A comparison between biostimulation and bioaugmentation in a solid treatment of anaerobic sludge: drug content and microbial evaluation

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Abstract

Emerging pollutants can reach the environment through the sludge of Wastewater Treatment Plants. In this work, the use of *Trametes versicolor* in biopiles at lab-scale was studied, evaluating its capacity to remove the most hydrophobic Pharmaceuticals and assessing the evolution of the biopiles microbial communities. The total removal of drugs at real concentrations from sewage sludge was assessed for non-inoculated and fungal inoculated biopiles, testing if the re-inoculation of the biopiles after 22 days of treatment would improve the removal yields. It was found that 2 out of the 15 initially detected pharmaceuticals were totally degraded after 22 days, and re-inoculated fungal biopiles achieved higher removal rates than non-re-inoculated fungal biopiles for single compounds and for all the drugs simultaneously: 66.45% and 49.18% re-inoculated and non-re-inoculated biopiles, respectively. Finally, the study of the bacterial and fungal communities revealed that fungal inoculated and non-inoculated biopiles evolved to similar communities adapted to the presence of those drugs.

Keywords

Abbreviations

BQL: Below Quantification Limit; **DGGE:** Denaturing Gradient Gel Electrophoresis; **DW:** Dry Weight; **EP:** Emerging Pollutant; **ITS:** Internal Transcribed Spacer; **ND:** Non-Detected; **PhAC:** Pharmaceutical Active Compound; **PPCP:** Pharmaceutical and Personal Care Products; **WHC:** Water Holding Capacity; **WRT:** White-Rot Fungi; **WWTP:** Wastewater Treatment Plant

29Bioremediation; *Trametes versicolor*; biopiles; pharmaceutical products; sewage sludge;
30microbial shifts.

31**1. Introduction**

32 The main residue of any Wastewater Treatment Plant (WWTP) is the sludge, which
33is originated during the solid-liquid separation (Fytili and Zabaniotou, 2008) performed
34in primary, secondary and tertiary treatments, and its composition and quantity depend
35on several factors such as the general operational methods and the geographic situation
36of the plant (Eddy *et al.*, 1991). Furthermore, the wastewater source had an important
37role not only in the formation of the sludge, but also in its final composition and
38physicochemical properties. The most common wastewaters treated in WWTPs have an
39urban, domestic and/or hospital origin (Harrison *et al.*, 2006).

40 The use of WWTP's sludge in agricultural and forestry activities has become an
41interesting valorisation method because of its ability to fertilise soils and the low cost of
42these materials compared to fertilisers. These actions improve the physicochemical
43properties of the land and can increase the crops yield (Singh and Agrawal, 2008).
44Nevertheless, the application of untreated sludge into soils can increase the potential
45risks for human and animal health (Dean and Suesst, 1985), as it can contain different
46types of pollutants. Consequently, sludge must be treated before its application into soil
47in order to remove micro-pollutants.

48 The sludge produced in a WWTP usually has a high concentration of solids –
49between 0.25 to 15 % in weight –, which is mainly composed of organic matter. Its
50treatment and disposal is one of the most complex and expensive problems during
51wastewater treatment. In general, sewage sludge must be stabilized, thickened and
52disinfected before its disposal out of the plant. Common techniques to stabilize the
53sludge are: anaerobic and aerobic digestion, lime stabilization, composting and heat

drying; while the general thickening treatments are: centrifugation, filtration and water evaporation (Eddy *et al.*, 1991; Ramalho, 1996). However, it has been proven that these traditional treatments are not capable of removing emerging pollutants (EPs) from the sludge (Clarke and Smith, 2011; Semblante *et al.*, 2015; Stasinakis, 2012).

Expensive tertiary or advanced treatments have been developed in the last years in order to decrease the presence of EPs in wastewater: adsorption into activated carbon, advanced oxidation (e.g. ozone and ultra-violet), UV photolysis, ion exchange and membrane filtration (Bolong *et al.*, 2009; Gavrilescu *et al.*, 2014). Nevertheless, these technologies have high implementation, operational and maintenance costs and have not yet been applied to sewage sludge (Heal the Ocean, 2001; USEPA, 2015).

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

The substrate plays an important role in biopiles inoculated with white-rot fungi (WRF), being one of the key factors for a successful mycoremediation application (Leštan *et al.*, 1996). In those cases, a lignocellulosic waste from agriculture, forestry and/or food industry is supplied as substrate, providing the essential lignocellulosic nutrients that the fungus needs to growth, and promoting the production of lignin-

modifying enzymes (Rodríguez Couto and Sanromán, 2005). The most studied and used lignocellulosic substrates are: sawdust, wood chips and barks, wheat straw, corn cobs, grape stalks, and olive oil waste (Kassaveti, 2008; Rigas *et al.*, 2007; Stahl and Aust, 1998). The correct selection of a ligninolytic substrate will lead to better pollutant removal by the inoculated WRFs, with low operational time and minor investments.

