Appl Microbiol Biotechnol (2016) 100:2401–2415
DOI 10.1007/s00253-015-7105-0

ENVIRONMENTAL BIOTECHNOLOGY

Continuous fungal treatment of non-sterile veterinary hospital effluent: pharmaceuticals removal and microbial community assessment

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Received: 15 September 2015 / Revised: 13 October 2015 / Accepted: 14 October 2015 / Published online: 6 November 2015

Abstract Source point treatment of effluents with a high load of pharmaceutical active compounds (PhACs), such as hospital wastewater, is a matter of discussion among the scientific community. Fungal treatments have been reported to be successful in degrading this type of pollutants and, therefore, the white-rot fungus *Trametes versicolor* was applied for the removal of PhACs from veterinary hospital wastewater. Sixty-six percent removal was achieved in a non-sterile batch bioreactor inoculated with *T. versicolor* pellets. On the other hand, the study of microbial communities by means of DGGE and phylogenetic analyses led us to identify some microbial interactions and helped us moving to a continuous process. PhAC removal efficiency achieved in the fungal treatment operated in non-sterile continuous mode was 44 % after adjusting the C/N ratio with respect to the previously calculated one for sterile treatments. Fungal and bacterial communities in the continuous bioreactors were monitored as well.

Keywords Veterinary hospital wastewater · Continuous treatment · Pharmaceutically active compounds · White-rot fungi

Marina Badia-Fabregat and Daniel Lucas contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00253-015-7105-0) contains supplementary material, which is available to authorized users.

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Introduction

Pharmaceutically active compounds (PhACs) are a group of organic micropollutants with raising concern among the scientific community due to their low biodegradability in activated sludge wastewater treatment (Joss et al. 2006; Verlicchi et al. 2012), their presence in surface waters, groundwater, and even tap water (Gros et al. 2012; Jurado et al. 2012), and their associated risks (Verlicchi et al. 2012). Joss et al. (2006) calculated that wastewater dilution, and, hence, dilution of PhAC concentration is detrimental for their biological removal by activated sludge since pseudo first order kinetics were observed for the biological degradation of PhACs. Therefore, treatment at source point (i.e., hospital effluents) could help increase removal of PhACs, taking into account that hospital wastewaters are considered to significantly contribute to PhAC load entering the wastewater treatment plants (WWTPs) (Santos et al. 2013). Some pieces of work dealing with hospital wastewater treatment can be found in the

literature (Kovalova et al. 2012; Verlicchi et al. 2010). However, there is no data about the presence of PhACs in wastewaters from veterinary hospitals.

Among all biological treatments, which are more cost-effective than physical-chemical ones, the use of white-rot fungi (WRF) has been extensively studied due to its ability to degrade a wide range of organic micropollutants more efficiently than conventional biological treatments. Some studies applying WRF for the degradation of dyes, polycyclic aromatic hydrocarbons (PAHs), pesticides and, most recently, a high variety of PhACs can be found in the literature (Asgher et al. 2008; Harms et al. 2011). However, most of them are performed at lab scale, and there are still few studies scaling up the process set-up from Erlenmeyer to bioreactor scale and even less working under non-sterile conditions. Some of them have dealt with synthetic wastewater (Libra et al. 2003; Nguyen et al. 2013) and fewer with real wastewater (Blánquez et al. 2008; Zhang and Geissen 2012). Moreover, in most of them, only the degradation of some spiked contaminants was monitored. Recently, degradation of PhACs at pre-existent concentrations in treatments using the WRF *Trametes versicolor* and dealing with real effluents, such as urban and hospital wastewater and reverse osmosis concentrate from a tertiary treatment in an urban WWTP, have been reported for the first time with promising results (Badia-Fabregat et al. 2015; Cruz-Morató et al. 2014; Cruz-Morató et al. 2013a). Some of those studies were carried out under non-sterile conditions in an attempt for approaching real conditions (Cruz-Morató et al. 2014; Cruz-Morató et al. 2013a). Nonetheless, all the experiments were in batch mode, and no continuous treatment with real effluents at pre-existing PhAC concentration has been reported so far. Moreover, in none of them, the co-existing microbial community has been assessed.

On the other hand, despite the advances in the recent years, fungal treatments are still far from the optimal operational conditions for treating real non-sterile effluents. Some drawbacks, such as the growth of unwanted microorganisms, are not yet solved. Therefore, the aim of the present work is to determine if PhAC degradation at already present concentrations in a fungal bioreactor is possible under non-sterile conditions for veterinary hospital wastewater (VHW) in batch and, for the first time, in continuous operation, with special emphasis in factors that could improve it. Thus, some parameters affecting degradation are discussed, such as microbial populations and nutrient requirements. To that purpose, the bacterial and fungal populations grown inside the bioreactors were analyzed by means of PCR-denaturing gradient gel electrophoresis (DGGE) analysis for a deeper understanding of microbial interactions affecting *T. versicolor* survival.

Materials and methods

Fungus, chemicals and wastewater

Trametes versicolor (ATCC#42530) was obtained from the American Type Culture Collection and was maintained by subculturing on petri dishes in malt extract (2 %) and agar (1.5 %) medium at 25 °C. Pellets production was done as previously described by Blánquez et al. (2006).

All the pharmaceutical and the corresponding isotopically labelled standards were of high purity grade (>90 %), and they 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). The individual standard solutions, as well as isotopically labelled standard solutions, were prepared according to Gros et al. (2012) The solvents, high-performace liquid chromatography grade methanol, acetonitrile, water (Lichrosolv), and formic acid (98 %), were provided by Merck (Darmstadt, Germany). Glucose, ammonium tartrate dibasic, malt extract, and other chemicals were purchased from Sigma-Aldrich (Barcelona, Spain).

VHW was obtained from a veterinary hospital located in the Universitat Autònoma de Barcelona campus (Bellatera, Barcelona, Spain) the same day that each bioreactor was set up and also once a week during continuous bioreactors operation. Table S1 shows wastewater characterization.

Fungal bioreactors and operating conditions

Two fungal biomass air-pulsed fluidized bed glass bioreactors (1.5 L) (Blánquez et al. 2006) were set up in parallel for each experiment (batch and continuous), one inoculated with *T. versicolor* (B-I and C-I for batch and continuous, respectively) and the other non-inoculated as a control (B-NI and C-NI). They were filled with 1.5 L of VHW, and temperature was set up at 25 °C, and pH was controlled to be constant at 4.5±0.5 by HCl 1 M or NaOH 1 M addition. Samples of approximately 250 mL, collected along bioreactors operation, were filtered by vacuum with Whatman GF/C filters and a 0.45-μm nylon filter (Millipore) from which 200 mL were then frozen until pharmaceutical characterization, and approximately 50 mL were used immediately for the other routine analyses (check the "Routine analyses" section). In the batch experiment, samples for microbial community analyses were also collected and frozen at −20 °C in 2 mL eppendorfs without any processing until DNA extraction.

Non-sterile fungal bioreactors operating in batch mode

Pellets of *T. versicolor* were added at 2 g L^{-1} dry cell weight (DCW) concentration. Bioreactors were operated in fed-batch

mode for nutrients supply. Glucose and ammonia tartrate were added in pulses of 0.6 min/h from a concentrated stock at a rate of 277 and 0.619 mg g DCW^{-1} day^{-1} respectively. Addition rate was adjusted in order to avoid glucose accumulation in the media. Samples from the bioreactors media were taken on days 0, 7, and 15.

Non-sterile fungal bioreactors operating in continuous mode

Fungal pellets were kept inside the bioreactor by means of a mesh. *T. versicolor* was initially added at 3.7 g DCW L^{-1}. However, every 2–5 days, approximately 1/3 of the biomass was replaced by fresh ones as determined by Blánquez et al. (2006). Hydraulic retention time (HRT) was 3.3 days. Initially, glucose and ammonia tartrate were added at a rate of 343 and 0.77 mg g DCW^{-1} day^{-1}, respectively in pulses of 0.6 min/h from a concentrated stock. Nutrient addition rate was adjusted along the process depending on the results. VHW in the feed storage tank was replaced every 2–3 days by fresh ones stored at 4 °C. VHW stored in the feed tank for 2–3 days is called "old wastewater." Twenty-four-hour samples (total volume of the effluent mixed) were taken from the effluent on days 0, 5, 10, 12, 14, 15, 17, 18, 21, and 26.

