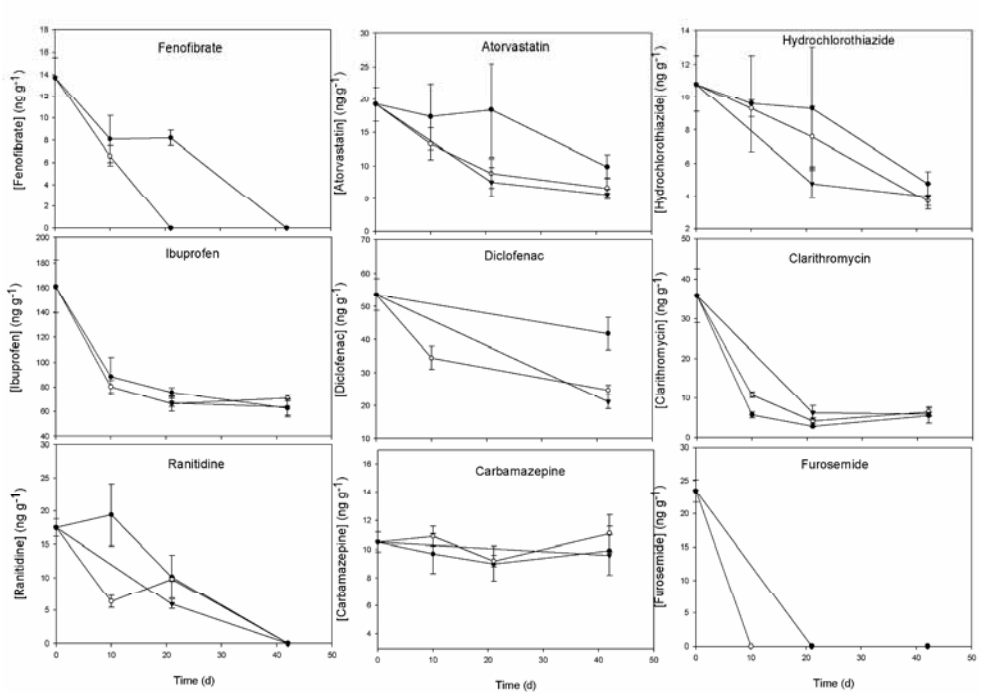


Figure 2
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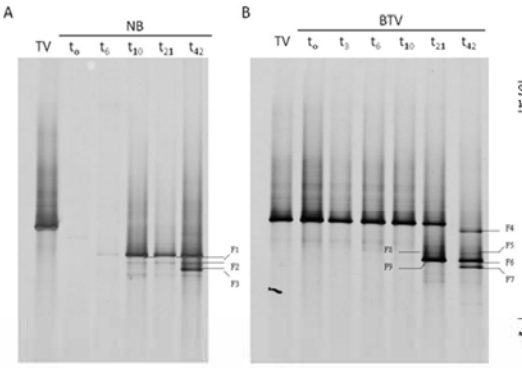



| Sequence length | DGGE band | Closest relative* | Identity (%) |
|---|---|---|---|
| 623 | F1/F5 | *Scopulariopsis brevicaulis* isolate 2737 | 99 |
| 624 | F2 | *Gymnascella dankaliensis* strain NRRL 1677 | 99 |
| 543 | F3/F7 | *Acremonium chrysogenum* | 91 |
| 622 | F4 | *Gymnoascus* sp. IFM 59328 | 99 |
| 624 | F6 | *Gymnascella dankaliensis* strain NRRL 1677 | 100 |
| 624 | F8 | *Scopulariopsis brevicaulis* isolate 2737 | 99 |
| 624 | F9 | *Gymnascella dankaliensis* strain NRRL 1677 | 99 |
| 506 | Not assigned | *Cephaliophora tropica* strain xsd08001 | 98 |

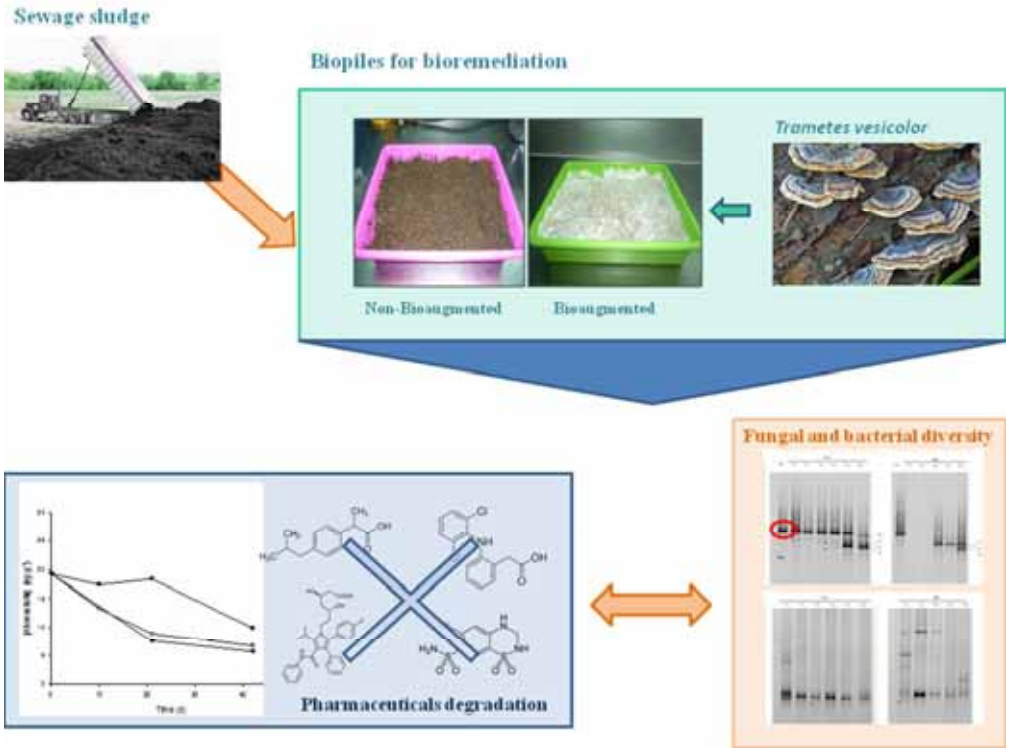
*Closest organisms in GenBank

| Sequence length | DGGE band | Closest relative* | Identity (%) | Order/Family[b] |
|---|---|---|---|---|
| 1478 | B1 | *Staphylococcus equorum* strain CTSP8 | 99 | Bacillales/ Staphylococcaceae |
| 1486 | B2 | *Oceanobacillus* sp. BSi20641 | 99 | Bacillales/Bacillaceae |
| 1477 | B3 | *Bacillus* sp. TH64 | 99 | Bacillales/Bacillaceae |
| 1476 | Not assigned | *Bacillus cereus* strain PPB6 | 100 | Bacillales/Bacillaceae |

*Closest organisms in GenBank

[b]Classified using the RDP Naive Bayesian Classifier.[17]

Figure 3
254x190mm (96 x 96 DPI)




TOC/Abstract art
254x190mm (96 x 96 DPI)