In previous studies, dry WWTP sludge was treated in biopiles inoculated with *Trametes versicolor* (Rodríguez-Rodríguez *et al.*, 2014). Straw was used as a substrate and microbial analysis demonstrated that *Trametes versicolor* can still be detected, at least, until day 22. From these results, a re-inoculation strategy for the biopiles after 22 days of treatment – in order to enhance the removal of pharmaceutical and personal care products (PPCPs) – was implemented. The aim of the present work was to determine if bioaugmentation with *T. versicolor* could be used to treat thermal dry WWTP sludge in biopiles systems using pine bark as a substrate. Although bark is more difficult to degrade, hydrolyze and colonize than straw, it is a better bulking material compared to straw and allows the scale-up of biopiles. Biopiles systems under non-sterile conditions were constructed using a non-spiked sludge, treating it for 42 days. A re-inoculation was carried out after 22 days of treatment, PPCPs removals were assessed and microbial community analysis were performed.

2. Materials and methods

2.1 Chemicals

All pharmaceutical compounds, methanol and acetonitrile were of high purity grade (> 90%) and were purchased from Sigma-Aldrich (Steinheim, Germany), US Pharmacopeia USP (MD, USA), Europea Pharmacopeia EP (Strasbourg, France) and Toronto Research Chemicals TRC (Ontario, Canada). Further information can be

consulted in Gros *et al* (2012). The individual standard solutions were prepared according to Gros *et al.* (2012).

2.2 Microorganisms

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

2.3 Sludge and lignocellulosic substrate

20 L of dry sewage sludge were collected from the final stage of the sludge processing system at El Prat de Llobregat WWTP (Spain) in January of 2014. This plant is designed to treat 419,000 $m^3 \cdot d^{-1}$ of wastewater for an equivalent population of two million inhabitants. It is a typical activated sludge (AS) plant that uses anaerobic digesters followed by dehydration and thermal drying techniques to treat the produced sludge. The initial water content of the sludge was 16.71 ± 0.03 %, and its water holding capacity was 1.19 ± 0.06 $gH_2O \cdot gDW^{-1}$. At the arrival to the laboratory the sludge was frozen (-20 ºC) until its use.

10 L of commercial decorative pine bark (*Pinus halepensis*) were bought from a local supplier and used as lignocellulosic substrate for the biopiles systems. The initial size of the pine barks ranged from ca. 2.5 cm to 10 cm. Pruning scissors were used in order to make the small pieces of pine bark not larger than 1.5 cm in the tests. The initial water content was 21.27 ± 0.43 %, and its water holding capacity was 1.28 ± 0.01 $gH_2O \cdot gDW^{-1}$. The substrate was kept at room temperature until its use.

2.4 Experimental procedures

2.4.1 Fungal mycelial suspension

Blended mycelial suspension and pellet suspension were prepared according to Blánquez *et al.* (2004). Mycelial suspension was made as follows: Erlenmeyer flasks (500 mL) with 150 mL of malt extract medium (2%) were inoculated with 1 cm^2 plugs

from agar cultures in Petri dishes and shook (orbital shakers: 135 rpm and r=25 mm) for 5 days at 25 ºC; the resulting fungal mass was homogenized (X10/20, Ystral GmbH) and stored in sterilized saline solution (0.85% NaCl) at 4 °C.

2.4.2 Water Holding Capacity and moisture content

The initial moisture of the sewage sludge from El Prat de Llobregat WWTP and the pine bark were determined weighing homogeneous volumes of sludge or substrate, and drying them for 24 hours at 105 ºC in an oven. They were expressed as percentage of moisture.

The water holding capacity (WHC) of the sewage sludge and the pine bark was determined as described by the European Committee for Standardization: metal cylinders with one of its ends covered with paper filter were filled (up to 2/3 parts of their volume) with sludge or substrate and placed in trays; water was added until it fully covered the sludge or substrate, without exceeding it; next, after two hours in contact with water, the cylinders with sludge or substrate were dried by capillarity for 30 min and weighted (wet weight); and finally, the cylinders were dried for 24 hours at 105 ºC in an oven and weighted (dry weight – DW). The WHC was calculated as the difference between the dry and the wet weights and expressed as grams of water by gram of dry sludge/substrate ($gH_2O \cdot gDW^{-1}$) (CEN, 1999).

2.4.3 Solid-phase experiments

Cultures were performed in Schott bottles (250 mL, 95 x 105 mm; GLS 80; Duran, Inc) equipped with 4-port screw caps (GL 18; Duran, Inc). Three ports of the caps were hermetically closed, while one was kept opened, using a 0.45 µm filter as passive air intake. First, 6 g of sterile lignocellulosic substrate (20 min at 120 ºC) were placed in each bottle and inoculated with 2 mL of mycelial suspension, setting humidity to 60% of the water holding capacity. After 7 days of static incubation (25 ºC), biopiles were

prepared adding 14 g of non-sterile dry WWTP sludge in every single pre-grown fungal culture and then homogenised though circular movements, without screwing the bottle; the moisture level was adjusted to 60%. Biopile cultures were incubated at 25 ºC in static conditions until sampling. After 22 days of incubation, half of the biopiles were re-inoculated with concentrated mycelial suspension, while same volume of water was added to the other half. Additionally, control cultures were performed with 6 g of sterile lignocellulosic substrate (20 min at 120 ºC) and 14 g of non-sterile dry WWTP sludge at 60% of the water holding capacity, but without *T. versicolor* inoculation. Triplicate cultures were sacrificed for analysis at every sampling time.