Analytical methods

Pharmaceuticals quantification

The analytical procedure performed is based on the method described by Gros et al. (2012) which allows quantification of 76 PhACs. Briefly, 50 mL of sample were pre-concentrated by solid phase extraction (SPE) using Oasis HLB (3 cc, 60 mg) cartridges (Waters Corp. Mildford, MA, USA), which were previously conditioned with 5 mL methanol and 5 mL HPLC grade water. Elution was done with 6 mL of pure methanol. The extracts were evaporated under nitrogen stream and reconstituted with 1 mL of methanol–water (10:90 *v*/*v*). Lastly, 10 μL of internal standard mix at 1 ng μL^{-1} were added in the extracts for internal standard calibration. Chromatographic separation was carried out with an ultra-performance liquid chromatography system (Waters Corp. Mildford, MA, USA), using an Acquity HSS T3 column (50×2.1 mm i.d. 1.7 μm particle size) for the compounds analyzed under positive electrospray ionization (PI) and an Acquity BEH C18 column (50×2.1 mm i.d., 1.7 μm particle size) for the ones analyzed under negative electrospray ionization (NI), both from Waters Corporation. The UPLC instrument was coupled to 5500 QqLit, triple quadrupole linear ion trap mass spectrometer (5500 QTRAP, Applied Biosystems, Foster City, CA, USA) with a Turbo V ion spray source. Two MRM transitions per compound were recorded by using the Scheduled MRM™ algorithm, and the data were acquired and processed using the Analyst 2.1 software.

Routine analyses

Glucose concentration was measured with a biochemical analyzer YSI 2700 SELECT (Yellow Spring Instruments). Laccase activity was measured through the oxidation of 2,6-dimetoxyphenol (DMP) by the enzyme laccase in a modified version of the method for the determination of manganese peroxidase of Kaal et al. (1993). The analysis process is based on the measure of the absorbance variance at 468 nm at 30 °C during 2 min in a Varian Cary 3 UV/Vis spectrophotometer. The reaction was done with 600 μL of sample, 200 μL of sodium malonate 250 mM at pH 4.5, and 50 μL of DMP 20 mM. Activity units per liter (U L^{-1}) are defined as the amount of DMP in micromoles per liter which are oxidized per minute (μmol DMP L^{-1} min^{-1}). The molar extinction coefficient of DMP was considered as 24.8 mM^{-1} cm^{-1} (Wariishi et al. 1992). Biomass pellet dry weight was determined after vacuum-filtering the cultures through pre-weighted glass-fiber filters (Whatman, Spain). The fungal amount was determined as the constant weight at 100 °C. Dissolved organic carbon (DOC), total suspended solids (TSS), and volatile suspended solids (VSS) were analyzed according to APHA (1995). The $N\text{-}NH_4^+$ concentration and chemical oxygen demand (COD) were analyzed by using commercial kits LCH303 and LCK114 or LCK314, respectively (Hach Lange, Germany). Clorur, nitrate, nitrite, and sulfate anions were quantified by a Dionex ICS-2000 ionic chromatograph. Conductivity was determined by a CRISON MicroCM 2100 conductometer and pH by a pH meter CRISON MicropH 2001. A Microtox® bioluminiscence assay with *Vibrio fisheri* was used to perform acute toxicity test of filtered samples at pH 7. The 50 % effective concentration (EC50) was measured after 15 min of exposure. Effluent toxicity was expressed in toxicity units (TU), calculated as TU= 100/EC50, and an effluent was considered toxic when its TU was over 25 as it is set by local sewage disposal regulation (Generalitat de Catalunya 2003).

Microbial community analysis

Homogenized samples were taken from the bioreactors at different times and stored at −20 °C. Total genomic DNA was extracted from approximately 4 mL for each sample using the Fast DNA® Spin kit for soil (MP Biomedicals, USA). Bacterial 16S rRNA genes and internal transcribed spacer (ITS) regions of fungal rRNA genes were amplified from total genomic DNA. Both cloning and band excision methodologies were used for phylogenetic assignment of the DGGE bands. Complete bacterial 16S rRNA genes were amplified for cloning and sequencing by using the forward primer Bact27-f and the universal reverse primer Uni1492-r (Weisburg et al. 1991). In the case of fungal ITS regions, the forward primer ITS1F (Gardes and Bruns 1993) and the

reverse primer ITS4 (White et al. 1990) were employed. The thermocycling programs were as described in Rodríguez-Rodríguez et al. (2012). For DGGE analysis, PCR products were generated using universal primers in both reactions: bacterial 16S rRNA gene primers U968-f and L1401-r (Nübel et al. 1996) and the fungal rRNA primers ITS1F and ITS2 (White et al. 1990). A 40-base GC clamp was attached to the primers U968-f and ITS1F at the 5′ end. The thermocycling programs were as described in Rodríguez-Rodríguez et al. (2012). The size and amount of PCR products was estimated by electrophoresis in 1 % agarose gels (*w*/*v*) using a DNA marker and SYBR® Safe or ethidium bromide staining.

DGGE analysis of the PCR products was performed with either a DCode system (Bio-Rad, Hercules, CA, USA) or an INGENYphorU (Ingeny, The Netherlands) machine. Gels containing 7.5–8 % (*w*/*v*) polyacrilamide (37.5:1 acrylamide/bis-acrylamide) with a denaturing gradient of 30–60 % or 40–80 %, respectively, for bacteria and 20–50 or 25–60 % for fungi was employed. Electrophoresis was performed for 16 h at 75–85 V in TAE buffer at 60 °C. Gels were then stained with silver nitrate and scanned in an Epson Perfection V750 PRO (Epson, USA) or with SYBR Gold (Invitrogen, Life Technologies) when DGGE bands were excised. Excised DGGE bands were re-amplified (22 cycles) and run in a DGGE gel until the bands were clear enough (3–6 cycles).

For cloning procedure, fragments previously amplified by PCR using the primers Bact27-f/Uni1492-r and ITS1F/ITS4 were purified by means of the PCR cleanup kit NucleoSpin Extract II (Macherey-Nagel, Germany). The fragments were then incorporated into a pGEM-T vector using the pGEM Easy Vector Systems kit (Promega, Madison, WI, USA). The vector was employed in the transformation of *Escherichia coli* competent cells JM109 Competent Cells (Nzytech). Positive transformants were selected after growth in LB medium supplemented with ampicillin, IPTG, and X-Gal. After PCR amplification with U968GC-f/L1401-r or ITS1FGC/ITS2, clones were screened in DGGE by means of comparing with the corresponding band patterns of the samples. Those clones matching different bands in the total community profile were selected, and their inserts were amplified by PCR using the pGEM-T vector-targeted sequencing primers Sp6 and T7, purified (NucleoSpin Extract II kit) and subjected to DNA sequence analysis. Inserts were bi-directionally sequenced with the primers Sp6/T7 at Eurofins MWG Operon (Ebersberg, Germany). Purification and sequencing of excised DGGE bands were performed by a commercial service (Macrogen Inc., South Korea) with the ITS1F without GC tail and R1401 primers.

For phylogenetic analysis, partial sequences were assembled using the CAP application included in the BioEdit v7.0.9 software package (Hall 1999). Chimeras were checked with Mallard 1.02 and Pintail 1.1 programs. Similarity searches for the assembled sequences were performed using the NCBI Blast search program within the GenBank database with Blast algorithm. Bacterial and fungal sequences have been submitted to the GenBank database under accession numbers KM392022–KM392055, KM355623–KM355667, and KM361323–KM361352. Those sequences that could not be deposited because their length was less than 200 bp can be found in the supplementary material (Tables S2 and S3).

Calculations and statistical analysis

For PhAC removal calculations, those compounds detected below quantification limit (BQL) were considered to have a concentration of ½ of the limit of quantification (LOQ) (EPA 2000). Mean and standard deviation (SD) were calculated using Microsoft® Excel 2011 functions. One-factor analysis of variance (ANOVA) and Student's *t* tests for statistical analysis were done with Sigmaplot 11.0; differences were considered as significant at $p<0.01$.

