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1**Study of the effect of the bacterial and fungal communities present in real wastewater**

2**effluents on the performance of fungal treatments**

3

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21

22**Abstract**

23The use of the ligninolytic fungi *Trametes versicolor* for the degradation of micropollutants

24has been widely studied. However, few studies have addressed the treatment of real

25wastewater containing pharmaceutically active compounds (PhAC) under non-sterile

26conditions. The main drawback of performing such treatments is the difficulty for the

27inoculated fungus to successfully compete with the other microorganisms growing in the

28bioreactor. In the present study, several fungal treatments were performed under non-sterile 29conditions in continuous operational mode with two types of real wastewater effluent, namely, 30a reverse osmosis concentrate (ROC) from a wastewater treatment plant and a veterinary 31hospital wastewater (VHW). In all cases, the setup consisted of two parallel reactors: one 32inoculated with *T. versicolor* and one non-inoculated, which was used as the control. The 33main objective of this work was to correlate the operational conditions and traditional 34monitoring parameters, such as laccase activity, with PhAC removal and the composition of 35the microbial communities developed inside the bioreactors. For that purpose a variety of 36biochemical and molecular biology analyses were performed: phospholipid fatty acids 37analysis (PLFA), quantitative PCR (qPCR) and denaturing gradient gel electrophoresis 38(DGGE) followed by sequencing. The results show that many indigenous fungi (and not only 39bacteria, which were the focus of the majority of previously published research) can 40successfully compete with the inoculated fungi (i.e., *Trichoderma asperellum*. overtook *T.* 41*versicolor* in the ROC treatment). We also showed that the wastewater origin and the 42operational conditions had a stronger impact on the diversity of microbial communities 43developed in the bioreactors than the inoculation or not with *T. versicolor*.

44

45**Keywords**

46Molecular biology, microbial communities, white-rot fungi, bioreactor, real effluents, 47pharmaceutically active compounds (PhAC)

48

49**1. Introduction**

50Special concern about the presence of pharmaceutically active compounds (PhACs) in surface 51waters arose two decades ago (Daughton and Ternes, 1999). PhACs are compounds that are 52specifically designed to be biologically active even at low concentrations. The main source of

53PhAC are the effluents of wastewater treatment plants (WWTPs), where conventional 54activated sludge treatment is not able to properly degrade them. For instance, diclofenac, a 55common analgesic and anti-inflammatory, has an average removal percentage of only 29% 56(Verlicchi et al., 2012). Therefore, alternative treatments should be found. The use of 57ligninolytic fungi has been studied based on their ability to degrade conventional pollutants 58(Pointing, 2001). The use of ligninolytic fungi immobilised enzymes has recently gained 59attention for the degradation of PhACs as they can overcome the problem of maintaining 60active microorganisms (Spina et al., 2015). However, it is common the need for a recurring 61supply of enzymes in order to maintain the activity of the enzymes for a long period of time.

62

63Successful results were obtained using the whole fungus *Trametes versicolor*, a well-known 64ligninolytic fungus, with degradation percentages greater than 90% for many PhACs in spiked 65experiments, as reviewed by Cruz-Morató et al. (2013b). *T. versicolor* has also been 66demonstrated to degrade PhAC in real wastewater (Badia-Fabregat et al., 2015a; Cruz-Morató 67et al., 2014, 2013a), even in continuous operation mode (Badia-Fabregat et al., 2015b). Many 68factors can affect the efficiency of the treatment, such as the configuration of the reactor, the 69chemical profile of the wastewater, the addition of nutrients and the pH (Anastasi et al., 702010). The two main drawbacks of fungal reactors when working under non-sterile conditions 71are the overtaking of the inoculated fungus by bacteria and the washing out of extracellular 72enzymes during continuous operation. However, it was reported that a continuous 73extracellular enzyme concentration is not crucial to achieve good degradation percentages 74(Anastasi et al., 2010; Badia-Fabregat et al., 2015b; Blánquez et al., 2004; Yang et al., 2013) 75and intracellular enzymes have also been reported to play a key role in the degradation of 76micropollutants (Marco-Urrea et al., 2009). Therefore, in the present study, we focus mainly 77on the competition between the inoculated fungus and other microorganisms.

78

79Most real wastewater treatment under non-sterile conditions has been conducted for textile 80wastewater (Blánquez et al., 2008; Hai et al., 2008; Libra et al., 2003; Lu et al., 2009) and, 81recently, urban and hospital wastewater (Badia-Fabregat et al., 2015a, 2015b; Cruz-Morató et 82al., 2014, 2013a). Treating urban wastewater with fungi is a greater challenge than treating 83textile wastewater due to the higher microbial titre of the former, leading to possible 84competition between the inoculated fungus and the indigenous microorganisms. Therefore, 85different strategies to avoid or minimise bacterial growth have been implemented in different 86studies. Some were successful but expensive, such as continuous ozonation of the media 87(Cheng et al., 2013), whereas others, such as maintaining an acidic pH, did not suppress 88bacterial growth (Libra et al., 2003). Fungal reinoculation was reported in previous studies for 89effective control of bacterial growth (Blánquez et al., 2006; Dhouib et al., 2006); thus, it was 90included in the treatments presented here.

91

92Little is known about fungal and bacterial interactions in liquid media (Weber et al., 2007; 93Yang et al., 2011) because the vast majority of studies on fungal-bacterial interactions are 94performed in soil (Mikesková et al., 2012; Rousk and Bååth, 2007). Usually, fungi are not 95taken into account in the microbiota characterisation of wastewater. However, they represent 96an important load (e.g., reaching approximately 100 colony forming units (CFU) in a landfill 97leachate effluent (Tigini et al., 2014)). In the present study, biochemical and molecular tools 98(phospholipid fatty acids analysis (PLFA), denaturing gradient gel electrophoresis (DGGE) 99and quantitative PCR (qPCR)) were used to study the microbial communities (both fungal and 100bacterial) during non-sterile fungal treatment of real effluents (reverse osmosis concentrate 101(ROC) and veterinary hospital wastewater (VHW)). The present study, thus, focuses on the 102microbial communities developed in the different treatments and their relationship with the

103operational parameters with the final aim to identify the optimal conditions for the 104development of the fungal activity in the near future. All treatments included a fungal-105inoculated bioreactor (I) and a non-inoculated bioreactor (NI) in parallel as a control. 106Therefore, the main aim of the study was to identify the microbial communities that 107developed in the continuously operating bioreactors and, for the first time, to statistically 108correlate them with PhAC removal, fungal survival and the operational parameters and data 109from traditional monitoring methods (mainly laccase activity, glucose consumption and visual 110aspects).

