# Environmental Science
## Processes & Impacts

Accepted Manuscript






ROYAL SOCIETY OF CHEMISTRY

rsc.li/process-impacts

Pharmaceuticals and Personal Care Products (PPCPs) are a widespread and heterogeneous group, which can achieved the environment trough wastewaters and sludge from Wastewater Treatment Plants (WWTPs). The reuse of wastewater and the use of WWTP sludge in some human activities can cause an accumulation of these pollutants in the water cycle. The present work exposes how to treat the sludge from a Membrane Biological Reactor (MBR) in order to reduce its PPCPs load. The treatment is based in a bioslurry system – at Erlenmeyer scale – inoculated with a non-genetically modified fungus, without any nutrient or chemical product addition.

# Degradation of pharmaceuticals from Membrane Biological Reactor sludge with *Trametes versicolor*

Guillem Llorens-Blanch[a], Marina Badia-Fabregat[a], Daniel Lucas[c], Sara Rodriguez-Mozaz[c], Damià Barceló[c, d], Taina Pennanen[e], Gloria Caminal[b], Paqui Blánquez[a]

[a] **Departament d'Enginyeria Química, Escola d'Enginyeria, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain.**

[b] **Institut de Química Avançada de Catalunya (IQAC) CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain.**

[c] **Catalan Institute for Water Research (ICRA), H2O Building, Scientific and Technological Park of the University of Girona, 101-E-17003 Girona, Spain.**

[d] **Water and Soil Quality Research Group Department of Environmental Chemistry, IDAEA-CSIC), Jordi Girona 18-26, 08034 Barcelona, Spain.**

[e] **Finnish Forest Research Institute (METLA), Jokiniemenkuja 1, FI-01301 Vantaa, Finland.**

## Abstract

Emerging contaminants are a wide group of chemical products that are found at low concentrations in the environment. These contaminants can be either natural – estrogens – or synthetics – like pesticides and pharmaceuticals –, which can get the environment through the water and sludge from Wastewater Treatment Plants (WWTP). The growth of *Trametes versicolor* on Membrane Biological Reactor (MBR) sludge in bioslurry systems at Erlenmeyer scale was assessed and its capacity for removing Pharmaceuticals and Personal Care Products (PPCPs) was evaluated. The ability of the fungus to remove hydrochlorothiazide (HZT) from liquid media cultures was initially assessed. Consequently, different bioslurry media (complete nutrient, glucose and no-nutrient addition) and conditions (sterile and non-sterile) were tested, and the removal

of spiked HZT was monitored in each condition. The highest spiked HZT removal was assessed under non-sterile conditions without nutrient addition (93.2 %). Finally, the removal assessment of a broad set of pharmaceuticals was performed in non-spiked bioslurry. Under non-sterile conditions, the fungus was able to completely degrade 12 out of the 28 drugs initially detected in the MBR sludge, reaching an overall degradation of 66.9 %. Subsequent microbial analysis showed that the microbial diversity increased after 15 days of treatment, but there was still some *T.versicolor* in the bioslurry. Results showed that *T.versicolor* can be used to remove PPCPs in bioslurry systems under non-sterile conditions, without extra nutrients in the media, and in matrices as complex as an MBR sludge.

## 1. Introduction

A Membrane Biological Reactor (MBR) is a system that combines a biological reactor with a filtration process, avoiding the need for a settler. These systems works with high hydraulic retention time, and high biomass concentration; leading to an improved pollutant biodegradation, and a significant sludge reduction [1]. However, the remaining sludge needs to be removed and treated before final disposal.

Pharmaceutical and personal care products (PPCPs) are part of the so-called emerging pollutants, a wide range of chemical products not yet legislated. Despite their low concentration in water and sludge, the impact that can cause to both environment and public health [2] is difficult to predict, due to lack of information about their toxicity and effects on ecosystems.

The use of WWTP's sludge in agricultural and forestry activities is becoming an interesting valorisation method, because its capability to fertilise soils, and the low economic impact of the operation. These actions improve the physical-chemical properties of the land, and can augment the crops yield [3]. Nevertheless, the application

of non-treated sludge into soils can increase the potential risks for human and animal health [4], since it can contain different types of pollutants. Consequently, sludge should be treated before its application into soil, in order to remove micro-pollutants.

The microorganism used in this study was *Trametes versicolor*, a white rot fungus able to produce extracellular enzymes, which can degrade lignin and are not substrate specific[5]. *T.versicolor* has been extensively studied in the degradation of organic pollutants e.g. dyes, pharmaceuticals and endocrine disruptors [6–8] from water, due to its ability to remove recalcitrant organic pollutants. Degradation of emerging pollutants from a heat dried sewage sludge has also been studied, under sterile [9,10] and non-sterile [11,12] conditions

The aim of this study was to evaluate the capacity of *T.versicolor* to remove PPCPs from MBR sludge. This sludge is highly diluted and has not been previously treated, so the microbial population is much higher than the present in previous works. Initially, the degradation ability of the fungus in MBR bioslurry was assessed in samples spiked with HZT, a hydrophilic drug from thiazide's group. HZT was chosen as target compound because was previously detected by Radjenović *et al.*[13] in a MBR pilot plant at the same WWTP of the present study, and due to lack of information about its behaviour in wastewater. The best operational conditions were chosen according to fungal sludge colonization and HZT removal. Finally, the best culture conditions were applied to treat non-spiked sludge under sterile and non-sterile conditions, in order to compare the role of the fungus and the autochthonous microorganisms of the MBR sludge in the degradation of PPCPs.

## 2. Materials and methods

### *2.1. Chemicals*

Hydrochlorothiazide (6-Chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide, 6-Chloro-7-sulfamyl-3,4-dihydro-1,2,4-benzothiadiazine 1,1-dioxide) was obtained from Sigma-Aldrich Co (St. Louis, MO); methanol and acetonitrile were HPLC-grade.

All other pharmaceutical standards and isotopically labeled compounds, used as internal standards, were of high purity grade (>90%). Compounds were purchased from Sigma– Aldrich (Steinheim, Germany), US Pharmacopeia USP (MD, USA), Europea Pharmacopeia EP (Strasbourg, France), Toronto Research Chemicals TRC (Ontario, Canada) and CDN isotopes (Quebec, Canada). Further information can be consulted at Gros *et al.* (2012). The individual standard solutions as well as isotopically labeled internal standard solutions were prepared according to Gros *et al* [14].

*2.2. Fungal strain*

The strain *T. versicolor* ATCC 42530 was obtained from the American Type Culture Collection, and maintained by subculturing every 30 days on 2% malt extract agar slants (pH 4.5) at 23 °C. Blended mycelial suspension and pellet suspension were prepared according to Blánquez *et al.* [15].

*2.3. Waste Water Treatment Plant*

The selected WWTP for this study is located in Terrassa (Catalonia, Spain), and has a treatment capacity of 15,000 $m^3d^{-1}$. After primary treatment, the water flow is divided into two streams: one driven to a MBR unit and another driven to an activated sludge reactor. The MBR has an internal configuration with a Total Suspended Solids (TSS) concentration around 4 – 5 $g \cdot L^{-1}$. It was designed to treat a 7,200 $m^3 \cdot d^{-1}$ stream with a HRT of 0.79 d. ZENON microfiltration membranes were equipped, with a nominal porosity of 0.10 μm.

*2.4. Sampling*

Homogeneous sludge samples were taken from the MBR's recirculation stream. Samples used for experiments under sterile conditions were autoclaved twice at 120 ºC for 30 min. For non-sterile experiments, samples were collected the same day.