Results

Non-sterile batch treatment: operational details and PhACs degradation

Two parallel bioreactors, one inoculated with *T. versicolor* (B-I) and the other one without fungi as a control (B-NI), were set up to assess fungal biodegradation of PhACs in a VHW under non-sterile conditions. An increase of laccase activity (around 70 U L^{-1}) in the fungal bioreactor on day 3 was observed, but after 7 days, no activity was detected (Fig. S1A), which led us to think that *T. versicolor* was not active. Initially, glucose was added at 276 mg g DCW^{-1} day^{-1} as previously calculated (Casas et al. 2013) and was totally consumed (Fig. S1). On day 5, glucose addition was increased to 1453 mg g DCW^{-1} day^{-1} when pellets started to disaggregate parallel to the laccase activity reduction. However, glucose addition rate was reduced again on day 8 when it was observed that glucose started to accumulate in the media. COD was around 400 mg O_2L^{-1} until day 7, reaching 2930 mg O_2L^{-1} after glucose input was augmented (Fig. S1A). However, it was far below the 11,560 mg O_2L^{-1} of COD accumulated in the control reactor at the end of the experiment (Fig. S1B), which indicates that metabolic products accumulated in it. VHW had very low acute toxicity, with a TU value in the Microtox® analysis of 1.64. After the treatment, B-NI bioreactor had a toxicity of 0.66 TU, whereas the effluent after fungal treatment showed no toxicity at all.

Regarding PhACs, 76 pharmaceutical compounds and some of their metabolites were analyzed in both bioreactors at 3 time points: at the beginning, after 7 days, and at the end of the treatment (15 days). They were selected according to

their widespread presence in wastewaters (Gros et al. 2012). Among them, 45 were detected in the VHW and/or in the effluent of the treatments. Antibiotics, anti-inflammatories, and analgesics were the compounds detected at higher concentrations (Fig. 1, initial time). Overall, antibiotics were found at 34.4 μg L^{-1}. The antibiotics found at highest concentration were cefalexin, ciprofloxacin, metronidazole, trimethoprim, and tetracycline. Analgesics and anti-inflammatories were found at 1.16 μg L^{-1}, with ketoprofen as the PhAC detected at highest concentration. Psychiatric drugs were also found among the highest concentrations (2.26 μg L^{-1}) with acridone, a carbamazepine metabolite, as the compound at the highest concentration.

Table S4 shows initial concentration and removal percentages of detected PhACs. In the B-I reactor, removal of 66 % of total analyzed compounds was achieved at the end of the treatment, whereas in the control B-NI reactor, no removal was observed (Fig. 1). Some antibiotics (with an overall removal of 57 % after 7 days and until 92 % after 14 days in B-I, and negligible removal in B-NI) such as ciprofloxacin, metronidazole and its hydroxilated metabolite, β-blocker carazolol, psychiatric drug diazepam, and anti-inflammatory naproxen were totally or partially removed in the fungal bioreactor, whereas no removal was observed in the control bioreactor. Other compounds were equally degraded in B-I and B-NI, among which were the antibiotics cefalexin and tetracyclin, psychiatric drugs sertraline and paroxetine, lipid regulator gemfibrozil, anti-hypertensive amlodipine, and diuretic furosemide. Some PhACs, like the antibiotics dimetridazole, azythromycin, and ronidazole and psychiatric drug olanzapine, were not significantly removed in any of the reactors. For some compounds, such as anti-inflammatories ketoprofen and piroxicam and β-blockers metoprolol, propranolol, and nadolol, their concentration increased after the treatments, but more in B-I bioreactor than in B-NI. Ketoprofen increase (until 5.7 and 2.3 μg L^{-1} at 15 days in B-I and B-NI reactors, respectively) even masks degradation of other anti-inflammatories in the overall removal calculation. The concentration of psychiatric drug carbamazepine and its metabolite acridone (detected already up to 1.07 μg L^{-1} initially), as well as lipid regulators atorvastatin and pravastatin and the antibiotic ciprofloxacin, increased preferentially in B-NI. Finally, only desloratadine was better removed in B-NI than in B-I after 7 days.

Evolution of bacterial and fungal populations in batch bioreactors

Results for evolution of microbial communities in the batch bioreactors B-I and B-NI are shown in Fig. 2 and Tables 1 and 2. It can be seen that the band corresponding to *T. versicolor* (F10) in B-I bioreactor almost disappeared after 15 days. Regarding other fungi, sequences related to the Ascomycota *Fusarium*, *Trichoderma*, *Rhinocladiella*, and *Phialemonium* were found in the B-I at both 7 and 15 days, instead of those related to the Basidiomycota *Trichosporon*, the main fungi in the B-NI. Some yeasts affiliated to *Galactomyces* were also detected at B-NI. It should be noted that higher diversity does not imply higher concentration. In the present work, microbial populations have only been identified but not quantified.

Regarding bacteria, microorganisms belonging to the orders *Burkholderiales* (β-proteobacteria), *Xanthomonadales* (γ-proteobacteria), and *Rhizobiales* (α-proteobacteria) were detected at B-I at the sample on day 7. Bacteria profiles of B-I and B-NI on day 15 were very similar. In both of them, two main bands were observed, belonging to γ- and α-proteobacteria (*Enterobacteriales* and *Rhodospirillales*, respectively).

Non-sterile continuous treatment: operational details and PhACs degradation

Results obtained in batch experiments encouraged us to further treat VHW in continuous mode under non-sterile conditions. The same setting of two parallel bioreactors, one inoculated with pellets of *T. versicolor* (C-I) and the other one non-

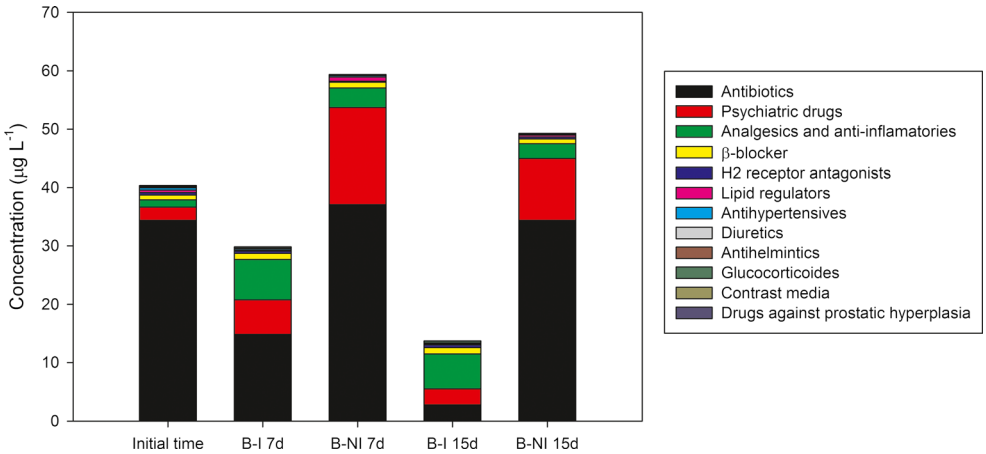

Fig. 1 Evolution of total PhACs grouped by therapeutic classes in the batch treatment inoculated with *T. versicolor* (B-I) and in the non-inoculated control bioreactor (B-NI) at 7 and 15 days of treatment in comparison with the initial concentration (the same for both reactors)