111

1122. Materials and methods

1132.1. Fungal strain and pellet production

114*Trametes versicolor* (ATCC#42530) was obtained from the American Type Culture Collection 115and was maintained by sub-culturing on Petri dishes in malt extract (2%) and agar (1.5%) 116medium at 25 ºC. Pellet production was performed as previously described by Blánquez et al. 117(2004). Briefly, a mycelial suspension was obtained by inoculating four 1 cm^2 plugs from the 118malt agar plate in a 500 mL Erlenmeyer flask containing 150 mL of malt extract medium (2%, 119adjusted to pH 4.5). Flasks were incubated at 25 ºC in an orbital shaker for 4-5 days. The 120obtained mycelial mass was ground with a homogeniser and the resulting mycelial suspension 121was stored in a sterile saline solution (8 g L^{-1} NaCl). This suspension was used to obtain 122pellets by inoculating 1 mL of the suspension in 250 mL malt extract medium (2%, adjusted 123to pH 4.5) in a 1 L Erlenmeyer flask. Flasks were incubated at 25 ºC in an orbital shaker for 5-1246 days.

125

1262.2. Wastewater

127The ROC,was obtained from a pilot plant located in Castell-Platja d'Aro WWTP, in the north-
128east of Spain. The pilot plant was described in detail by Dolar et al. (2012); briefly, it consists
129of a membrane bioreactor (MBR) that treats urban wastewater, followed by a reverse osmosis
130unit. The volume treated in the pilot plant is 200 L h^{-1}. The obtained permeate is two thirds of
131it and, the other third, is concentrate. The ROC was sampled on April 2013 and was stored for
132a month at 4 ºC until its use in the bioreactor experiments. VHW was sampled twice from a
133veterinary hospital located on the Universitat Autònoma de Barcelona campus (Bellaterra,
134Barcelona, Spain): on the day that the bioreactor was set up and after a week. The pertinent
135wastewater in the feed storage tank was replaced by fresh water stored at 4ºC every 2-3 days
136for VHW and 3-5 days for ROC. The characterisation parameters of the ROC and VHW
137samples are presented in Table S1.
138
139**2.3. Overview of the experimental design**
140Three experiments were performed: two on VHW (VHW1 and VHW2) and one on ROC. Two
141air-pulsed fluidised bed glass bioreactors were used for each experiment. One bioreactor was
142inoculated with *T. versicolor* (I), and the other was not inoculated and served as a control
143(NI). The operational conditions are described in Section 2.4. The conditions were similar for
144the VHW1 and ROC treatments and were modified for the VHW2 experiment to improve *T.*
145*versicolor* survival. For each bioreactor (I and NI) and experiment, various analyses were
146performed, as summarised in Table 1: PhACs were quantified to determine their degradation
147during the experiments; the toxicity of the samples was evaluated by means of a standard
148Microtox analysis; DGGE-sequencing was used to identify the microorganisms present in the
149samples; the ratio of fungi to bacteria was determined by PLFA; and the ratio of *T. versicolor*
150vs total fungi was determined by qPCR. PhAC concentration, toxicity analysis and DGGE
151results for VHW2 were previously reported by Badia-Fabregat et al. (2015b). The PhAC

152concentrations of the VHW1 treatments were not analysed due to the suspicion of poor fungal 153performance (see Section 3). All the results from the VHW and ROC experiments were 154processed by principal component analyses (PCA) and detrended correspondence analyses 155(DCA) to determine the correlations between the microbial community and operational 156conditions.

157

158**2.4. Bioreactors and operating conditions**

159The air-pulsed fluidised bed glass bioreactors had a working volume of 1.5 L (Blánquez et al., 1602004). Each bioreactor can be divided in two areas: a lower part, with an inlet diameter of 7.5 161cm, where the air pulses enter through a porous plate, and an upper part, with an inlet 162diameter of 14 cm, which allows better air diffusion and the insertion of probes. The 163electrovalve was controlled by a cyclic timer that produced air pulses at a frequency of 1 sec 164open every 4 sec. The temperature was set to 25 ºC and the pH was kept constant at 4.5 by 165adding 1 M HCl or 1 M NaOH. Glucose and ammonium tartrate were added in pulses from a 166concentrated stock. The main operational parameters of each experiment are provided in Table 1671. The conditions were initially set based on studies performed under sterile conditions 168(Blánquez et al., 2006; Casas et al., 2013) because no publications have reported optimal 169conditions for non-sterile continuous fungal treatment. Therefore, the nutrient additions and 170cellular retention time (CRT) were changed during the experiments according to the 171monitoring results (see Section 2.7) to improve the performance of *T. versicolor*. Moreover, 172the hydraulic retention time (HRT) effect was studied and was modified from 3 to 2 days for 173the VHW1 and ROC treatments (Table 1). For VHW2, it was maintained at approximately 3 d 174to exclude it as a variable to simplify the interpretation of results. Each treatment was run for 175approximately one month. Fungal pellets were kept inside the bioreactor by means of a mesh.

176Usually (see Table 1), 1/3 of the biomass was replaced with fresh biomass every 5 days
177(partial biomass renovation), corresponding to a CRT of 15 days (Blánquez et al., 2006).
178
179Liquid samples were collected from the outlet effluent storage bottle, which was replaced
180every 24 hours. A 60 mL sample was kept frozen at -20 ºC until microbial analysis was
181performed, and 250 mL was sequentially vacuum filtered with Whatman GF/C filters and a
1820.45 µm nylon filter (Millipore), and 200 mL was then stored at -20ºC until pharmaceuticals
183characterisation and 50 mL was used immediately for the other analyses. Samples of fungal
184pellets washed with distilled water were also taken during the VHW2 and ROC experiments
185at every partial biomass renovation.
186
187**Table 1**.Summary of operational parameters and results of continuous fungal treatments.

	VHW1	VHW2[a]	ROC
HRT (d)	3 and 2	3.3	3 and 2
Length of treatment (d)	30	26	24
CRT (d)	15	Variable	15
Glucose initial feed rate (mg g DCW^{-1} d^{-1})	200	343	192
Ammonia initial feed rate (mg g DCW^{-1} d^{-1})	0.45	0.77	0.43
C/N ratio (mol/mol)	1326[b]	1326[b], decreased to 7.5 at day 12	1326[b]
Initial fungal biomass (g DCW L^{-1})	4.4	3.7	3.6
Final fungal biomass (g DCW L^{-1})	2.8	3.6	3.3

188[a] Badia-Fabregat et al. (2015b); [b] Optimal C/N ratio for sterile conditions (Casas et al., 2013)
189n.a.: not analysed; HRT: hydraulic residence time; CRT: cellular residence time; DCW: dry
190cell weight
191
192**2.5. Microbial community analysis**
193*2.5.1. Phospholipid fatty acids analysis (PLFA)*

194For PLFA extraction, 40 mL of liquid samples was centrifuged for 20 min at 10000 g, and the
195supernatant was filtered through a 0.7 µm glass-fibre filter (Millipore). The cell pellet and
196filter were extracted together according to Frostegård et al. (1993). Then, the samples were
197analysed by gas chromatography (Hewlett Packard Series II 5890 equipped with a flame
198ionisation detector and a 50 m HP-5 capillary column) following the protocol described by
199Pennanen et al. (1996).