*2.5. Experimental procedures*

*2.5.1. Liquid media cultures*

Erlenmeyer flasks (0.5 L) were used to perform liquid cultures. Each flask was filled with 100 mL of sterile media consisting of glucose (8 $g \cdot L^{-1}$), ammonium tartrate (3.3 $g \cdot L^{-1}$), dimethyl succinic (1.168 $g \cdot L^{-1}$), macronutrients (100 $mL \cdot L^{-1}$), and micronutrients (10 $mL \cdot L^{-1}$) [16]. All flasks were inoculated with *T.versicolor* pellets (0.55 gDW), and spiked with HZT (10 $mg \cdot L^{-1}$). Incubation was carried out in orbital shakers (130 rpm) at 25 ºC. Experiments also included abiotic controls, which contained 20 mL of water instead of pellets, and killed controls, that consisted at first of inoculated cultures with sodium azide (0.2 $g \cdot L^{-1}$) and then of autoclaved cultures (120 ºC ~ 30min). All the experiments were run in triplicate.

*2.5.2. Spiked Bioslurry*

Different kinds of experiments were carried out: cultures with defined media, cultures with only glucose (8 $g \cdot L^{-1}$) as nutrient source, and cultures without any nutrient. The experiments were conducted under sterile conditions (120 ºC for 30 min), except for cultures without nutrients where sterile and non-sterile conditions were tested. Erlenmeyer flasks (0.5 L) were used and filled with 100 mL of MBR sludge, with nutrients or glucose when required. All the experiments were run in triplicate.

Flasks were inoculated with 0.55 gDW of pellets, and spiked with 10 $mg \cdot L^{-1}$ of HZT. Initial pH (7.0 – 8.1) was adjusted to 4.50, and cultures were incubated at 25 ºC in an orbital shaker (130 rpm). Degradation experiments included abiotic controls,

containing 20 mL of water instead of pellets, as well as heat-killed controls – as described in 2.5.1.

*2.5.3. Non-spiked Bioslurry*

Erlenmeyer flasks (0.5 L) were filled with 100 mL of non-sterile MBR sludge with the pH initially adjusted to 4.5; afterwards no further pH control and adjustment was carried out. Experimental cultures were inoculated with 0.55 gDW of pellets, killed controls were inoculated with 0.55 gDW of heat-killed pellets, and in abiotic cultures 20 mL of water replaced the pellets. All the cultures were incubated at 25 ºC and 130 rpm in orbital shakers. All the experiments were made in triplicates.

*2.6. Analytical methods*

*2.6.1. Sludge characterization*

TSS was assessed drying 25 mL of homogeneous samples at 105 ºC for 24 h. Dried samples were burned at 550 ºC during 30 min to assess the Volatile Suspended Solids (VSS)[17]. Total Carbon (TC) and Total Organic Carbon (TOC) were analysed using a 1020A Total Organic Carbon Analyser (O·I·Analytical, TX, USA). Total Ammonia Nitrogen (TAN) was assessed using the LCK 303 Ammonium cuvette test kit (HACH-Lange, UK).

*2.6.2. Glucose quantification*

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

*2.6.3. Laccase activity*

Although the relation between Laccase expression and degradation of some compounds is not clear, its activity has been used as presence indicator of *T.versicolor*, because is the main enzyme produced, and the determination method is simple.

Culture's extracts were taken and centrifuged at 15,000 *g* for 15 min. Enzymatic activity was measured using a modified version of the method for manganese peroxidase determination [18]. 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 ºC. Results were expressed as activity units per litter ($U \cdot L^{-1}$). One U was defined as the number of DMP micromoles oxidized per min. The DMP extinction coefficient was 24,800 $M^{-1}cm^{-1}$.

*2.6.4. Hydrochlorothiazide quantification*

Based on the work of Wankhede *et al.* [19], a method to extract and quantify HZT from liquid samples was developed. Homogeneous samples (1 mL) were collected into glass vials, shook at 35 Hz for 2 min (ZX3, VELP Scientifica, Spain), and filtered (Millipore Millex-GV 0.22 µm) prior their analysis.

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

*2.6.5. Total pharmaceutical products quantification*

Each culture was frozen in thin layer (CHRIST cooling bath CB 18-40, Wiegand International, Hamburg, Germany) and then maintained at -80 ºC for 24h prior lyophilization (Virtis Sentry freeze-drying equipment, Gardiner, NY). 0.2 grams of the

sample were extracted using an accelerated solvent extraction (ASE) system as described in detail by Jelić *et al* [20]. Concentrated extracts were diluted in water up to 500 mL and then, were filtered through 0.45μm nylon membrane filters (Whatman, UK) in order to retain suspended solids. A certain volume of the chelating agent EDTA was added to all of the samples to a final concentration of 3% (ml solute $ml^{-1}$ solution), as it is well known that improves the extraction of some pharmaceuticals. Clean-up of the samples was performed by SPE (Solid Phase Extraction) using a Baker (J.T.Baker®) system and Oasis HLB 3cc, 60 mg, extraction cartridges (Waters Corp. Mildford, MA, USA). Cartridges were conditioned using 5 mL of methanol followed by 5 mL of HPLC grade water at 1 mL min-1; then 50 mL of each sample were loaded at 1 mL min-1. Elution of the samples was performed passing 6 mL of pure methanol at a flow rate of 2 mL min-1 through the cartridges. The extracts were evaporated under nitrogen stream using a Reacti-Therm 18824 system (thermo Scientific) and reconstituted with 1 mL of methanol-water (10:90 v/v). Lastly, 10 μL of standard of internal standard mix at 10 ng $\mu L^{-1}$ were added to the extracts for internal standard calibration and to compensate, if it was necessary, a possible matrix effect.

Chromatographic separation was carried out with a Ultra-Performance liquid chromatography (UPLC) system (Waters Corp. Mildford, MA, USA) equipped with a binary solvent system (Mildford, MA, USA) and a sample manager, using an Acquity HSS T3 column (50 mm x 2.1 mm i.d. 1.7 μm particle size; Waters Corp. Mildford, MA, USA) for the compounds analyzed under positive electrospray ionization (PI) and an Acquity BEH C18 column (50 mm×2.1 mmi.d., 1.7 μm particle size) for the ones analyzed under negative electrospray ionization (NI), both purchased from Waters Corporation. The UPLC instrument was coupled to a 5500 QqLIT, quadrupole–linear ion trap mass spectrometer (5500 QTRAP, Applied Biosystems, Foster City, CA, USA)

with a Turbo V ion spray source. All transitions were recorded by using the Scheduled MRMTM algorithm and the data were acquired and processed using Analyst 2.1 software. Elimination rates were calculated comparing initial and final concentration of each pharmaceutical compound and expressed as removal percentage. Those pharmaceuticals that were non-detected (ND) at the end of the assay in the experimental cultures, but detected in the control samples, were considered as fully degraded in those experiments. In addition and just for removal calculations, those compounds detected below quantification limit (BQL) were considered to have a concentration equal to their detection limit divided by two [21,22].

### *2.7. Microbial community analysis*

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

Total DNA was extracted from 50-100 mg of lyophilized samples with FastDNA SPIN Kit for Soil (MP Biomedicals) following the procedure described by the company. Fragments of bacterial 16S and fungal Internal Transcribed Spacer (ITS) region of 18S rDNA were PCR amplified by DreamTaq polymerase (Thermo Scientific). Universal primers were used in both reactions: ITS1F forward (5' CT TGG TCA TTT AGA GGA AGT AA 3')[23] and ITS2 reverse (5' GCT GCG TTC TTC ATC GAT GC 3')[24] for fungi and F1055 forward (5' ATG GCT GTC GTC AGC T 3') and R1378 reverse (5' CG GTG TGT ACA AGG CCC GGG AAC G 3') for bacteria. A GC clamp (5' CCC CCC CCC CCC CGC CCC CCG CCC CCC GCC CCC GCC GCC C 3') was attached to the primers ITS1F and R1378 at the 5' end. The PCR program for fungi was 5 min at 95ºC followed by 40 cycles of 30 sec at 95ºC, 40 sec at 55ºC and 1 min at 72ºC, ending with a final elongation step of 5 min at 72ºC. Bacterial program was the same except for the hibridation temperature which was 56ºC. The length and

ammount of PCR products were estimated in 1% agarose gel with DNA ladder and labeled with ethidium bromide.