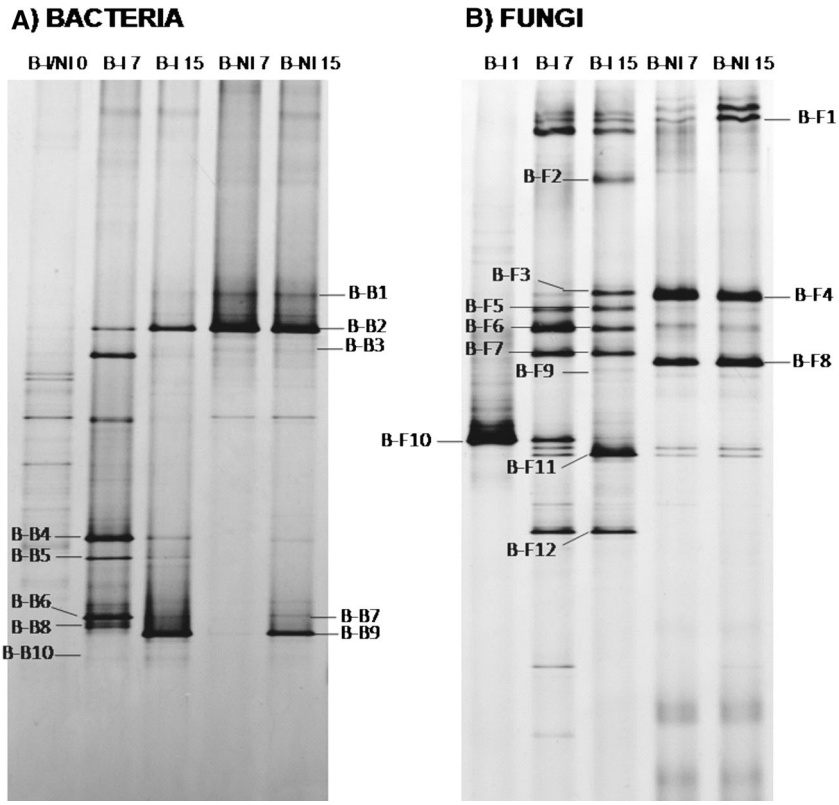

Fig. 2 DGGE profiles of PCR amplified **a** bacterial 16S rRNA fragment and **b** fungal ITS fragment. Samples are from the non-sterile batch bioreactor. *I* correspond to bioreactor inoculated with *T. versicolor* and *NI* to non-inoculated reactor. 0, 1, 7, and 15 correspond to the time of sampling

inoculated as control (C-NI), was performed. Bioreactors were operated at an HRT of 3.3 days. From the DGGE results of *T. versicolor* predominance, cellular retention time (CRT) was fixed between 6 and 15 days, with partial biomass renovation every 2–5 days, depending on visual evolution of the pellets consistency. As shown in Fig. S2A and S2B, initial addition of glucose was 343 mg g DCW^{-1} day^{-1}, in a similar amount per DCW as in the batch bioreactors, but it was increased until 1040 mg g DCW^{-1} day^{-1}, thus trying to avoid fungal lysis (Fig. 3). However, fungal biomass was not stabilized until the addition of a pulse of 4.9 g of ammonia tartrate on day 12 and the following decrease in the C/N ratio in the nutrient stock after day 19, when ammonia from the pulse was depleted. The amount of ammonium tartrate used for the pulse and C/N ratio in the feeding nutrient stock corresponded to the one used when working in synthetic media (Marco-Urrea et al. 2010), being glucose added at 480–1040 mg g DCW^{-1} day^{-1} and ammonium tartrate at 217–433 mg g DCW^{-1} day^{-1} from day 19 onward. Since the change in the C/N ratio on day 12 until the end of operation on day 26, the amount of fungal biomass in the C-I reactor was stabilized. Laccase activity oscillated between 0 and 90 U L^{-1} until day 19. From that moment onward, laccase activity remained below 10 U L^{-1} despite being the period when fungal pellet biomass was maintained. Acute toxicity was analyzed by Microtox®, and TU for veterinary hospital fresh wastewater were 10, 11, and 20 for samples at initial time, 10 and 18 days, respectively. However, all of them were below the value of 25 TU when an effluent is considered as toxic (Generalitat de Catalunya 2003). Old wastewater stored in the storage tank decreased their toxicity to 1–2 TU, denoting some degradation in the storage tank. At 11 days, toxicity in the reactors was also 1–2 TU, while after 26 days, acute toxicity was totally removed (Table S5).

Regarding PhACs degradation, no statistical significant removal was detected during the operation except at the last part of the continuous treatment (from day 21 to 26), when removal in C-I bioreactor was 44 %, whereas in the C-NI, global concentration of PhACs increased (Fig. 4). It is noteworthy that no stationary state was achieved because different nutrient additions were being tested during the process to determine favorable conditions for fungal survival. Therefore, removal data for continuous treatments were calculated at the best performance point of fungal bioreactors, which was at the end of the operation (26 days). However, the decreasing tendency in PhAC concentration in C-I can be observed already from the 14th day, just after ammonia pulse, when lysis of *T. versicolor* was stopped. Analgesics and anti-inflammatories were the PhACs detected at higher concentration in the samplings for continuous treatment (Table S6). Concentration decrease after ammonia addition can be observed as well in their profile (Fig. S3). That profile is dominated by salycilic acid, the compound detected at highest concentration. Regarding antibiotics, their concentration was much lower than in the previous

Table 1 Phylogenetic affiliation of 16S rRNA bacterial sequences corresponding to the different bands of DGGE patterns in batch and continuous treatments

DGGE Band	Seq. length	Closest relative[a]	Accession number	Length coverage	Identity (%)	Class/Order[b]
B-B1	1464	*Klebsiella pneumoniae* strain Kp52.145	FO834906	1460/1464	99	*γ-Proteobacteria/ Enterobacteriales*
B-B2	1465	Enterobacter sp. LRC22 *Raoultella ornithinolytica* strain B6	JF772056 CP004142	1463/1465 1462/1465	99	*γ-Proteobacteria/ Enterobacteriales*
B-B3	1463	*Klebsiella pneumoniae* strain MBR11	JX966429	1462/1464	99	*γ-Proteobacteria/ Enterobacteriales*
B-B4	1469	*Luteibacter* sp. enrichment culture clone 35Fe215 *Dyella yeojuensis* strain rif200829	KF287733 FJ527678	1464/1469 1453/1469	99	*γ-Proteobacteria/ Xanthomonadales*
B-B5	1409	Uncultured *Agrobacterium* sp. clone CBXB23 *Agrobacterium tumefaciens* strain NBRC 15293	GU569105 AB680824	1395/1409 1392/1409	99	*α-Proteobacteria/ Rhizobiales*
B-B8 B-B10	1457	*Burkholderia gladioli* strain CACua-73	HQ023278	1456/1457 1438/1457	99	*β-Proteobacteria/ Burkholderiales*
B-B7	1414	*Acidomonas methanolica* strain: NBRC 104435	AB682176	1406/1414	99	*α-Proteobacteria/ Rhodospirillales*
B-B9	1414	*Acidomonas methanolica* strain: NBRC 104435	AB682176	1410/1414	99	*α-Proteobacteria/ Rhodospirillales*
C-B2	334	Uncultured bacterium clone ncd2574c06c1	JF226179	329/334	99	*Unclassified proteobacteria*
C-B4	339	Uncultured soil bacterium clone SM11 *Micavibrio aeruginosavorus* EPB	EU339599 CP003538	334/339 298/325	99 92	*Unclassified proteobacteria*
C-B5	337	Uncultured alpha proteobacterium *Acidocella aluminiidurans* strain NBRC 104303	AB809968 NR_114266	337/337 336/337	100 99	*α-Proteobacteria/ Rhodospirillales*
C-B6	351	*Klebsiella* sp. Enrichment culture clone F-2 *Klebsiella pneumoniae* str. Kp52.145	KJ465989 FO834906	351/351	100	*γ-Proteobacteria/ Enterobacteriales*
C-B7	377	*Klebsiella* sp. Enrichment culture clone F-2 *Klebsiella pneumoniae* str. Kp52.145	KJ465989 FO834906	377/377	100	*γ-Proteobacteria/ Enterobacteriales*
C-B8	346	Uncultured bacterium clone ZBAF2-82	HQ682040	346/347	99	*Unclassified Proteobacteria*
C-B9	317	Uncultured bacterium clone ZBAF2-82	HQ682040	317/317	100	*Unclassified Proteobacteria*
C-B10	340	Uncultured bacterium clone UVaBiofilter-V26 *Prosthecobacter fluviatilis* strain HAQ-1	KJ002532 NR_041608	335/340 327/341	99 96	*Verrucomicrobiae/ Verrucomicrobiales*
C-B11	335	Uncultured bacterium clone eff4	JN245720	331/335	99	*Unclassified α-Proteobacteria*
C-B12	341	Bacterium NLAE-zl-P34 *Acetoanaerobium noterae* strain NOT-3	JQ606884 NR_104848	341/341	100	*Clostridia/ Clostridiales*
C-B13	341	*Acetoanaerobium* sp. WJDL-Y2 *Acetoanaerobium noterae* strain NOT-3	KF176997 NR_104848	340/341	99	*Clostridia/ Clostridiales*
C-B14	353	Uncultured bacterium A1Q1_fos_291 *Acetoanaerobium noterae* strain NOT-3	JX649891 NR_104848	353/353 352/353	100 99	*Clostridia/ Clostridiales*
C-B15	335	*Parachlamydia acanthamoebae* UV-7 strain UV-7	NR_074972	335/335	100	*Chlamydiae/ Chlamydiales*
C-B16	336	Uncultured *Peptostreptococcaceae* bacterium clone CatInokulum014	KJ600240	336/336	100	*Clostridia/ Clostridiales*
C-B17	336	*Micavibrio aeruginosavorus* EPB	CP003538	336/336	100	*Unclassified proteobacteria*
C-B18	327	*Micavibrio aeruginosavorus* EPB	CP003538	327/327	100	*Unclassified proteobacteria*
C-B19	338	Uncultured bacterium isolate DGGE gel band MFC7-1 *Clostridium sticklandii* str. DSM 519	EU272913 FP565809	337/338 335/338	99	*Clostridia/ Clostridiales*
C-B21	319	Uncultured alpha proteobacterium clone S-30-48	KF827415	313/319	98	*Unclassified Proteobacteria*
C-B22	305	Uncultured bacterium clone ncd1306f09c1 *Dongia mobilis* strain LM22	JF107942 NR_116676	272/307 263/300	89 88	*Unclassified Proteobacteria*
C-B23	331	*Burkholderia* sp. WR43 *Burkholderia nodosa* strain Br3470	AB365791 AM284972	327/331 325/331	99 98	*β-Proteobacteria/ Burkholderiales*
C-B25	304	Uncultured *Verrucomicrobiaceae* bacterium clone A03-06C	FJ542838	292/304	96	*Verrucomicrobiae/ Verrucomicrobiales*
C-B26	333	*Burkholderia* sp. WR43 *Burkholderia nodosa* strain Br3470	AB365791 AM284972	330/333 328/333	99 98	*β-Proteobacteria/ Burkholderiales*