200

201To determine the bacterial biomass, the following fatty acids were considered: i15:0, a15:0,
20215:0, i16:0, 16:1ω9, i17:0, a17:0, 17:0, cy17:0, 18:1ω7 and cy19:0. To determine the fungal
203biomass, only 18:2ω6,9 was considered because it is reported to correlate well with ergosterol
204and fungal qPCR (Bååth and Anderson, 2003; Landeweert et al., 2003). To calculate the
205approximate amount of C that can be assigned to bacteria and fungi, conversion factors
206obtained from the literature were applied: 363.6 nmol bacterial PLFA/mg C for bacteria
207(Frostegård and Bååth, 1996) and 11.8 nmol fungal PLFA/mg C for fungi (Klamer and Bååth,
2082004).

209

210*2.5.2. Nucleic acid extraction, PCR-DGGE, sequencing and phylogenetic analyses*

211Total DNA was extracted from 4 mL samples using a FastDNA SPIN Kit for Soil (MP
212Biomedicals) following the procedure described by the company. Fragments of the bacterial
21316S and fungal internal transcribed spacer (ITS) region of 18S rDNA were PCR amplified by
214DreamTaq polymerase (Thermo Scientific). Universal primers were used in both reactions:
215U968 forward (5’ ACC GCG AAG AAC CTT AC 3’) and R1401 reverse (5’ CGC TGT GTA
216CAA GAC CC 3’) for bacteria (Nübel et al., 1996) and ITS1F forward (5’ CT TGG TCA TTT
217AGA GGA AGT AA 3’) (Gardes and Bruns, 1993) and ITS2 reverse (5’ GCT GCG TTC TTC
218ATC GAT GC 3’) (White et al., 1990) for fungi. A GC clamp (5’ CCC CCCCCCCCC CGC

219CCC CCG CCC CCC GCC CCC GCC GCC C 3’) was attached to the primers ITS1F and 220U968 at the 5’ end. The PCR programmme for fungi was 5 min at 95 ºC followed by 40 221cycles of 30 sec at 95 ºC, 40 sec at 55 ºC and 1 min at 72 ºC, with a final elongation step of 5 222min at 72 ºC. The bacterial programme was the same, except for the annealing temperature, 223which was 56 ºC. The length and amount of PCR products were estimated in 1% agarose gel 224labelled with ethidium bromide. A DNA ladder was added as a control.

225

226DGGE was performed in an INGENYphorU (Ingeny, The Netherlands) system. The gel 227contained 7.5% acrylamide/bisacrylamide (37:5:1). The urea gradients for optimal separation 228of the bands were 40-80% for bacteria and 25-60% for fungi. Electrophoresis was performed 229for 16 hours at 75 V in 1x TAE buffer at 60 ºC. Gels were stained with SYBR Gold 230(Invitrogen, Life Technologies). Selected DGGE bands were excised, reamplified (22 cycles) 231and re-run in a DGGE gel until the bands were sufficiently clean (after 3-6 repetitions) for 232sequencing. Purification and sequencing of the PCR products amplified without the GC clamp 233was performed by a commercial service (Macrogen Inc., South Korea). Partial fungal and 234bacterial DGGE-derived sequences were aligned with sequences retrieved from the databases 235of GenBank/EMBL/DDBJ with the Blast algorithm (Tables S3 and S5). Bacterial and fungal 236sequence data were deposited at the GenBank database under Accession Numbers 237KM355623-KM355667 and KM361323-KM361352, respectively. Sequences that could not 238be deposited because their length was less than 200 bp can be found in the supplementary 239material (Tables S4 and S6).

240

241*2.5.3. Quantitative PCR (qPCR)*

242qPCR was performed for the total fungi and specific for *T. versicolor* with ITS1F and ITS2 243primers and those described by Eikenes et al. (2005) in the ITS1 region for *T. versicolor,*

244respectively. The 20 µL of reaction mixture contained 10 µL of Maxima SYBR Green qPCR
245Master Mix (Fermentas), 0.375 µM of each primer and 1 µL of DNA. The reactions were
246performed on a Rotor-gene 6000 (Corbett Research, Australia) apparatus using the
247temperature programme described in the article of Eikenes et al. (2005) for *T. versicolor* and
248the programme described at Rajala et al. (2013) for total fungi. Standard curves were
249performed with known amounts of *T. versicolor* (CT= -3.126*log(conc)+32.221, efficiency
2501.089) and *Heterobasidion annosum* (CT= -3.748*log(conc)+36.037, efficiency 0.848),
251respectively.
252

253**2.6. Analysis of pharmaceuticals**

254The analytical procedure performed for the VHW2 samples was based on Gros et al. (2012).
255Briefly, 50 mL of sample was pre-concentrated by solid-phase extraction (SPE) using Oasis
256HLB (3 cc, 60 mg) cartridges (Waters Corp. Mildford, MA, USA), which were previously
257conditioned with 5 mL methanol and 5 mL HPLC grade water. Elution was performed with 6
258mL of pure methanol. The extracts were evaporated under nitrogen stream and reconstituted
259with 1 mL of methanol-water (10:90 v/v). Lastly, 10 µL of internal standard mix at 1 ng μL^{-1}
260was added to the extracts for internal standard calibration. Chromatographic separation was
261performed with an ultra-performance liquid chromatography system (Waters Corp. Mildford,
262MA, USA), using an Acquity HSS T3 column (50 mm x 2.1 mm i.d. 1.7 µm particle size) for
263the compounds analysed under positive electrospray ionisation (PI) and an Acquity BEH C18
264column (50 mm × 2.1 mm i.d., 1.7 µm particle size) for compounds analysed under negative
265electrospray ionisation (NI), both from Waters Corporation. The UPLC instrument was
266coupled to a 5500 QqLit, triple quadrupole–linear ion trap mass spectrometer (5500 QTRAP,
267Applied Biosystems, Foster City, CA, USA) with a Turbo V ion spray source. Two MRM