Denaturing Gradient Gel Electroforesis (DGGE) were performed in an INGENYphorU (Ingeny, The Netherlands) machine. Urea gradients were adjusted in order to optimize separation of the bands, being the final gradients 40-80% for bacteria and 25-60% for fungi, and 7.5% acrylamide/bisacrylamide (37:5:1) both of them. Electroforesis were performed during 16 hours at 75 V in 1x TAE buffer at 60ºC. Gels were stained with SYBR Gold (Invitrogen, Life Technologies). Selected DGGE bands were excised, reamplified (22 cycles) and run in a DGGE gel until the bands were clear enough (3-6 cycles). Purification and sequencing were performed by a commercial service (Macrogen Inc., South Korea) with the ITS1F without GC tail and F1055 primers. Partial fungal and bacterial DGGE-derived sequences were aligned with sequences retrieved from databases of GenBank/EMBL/DDBJ with Blastn algorithm. Bacterial sequence data have been deposited to GenBank database under Accession Numbers from KJ599735 to KJ599740. Fungal sequences could not be deposited because their length was less than 200 bp. Their sequences can be found in the supplementary material (Table S1).

*2.7.2. Quantitative PCR (qPCR)*

Quantitative PCR (qPCR) were performed for total fungi and specific for *T. versicolor*. The primers used were the same described in the last section but without GC clamp for total fungi (ITS1F and ITS2) and those described by Eikenes *et al.* [25] in the ITS1 region for *T. versicolor*. The 20 µL of the reaction mixture contained 10 µL of Maxima SYBR Green qPCR Master Mix (Fermentas), 0.375 µM of each primer and 1 µL of DNA. The reactions were carried out on a Rotor-gene 6000 (Corbett Research) apparatus using the temperature program described in the article of Eikenes *et al* for *T.*

*versicolor* and the program described at Rajala *et al.* [26] for total fungi. Standard curves were performed with known ammounts of *T.versicolor* (CT = -3.126*log(conc)+32.221, efficiency 1.089) and *Heterobasidion annosum* (CT= -3.748*log(conc)+36.037, efficiency 0.848), respectively.

## 3. Results and Discussion

### *3.1. HZT degradation studies in liquid cultures*

HZT degradation experiments in spiked media cultures were carried out at optimal growth conditions for *T.versicolor*. Analyses on glucose consumption, laccase activity, and HZT degradation were performed.

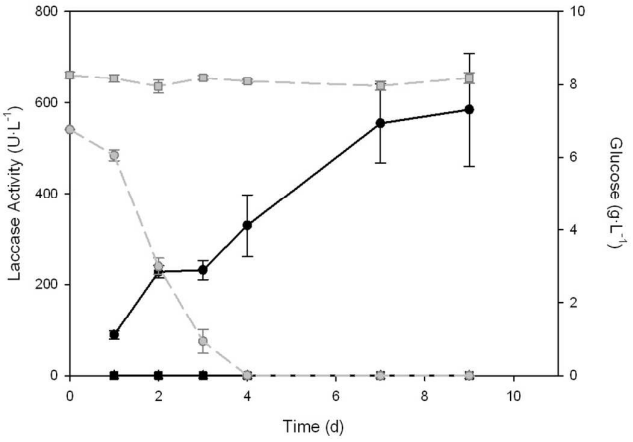


**Fig. 1.** Laccase activity and glucose consumption of liquid media cultures. Laccase activity: experimental cultures —●—, abiotic controls —■— and killed controls —▲— (graphs of both controls are overlapped because the activity is 0). Glucose consumption: experimental cultures - -●- - and abiotic controls - -■- -. Error bars represent standard error of triplicates. In this experiment killed controls were made with sodium azide.

Figure 1 shows the measured laccase activity, and glucose consumption for experimental and abiotic cultures. Glucose analyses for killed controls exhibit unexpected results due to interferences caused by sodium azide (according YSI 2700 owner's manual). Glucose concentration remained constant for abiotic controls, and fell rapidly for experimental cultures until the day 4. Experimental cultures also showed an

increasing trend of the laccase activity, reaching the highest point the day 9 (ca. 600 $U \cdot L^{-1}$). This confirmed that *T.versicolor* had oxidative capacity under the selected conditions. Similar results have been reported for the same medium and fungus with other compounds. For instance, Jelic *et al.* obtained a comparable laccase activity in the degradation of carbamazepine [27], and Cruz-Morató *et al.* obtained lower laccase production in the degradation of clofibric acid [28]. However, previous reports had demonstrated that pollutants degradation can be achieved without laccase involvement [16,29].

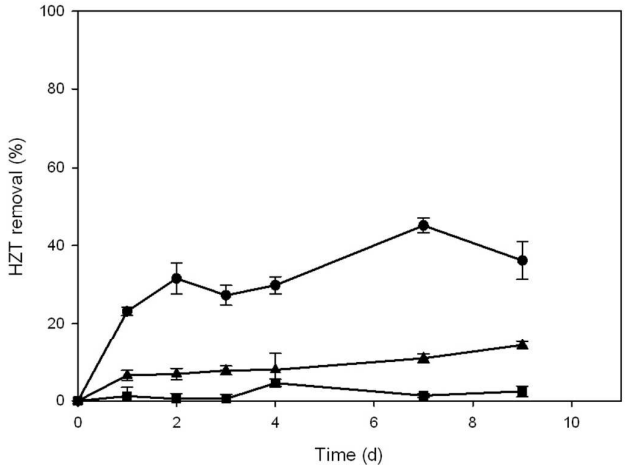


**Fig. 2.** HZT degradation trends in liquid media cultures: experimental cultures, abiotic controls and killed controls. Error bars represent standard error of triplicates.

HZT degradation results are presented in figure 2. Nearly 45 % of measured HZT is eliminated from media when experimental and abiotic cultures are equated. It can be considered that 10 % of the compound has been adsorbed on fungal biomass, and 35% has been degraded, as minimum, when killed and abiotic controls are compared.

*3.2. HZT degradation in spiked bioslurry*

Further experiments were designed to test both the ability of *T.versicolor* to grow on liquid MBR sludge, and its degradation capacity using HZT as target compound. Different culture medias were used: one with all the nutrients listed for

liquid media cultures, another with only glucose, and finally one without any additional nutrient; calling them from now as complete media, glucose media and no-nutrient media, respectively. Also, sterile and non-sterile conditions were tested.

Initially, the inactivation of the fungus was made with sodium azide instead of heat, in order to avoid an improved pollutant adsorption onto fungal biomass, because heat not only deactivates enzymes but also breaks the fungal membrane, affecting the adsorption. However, sodium azide did not completely inactivate cultures, and some HZT degradation (data not shown) was observed in killed controls. Sodium azide inhibits the oxygen uptake of the fungus, but some enzymes can remain active, as it was reported for diclofenac degradation by Badia-Fabregat *et al* [30]. Subsequently, sodium azide was substituted by heat in the fungus' deactivation in killed control cultures.

*3.2.1.* Sterile conditions

The effect of media composition in the degradation of spiked HZT in sterile bioslurry systems was the first aspect tested. As it can be seen in figure 3, both complete and glucose media experimental cultures consumed all the glucose by days 2 and 3 respectively, and both showed a similar laccase activity trends, reaching a peak the day 4 (complete media: 322 ± 66 $U \cdot L^{-1}$; glucose media: 198 ± 90 $U \cdot L^{-1}$). No-nutrient media also exhibited a gradual growth of the laccase production from day 2 until 4 (331 ± 46 $U \cdot L^{-1}$), and then levelled out until day 7.