[a] Closest organism at GenBank and, when possible, cultured closest match

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

sampling campaign for batch bioreactors. The compounds detected at higher concentrations were metronidazole and its hydroxylated metabolite, mainly in the wastewater fed during day 18 to 26. When concentration in the entrance was lower (days 0–18), no statistically significant degradation was observed, neither in C-I nor in C-NI bioreactors. However, after the 21st day, around 40–50 % degradation was detected in C-I bioreactor, whereas an increase in the concentration was observed in C-NI. The low biodegradable contrast media iopromide was only detected in the entrance wastewater from day 10 to 18. The concentration of iopromide in the effluent of C-I and C-NI increased progressively from the 10th to 15th day, followed by a decrease in C-I due to fungal degradation, whereas in C-NI, it continued to increase (Fig. S3). Psychiatric drugs had the same concentration profile in the entrance as iopromide, with a peak of approximately 2.5 μg L^{-1} between the 10th and 18th due to the increase of diazepam in the VHW. That increase was not observed in the effluent of the reactors, which showed a diazepam concentration of around 0.1–0.3 μg L^{-1}. However, diazepam degradation can not be assured as adsorption in the sampling system was observed previously (Badia-Fabregat et al. 2015). Carbamazepine was never detected in the fed wastewater; however, it was detected in the effluent of the bioreactors. Those increases in the concentration of some compounds are discussed in the "Removal of PhACs and toxicity in the fungal treatment and importance of the addition of external nutrients section." Diuretic furosemide was highly removed in the fungal bioreactor (83 % at 26 days), whereas its concentration increased in the C-NI reactor in the second half of the treatment. The H2 receptors antagonist ranitidine was neither detected in the fresh water, but its concentration increased in the storage tank and also in the C-NI bioreactor. However, in the C-I bioreactor, it was totally removed. Other changes in PhAC concentration were detected between fresh and old wastewater in the storage tank (Table S6). In the antihypertensives amlodipine and irbesartan, similar deconjugation processes might occur.

Taking into account removal percentages on day 26 (Table S6), PhACs can be grouped as compounds better removed in C-I than in C-NI (i.e., salicylic acid, metronidazole, diazepam, furosemide, clopidrogel and ranitidine), equally removed in both reactors (ibuprofen), and not statistically significantly removed in any of them (ketoprofen). Among those whose concentration increased, piroxicam, diclofenac, and carbamazepine increased more in C-I and metronidazole-OH increased more in C-NI.

Evolution of bacterial and fungal populations in continuous bioreactors

Figure 5 shows the DGGE profiles of fungi and bacteria, respectively. Phylogenetic affiliation of the selected bands can be found at Tables 1 and 2. *T. versicolor* was detected in all samples. Liquid sample at day 1 of C-NI was discarded for further analysis due to PCR contamination suspicion. Other fungi detected at C-I and C-NI bioreactors were similar, mainly *Candida spp*. and *Fusarium spp*. A fungus belonging to *Xylariales sp*. and an unknown fungus (88 % of identity with *Ophiostoma*) was grown at C-NI as well. Some sequences, such as that belonging to F11 band, could not be cleaned enough for sequencing. Similar bacterial communities were also found at C-I and C-NI, as what happened with fungi. Thus, many unclassified proteobacteria, *Enterobacteriales sp*., *Burkholderiales sp*., and *Verrucomicrobiales sp*. were detected. *Chlamydiales sp*. and *Rhodospirillales* were also found at day 26 at C-NI. Both fungal and bacterial diversity (accounted as diversity of DGGE bands) in the continuous reactors increased along the treatment.

Discussion

PhAC concentration in the VHW

Although urban and human hospital wastewaters have been extensively studied regarding PhAC concentration (Verlicchi et al. 2012; Verlicchi et al. 2010), a VHW has been characterized for the first time in the present study. As shown in Table S1, regarding physicochemical characteristics, VHW has the same values than average urban wastewater. The effluent presented certain inter-day variability regarding PhAC concentration, with particular peaks of high concentration of i.e., the contrast media iopromida, the psychiatric drug diazepam, the antibiotics metronidazole, cefalexin, and ciprofloxacin, and the analgesic salycilic acid. However, in general, antibiotics, anti-inflammatories, and analgesics were the compounds detected at higher concentrations in all the samples for both batch and continuous treatments (Fig. 1, initial time, and Tables S4 and S5). This is in agreement with the type of drugs mostly prescribed at the hospital, according to the veterinary hospital staff. However, although not prescribed for veterinary treatment, psychiatric drugs were also found among the highest concentrations, even though they were lower than those reported in hospital and urban wastewaters (Cruz-Morató et al. 2014; Cruz-Morató et al. 2013a; Gros et al. 2012; Santos et al. 2013) because wastewater from the human activities of the veterinary hospital was collected as well. The overall antibiotic concentration was in the same order of magnitude to what is reported for human hospitals, with an average value of 11 μg L^{-1} (Verlicchi et al. 2010). Ciprofloxacin and metronidazole, the antibiotics found at highest concentrations, are also among those highly detected at hospital wastewaters (Santos et al. 2013; Verlicchi et al. 2010). Analgesics and anti-inflammatories were at lower concentration than expected (1.16–5.66 μg L^{-1}), since they are reported to be at concentrations around 100 μg L^{-1} in hospital wastewaters

Table 2 Phylogenetic affiliation of ITS fungal sequences corresponding to the different bands of DGGE patterns in batch and continuous treatments