268transitions per compound were recorded using the Scheduled MRM™ algorithm, and the data
269were acquired and processed using Analyst 2.1 software.
270
271**2.7. Monitoring analyses**

272The glucose concentration was measured using a YSI 2700 SELECT (Yellow Spring
273Instruments) biochemical analyser. Laccase activity was determined spectrophotometrically at
274468 nm by monitoring the oxidation of 2,6-dimethoxyphenol (DMP) in a modified version of
275the method for the determination of manganese peroxidase of Kaal et al. (1993). The reaction
276mixture contained 600 µL of sample, 200 µL of 250 mM sodium malonate at pH 4.5, and 50
277µL of 20 mM DMP. The molar extinction coefficient of DMP was taken as 24.8 mM DMP^{-1}
278cm^{-1} (Wariishi et al., 1992). One activity unit was defined as the amount of enzyme able to
279oxidise 1 micromole of DMP per minute under the assay conditions. The biomass pellet dry
280weight was determined after vacuum-filtering the cultures through pre-weighed glass-fibre
281filters (Whatman, Spain). The fungal amount was determined as the constant weight at 100
282ºC. Additionally, the visual evolution of the biomass in the bioreactors, mainly the colour and
283consistency of the fungal pellets, was routinely checked. Dissolved organic carbon (DOC) and
284total suspended solids (TSS) were analysed according to APHA (APHA-AWWA-WEF, 1995).
285The $N\text{-}NH_4^+$ concentration and chemical oxygen demand (COD) were analysed by using
286commercial kits LCH303 and LCK114 or LCK314, respectively (Hach Lange, Germany).
287Chloride, nitrate, nitrite and sulphate anions were quantified by a Dionex ICS-2000 ionic
288chromatograph. Phosphate was analysed by a phosphate analyser (Hach Lange 115 VAC
289Phosphaxsc). Conductivity was determined by a CRISON MicroCM 2100 conductivity meter,
290and pH was measured by a CRISON MicropH 2001 pH meter. A Microtox assay with the
291bioluminescent bacteria *Vibrio fischeri* was used to perform the acute toxicity test. The 50%

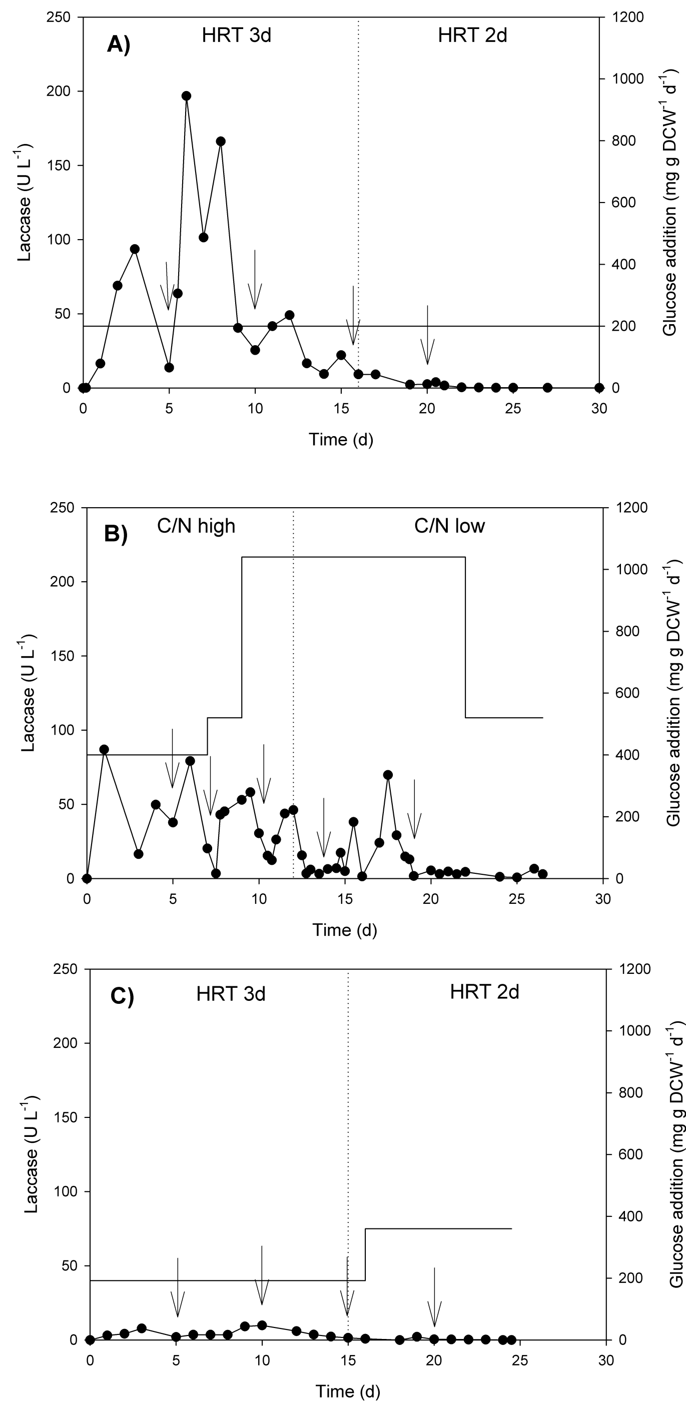
292effective concentration (EC_{50}) was measured after 15 min of exposure with a filtered sample
293at pH 7. Effluent toxicity was expressed in toxicity units (TU), calculated as $TU=100/EC_{50}$.
294
295**2.8. Calculations and statistical analysis**
296PCA was performed for the PLFA results on a correlation matrix using the area percentage of
297the respective PLFA (% of the summed area of all PLFA peaks used in the analyses). DGGE
298gel images were analysed with GelCompar II (ver. 5.1.; Applied Maths BVBA, Belgium),
299generating a binary matrix (presence/absence of band) that was further analysed by DCA on a
300Jaccard distance matrix (including all 71sequenced and non-sequenced fungal and bacterial
301bands and 46 samples). The binary matrices for the bacteria and fungi were combined for all
302samples to perform the analysis, similar to the approach of PLFA. PCA and DCA were
303performed with the PC-ORD 5.0 program. Thirteen bioreactor variables (the full list can be
304found in supplementary material), such as time, glucose concentration and COD were tested
305for their possible axis distribution explanation.
306
307For the pharmaceutical removal calculations, compounds below the quantification limit were
308considered to have a concentration of ½ the limit of quantification (LOQ) (EPA, 2000). The
309mean and standard deviation were calculated using Microsoft® Excel 2011 functions. One-
310factor analysis of variance (ANOVA) was performed for experimental data with Sigmaplot
31111.0.
312
313**3. Results**
314**3.1. Performance of non-sterile fungal bioreactors in continuous operation**
315*3.1.1. Bioreactor operation and monitoring*