The fungus' ability to survive and remove drugs in a bioslurry system was previously studied by Rodríguez-Rodríguez *et al.* [31]. The authors tested the capability of *T.versicolor* to degrade naproxen and carbamazepine in bioslurry systems made by adding water to dry WWTP sludge, obtaining different concentrations: 100 to 600 $g \cdot L^{-1}$. The present study also tested the ability of *T.versicolor* to grow and eliminate drugs in

bioslurry systems, but with naturally wet sludge to avoid the addition of water. Despite the selected liquid-sludge was poor in solids (4 $g \cdot L^{-1}$), similar results were obtained.

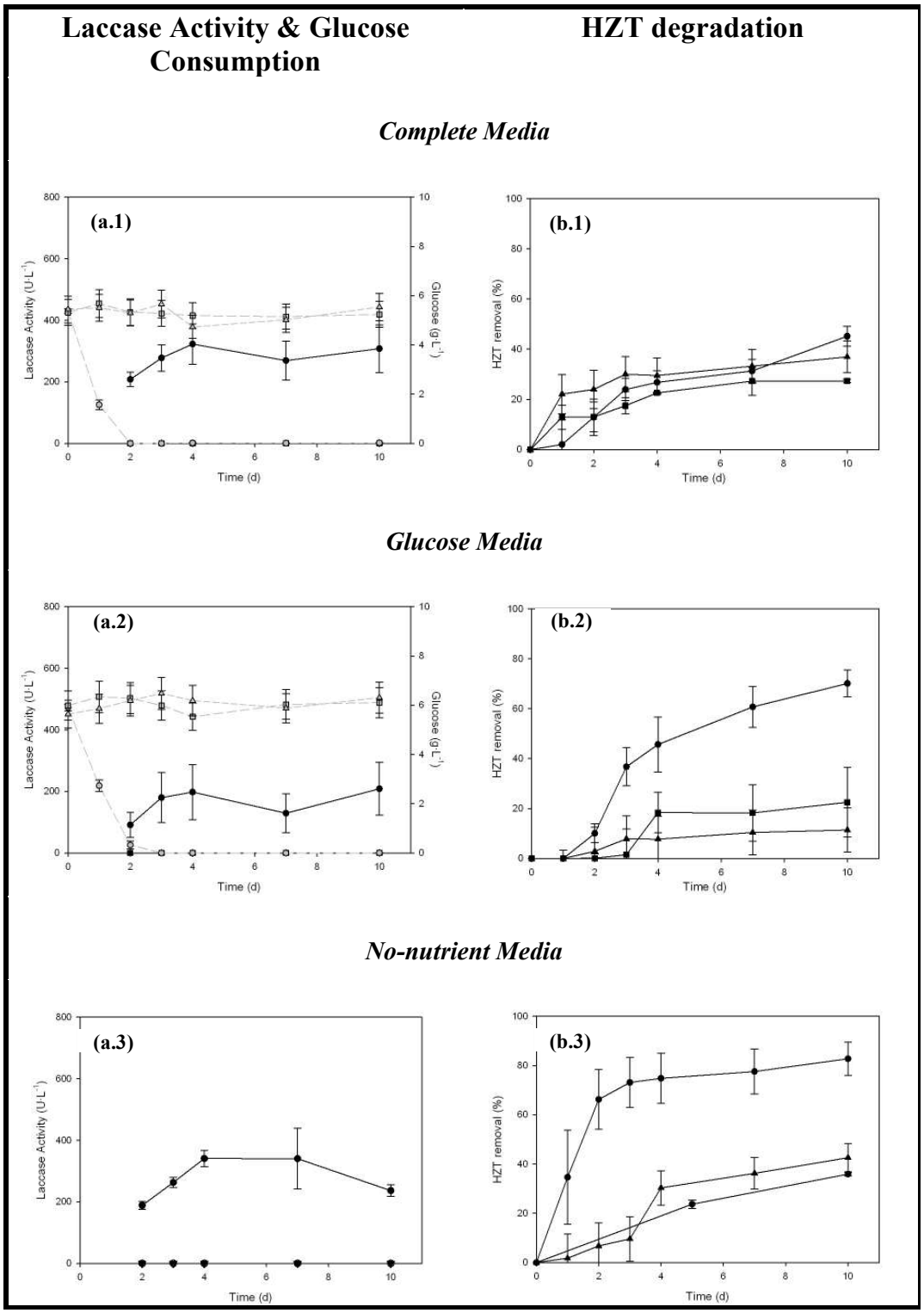

**Fig. 3.** Laccase activity, glucose consumption (a) and HZT degradation (b) in sterile bioslurry cultures with complete (1), glucose (2) and no-nutrient (3) medias. Laccase activity and HZT degradation profiles: experimental cultures ●, abiotic controls ■ and killed controls ▲. Glucose concentration (only for complete and glucose medias): experimental cultures ○, abiotic controls □ and killed controls △. Error bars represent standard error of triplicates.

In figure 3 it can be seen that *T.versicolor* is able to degrade spiked HZT in bioslurry systems. Some decrease in HZT concentration in killed and abiotic controls was observed in all three media conditions. In complete media experiments, both controls followed a similar trend, reaching a final HZT concentration 37 % lower than the initial. The different final HZT concentration in abiotic (6.06 $\mu g \cdot mL^{-1}$), and killed control (5.25 $\mu g \cdot mL^{-1}$) indicates that part of the drug was adsorbed on the biomass. Glucose and no-nutrient medias control showed no statistically differences between killed and abiotic controls, with removals at the end of the experiment ca. 11.4 % in glucose media and ca. 34.7 % in no-nutrient media.

Complete media experimental culture showed a final HZT degradation by *T. versicolor* of 13.8 %, with a 9.1 % of the drug adsorbed in the fungal biomass. This degradation was higher in the glucose media experimental culture, were a 71.4 % of the drug was degraded, but adsorption could not be measured due to controls' behaviour. No-nutrient media experimental culture showed a similar HZT degradation (69.1 %), with an adsorbed fraction of 4.3 %. However, the degradation in the no-nutrient media was faster, reaching already 66.7 % of degradation at 2 days of experiment. ANOVA analyses (Table S2 in supplementary material) showed that there is a significant statistical difference between tested groups. Consequently, it can be concluded that the media affects the degradation of the selected drug. HZT was degraded in all experimental cultures, but the highest rate was obtained in systems without nutrient additions. Furthermore, according to table 1 it can be assumed that the sludge has

enough carbon and nitrogen to become the main source of nutrients for the fungus. According to these results and taking into account further applications of the technology, the no-nutrient media was selected for subsequent experiments.

Table 1. MBR sludge physical characteristics

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

### *3.2.2. Non-sterile conditions*

After assessing the effect of media in HZT degradation, non-sterile conditions were tested for no-nutrient cultures. The aim was to determine if *T.versicolor* could degrade spiked HZT in competition with the autochthonous sludge microorganisms.

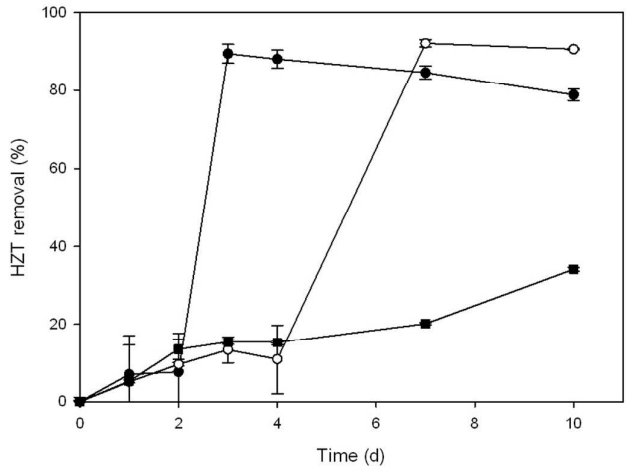


**Fig. 4.** HZT concentration in non-sterile flask-bioslurry cultures with no-nutrient media: experimental cultures (non-sterile, inoculated) —●—, raw sludge controls (non-sterile, non-inoculated) —○— and abiotic controls (sterile, non-inoculated) —■—. Error bars represent standard error of triplicates.