DGGE band	Seq. length	Closest relative[a]	Accession number	Length coverage	Identity (%)
B-F1	368	*Galactomyces candidum* strain CBS 180.33	JN974289	366/368	99
B-F2	603	*Pichia guilliermondii* strain S0201	EF643576	601/603	99
B-F3	552	*Phialemonium curvatum* strain: CBS 490.82	AB278180	547/552	99
B-F4	527	Uncultured fungus isolate RFLP27 *Trichosporon laibachii* strain CBS 5790	AF461588 EU559348	527/527 511/511	100
B-F5	565	*Fusarium solani* strain 6.M8	FJ224382	559562	99
B-F6	563	Uncultured *Fusarium* sp. clone PA1912 *Fusarium solani* culture-collection UOA/HCPF:12649	GQ280338 KC254048	562/563 557/565	99
B-F7	565	*Fusarium solani* culture-collection UOA/HCPF:12649	KC254048	565/565	100
B-F8	524	Uncultured eukaryote clone N414T_268 *Trichosporon dermatis* culture-collection UOA/HCPF	GU941914 KC254108	521/524 519/524	99
B-F9	523	*Trichosporonales* sp. LM88	EF060462	523/524	99
		Trichosporon jirovecii strain ATCC 34499	HM802131	510/510	100
B-F10	619	*Trametes versicolor* sample ID: MQN028	AB811868	619/619	100
B-F11	612	*Rhinocladiella similis* culture-collection UOA/HCPF:11700	KC254071	610/612	99
B-F12	599	*Trichoderma asperellum* strain ZJSX5001	JQ040323	598/599	99
C-F1	179	Candida sp. NRRL Y-27161	DQ911459	164/164	100
C-F2	227	*Candida palmioleophila* strain ATCC 96299	KC479687	227/227	100
C-F4	188	Uncultured eukaryote clone NS31T_257 *Rhodotorula mucilaginosa* strain ATCC 4056	KJ182680 KC881070	188/188	100
C-F5	172	Xylariales sp. HP-2011b	HE599293	172/172	100
		Phialemonium curvatum strain UTHSC R-3447	AY818323	171/172	99
C-F6	172	Xylariales sp. HP-2011b	HE599293	172/172	100
		Phialemonium curvatum strain UTHSC R-3447	AY818323	171/172	99
C-F7	213	Exophiala sp. CCFEE 5933 *Exophiala equina* CBS 119.23	JX681050 NR_111627	213/213	100
C-F8	188	*Fusarium solani* voucher CEQCA-O0484	KC771504	188/188	100
C-F9	188	*Fusarium solani* voucher CEQCA-O0484	KC771504	188/188	100
C-F10	188	Uncultured soil fungus isolate DGGE gel band F3 *Fusarium solani* strain DAOM 215455	KJ562397 JN942906	188/188	100
C-F13	220	*Trametes versicolor* culture-collection ICMP:19973	KF727428	220/220	100
C-F14	202	*Trametes versicolor* culture-collection ICMP:19973	KF727428	202/202	100
C-F15	222	*Trametes versicolor* culture-collection ICMP:19973	KF727428	222/222	100
C-F16	241	*Exophiala oligosperma* strain MH2012-17	AB777520	239/239	100
		Exophiala oligosperma CBS 725.88	NR_111134	239/240	99
C-F17	239	*Exophiala oligosperma* strain MH2012-17	AB777520	239/239	100
		Exophiala oligosperma CBS 725.88	NR_111134	239/240	99
C-F18	212	Uncultured exophiala clone CBB3.14	KC876175	200/212	94
		Exophiala moniliae CBS 520.76	NR_111448	200/215	93
C-F19	202	Heterobasidion sp. NL-2013 isolate PFC 5320 *Heterobasidion parviporum* strain B1142	KC492958 GQ162421	202/202	100
C-F20	201	*Trichoderma asperellum* strain TR08	AB935955	201/201	100
C-F21	132	*Ophiostoma stenoceras* strain CBS103.78	AF484449	106/121	88

[a] Closest organism at GenBank and, when possible, cultured closest match

(Cruz-Morató et al. 2014; Verlicchi et al. 2010). Overall PhACs levels were in general lower than those reported for hospital wastewaters (Kovalova et al. 2012; Verlicchi et al. 2010) probably due to the higher use of water (and therefore dilution of wastewater) for cleaning the veterinary facilities, and the fact that urine from big animals (i.e., horses) was collected with straw and disposed separately. In conclusion, although not an extensive sampling campaign has been carried out for the VHW, the results hereby presented pointed that the type of PhACs and the range of concentration in that kind of

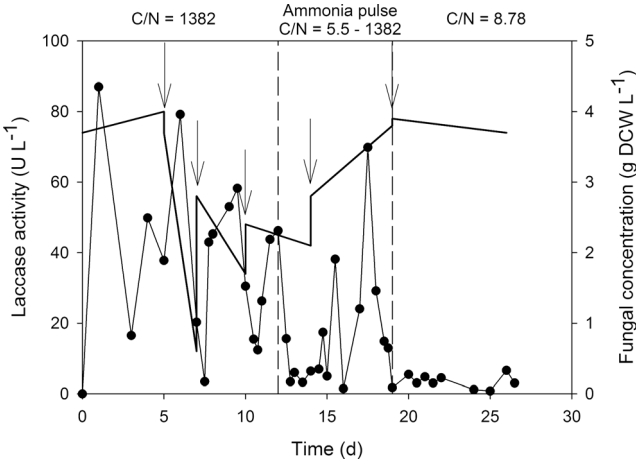

Fig. 3 Profiles of (*black circle*) laccase activity and (*horizontal bar*) fungal pellets concentration in *T. versicolor* inoculated continuous bioreactor (C-I). *Vertical dashed lines* mark changes in the C/N ratio of the added nutrients and *arrows* mark renovation points of 1/3 of the old pellets by fresh biomass

effluents can be in between those detected in urban and hospital wastewater effluents and, thus, some treatment at source point might be interesting to implement.

Removal of PhACs and toxicity in the fungal treatment and importance of the addition of external nutrients

T. versicolor has been reported to be able to degrade a wide range of xenobiotics, including many PhACs (Cruz-Morató et al. 2013b). Therefore, it was inoculated in air-pulsed fluidized bioreactors with the aim of achieving the degradation of PhACs present in the VHW. However, the degradation of PhACs needs other carbon sources, irrespective of whether their degradation is cometabolic or not, due to their low concentration. Moreover, previous studies reported that C and N already present in an urban wastewater were not enough for

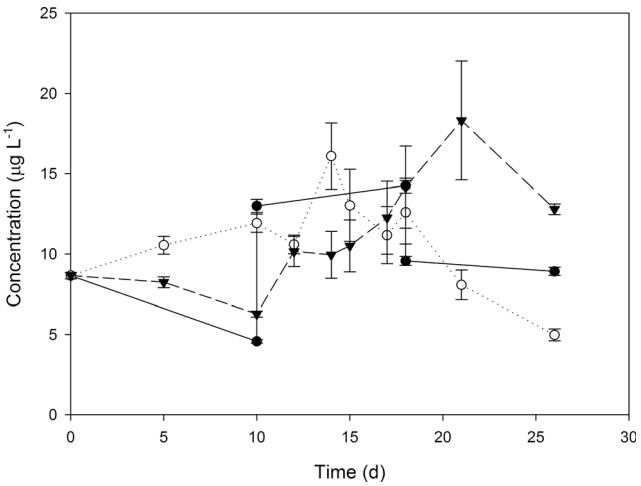

Fig. 4 Total PhACs concentration in the (*black circle*) inlet and the effluent of (*white circle*) C-I and (*inverted black triangle*) C-NI in the continuous VHW treatment

the survival of *T. versicolor* under non-sterile conditions (Cruz-Morató et al. 2013a). Zhang and Geissen (2012) also pointed the need for external supply of both carbon and nitrogen in a treatment with *Phanerochaete chrysosporium*, another well-studied WRF. Therefore, glucose and ammonia tartrate were added in the bioreactor at the previously optimized minimum feed rate (Casas et al. 2013). However, those rates were calculated in sterile conditions, and the consumption of nutrients by the other microorganisms growing in the bioreactor as well and competing with *T. versicolor* resulted in them being insufficient for the survival of the innoculated fungus. Although, removal at B-I was 66 %, whereas at B-NI there was no removal at all. Therefore, the inoculation with *T. versicolor* constituted an efficient treatment for the removal of some of the compounds, as already reported for other effluents (Cruz-Morató et al. 2014; Cruz-Morató et al. 2013a). As an example, metronidazole and diazepam, two examples of hardly biodegradable PhACs that were present in all sampled wastewaters at considerable concentration, were better removed by the fungal bioreactor at both treatments (batch and continuous).

When working in continuous mode, the increase in the glucose and ammonium tartrate feeding rate, trying to avoid fungal lysis (Fig. 3), did not help in the degradation of PhACs and survival of *T. versicolor*. Only after the decrease in the C/N ratio was a stable concentration of fungus maintained, and a decrease in the total PhAC concentration was observed, highlighting the relationship between *T. versicolor*, the main fungus detected in the pellets (Fig. 5b) and the PhAC degradation. Such observation points to the fact that not only the external supply of carbon and nitrogen is important but that also the ratio between them might affect the microorganisms developing in the bioreactor and the chance for *T. versicolor* to compete with them. Further studies in that direction should be performed to optimize the nutrients needed in non-sterile treatments in order to minimize the use of chemicals and, in addition, the economic expenditures. An advisable value to start the optimization might be a C/N ratio of around 10 mol C/mol N for this particular fungi and taking always into account the features of the wastewater.