316The performance of three bioreactors inoculated with the fungus *T. versicolor* to treat ROC
317and VHW was compared with non-inoculated bioreactors. The extracellular enzyme activity
318was used as a possible indicator of fungal activity, although some previous works found no
319clear relationship between laccase activity and good removal percentages (Blánquez et al.,
3202004; Yang et al., 2013). As shown in Fig. 1, the laccase activity was not stable over time in
321any reactor, with peaks only after some partial fungal renovations, as already found by Badia-
322Fabregat et al. (2015b). The laccase activity was higher for VHW than ROC. However, in
323both cases it was lower than expected for a mixed culture, where it has been reported to be
324induced due to inter-specific interactions (Baldrian, 2004; Freitag and Morrell, 1992). In fact,
325for VHW1-I and ROC-I at an HRT of 2 days, the laccase activity was mostly less than 1 U L^{-1}.
326Nevertheless, for VHW2-I, decreased laccase activity occurred when *T. versicolor* was more
327active in terms of removal percentage (Badia-Fabregat et al., 2015b). Therefore, decreased
328laccase activity might not be related to a lack of optimal conditions for the longer survival of
329*T. versicolor*. Some laccase activity (<5 U L^{-1}) was observed in the ROC-NI control bioreactor
330from day 16 until the end of the operation (Fig. S1). Therefore, we confirm that laccase
331activity is not a suitable indicator of good performance for fungal treatment, as we previously
332suggested (Badia-Fabregat et al., 2015b).

333

334With respect to the other monitored parameters, glucose was completely consumed in all
335fungal (I) bioreactors, whereas in the control (NI) bioreactors, it accumulated in the media
336during the first 6-9 days of operation until indigenous microorganisms started to grow inside
337the reactor (data not shown). COD was lower in all I reactors than NI reactors for all three
338treatments because of the higher accumulation of metabolic products in the latter (i.e.,
339reaching approximately 10 g O_2 L^{-1} in the VHW2-NI treatment, see Fig. S2). Nevertheless,
340COD was always higher in the bioreactor effluent than in the initial wastewater.

341



342

343**Figure 1.** Evolution of (●) laccase activity at **A)** VHW1, **B)** VHW2 and **C)** ROC fungal 344inoculated (I) continuous reactors. Arrows shows the days where 1/3 old fungal biomass was 345changed by fresh one.Continuous line shows the gluccose addition rate. Dotted vertical line 346marks the change of HRT from 3 to 2 days or the change in the C/N ratio of the fed nutrients

347(at VHW2 HRT was 3d during all the operation). Data of **B)** is from Badia-Fabregat et al.
348(2015b).

349

350Taking into account that the extracellular enzyme activity and gluccose consumption rate
351might not be optimal indicators of *T.* versicolor activity, visual examination of bioreactors has
352also been reported as a possible indicator of performance. The visual appearance of the
353bioreactor biomass varied over time and between treatments (Fig. S3 and S4). For VHW1-NI
354between days 23 and 29, a black biofilm developed on the bioreactor inner walls. Fungal
355pellets of VHW1-I were also black at the end of the treatment, which made us suspect failure
356of *T. versicolor* to succeed against contamination. For VHW2-I, the *T. versicolor* pellets lysed
357at day 6 but were recovered probably due to a change in the nutrient addition (Badia-Fabregat
358et al., 2015b). The higher supply of nutrients and the later change in the carbon/nitrogen
359(C/N) ratio allowed the recovery of the fungus and made the bioreactor brownish due to the
360production of melanin-like pigments (Song, 1999; Temp and Eggert, 1999). For ROC-I, a
361pink and green biofilm appeared on the reactor walls, whereas for ROC-NI, the biofilm was
362brown and white. The fungal biomass also decreased until 1.4 g DCW L^{-1} (data not shown)
363between days 15 and 20 for ROC-I for unknown reasons. Increasing the nutrient feed to 360
364mg glucose g^{-1} d^{-1} at day 16 and partial renewal of the biomass at day 20 recovered the fungal
365biomass. However, as shown in Section 3.2, recovery was not achieved and colonisation of
366fungal pellets was observed.

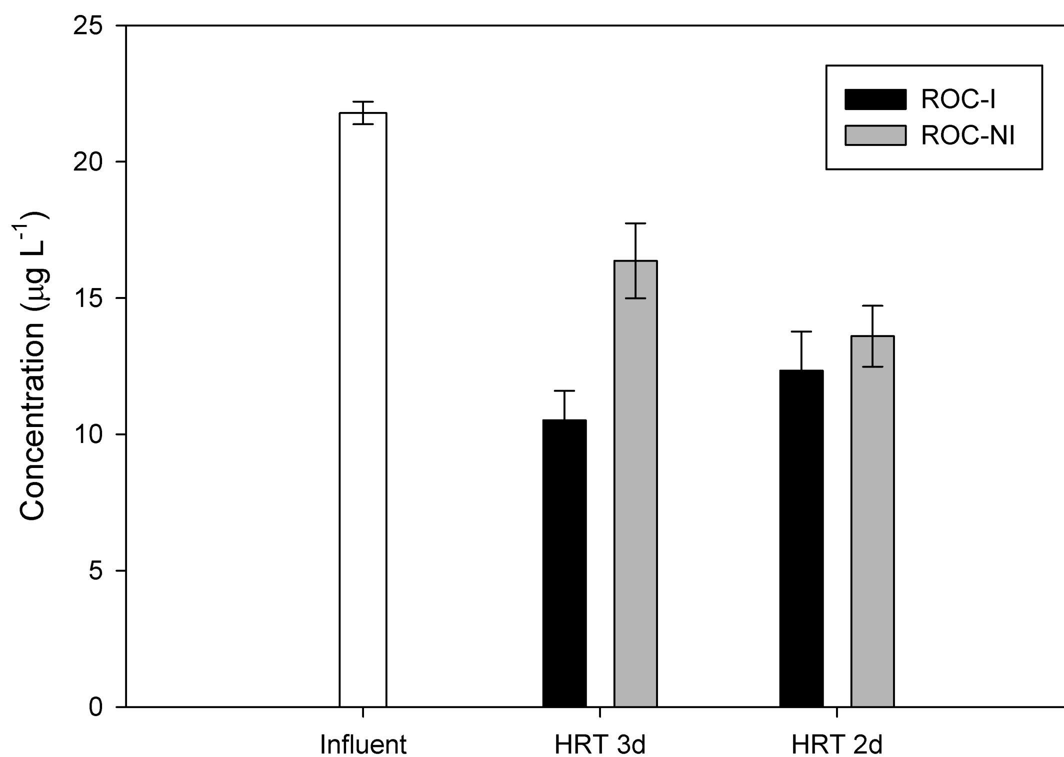
367

368*3.1.2. Degradation of pharmaceuticals*

369PhACs were analysed in the VHW2 and ROC experiments but not in the VHW1 experiment
370due to the suspicion of poor fungal performance in the latter. Then, the main purpose of
371VHW1 was to compare the microbial analysis with that of VHW2 to see whether there were