The measured laccasse activity was negligible, less than 0.5 $U \cdot L^{-1}$. This reduction could be caused as a result of the competition between fungus and autochthonous sludge microorganisms. Therefore, inhibitory effects should be considered to explain this reduction.

In figure 4 it can be observed that HZT degradation is higher than in previous experiments. Abiotic controls showed a HZT removal of 34 %, which was previously observed. When HZT degradation yields are compared, ANOVA analyses (Table S3 in supplementary material) indicate there are no significant differences between inoculated and non-inoculated cultures with *T.versicolor*. However, HTZ degradation in experimental culture was faster than in raw sludge control, reaching the maximum degradation at days 3 and 7 of the experiment, respectively. In the case of the experimental culture, it means a fungal degradation of 93.2 % the day 3.

Therefore, even though it is possible to eliminate spiked drugs from MBR liquid-sludge with its autochthonous microorganisms, the bioslurry treatment with the fungus improves this elimination by reducing the time needed. Although there are not significant differences between inoculated and non-inoculated cultures in terms of overall spiked HZT removal, it is still unknown what happens with HZT and other drugs at real concentrations.

### *3.3. Pharmaceuticals degradation in non-spiked bioslurry*

Radjenovic *et al.* [32] showed that pharmaceutically active compounds are more easily eliminated in MBR systems than in conventional activated sludge reactors, and that some compounds such as HZT by-pass the reactor without any change in their concentration. In order to assess if the fungus can improve the removal of pharmaceuticals in the outlet of MBR systems, a non-spiked experiment was carried out.

The aim was to determine the efficiency of fungus to eliminate PPCPs at real concentration in bioslurry systems. Two experimental groups were developed to assess total PPCPs elimination from MBR sludge: one heat sterilized before fungal inoculation and the other non-sterilized. Non-inoculated controls under non-sterile conditions were also included. Treatments were carried out in Erlenmeyer flasks with MBR sludge sampled the same day, and no drugs were spiked.

Laccase activity trends were similar to previous experiments. Non-sterile cultures showed negligible activity, while sterile cultures showed activities above 200 $U \cdot L^{-1}$. Table 2 shows detected pharmaceuticals at initial MBR sludge and their removal yields after 15 days of bioslurry treatment, and table 3 presents a summary of PPCPs removal sorted by concentration range. In summary, out of the 50 pharmaceuticals analysed, 40 were detected in raw sludge's samples at the beginning of the experiment (11 of them below quantification limits), which represents a total amount of 10,151.5 ± 574.1 $ng \cdot L^{-1}$. The highest concentrations in MBR sludge were found for the antibiotics ciprofloxacin (3,726.8 ± 229.9 $ng \cdot L^{-1}$) and ofloxacin (2,921.4 ± 173.6 $ng \cdot L^{-1}$). More than 66% of initial detected drugs were removed from both inoculated bioslurry, while non-inoculated removed approximately 54% of the initially detected drugs.

**Table 2.** Detected pharmaceuticals in MBR sludge and its removal yields after bioslurry treatment

| **Pharmaceutical** | **Initial concentration ± standard deviation** ($ng \cdot L^{-1}$) | **Removal yields of the cultures (%)** | | |
|---|---|---|---|---|
| | | Inoculated (sterile conditions) | Inoculated (non-sterile conditions) | Non-inoculated (non-sterile conditions) |
| *Analgesics and anti-inflammatory drugs* | | | | |
| Ketoprofen | 168.2 ± 9.0 | 39.2 | 78.7 | * |
| Phenazone | 88.0 ± 4.2 | 45.0 | * | * |
| Acetaminophen | 77.4 ± 1.0 | 67.6 | 67.6 | 67.6 |
| Codeine | 31.9 ± 1.3 | 39.6 | 100.0 | 91.2 |
| Propyphenazone | 6.5 ± 0.3 | 72.9 | 72.9 | 72.9 |
| Piroxicam | 5.1 ± 0.6 | 100.0 | 100.0 | 100.0 |

| | | | | |
|---|---|---|---|---|
| *Antihypertensive* | | | | |
| Valsartan | 135.2 ± 1.9 | 36.7 | 88.3 | 88.3 |
| *Anthelmintic* | | | | |
| Levamisol | 10.7 ± 0.5 | 100.0 | 4.1 | 30.7 |
| *Anti-H2* | | | | |
| Ranitidine | 31.9 ± 3.9 | 100.0 | 100.0 | 92.4 |
| *Calcium Channel Blockers* | | | | |
| Diltiazem | 43.8 ± 0.7 | 100.0 | 100.0 | 100.0 |
| Norverapamil | 18.7 ± 0.3 | 0.0 | 71.3 | 9.2 |
| Verapamil | 16.4 ± 0.3 | 100.0 | 100.0 | 90.7 |
| *Antibiotics* | | | | |
| Ciprofloxacin | 3726.8 ± 229.9 | 89.2 | 61.0 | 46.5 |
| Ofloxacin | 2921.4 ± 173.6 | 80.5 | 64.5 | 41.5 |
| Azithromycin | 594.7 ± 43.0 | 98.6 | 96.7 | 92.8 |
| Sulfamethoxazole | 158.2 ± 2.7 | 95.0 | 95.7 | 92.7 |
| Clarithromycin | 75.9 ± 1.6 | 95.9 | 100.0 | 42.4 |
| Trimethoprim | 47.2 ± 8.5 | 91.1 | 100.0 | 78.7 |
| *Antiplatelet drug* | | | | |
| Clopidrogel | 11.1 ± 0.3 | 100.0 | 100.0 | 100.0 |
| *Contrast medium* | | | | |
| Iopromide | 490.5 ± 43.2 | 47.9 | 100.0 | 100.0 |
| *Diuretics* | | | | |
| Hydrochlorothiazide | 407.5 ± 9.3 | 99.1 | 38.1 | * |
| Furosemide | 356.0 ± 10.0 | 78.7 | 24.4 | 71.7 |
| *Psychiatric drugs* | | | | |
| Citalopram | 295.4 ± 13.3 | 91.8 | 47.9 | 42.1 |
| Venlafaxine | 233.1 ± 7.7 | 66.8 | 4.8 | * |
| Lorazepam | 84.8 ± 2.0 | 100.0 | 44.0 | 38.4 |
| Carbamazepine | 66.9 ± 2.1 | 49.0 | * | * |
| Trazodone | 34.1 ± 1.3 | 100.0 | 100.0 | 100.0 |
| Olanzapine | 14.3 ± 1.6 | 100.0 | 100.0 | 79.6 |
| **Total:** | 10,151.5 ± 574.1 | | | |

* Removal not assessed, final concentration was higher than the initial

In general, partial or total drug removal was observed, but 5 pharmaceuticals showed negative elimination rates (ketoprofen, phenazone, carbamazepine, HZT, and venlafaxine). This can be explained by the occurrence of conjugates in the bioslurry. Kovalova *et al.* [33] also observed the occurrence of conjugation/deconjugation processes in the pharmaceuticals removal in MBR systems. Some conjugates are formed by human metabolism to increase the solubility, and excretion of the drug, which can be

discomposed after a biological treatment back into their original compound, leading to a higher concentration of some drugs in the effluent than in the influent. Otherwise, fungi can also conjugate pharmaceuticals during biodegradation treatments [8]. Since the evaluation of PPCPs' impact in water is still in their early stages, few data of their behaviour in sludge can be found. More attention has been paid to Endocrine Disrupting Compounds (ECDs), due to male fish feminization and the possible alteration of human tissues' development [34], among others. Xu *et al.* [35] found conjugated estrogens in the influents of some sewage treatment plants that could be deconjugated by intestinal bacteria in the sewer lines. These authors also obtained negatives removals that were attributed to sludge desorption, deconjugation of conjugates, and biotransformation between compounds. Therefore, negative pharmaceuticals' removal in bioslurry systems could be related to one of the above mechanisms, but more research is needed in this field.