2.5 Analytical methods

2.5.1 Laccase activity

Laccase from biopile systems was 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. The supernatants were centrifuged at 15,000 *g* for 15 min. Enzymatic activity was measured using a modified version of the method for manganese peroxidase determination (Wariishi *et al.*, 1992). The reaction mixture consisted of 200 µL sodium malonate (250 mM, pH 4.5), 50µL 2,6-dimethoxyphenol (DMP, 20 mM), and 600 µL sample. Dimethoxyphenol (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 liter ($U \cdot L^{-1}$). One U was defined as the number of DMP micromoles oxidized per min. The DMP extinction coefficient was 24,800 $M^{-1}cm^{-1}$.

2.5.2 Total PPCPs quantification

Biopile cultures were frozen (-80 ºC) for 24 hours without further pre-treatment. 1.0 g of grounded biopiles was extracted using an accelerated solvent extraction system (Jelić *et al.*, 2009). The concentrated extracts were diluted in 500 mL of water and

filtered through 0.45 μm nylon membrane filters (Whatman, UK) in order to retain the suspended solids. A certain volume of the chelating agent EDTA was added to all of the samples to a final concentration of 3% (ml solute · ml^{-1} solution), which is well known to enhance the extraction of some pharmaceutical active compounds (PhACs). The clean up of the samples was performed by solid phase extraction using a Baker (J.T. Baker®) system and Oasis HLB 3 cc, 60 mg, extraction cartridges (Waters Corp. Mildford, MA, USA). The 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 by 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). Finally, 10 μL of the internal standard mix at 10 ng·μL^{-1} were added in the extracts for calibration and to compensate for a possible matrix effect, if necessary.

Chromatographic separation was performed using an UPLC system (Waters Corp. Mildford, MA, USA) equipped with a binary solvent system (Mildford, MA, USA) and a sample manager equipped with an Acquity HSS T3 column (50 mm × 2.1 mm, 1.7 μm particle size; Waters Corp. Mildford, MA, USA) for the compounds analysed under positive electrospray ionization and with an Acquity BEH C18 column (50 mm × 2.1 mm, 1.7 μm particle size) for the ones analysed under negative electrospray ionization, both purchased from Waters Corporation. The UPLC instrument was coupled to a 5500 QqLIT, quadrupole–linear ion trap mass spectrometer (5500 QTRAP, Applied Biosystems, Foster City, CA, USA) with a Turbo V ion spray source. All the transitions were recorded using the Scheduled MRM™ algorithm, and the data were acquired and processed using Analyst.

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

2.6 Microbial community analysis

2.6.1 Denaturing gradient gel electrophoresis

Total DNA was extracted from lyophilized samples with PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc) following the procedure described by the manufacturer. Additionally, PCR inhibitors were removed with OneStep™ PCR Inhibitory Kit (Zymo Research, Inc). Fragments of bacterial 16S and fungal Internal Transcribed Spacer (ITS) region of 18S rDNA were PCR amplified by Taq DNA polymerase (Invitrogen). For bacteria, the primers 341f-GC (5' CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCC TAC GGG AGG CAG CAG 3') (Muyzer *et al.*, 1993) and 907r (5' CCG TCA ATT CMT TTG AGT TT 3') (Muyzer *et al.*, 1995) were used. Fungal DNA was amplified using two sets of primers in two rounds: first, the primers EF4 (5' GGA AGG GRT GTA TTT ATT AG 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3'); and second, the primers ITS1f-GC (5' CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCT TGG TCA TTT AGA GGA AGT AA 3') and ITS2 (5' GCT GCG TTC TTC ATC GAT GC 3') (Gardes and Bruns, 1993). The PCR programs for bacteria and fungi are shown in supplementary table 1.

Denaturing Gradient Gel Electrophoresis (DGGEs) was performed in a Dcode Universal Mutation Detection System (Bio-Rad). Urea-formamide denaturant gradients were adjusted in order to optimize the bands separation, being the final gradients 30-

170% for bacteria and 15-55% for fungi, and 6% of acrylamide gel for both.
Electrophoreses were performed during 16 hours at 75 V in 1x TAE buffer at 60 ºC.
Gels were stained with ethidium bromide (1 μg·mL^{-1}). Selected DGGE bands were
excised and re-amplified. Purification and sequencing were performed by a commercial
service (Macrogen Inc., South Korea) with the primers: 341f for bacteria, and ITS1f for
fungi. Sequences were manually trimmed and quality checked using FinchTV 1.4.0
(Geospizza, Inc.) and further checked for chimeras using the UCHIME algorithm
(Edgar et al., 2011). Curated sequences were deposited in the GenBank database from
the National Center for Biotechnology Information under the accession numbers
MF383380— MF383399 and MF398478—MF398488 for Bacteria and Fungi,
respectively.