372differences between a well-performing bioreactor and a poorly performing bioreactor and to 373identify the microorganisms that caused the failure of the bioreactor. The pharmaceuticals 374removal results for VHW2 can be found in a previous article (Badia-Fabregat et al., 2015b). 375Briefly, 44% removal of total PhACs was achieved at an HRT of 3 days after decreasing the 376C/N ratio whereas in the non-inoculated bioreactor, no removal, or even an increase in the 377concentration of PhACs, was observed.

378



379

380**Figure 2.** PhACs concentration at the wastewater (influent) and at every HRT for each 381bioreactor (ROC-I and ROC-NI). Error bars represents standard deviation of 4 samples taken 382at hydraulic steady state.

383

384For the pharmaceutical removal evaluation of the ROC experiment, the mean values of 4 385samples for each HRT (at 12, 13, 14 and 15 days for an HRT of 3 days; and at 21, 22, 23 and

38624 for an HRT of 2 days) were compared with the initial ROC concentration in the influent.
387The PhAC concentration in the ROC and the degradation percentages of specific compounds
388are presented in Table S7. Fig. 2 shows significant differences in the fungal bioreactor at HRT
3893d compared to the non-inoculated control (degradation of 52% in I and 25% in NI), showing
390that degradation was presumably due to fungal action. For an HRT of 2d there were no
391statistically significant differences between treatments due to a decrease in the degradation
392percentage in ROC-I (43% removal) and an increase in ROC-NI (38%). However, there were
393significant differences with respect to the influent in both reactors. The decrease in the
394removal in ROC-I at an HRT 2d was due to the decrease in the HRT or the biomass
395conditions, or a combination of both.

396

397*3.1.3. Toxicity of bioreactors effluents*

398Microtox analysis of the acute toxicity showed no toxicity in all of the reverse osmosis
399concentrates and ROC-I effluent (Table S8). However, an increase (5 TU) in toxicity was
400observed for ROC-NI. This might be attributed to toxic compounds produced in the control
401bioreactor. However, the values were always less than 25 TU, the threshold value for an
402effluent to be considered toxic (Generalitat de Catalunya, 2003). Therefore, the compounds
403were either not highly toxic or were present at low concentrations. VHW was not toxic (1-20
404TU) nor were the effluents of the I and NI bioreactors (0-3 TU).

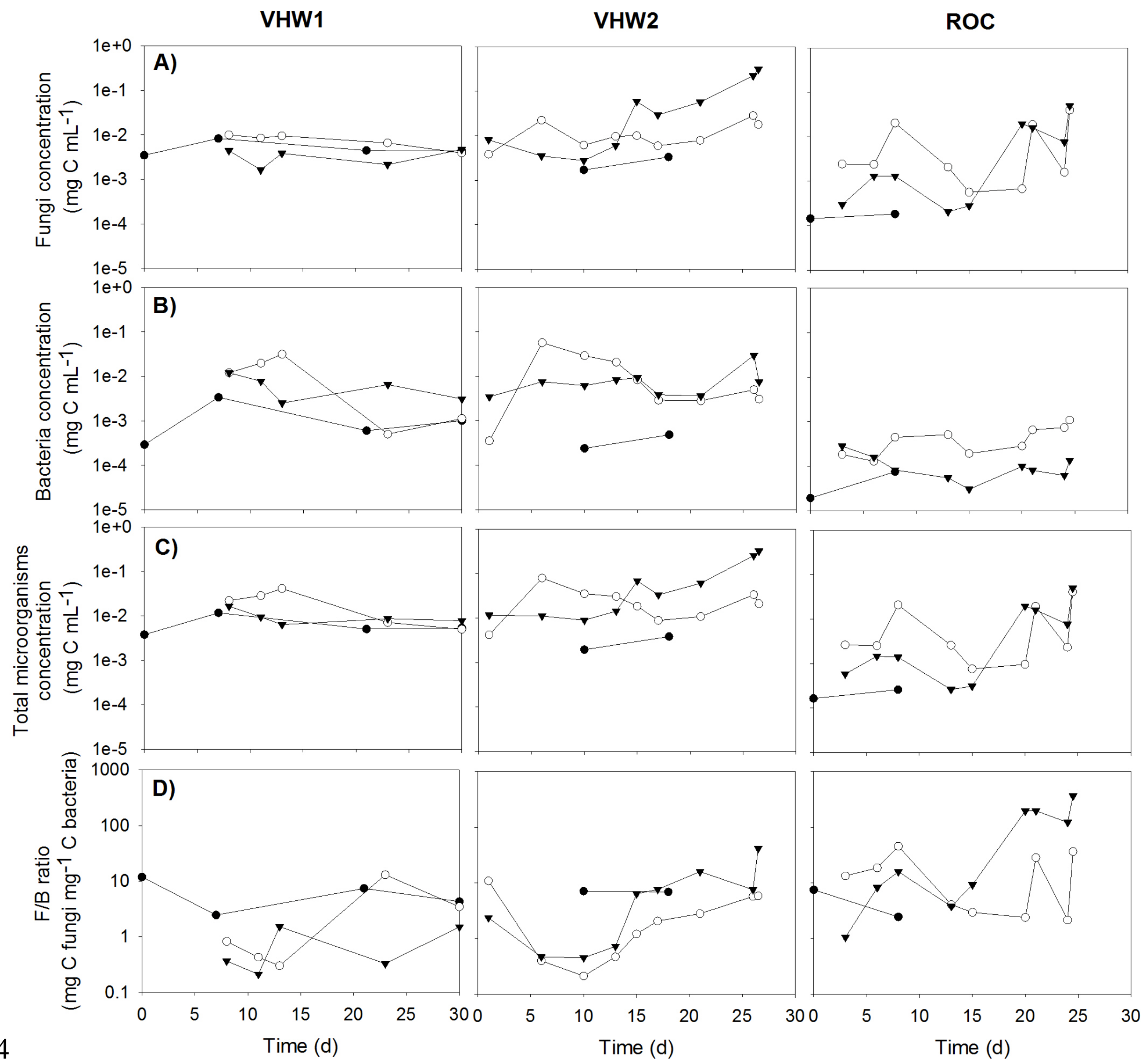
405

406**3.2. Microbial communities study**

407*3.2.1. Fungal/Bacterial ratio by PLFA analysis*

408PLFA analyses were performed to quantify the total bacteria and total fungi at different times
409in each experiment. For the sake of clarity, it has to be taken into account that *T. versicolor*
410pellets were kept inside the reactor and samples were from the effluent, therefore, only fungi