**Table 3.** Summary of pharmaceutical removal yield sorted by initial concentration range at non-spiked MBR bioslurry treatments. Compounds that their concentration increased after treatment are not included.

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

Figure 5 compares the initial and final concentrations of the detected pharmaceuticals. For an easier understanding of the data, drugs had been grouped by initial concentration into three ranges: 0-50 $ng \cdot L^{-1}$, 50-200 $ng \cdot L^{-1}$ and more than 200 $ng \cdot L^{-1}$. ANOVA analysis had not been carried out because of the complexity of the

matrix, the elevated number of pharmaceuticals in the MBR sludge, and the differences in drugs concentration – up to 3 orders of magnitude.

When inoculated and non-inoculated cultures under non-sterile conditions are compared, it can be seen that there are 3 principal behaviours: (1) inoculated cultures leads to lower drug concentration, (2) inoculated and non-inoculated cultures gets the same final drug concentration, and (3) non-inoculated cultures leads to lower drug concentration. The predominant behaviour is (1) and can be noticed for 13 drugs (ciprofloxacin, ofloxacin, azithromycin, citalopram, sulfamethoxazole, lorazepam, clarithromycin, trimethoprim, ranitidine, codeine, norverapamil, verapamil and olanzapine – figures 5a, 5b and 5c) of which 6 are fully degraded. In contrast, only 2 drugs (furosemide and levamisol – figures 5a and 5c) are better removed in (3) non-inoculated cultures; and none of both drugs are fully degraded. Finally, the behaviour (2) where both mentioned cultures get equal final concentration is given in 8 pharmaceuticals (iopromide, valsartan, acetaminophen, diltiazem, trazodone, clopidrogel, propyphenazone and piroxicam – figures 5a, 5b and 5c) of which 5 are fully degraded. Other two observable behaviours are the deconjugated drugs, which appear with a higher concentration after the treatment (figures 5a and 5b), and the case where the final concentration of 5 drugs (iopromide, ketoprofen, valsartan, codeine and norverapamil – figures 5a, 5b and 5c) in inoculated cultures under sterile conditions is higher than the other two treatments.

In summary, for any concentration range the cultures inoculated with *T.versicolor* under non-sterile conditions have obtained better results in the PPCPs' removal than non-inoculated cultures.

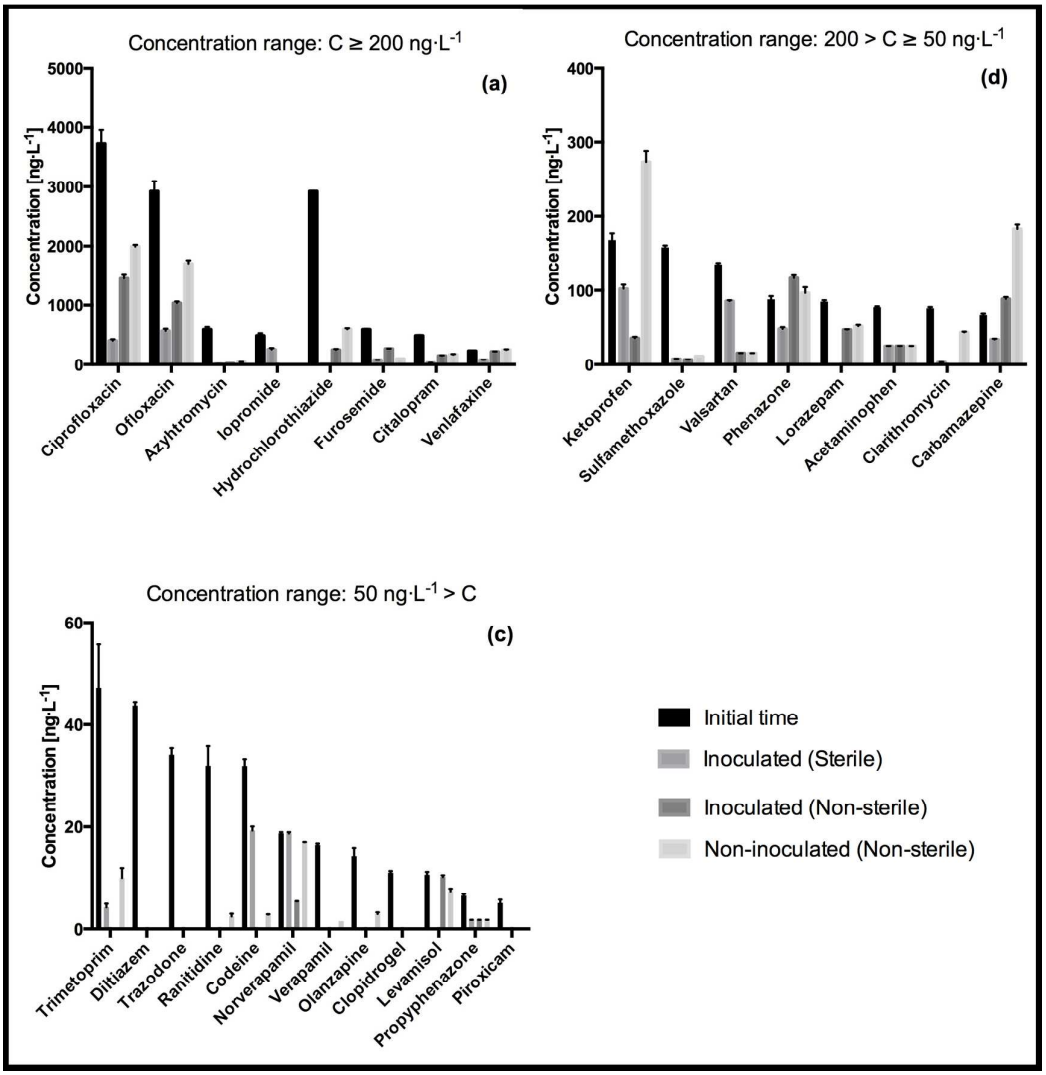


**Fig. 5.** Concentration of the pharmaceuticals in the initial MBR sludge and after 15 days of treatment in the 3 bioslurry systems (inoculated under sterile conditions, inoculated under non-sterile conditions and raw sludge controls). The pharmaceuticals had been grouped into 3 groups according to the initial concentration in the sludge: (a) more than 200 ng·L$^{-1}$, (b) between 200 and 50 ng·L$^{-1}$ and (c) below 50 ng·L$^{-1}$.

Should be noted that due to the nature of an experiment conducted in Erlenmeyer flasks, the monitoring and adjustment of pH was not feasible. pH has been proved as an important parameter for the fungus role in the degradation of pollutants[36] as well as the fungus growth[37]. Moreover, during the development of the experiment researchers noticed that fungus pellets were not visible in cultures under non-sterile

conditions after 5-7 days of treatment. This, together with the negligible laccase activity, resulted in a microbial analysis of the cultures, in order to find out if *T.versicolor* was able to survive in these conditions, and the evolution of microbial diversity.