2.6.2 Real-time qPCR

To quantify the contribution of *T. versicolor* to the whole community, a quantitative PCR reaction was performed. The qPCR assay was performed using a specific primer set targeting *T. versicolor* (Eikenes *et al.*, 2005) and the following amplification program: 95ºC for 10 min and 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min. Reactions contained 1× ssoAdvanced Universal SYBR Green Supermix (Bio-Rad), 0.3 μM of each forward and reverse primers and 36 ng of sample DNA in a final volume of 20 μL. All reactions were run in triplicate in a CFX95 Real-Time System (Bio-Rad) with amplification efficiency of 93.5% and R2 value of 0.999. Calibration curve was prepared using a purified amplicon (302 bp) from the ITS region of *T. versicolor* ATCC 42530.

2.7 Statistical analysis

254 Descriptive statistics were calculated using Microsoft Excel 2011 and SOFA
255Statistics (v. 1.4.3.). One-factor ANOVA procedure with $p<0.05$ was utilised, and
256Dixon's Q-test was used in order to identify and reject outlier values.

257**3 Results and Discussion**

258 It has been demonstrated that different lignocellulosic substrates can be used to
259grow WRF in bioremediations systems; however, not all the described substrates are
260able to act as bulking material inside fungal biopiles systems. In this work pine bark
261was used as co-substrate. It was proved that this material is an effective bulking material
262in solid state fermentation, and it was already reported that fungi could grow in this type
263of wood (Valentín *et al.*, 2010). The selected barks were of *Pinus halepensis*, since in
264our region (Catalonia) the most common forests are of Aleppo pine (CREAF, 1998), and
265it has the lowest economical value (CTFC, 2015).

2663.1 Total drugs removal

267 Biopiles were constructed with pre-grown fungi onto sterile pine barks and non-
268sterile thermal dried WWTP sludge. The fungus activity was monitored by means of
269laccase activity, as *T. versicolor* has been reported to produce large amounts of this
270enzyme (Couto *et al.*, 2002; Lorenzo *et al.*, 2002; Pazarlıoğlu *et al.*, 2005). The
271maximum enzymatic activity was achieved by day 10 with 0.007 ± 0.002 $U \cdot g^{-1}$, which
272decreased to nil by day 22. After re-inoculating half of the experimental cultures,
273laccase activity was maintained without any fluctuation for these systems, but it was
274null for non-re-inoculated biopiles. These laccase activities were low if compared with
275other studies, for instance activities 1 and 4 orders of magnitude higher were achieved
276for small biopiles with soil (Borràs *et al.*, 2010) and for biopiles with sewage sludge
277(Rodríguez-Rodríguez *et al.*, 2010); however, these researchers used wheat straw as
278ligninolytic substrate, which has been proved to be more easily degradable by the

fungus. Also, the phenolic groups of the pine barks could inhibit the laccase expression. Although the laccase activity was low, the degradation pathway not only involved the fungal co-metabolism (ligninolytic enzymes), but also its metabolism and detoxification mechanisms (cytochrome P450) (Badia-Fabregat *et al.*, 2014; Corvini *et al.*, 2006; Reddy, 1995). Additionally, the competition with the sludge's autochthonous populations could lead to a lower laccase expression so we performed a microbial community analysis to assess potential interactions between the indigenous populations and *T. versicolor*.

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

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

304removal with the same analytical method as in the present work (Rodríguez-Rodríguez
305*et al.*, 2012), detecting only 9 PPCPs in the sludge, 3 of which were also detected in the
306present experiment, but at different concentrations: atorvastatin (13.7 ± 1.8 $ng \cdot g^{-1}$),
307carbamazepine (10.5 ± 0.7 $ng \cdot g^{-1}$) and ibuprofen (161.0 ± 21.4 $ng \cdot g^{-1}$).

308 In a second study of Rodríguez-Rodríguez *et al.* (2014), the authors found 21
309pharmaceutical compounds, 8 of them detected in the current biopiles: amlodipine
310(13.66 $ng \cdot g^{-1}$), carbamazepine (5.13 $ng \cdot g^{-1}$), citalopram (309.52 $ng \cdot g^{-1}$), codeine (9.28
311$ng \cdot g^{-1}$), gemfibrozil (86.17 $ng \cdot g^{-1}$), ibuprofen (203.93 $ng \cdot g^{-1}$), olanzapine (19.58 $ng \cdot g^{-1}$)
312and venlafaxine (134.05 $ng \cdot g^{-1}$). Therefore, although the sludge was produced in the
313same WWTP and collected from the same point, the pollutants content and
314concentrations in the sewage sludge varied among time (this can be appreciated
315comparing the results found by other authors – listed in the two paragraphs above – and
316the values found in the present study – table 1); implying that EPs treatment systems
317should not be specific for certain compounds and concentration ranges. In this regard,
318fungal biopiles were proved to remove a wide variety of PPCPs without no or minimal
319changes in their configuration.