Another remarkable issue regarding PhAC removal is the increase in the concentration of some compounds. For example, in the continuous treatment, carbamazepine was never detected in the fed wastewater, whereas it was detected in the effluent of the bioreactors. Those increases have been already reported (Badia-Fabregat et al. 2015; Cruz-Morató et al. 2013a), and this aspect has been recently deeply discussed for a similar treatment of reverse osmosis concentrate (Badia-Fabregat et al. 2015). Deconjugation or other transformation reactions were postulated as the main factors for the increase of carbamazepine and ketoprofen among other compounds. Kovalova et al. (2012) also found an increase in carbamazepine concentration among other compounds after an MBR

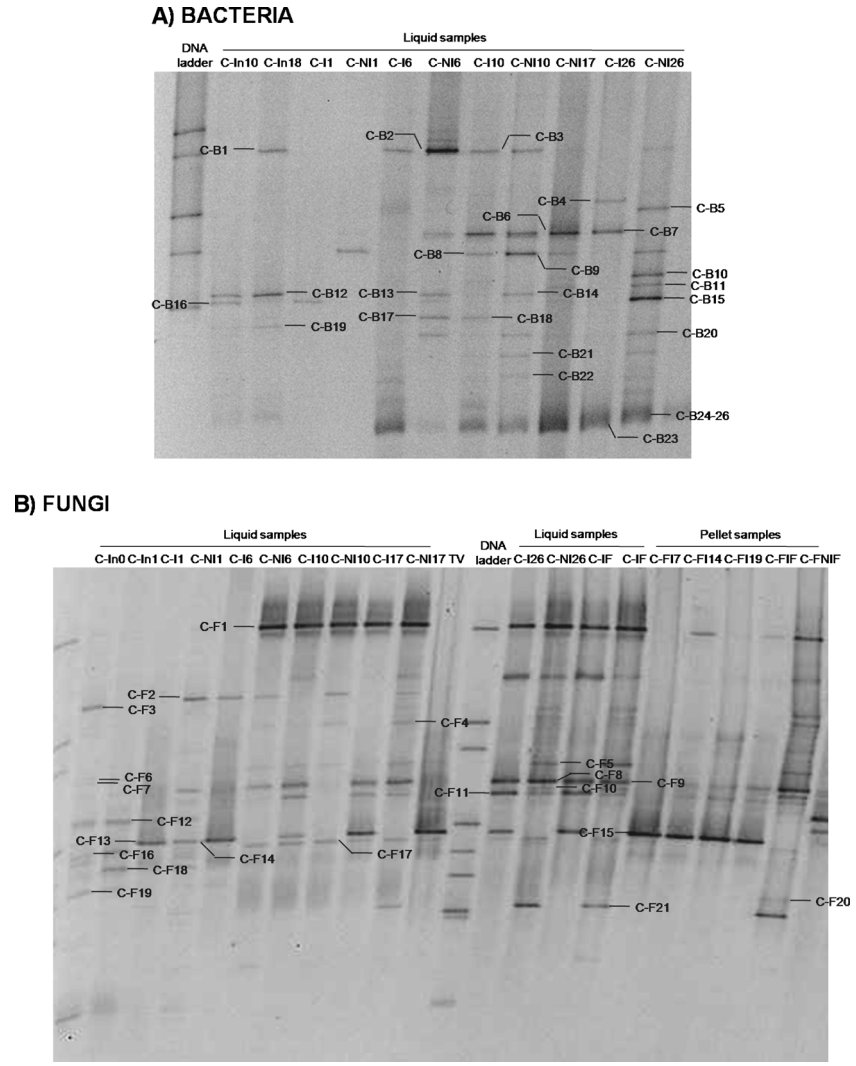

Fig. 5 DGGE profiles of PCR amplified **a** bacterial 16S rRNA fragment and **b** fungal ITS fragment. Samples are from the non-sterile continuous bioreactor. *In* correspond to VHW feed to the reactors, *I* to bioreactor inoculated with *T. versicolor*, *NI* to non-inoculated reactor, and *TV* to *T. versicolor* pure culture samples. Numbers of the columns correspond to the time of sampling

treatment of hospital wastewater due to the combined effect of deconjugation and low biodegradation. In the present study, taking into account that those increases also occurred in the non-inoculated controls, other factors beyond *T. versicolor*, such as transformation by means of bacteria or other fungi might be responsible for it. It would be very interesting to try to include conjugated compounds in the chemical analysis in order to be able to monitor all kind of treatments more accurately.

In an attempt to cover the degradation or transformation of those toxic compounds not included in the chemical analysis, a general test for acute toxicity (Microtox) was performed. VHW presented toxicity values between 1 and 20 TU, already below local legislation limits (Generalitat de Catalunya 2003). After the fungal treatments, the effluents had no toxicity at all, which means that *T. versicolor* was able to degrade those toxic micropollutants present in the wastewater, and no toxic metabolites were produced or, at least, not at significant concentrations.

Role of *T. versicolor* and the competing microorganisms in the degradation of PhACs

According to the DGGE results, the inoculated *T. versicolor* was active during the first 7 days in the B-I reactor. However, between days 7 and 15, it lost its predominance among fungi. On the other hand, total PhAC removal during the first period was 26 %, whereas in the second period, it reached up to 66 %. These results might point out that not only *T. versicolor* was the responsible for the removal of some PhACs but that some synergies were also taking place between different organisms. However, inoculation with the fungus might be crucial to achieve those synergies because in the B-NI reactor, there was no removal at all of total PhACs. In the continuous reactors, the pattern seems to be the same: if there has not been active *T. versicolor* in the bioreactor, no PhAC removal is observed. That is, in the C-NI reactor, there is no significant PhAC removal nor in the C-I reactor during the first 15 days, when *T. versicolor* pellets were lysing (Fig. 3) due to the lack

of appropriate nutrients. It was not until the change in the C/N ratio of the nutrients, favoring the survival of *T. versicolor*, that the degradation of PhACs seemed to differ from the non-inoculated control. The detection of DGGE bands corresponding to *T. versicolor* even when deterioration of the system was observed (samples taken on days 6 and 10) might be due to the partial biomass renovations (in contrast to the non-fungal renovations in batch treatment) and that analysis was based on genomic DNA template instead of more responsive RNA (Rajala et al. 2011).

Taking into account that PhAC degradation in the fungal batch treatment seems to be performed by other microorganisms apart from *T. versicolor*, especially after day 7, PCR-DGGE analyses of bacteria and fungus were performed. The presence of *T. versicolor* seems to inhibit the growth of other fungi, especially other basidiomycetes. However, the conditions in B-I reactor seemed to favor other fungi, such as *Trichoderma* spp., some strains of which were previously reported to inhibit *T. versicolor* (Bruce and Highley 1991). Nevertheless, in the continuous treatment, *Trichoderma spp*. were not significantly found at any VHW bioreactor, pointing to some differences between batch and continuous operation regarding the conditions for microbial community development. Contrary to the previous work pointing toward the dominance of *T. versicolor* over *Fusarium oxysporum* (Ruiz-Dueñas and Martínez 1996), in the present study, related fungi were detected in presence of *T. versicolor*. This could imply that depending on the media conditions and other microorganisms coming along in the waste water, dominant species can and will vary case-by-case.

It can be noted that fungi growing in all the bioreactors were mainly Ascomycetes, unlike in the B-NI treatment where Basidiomycetes were predominant. Almost all genera of fungi detected in both I and NI bioreactors are reported to degrade organic pollutants. For example, *Fusarium* and *Trichoderma* can degrade PAHs (Verdin et al. 2005) and *Trichosporon* can degrade dyes (Saratale et al. 2009). *Fusarium* is a ubiquitous plant pathogen fungus and was found, for example, in olive-mill wastewater lagoons (Millán et al. 2000). *Rhinochladiella* was also found there, but its growth was limited; however, in another study, some signs of organic micropollutant transformation were provided (Fujii and Kikuchi 2005). Therefore, taking into account that *T. versicolor* was overtaken by other fungi and/or bacteria between days 7 and 15 at B-I reactor, further degradation of PhACs might be due to the activity of fungi belonging to the genera *Fusarium*, *Trichoderma*, and/or *Rhinocladiella*. In fact, Lu et al. (2009) reported a biofilm inoculated with a microbial consortium with decolorizing strains *Fusarium oxysporum* and *Candida tropicalis* among others that after 4 months in operation, the main fungi turned out to be *Candida* and a non-decolorizing species of *Trichoderma. Candida* spp. were present from days 6–10 onward in the continuous treatments. This is in accordance with the previous observations, as it was found to be one of the main genera at urban WWTP, together with *Rhodotorula* spp., *Trichosporon* spp., and other five unidentified genera (Yang et al. 2011).