### *3.3.1. Microbial analysis*

DGGE profiles of fungal and bacterial PCR products at initial time before inoculation and at the end of the fungal inoculated non-sterile experiment are shown in figure 6. Their phylogenetic affiliations are presented in the Tables 4 and 5. Initially, in the MBR sludge, there were two main fungal species (F3 and F4). However, after 15 days of experiment, the microbial diversity of the mixture increased. In the bacterial profile, more bands were obtained in the 15d samples than initially as well. All fungal bands at exception of that belonging to *T. versicolor* (F5) correspond to unknown fungi, as the best alignments gave identities of only 79 and 77% to different uncultured fungi for F1/F2 and F3/F4 respectively. The identity between F1 and F2 is 81% while between F3 and F4 is 97%. Bacteria found in the bioslurry treatment belonged to diverse classes, from *Alphaproteobacteria* (B1) to *Chloroflexi* (B6), going through *Gammaproteobacteria* (B3), *Holophagae* (B2), *Bacteroidetes* (B4) and some unclassified bacteria (B5). All sequencies retrieved a match with 95% of identity or higher with sequencies already deposited in the GenBank, however most of them were from uncultured bacteria. Sequences identified in the initial sludge were related with treatment of wastewater or degradation of selected pollutants: the closest organism for B3 was found in a MBR treating VOCs at Valladolid, Spain [38] and the closest for B6 was found in a biotrickling filter also in Valladolid [39]. Sequences found after 15 days of treatment were more related with soil bacteria such as B1, B2 [40] and B4 [41].

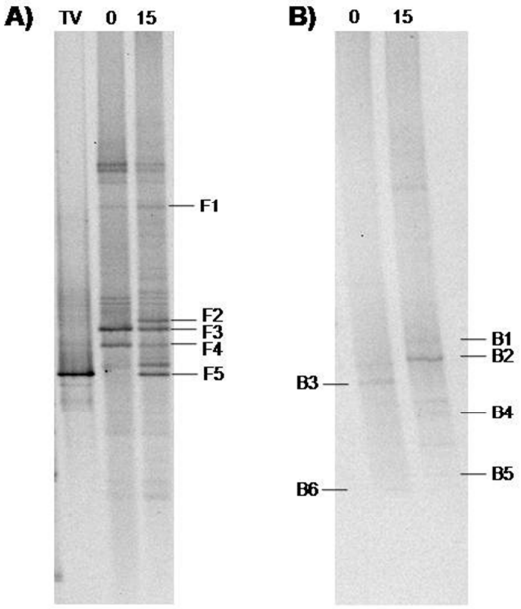


**Fig. 6**. DGGE profiles of **A)** fungal PCR-amplified ITS fragments of 18S rDNA and **B)** bacterial PCR-amplified 16S fragments rDNA from bioslurry experiments at initial time (0) and after 15 days (15) of fungal inoculated non-sterile treatment. TV corresponds to the amplified product of a *T. versicolor* pure culture.

**Table 4**. Phylogenetic affiliation of the retrieved fungal ITS sequences of the selected DGGE bands

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

[a] Closest organism at GenBank

**Table 5.** Phylogenetic affiliation of the retrieved bacterial 16S sequences of the selected DGGE bands

| DGGE Band | Seq. length | Closest relative[a] | Covery | Identity (%) | Accession number | Class/Order[b] |
|---|---|---|---|---|---|---|
| B1 | 241 | Uncultured Asticcacaulis sp. clone AS85P1 | 228/241 | 95 | KC172633 | Unclassified α-proteobacteria |
| | | Asticcacaulis excentricus strain CB 48 | 226/241 | 94 | NR_074137 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| B2 | 265 | Uncultured bacterium clone H-HN-E4-Min_444417 | 262/266 | 98 | JQ054706 | Holophagae/ Holophagales |
| | | Acidobacterium sp. ORAC | 258/265 | 97 | FN689719 | |
| B3 | 280 | Uncultured bacterium isolate DGGE gel band 18 | 272/278 | 98 | JX627832 | γ-proteobacteria/ Xanthomonadales |
| | | Dokdonella soli strain KIS28-6 | 271/278 | 97 | NR_044554 | |
| B4 | 266 | Uncultured bacterium | 256/266 | 96 | AB087366 | Unclassified Bacteroidetes |
| | | Bacteroidetes bacterium N2 | 249/266 | 94 | AB540001 | |
| B5 | 254 | Uncultured bacterium clone ncd265f05c1 | 248/252 | 98 | HM270445 | Unclassified Bacteria |
| B6 | 246 | Uncultured bacterium isolate DGGE gel band 14 | 246/246 | 100 | JQ038792 | Chloroflexi/ Chloroflexales |
| | | Kouleothrix aurantiaca strain:MYSI-A | 223/246 | 91 | AB079639 | |

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

However, the main conclusion one can deduce from the results is that after 15 days of treatment there was still some *T. versicolor* in the bioslurry mixture. With DGGE analysis it is only possible to determine presence and absence of the species. Therefore, in order to quantify *T. versicolor* in relation to total fungus, quantitative PCR was performed. At 15 days, total fungi increased 87% with respect to the initial time, what means almost doubling the ammount. However, *T.versicolor* only accounts for the 7.4% of the total fungi at 15 days (Figure 7). Those low values agree with the visual disappearance of *T.versicolor* at that experimental time. These results are in concordance with the findings of Rodríguez-Rodríguez *et al.* [11] working with *T. versicolor* in biopiles. They found that after 21 days of treatment other fungi replaced *T. versicolor*. Therefore, although matrices are different, similar reinoculation strategy [12] could be also applied at bioslurry treatment to achieve higher removal percentages.

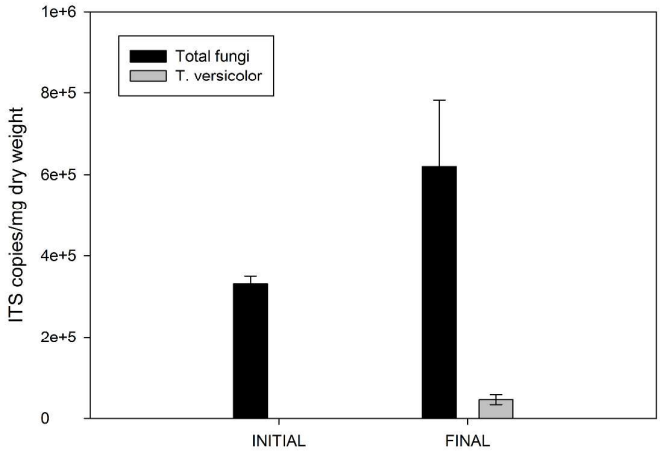


**Fig. 7**. Ammount of total fungi and *T. versicolor* (related to ITS copies/mg dry weight) at initial time and after 15 days of inoculated non-sterile bioslurry treatment.

## 4. Conclusion

This work has proved that liquid MBR sludge can be treated with *T.versicolor* at Erlenmeyer scale. The fungus has grown under non-sterile conditions without any extra nutrients, and has been able to remove a widely range of emerging pollutants. The activity of the MBR sludge's autochthonous microorganisms is enough to eliminate spiked HZT, but when it is treated with *T. Versicolor* the time to eliminate the drug is reduced. Even though the autochthonous microorganisms of the MBR sludge were able to remove some of the measured pharmaceuticals, the cultures with *T.versicolor* resulted in an improved removal of these pollutants.

MBR sludge has been successfully treated at Erlenmeyer scale, but the low solids content of the sludge makes difficult to assess whether the solid or just the liquid is being treated, maybe the two phases should be separated for the analysis. Moreover, the studies carried out at Erlenmeyer scale without pH control can limit the fungal activity. Despite these drawbacks, the degradation process, which at industrial scale should be carried out under non-sterile conditions, presents better results inoculating *T.*

*Versicolor*. The fungus remains active even its presence decreases compared to other fungi after 15 days of treatment. It has been observed that degradation occurs mainly during the first seven days, therefore *Trametes* would be active and a reinoculation strategy between 10 and 15 days of treatment could be applied in order to increase the degradation of these compounds.