320 It can be said that, in the present study, partial or total PhACs removals have been
321achieved. Olanzapine and diltiazem were the only drugs completely removed, both after
32222 days of treatment. For non-re-inoculated biopiles, 5 drugs (not taking into account
323the compounds completely removed) were eliminated with removal efficiencies higher
324than 75%: atorvastatin, paroxetine, sertraline, sulfamethoxazole and
325hydrochlorothiazide, whereas for re-inoculated cultures 8 PhACs experienced removals
326higher than 75%: amlodipine, atorvastatin, paroxetine, sertraline, sulfamethoxazole,
327trazodone, fluoxetine and hydrochlorothiazide. On the contrary, 5 compounds showed
328removal yields lower than 20% for non-re-inoculated biopiles: ibuprofen, oxycodone,

codeine, tamsulosin and citalopram; while in re-inoculated cultures 2 drugs experienced removals lower than 20%: venlafaxine and carbamazepine. Furthermore, 4 drugs showed higher concentrations after 22 days of treatment (i.e. ibuprofen, oxycodone, codeine and carbamazepine) and 3 drugs after 42 days (i.e. venlafaxine and carbamazepine in non-re-inoculated cultures; codeine in the re-inoculated). This can be explained by the occurrence of conjugates. Conjugation is a mechanism used by several organisms to detoxify xenobiotics, which involves the covalent addition of a molecule to a compound. In the human liver, the conjugation leads to the formation of water-soluble compounds that can be excreted through urine, being glucuronidation the most common conjugation pathway and often the initial step of biotransformation of xenobiotics. The analysis is only able to detect and quantify the non-conjugated molecules, showing higher concentrations after the treatment than at the beginning. Although conjugation/deconjugation processes of PPCPs have mainly been observed in liquid environments, it has been described that it could also take place in river sediments and sewage sludge (Andersen *et al.*, 2003; Gomes *et al.*, 2004; Labadie and Hill, 2007; Matejícek *et al.*, 2007; Petrovic *et al.*, 2002; Ternes *et al.*, 2002).

The concentration of each pollutant in the biopiles is shown in figure 1. In general, important concentration decreases were obtained for all the detected compounds except for citalopram, venlafaxine (both psychiatric drugs; plot a in figure 1) and ibuprofen (plot b in figure 1). Citalopram is a largely consumed antidepressant that can interfere both the behaviour regulation and neuroendocrine signals of aquatics organisms. Since only a moderate biodegradability (ca. 50%) has been achieved in traditional WWTP, further tertiary treatments are being studied in order to increase its removal (Calisto *et al.*, 2014; Hörsing *et al.*, 2012). Similarly, venlafaxine is a widely prescribed antidepressant that affects the brain serotonin levels of fishes. As this drug has been

poorly removed in conventional WWTP, advanced treatments such as ozonization are being studied (Bisesi *et al.*, 2014; Li *et al.*, 2015; Rúa-Gómez and Püttmann, 2013). Finally, ibuprofen is an analgesic used worldwide that is inadequately removed in regular WWTP. Its degradation in sewage sludge, wastewater, surface and ground water has been broadly studied (Collado *et al.*, 2012; Evgenidou *et al.*, 2015; Kosma *et al.*, 2014).

Rodríguez-Rodríguez *et al.* (2014) observed similar removal patterns for single and overall compounds while studying re-inoculation strategies for larger fungal biopiles. However, the authors did not assess how the microbial communities of the sludge evolved and affected both the removal percentage and the biopiles colonization by *T. versicolor*. Thus, the influence of the autochthonous WWTP sludge populations during the fungal bioremediation strategy is studied in this work.

3.2 Microbial community evolution

PCR-DGGE fingerprints were obtained in order to determine the microbial diversity in the biopile systems. Fungal and bacterial characterization were carried out in 5 different time-points for inoculated cultures (experimental group): 0, 10, 22, 23 (only for re-inoculated biopiles) and 42 days. Only times 0, 22 and 42 days were examined in non-inoculated cultures (control group).

DGGE profiles are presented in figures 2 and 3, showing different community structures along time. It can be observed that *T. versicolor* was the predominant fungi in the biopiles after the inoculation at time 0, and it remained as the prevailing one at least until day 23 – the day after half of the experimental biopiles were re-inoculated. At the end of the experiments (day 42) *T. versicolor* was not the predominant fungus in the biopiles, although PPCPs removal improved in the re-inoculated ones.

As expected, the inoculation of the sludge with *T. versicolor* changed the fungal populations in comparison with the non-inoculated controls as it is reflected in the DGGEs profiles. Interestingly, bacterial communities were not affected by the fungal inoculation and both the experimental and control piles followed similar dynamics. Nevertheless, it should be noted that other parameters such as the experimental design and the PPCPs contained in the sludge could have also affected the microbial populations dynamics.