Regarding bacteria, B-I profile at the end of the treatment was similar to the B-NI. The reason might be that there was almost no *T. versicolor* at that point, and bacteria that might be inhibited by *T. versicolor* could proliferate in the B-I bioreactor as well. Those bacteria probably belonged to the genera of *Enterobacteriales* and *Rhodospirillales* (bands of B-I 15d were not sequenced, assignment only by position). In the sample on day 7, although there are some bands that could not be identified, *Enterobacteriales* seem to be present in fewer amounts than in the absence of *T. versicolor* (B-I 15d and B-NI). That could imply that the presence of *T. versicolor* might help in the elimination of pathogens belonging to the genera of *Enterobacteriales*. However, there is no other reports supporting this hypothesis, and more studies should be performed in that respect. Moreover, *Enterobacteria spp*. were present at each continous bioreactor, independently if it was inoculated by *T. versicolor* or not. *Burkholderia spp*. were also present at both continuous bioreactors and also in the batch bioreactor. Results are consistent because *Burkholderia* belong to β-proteobacteria, which are the major populations in environmental samples and also aerobic MBRs (Ayache et al. 2013).

No specific bacteria can be totally related with the dead of *T.versicolor* around day 6 of C-I because the majority of the bacteria detected at that moment were also present during all the treatment, also when *T. versicolor* recovered. The single band only present at days 6 and 10 was assigned to an unclassified proteobacteria (band C-B2, Fig. 5a)

Regarding the possible involvement of bacteria in the degradation of PhACs, as band pattern is the same at B-I 15d and B-NI 15d, the possible role of bacteria in the fungal reactor might be the further degradation of fungal metabolites (Mikesková et al. 2012). In the work of Lu et al. (2009), the main bacteria found after 4 months of operation of a biofilm inoculated with a mixed consortium of fungi and bacteria were α-proteobacteria (*Rhizobiales* and *Rhodobacterales*). However, in general, there is little data in the literature regarding microbial interactions between *T. versicolor* (or WRF in general) and other microorganisms and, as stated before, with contradictory results. Moreover, no relationship between certain detected microorganisms and degraded PhACs could be established. Even regarding *T. versicolor*, although the overall concentration decreased, the degradation percentages of each compound can vary depending on the culture conditions, the wastewater, or the initial concentration, among other factors.

In the continuous bioreactors, higher diversity was generally found at the samples of the last days of the treatment, probably due to the much higher HRT than conventional growht rates, which allows proliferation of many types of

microorganisms. However, no clear DGGE pattern change was observed between before and after the C/N ratio change in the added nutrients. Therefore, as DGGE analysis are only qualitative, improvement in the *T. versicolor* survival might be due to quantitative differences between *T. versicolor* and the other microorganisms. Thus, although this work shows some preliminary results about microbial community development in wastewater treatments inoculated with white-rot fungi, more work should be done regarding multiple interactions and activities between fungi and bacteria in fungal treatments and their relationship with xenobiotics degradation. Moreover, it should also be taken into account that microbial community can be highly case-dependent, as shown in here. Thus, in the future, analysis of many experimental data might lead to a better correlation between operational factors and presence of certain microorganisms, being this work one of the first steps in that direction.

Role of laccase activity in the degradation of PhACs and as indicator of *T. versicolor* activity

Laccase has been reported to be the main linginolytic extracellular enzyme expressed by *T. versicolor* under similar culture conditions (Font et al. 2003). This does not mean that laccase, although it can participate, is the main enzyme involved in the degradation of PhACs. In fact, it was previously reported that extracellular enzymes are not crucial for achieving good degradation percentages of xenobiotics (Blánquez et al. 2004; Yang et al. 2013). Taking that into account, in the present study, extracellular laccase activity was analyzed and studied as a valid indicator for the *T. versicolor* activity. In the batch treatment, a positive correlation between laccase activity and *T. versicolor* survival (as shown by DGGE results) can be found. On the other hand, absence of laccase activity does not seem to affect PhAC degradation as there was still a significant degradation rate between days 7 and 15. Therefore, degradation could be assigned to other enzymes such as cytochrome P450 (Marco-Urrea et al. 2010) or to other microorganisms growing in the bioreactor. Taking into account the molecular biology results, it is likely to be due to the activity of other microorganisms.

On the other hand, in the fungal continuous treatment, laccase activity remained below 10 U L^{-1} after the change of the C/N ratio in the nutrients, despite being the period when fungal pellet biomass was maintained (*T. versicolor* was still present at both pellets and liquid samples according to DGGE analysis). It confirms, in this case, the decoupling between fungal degradation and presence of extracellular enzymes (Blánquez et al. 2008). Extracellular enzymes can be produced but degraded or inactivated by products from other microorganisms (Yang et al. 2013). In conclusion, extracellular laccase might not be a good indicator of *T. versicolor* activity, and it does not seem to be important for the degradation of PhACs either (at least in our continuous operating conditions).

Strategies for fungal survival and future perspectives

According to DGGE results of batch treatment and previous experiments (Blánquez et al. 2006), biomass was partly renewed every 2–5 days in the continuous treatment, depending on visual evolution, which results in a CRT between 6 and 15 days. This CRT should be optimized in the future at the same time as the amount of nutrients and its C/N ratio. The amount of external nutrients to keep *T. versicolor* alive and active over the rest of microorganisms and to limit the renovation of biomass should be found. However, addition of nutrients cannot be in excess for both the higher costs (even if using waste subproducts) and the increase in the COD of the effluent that an accumulation of metabolic products in the media involves, as happened in the B-NI reactor (Fig. S1B and S2B).

On the other hand, there is a lack of studies about bacterial and fungal interactions in fungal treatments for the degradation of xenobiotic compounds. In the present work, microbial community analysis showed that after 15 days of batch treatment, *T. versicolor* lost its predominance. However, other fungi, such as *Fusarium* and *Trichoderma*, which could also degrade PhACs, were detected in the inoculated reactor but not in the control one. Strategies inoculating with some fungal consortium, in a similar way to what Lu et al. (2009) did, might be a good alternative and should be studied in depth.

Therefore, further optimization on nutrient requirements under non-sterile conditions and comprehensive studies on the interactions between fungi and bacteria are needed, although promising results regarding PhAC degradation in real wastewaters and non-sterile conditions arise from the present work.

Acknowledgments Authors want to acknowledge the UAB veterinary hospital staff for their kind permission and help for the samplings. This work has been funded by the Spanish Ministry of Economy and Competitiveness and FEDER (projects CTM2013-48545-C2 and AIB2010PT-00169) and supported by the Generalitat de Catalunya (Consolidated Research Groups 2014-SGR-476 and 2014-SGR-291). The Department of Chemical Engineering of the Universitat Autònoma de Barcelona (UAB) is a member of the Xarxa de Referència en Biotecnologia de la Generalitat de Catalunya. M. Badia-Fabregat and D. Lucas acknowledge the predoctoral grants from UAB and from the Spanish Ministry of Education, Culture and Sports (AP-2010-4926), respectively. The authors also thank the Portuguese Foundation for Science and Technology (FCT) Strategic Project PEst-OE/EQB/LA0023/2013, Project FCOMP-01-0124-FEDER-027462 co-funded by Operational Competitiveness Programme, FEDER, and Project "BioEnv—Biotechnology and Bioengineering for a sustainable world," REF. NORTE-07-0124-FEDER-000048, co-funded by Programa Operacional Regional do Norte (ON.2 – O Novo Norte), QREN, FEDER.

Compliance with ethical standards All authors of the article declare that they have no conflict of interest. This article does not contain any studies with human participants or animals performed by any of the authors.

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