411in the liquid phase (not in the pellets) were quantified. The results for VHW1 are shown in the
412first column of Fig. 3. The fungal and bacterial concentrations in the effluent were maintained
413similar to that of the feed (Fig. 3, first column). For VHW2 (Fig. 3, second column), the
414growth of microorganisms was more notable, probably due to the higher supply of nutrients.
415A bacteria peak was observed at day 6 for VHW2-I, when *T. versicolor* was lysed. However,
416in both the I and NI bioreactors, the increase in the fungal/bacterial (F/B) ratio from day 10
417shows that the decrease in the C/N ratio favoured the growth of fungi. For ROC (Fig. 3, last
418column), microorganism concentration in the wastewater was considerably lower than for
419VHW, as expected, due to the origin of each wastewater (RO concentrate from a MBR
420effluent and raw wastewater, respectively). Inside the bioreactors, the fungal and bacterial
421concentrations increased, but bacteria did not reached the levels obtained for the VHW
422bioreactors. Therefore, F/B ratio was the highest for the ROC treatment.
423



424

425**Figure 3.** Evolution of **A)** fungal carbon, **B)** bacterial carbon, **C)** total biological carbon and 426**D)** ratio fungi/bacteria in the three experiments (VHW1, VHW2 and ROC). Symbols: (●) 427influent wastewater, (○) fungal inoculated bioreactor (I) effluent and (▼) non-inoculated 428control bioreactor (NI) effluent.

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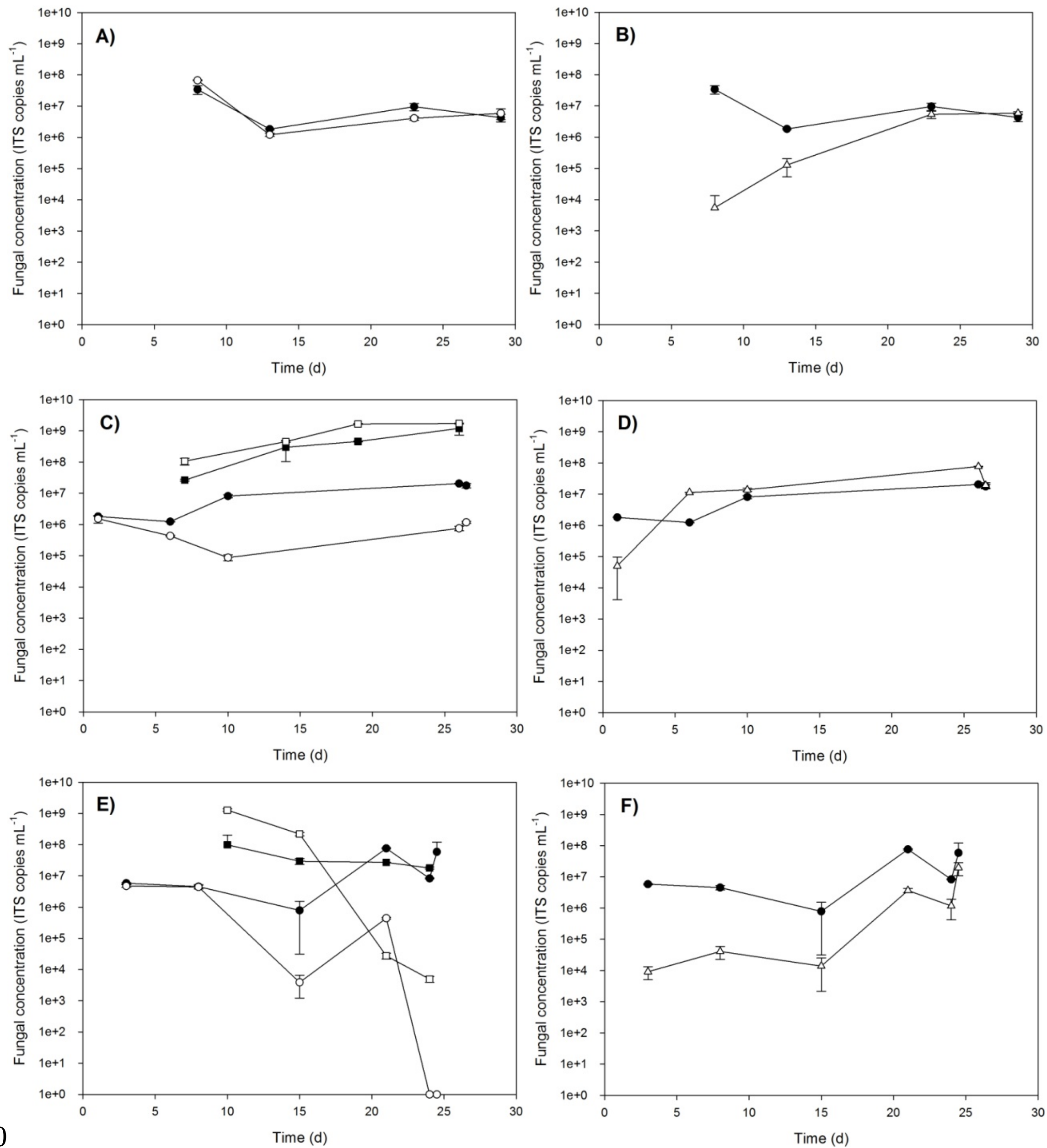

430

431**Figure 4.** Total fungi and *T. versicolor* quantification by qPCR in the different treatments. 432Comparison of total fungi and *T. versicolor* of liquid and pellets in *T. versicolor* inoculated 433reactors containing **A)** VHW1, **C)** VHW2 and **E)** ROC, and comparison of total fungi 434developed in *T. versicolor* inoculated reactors and non-inoculated control reactors of **B)** 435VHW1, **D)** VHW2 and **F)** ROC. Symbols: (●) Total fungi at liquid sample of fungal reactor, 436(○) *T. versicolor* at liquid sample of fungal reactor, (■) Total fungi at pellets sample of fungal 437reactor, (□) *T. versicolor* at pellets sample of fungal reactor and (△) Total fungi at liquid 438sample of non-inoculated control reactor.