Previous to this work, the increase in the number of microorganisms and the change of the microbial population in soils due to bioremediation processes, such as bioaugmentation and biostimulation techniques, was reported in polluted soils. For instance, Lin *et al*. (2010) noticed that, while treating soil polluted with diesel and fuel oils in biopiles, the microbial presence not only increased, but also changed, leading to communities adapted to degrade recalcitrant contaminants. Wang *et al.* (2011) observed similar microbial community tendencies when composting polluted soil with hydrocarbons followed by a rhizodegradation process. Betancur-Corredor *et al.* (2015) found a similar microorganism increase during the biostimulation of soil with pesticides, and Kao *et al.* (2015) noted a microbial community shift when polluted groundwater with chlorinated products was also biostimulated. These observations corroborate the community change experienced not only in the biopiles without fungal inoculation, but also the biopile bioaugmented with *T. versicolor*.

On one hand, dry sludge in non-inoculated biopiles was stimulated by adding water and pine bark, which offered a better fluid transport and an additionally carbon source, resulting infungal and bacterial populations well-adapted to the culture conditions and the presence of PPCPs, leading to the assessed PPCPs removal. On the other hand, in biopiles where *T. versicolor* was inoculated, the sludge was not only stimulated by the

addition of a bulking material and water, but also with an organism that mineralised the lignocellulosic substrate and certain pollutants of the sludge (Dehorter and Blondeau, 1992; Marco-Urrea *et al.*, 2008; Sack *et al.*, 1997; Tuomela *et al.*, 1998), making them a more accessible source of C and N for other organisms. Regarding PhACs, few studies about mineralisation of pharmaceuticals by *T. versicolor* are available in the bibliography, however Marco-Urrea et al., 2010 suggest possible mineralization of ketoprofene. Furthermore, the addition of the substrate and the fungus changed the aerobic/anaerobic conditions of the sludge, exposing anaerobic organisms to O_2. This would explain the rapid shift of the bacterial community (figure 3), as obligate anaerobes would have died, have produced spores or were a minority member of the community population. In the fungal community, the prevalence of *T. versicolor* during the first half of the experiment was due to three main reasons: (i) it was inoculated in high amounts, (ii) the normal fungi strategy to prevent the colonisation by other fungi (Cano and Bago, 2005), and (iii) the different colonisation strategies of each fungus (Mucha *et al.*, 2014). Briefly, *Trametes* is specialized in the colonization of decaying wood and that confers him advantage over generalist fungi, surface saprophytes or endophytes that need wounds, specific tissues or insect vectors to colonize. The demise of the fungus could be due to the interaction with the autochthonous organisms of the system (Ejechi and Obuekwe, 1994).

Finally, in order to elucidate the fate of *T.versicolor* in the biopiles, a real-time qPCR assay was performed. Three samples from initial (d0) and both non-reinoculated and re-inoculated end points (d42 and d42Re) were processed and results are shown in Figure 4. As it was suspected, *T. versicolor* did not disappear from the biopiles, but its concentration was reduced at least 100-fold even when re-inoculated; justifying why it was not detected in the DGGE after 23 days (d23).

3.2.1 Fungal community

As can be seen in figure 2 and table 2, the predominant bands in fungal DGGE belonged to *T. versicolor* in inoculated cultures during the first 23 days.

In contrast, non-inoculated biopiles did not show any predominant band, and different fungi were observed in every time-point; being *Trichosporon asahii* and *Meyerozyma sp.* the only fungi observed twice in these cultures (by days 22 and 42 for both). In fact, all fungi detected in control cultures at initial time (day 0) existed also in inoculated cultures, but they were not detected in the PCR-DGGE fingerprints as *T. versicolor* was in a far greater concentration (>99% of the community population); being almost the only one amplified. Moreover, and given the source of the sewage sludge, the biopiles fungi likely came from the ligninolytic substrate. Particularly, 6 out of the 11 fungal genera detected are known to decompose or take advantage of lignocellulosic materials: *Acremonium.* (saprophyte organisms that decompose plants), *Pseudallescheria* (saprophytic fungi that lives in soils rotting vegetation), *Peniophora* (plant parasites, saprophytes or symbiotic organisms), *Rhodotorula* (which can grow in water, soil and air), *Aspergillus* (used industrially) and *Coriolopsis* (from the same order of *T. versicolor*, this fungus also decomposes decaying wood), which was the only one found the day 42 (Jimenez-Quero *et al.*, 2017; Maddison *et al.*, 2007; Malloch, 2013; Nordberg *et al.*, 2014; Robert *et al.*, 2013).