**Acknowledgements**

This work has been supported by the Spanish Ministry of Economy and Competitiveness (projects CTQ2010-21776-C01 and CTQ2010-21776-C02), co-financed by the European Union through the European Regional Development Fund (ERDF) and supported by the Generalitat de Catalunya (Consolidated Research Groups: Biodegradation of Industrial Pollutants and Waste Recovery Group – 2009-SGR-656 – and Catalan Institute for water Research - 2014 SGR 291). The Department of Chemical Engineering of the Universitat Autònoma de Barcelona (UAB) is member of the Xarxa de Referència en Biotecnologia de la Generalitat de Catalunya. Daniel Lucas acknowledges the pre-doctoral grant from the Spanish Ministry of education, culture and sports (AP-2010-4926). Guillem Llorens-Blanch acknowledges the pre-doctoral grant from the Spanish Ministry of economy and competitiveness, secretariat of state for research, development and innovation (BES-2011-046090).

## 5. References

1 J. Sipma, B. Osuna, N. Collado, H. Monclús, G. Ferrero, J. Comas and I. Rodriguez-Roda, *Desalination* 2010, **250**, 653–659.

2 B.E. Erickson, *Environ. Sci. Technol.* 2002, **36**, 140A–145A.

3 R. P. Singh and M. Agrawal, *Waste Manag.* 2008, **28**, 347–358.

4 R.B. Dean and M.J. Suesst, *Waste Manag. Res.* 1985, **3**, 23–26.

5 H. Singh, in *Mycoremediation : fungal bioremediation*, Wiley-Interscience, New Jersey, 1st edn, 2006, chp 1, pp. 2-4, 16-20.

6 P. Blánquez, G. Caminal, M. Sarrà and T. Vicent, *Chem. Eng. J.* 2007, **126**, 163–169.

7 E. Marco-Urrea, J. Radjenović, G. Caminal, M. Petrović, T. Vicent and D. Barceló, 2010, **44**, 521–532.

8 M. Badia-Fabregat, C. E. Rodríguez-Rodríguez, P. Gago-Ferrero, A. Olivares, B. Piña, M.S. Díaz-Cruz, T. Vicent, D. Barceló and G. Caminal, *J. Environ. Manage.* 2012, **104**, 114–120.

9 C. E. Rodríguez-Rodríguez, A. Jelić, M. Llorca, M. Farré, G. Caminal, M. Petrović, D. Barceló and T. Vicent, *Bioresour. Technol.* 2011, **102**, 5602–5608.

10 C. E. Rodríguez-Rodríguez, E. Barón, P. Gago-Ferrero, A. Jelić, M. Llorca, M. Farré, M. S. Díaz-Cruz, E. Eljarrat, M. Petrović and G. Caminal, *J. Hazard. Mater.* 2012, **233–234**, 235–243.

11 C. E. Rodríguez-Rodríguez, A. Jelic, M. A. Pereira, D. Z. Sousa, M. Petrovic, M. M. Alves, D. Barcelo and T. Vicent, *Environ. Sci. Technol.*, 2012, **46** , 12012–12020

12 C. E. Rodríguez-Rodríguez, D. Lucas, E. Barón, P. Gago-Ferrero, D. Molins-Delgado, S. Rodríguez-Mozaz, E. Eljarrat, M. Silvia Díaz-Cruz, D. Barceló and G. Caminal, *Bioresour. Technol.* 2014, **168**, 180–189.

13 J. Radjenović, M. Petrović and D. Barceló, *Water Res.* 2009, **43**, 831–841.

14 M. Gros, S. Rodríguez-Mozaz and D. Barceló, *J. Chromatogr. A* 2012, **1248**, 104–121.

15 P. Blánquez, N. Casas, X. Font, X. Gabarrell, M. Sarrà, G. Caminal and T. Vicent, *Water Res.* 2004, **38**, 2166–2172.

16 E. Marco-Urrea, M. Pérez-Trujillo, T. Vicent and G. Caminal, *Chemosphere* 2009, **74**, 765–772.

17 A.P.H.A., A.W.W.A. and W.E.F., *Standard methods for the examination of water and wastewater*, 2012; **22**.

18 H. Wariishi, K. Valli and M. H. Gold, *J. Biol. Chem.* 1992, **267**, 23688–23695.

19 S. Wankhede, M. Tajne, K. Gupta and S. Wadodkar, *Indian J. Pharm. Sci.* 2007, **69**, 298–300.

20 A. Jelić, M. Petrović and D. Barceló, *Talanta* 2009, **80**, 363–371.

21 T. M. Gilliam and C. C. Wiles, in *Stabilization and Solidification of Hazardous, Radioactive, and Mixed Wastes*, ASTM, Fredericksburg, 1996, vol. 3, pp. 731.

22 P. K. Hopke, C. Liu and D. B. Rubin, *Biometrics* 2001, **57**, 22–33.

23 M. Gardes and T. D. Bruns, *Mol. Ecol.* 1993, **2**, 113–118.

24 T. White, T. Bruns, S. Lee and J. Taylor, in *Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR protocols: a guide to methods and applications*, ed. M. Innis, D. Gelfand, J. Sninsky and T. White, Academic Press, San Diego, 1990, pp. 315–322.

25 M. Eikenes, A. M. Hietala, G. Alfredsen, C. Gunnar Fossdal and H. Solheim, *Holzforschung* 2005, **59**, 568.

26 T. Rajala, S. M. Velmala, T. Tuomivirta, M. Haapanen, M. Müller and T. Pennanen, *Fungal Biol.* 2013, **117**, 182–190.

27 A. Jelić, C. Cruz-Morató, E. Marco-Urrea, M. Sarrà, S. Perez, T. Vicent, M. Petrović and D. Barcelo, *Water Res.* 2012, **46**, 955–964.

28 C. Cruz-Morató, A. Jelić, S. Perez, M. Petrović, D. Barceló, E. Marco-Urrea, M. Sarrà and T. Vicent, *Biochem. Eng. J.* 2013, **75**, 79–85.

29 N. H. Tran, T. Urase and O. Kusakabe, *J. Water Environ. Technol.* 2010, **8**, 125–140.

30 M. Badia-Fabregat, M. Rosell, G. Caminal, T. Vicent and E. Marco-Urrea, *Chemosphere* 2014, **103**, 336-342.

31 C. E. Rodríguez-Rodríguez, E. Marco-Urrea and G. Caminal, *Bioresour. Technol.* 2010, **101**, 2259–2266.

32 J. Radjenović, M. Petrović and D. Barceló, *Water Res.* 2009, **43**, 831–841.

33 L. Kovalova, H. Siegrist, H. Singer, A. Wittmer and C. S. McArdell, *Environ. Sci. Technol.* 2012, **46**, 1536–1545.

34 M. Auriol, Y. Filali-Meknassi, R. D. Tyagi, C. D. Adams and R. Y. Surampalli, *Process Biochem.* 2006, **41**, 525–539.

35 Y. Xu, N. Xu, N. R. Llewellyn and H. Tao, H. *Environ. Sci. Process. Impacts* 2014, **16**, 262–270.

36 A. Folch, M. Vilaplana, L. Amado, T. Vicent and G. Caminal, *J. Hazard. Mater.* 2013, **262**, 554–560.

37 E. Borràs, P. Blánquez, M. Sarrà, G. Caminal and T. Vicent, *Biochem. Eng. J.* 2008, **42**, 61–66.

38 R. Lebrero, D. Volckaert, R. Pérez, R. Muñoz and H. A. Van Langenhove, *Water Res.* 2013, **47**, 2199–2212.

39 R. Lebrero, E. Rodríguez, J. M. Estrada, P. A. García-Encina and R. Muñoz, *Bioresour. Technol.* 2012, **109**, 38–45.

40 S. A. Turlapati, R. Minocha, P. S. Bhiravarasa, L. S. Tisa, W. K. Thomas and S. C. Minocha, *FEMS Microbiol. Ecol.* **2013**, *83*, 478–493.

41 K. Toyota and S. Kuninaga, *Appl. Soil Ecol.* **2006**, *33*, 39–48.