Furthermore, 8 of the detected fungi have been reported to be able to degrade and/or to live in habitats with EPs (Ahalya *et al.*, 2003; Ertuğrul *et al.*, 2009; Huang and Huang, 1996; Lakshmi and Das, 2013; Lakshmi *et al.*, 2013; Matheus *et al.*, 2000; Moreira Neto *et al.*, 2011; Pointing, 2001; Viegas *et al.*, 2014), but only 3 of them have endured until the end of the experiment of the present study: *Coriolopsis gallica*, which has been studied in the degradation of organic pollutants in liquid media (Bressler *et al.*,

4532000; Yagüe *et al.*, 2000), and *Meyerozyma* sp. and *Wickerhamomyces anomalus*, two
454Saccharomycetales that have been reported to degrade oil products (Goulart *et al.*,
4552014).

456 Additionally, the 4 fungi that had mycostatic abilities persisted until the end of the
457experiment: *Trichosporon asahii* (from the Tremellales order) is a yeast that parasitises
458other fungi (El-Tarabily, 2004), and *Wickerhamomyces anomalus*, *Meyerozyma* sp. and
459*Meyerozyma guilliermondii*, which can produce mycocin killer toxins (Coda *et al.*,
4602013; Druvefors *et al.*, 2005; Parafati *et al.*, 2015; Walker, 2010).

4613.2.2 Bacterial community

462 At the beginning of the experiment (day 0), both cultures mainly consisted of
463organisms from the Clostridiales order (*Clostridium* sp. and *Intestinibacter bartlettii*);
464which are Gram-positive obligate anaerobes capable of forming heat and desiccation-
465resistant endospores (Gupta, 2015). The demise of those organisms was expected after
466the addition of the ligninolytic substrate in control cultures, which caused the input of
467O_2. While facultative anaerobes could survive, obligate anaerobes such as *Clostridium*
468spp and *Intestinibacter bartlettii* either perished or decreased their relative abundance to
469less than 1% of the population.

470 As can be seen in figure 3 and table 3, both inoculated and non-inoculated biopiles
471showed a similar bacterial community along all the operation, *Lysobacter* being the
472predominant genus in bacterial DGGE. This bacterium belongs to the Xanthomonadales
473order, which are characterised for being Gram-negative sporulating phytopathogens
474(Gupta, 2015). *Lysobacter* sp has been found during the treatment of synthetic effluents
475with PPCPs in an experimental sequencing batch bioreactor inoculated with activated
476sludge from a WWTP in Portugal (Amorim *et al.*, 2014); and in an anoxic/oxic-
477membrane bioreactor (MBR) at lab-scale inoculated with activated sludge from a

WWTP in China (Gao *et al.*, 2014). Additionally, *Lysobacter* sp. has been identified in the bioremediation processes of surfactants in river sediments (Wang *et al.*, 2014) and soils polluted with pesticides (Betancur-Corredor *et al.*, 2015).

Five different phylotypes related to the Bacillales order were also described. They are close to the Clostridiales order as both are encompassed in the Firmicutes phylum. These bacteria were only detected during the firsts days and until day 22, after which no more species of this order were identified. The species from the Bacillales order have been found as one of the dominant orders in mixed liquor suspended solids of a lab-scale sequencing batch bioreactor inoculated with anaerobic sludge (Jena *et al.*, 2015), and they have also been detected in compost piles made of anaerobic sludge and wheat residues as bulking material (Li *et al.*, 2014). Furthermore, it has been reported that not only some Bacillales in compost piles degrade cellulose, solubilized lignin and colonized the lignocellulosic bulking material, but they can also increase the degradation of organic pollutants (Li *et al.*, 2013; Martins *et al.*, 2013).

Other microorganisms from many bacterial orders aside from *Lysobacter* were also detected at intermediate and final stages and are worth mentioning: *Brevibacterium siliguriense* and *B. vesicularis*, aerobic Gram-negative non-sporulating microorganisms from the Caulobacterales order (Bhatawadekar and Sharma, 2011; Collins, 2006; Dass *et al.*, 2002); *Dietzia* sp., a Gram-positive bacteria of the Corynebacteriales order; *Salinimicrobium* sp., a Gram-negative Flavobacteriales; and *Pedobacter bauzanensis*, a Gram-negative from the Sphingobacteriales order.

In general, all these bacterial species have been found in WWTP (Beale *et al.*, 2015; Friha *et al.*, 2014; Li *et al.*, 2011; Wagner and Loy, 2002) and reported to degrade organic pollutants in wastewaters, sludges or soils (Jin *et al.*, 2012; Mao *et al.*, 2012;

Maqbool *et al.*, 2012; Pérez *et al.*, 2015; Satheesh Babu *et al.*, 2015; Sun *et al.*, 2015). Interestingly, Caulobacterales representatives were not detected in non-inoculated cultures at 42 days and they are precisely the only group in which contribution in the removal of environmental pollutants has not been described yet. Members of this taxonomical group have been associated with cellulose metabolism and its disappearance could be related with shifts in fungal populations.

4 Conclusions

The present work has proved that *T. versicolor* is able to degrade drugs from thermal dried sewage sludge in biopiles systems under non-sterile conditions, using pine bark as lignocellulosic substrate. The percentage of drugs removal in fungal inoculated biopiles was 66.45%, while for non-inoculated biopiles was 49.18%.

T. versicolor was the most abundant fungi in inoculated biopiles at least until day 22; after which, its presence was reduced at least 100-fold, not being the dominant specie at the end of the experiment. Interestingly, the fungus was unable to colonize the biopiles after being re-inoculated but the global drugs concentration decreased.

Fungal community in inoculated biopiles shifted from one predominant specimen to three prevalent species: one lignocellulosic decomposer and two fungi with mycostatic abilities. In control groups, a diverse community ended up with two predominant species (*T. asahii* and *Meyerozyma* sp.), both harbouring fungal inhibitory systems and pollutant removal abilities.

The bacterial community was not directly affected by the inoculation of *T. versicolor*; however, the addition of the lignocellulosic substrate led to an aeration of the original anaerobic sludge.

Further research should be carried out in order to: (i) evaluate the degradation rate of each compound, and (ii) determine if biopiles re-inoculation should be performed at an

early stage (i.e. while the fungus is not only still active, but also the predominant organism) or with previously colonized substrate, improving the viability of the fungus.

5 Acknowledgments

This work has been supported by the Spanish Ministry of Economy and Competitiveness (project CTQ2010-21776-C01) and supported by the Generalitat de Catalunya (Consolidated Research Groups 2014-SGR-476 and 2014-SGR-559). 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. Guillem Llorens-Blanch and Eloi Parladé acknowledges the pre-doctoral grants from the Spanish Ministry of Economy and Competitiveness, Secretariat of State for Research, Development and Innovation (BES-2011-046090) and the Universitat Autònoma de Barcelona (PIF2014-2015 D040902/D10535), respectively.

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Table 1. Detected PhACs in Biopiles systems and its removal yields before and after re-inoculation

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

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

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Table 2. Sequence information for the DGGE bands obtained analysing the fungal community

Band code	Lane/s	Accession number	Closest related sequence (% similarity)	Order
A	20-22	KP975532	*Aspergillus oryzae* (100)	Eurotiales
B	7	KF669512	*Acremonium* sp. (99)	Hypocreales

C	19	KP132722	*Pseudallescheria ellipsoidea* (100)	Microascales
D	16	AY684172	*Coriolopsis gallica* (99)	Polyporales
E	1-13; 19-22	KP761168	*Trametes versicolor* (100)	Polyporales
F	18	LN808982	*Peniophora cinerea* (100)	Russulales
G	22-24	KR054629	*Meyerozyma* sp. (100)	Saccharomycetales
H	14	KJ451713	*Wickerhamomyces anomalus* (99)	Saccharomycetales
I	15; 20-22	KR085964	*Meyerozyma guilliermondii* (100)	Saccharomycetales
J	17	LN833560	*Rhodotorula mucilaginosa* (99-100)	Sporidiobolales
K	22-23	KP658861	*Trichosporon asahii* (99-100)	Tremellales

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Table 3. Sequence information for the DGGE bands obtained analysing the bacterial community

Band code	Lane/s	Accession number	Closest related sequence (% similarity)	Order
A	3-6	LN774422	*Bacillus humi* (94)	Bacillales
B	8	KP670289	*Bacillus* sp. (97)	Bacillales
C	23	HQ603002	*Sporosarcina* sp. (98)	Bacillales
D	8	KT261256	*Staphylococcus nepalensis* (99)	Bacillales
E	21-23	KR732655	*Staphylococcus stepanovicii* (95)	Bacillales
F	21-23	LC082101	*Brevibacterium siliguriense* (100)	Caulobacterales
G	1-6; 8; 10; 12; 14-17	LC068966	*Brevibacterium* sp. (100)	Caulobacterales
H	10-15	KP895785	*Brevundimonas vesicularis* (97)	Caulobacterales
I	18-20	AB971795	*Clostridium celatum* (98)	Clostridiales
J	1-3;18-20	KF528156	*Clostridium cellulovorans* (94)	Clostridiales
K	1-3	KJ722507	*Clostridium glycolicum* (98)	Clostridiales
L	1-3	EU089965	*Clostridium maritimum* (97)	Clostridiales
M	1-3;18-20	KJ722512	*Clostridium ruminantium* (99)	Clostridiales
N	1-3;18-20	AB610575	*Clostridium* sp. (99)	Clostridiales
O	1-3	FJ424481	*Clostridium* sp. (100)	Clostridiales
P	1-3;18-20	NR_027573	*Intestinibacter bartlettii* (97)	Clostridiales
Q	8-17	KR181931	*Dietzia* sp. (98)	Corynebacteriales
R	9-17; 22-26	HG008896	*Salinimicrobium* sp. (95)	Flavobacteriales
S	4-17; 21-26	NR_117231	*Pedobacter bauzanensis* (97)	Sphingobacteriales
T	2-17; 21-26	DQ490982	*Lysobacter* sp. (100)	Xanthomonadales

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