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440*3.2.2. qPCR of* T. versicolor *and total fungi*

441qPCR for the total fungi and specific for *T. versicolor* was performed using liquid samples 442and fungal pellets. As shown in Fig. 4A, 4C and 4E, fungi other than *T. versicolor* developed 443in the liquid of all bioreactors except VHW1. In fact, throughout the VHW1 treatment, the *T.* 444*versicolor* amount was similar to the total fungi, in contrast to what was expected when 445considering the black colour of the pellets due to the growth of other microorganisms. In 446contrast, for ROC, *T. versicolor* decreased and even disappeared from the liquid sample at day 44724 and decreased by more than 4 orders of magnitude in the pellet samples. Therefore, other 448fungi were growing in the original pellets of *T. versicolor*. The increase in *T. versicolor* 449observed at 21 days in the liquid was probably due to the 1/3 pellet renovation on day 20. 450When comparing fungal bioreactors (I) with the controls (NI) (Fig. 4B, 4D and 4F), fungal 451concentration in I was stable, whereas in the NI, it increased until reaching the same 452concentration as I. In general, the results from the total fungi qPCR analyses are in accordance 453with those obtained by PLFA.

454

455*3.2.3. Microbial diversity by DGGE*

456The DGGE results for *T. versicolor* are in agreement with those of the qPCR. *T. versicolor* 457was found in every sample of the I bioreactors, except for the ROC experiment from day 15 458onwards (Fig. 5). Sequencing of the fungal DGGE profiles indicated that the fungus that 459competed with *T. versicolor* and finally prevailed in ROC-I was most likely *Trichoderma* 460*asperellum*. At the same time, in ROC-NI, the predominant fungus was a *Penicillium sp.*, 461which was probably responsible for the brown and white biofilm on the reactor inner wall 462(Fig. S4). On the other hand, at the beginning of the experiment (until day 15) the dominant 463fungus in the NI bioreactor was *Fusarium oxysporum* with presence of *Rhodotorula sp.* as

67

464well. The laccase activity detected from day 16 onwards was likely produced by the 465Ascomycete *Penicillium sp.*

466

467On the other hand, *Trichoderma* spp. were not found either VHW1 or VHW2. For VHW1-I, 468*Candida* sp., *Exophiala equina* and *Scytalidium lignicola* grew in the bioreactor whereas 469*Exophiala oligosperma* and also *Fusarium* sp, developed in VHW1-NI.

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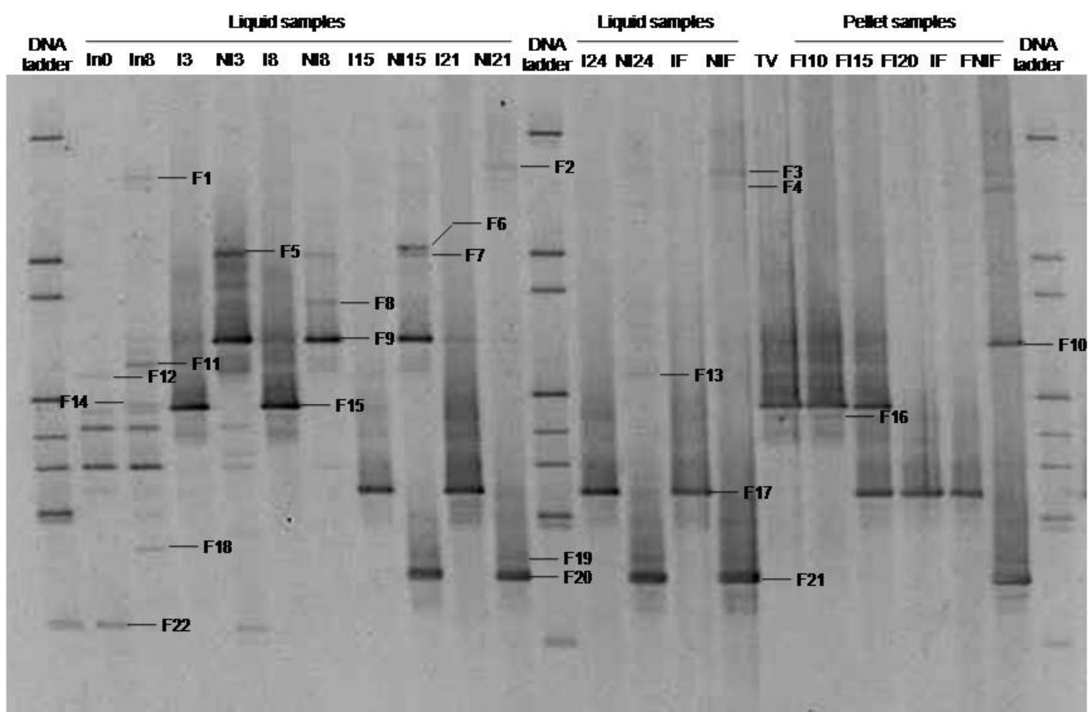
471 

472**Figure 5.** DGGE gel of ITS1F-ITS2 fungal fragments PCR amplified from samples of ROC 473experiment. Lanes legend: I: fungal inoculated reactor; NI: non-inoculated control reactor; In: 474inlet wastewater; numbers: experimental day of sampling; F (at the end): final time of 475operation (25 d); F (at the beginning): fungal pellet or biofilm sample of I and NI bioreactor 476respectively; TV: pure *T. versicolor*. Important bands for the discussion of results: F9: 477*Fusarium oxysporum*; F15: *Trametes versicolor*; F17: *Trichoderma asperellum*; F20 and F21: 478*Penicillium* sp. Closest relatives for the other bands can be found at Table S3.

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480Difficulties were encountered in the amplification of the ribosomal DNA of some bacterial
481communities, especially for the ROC samples, due to the lower amount of bacteria. Only an
482*Enterobacter* sp. and *Clostridia* were identified in ROC-NI, and *Clostridia, γ-proteobacteria*
483(*Enterobacteriales* and *Xanthomonadales*) and some unidentified bacteria were found in
484ROC-I. In VHW1, some *Enterobacter sp.* initially developed in both reactors (I and NI). The
485proteobacteria *Luteibacter* and *Burkholderia* also grew in VHW1-I, whereas in VHW1-NI,
486another *Enterobacter* replaced the previous one. In the feed water and in some isolated VHW
487reactor samples, some *Clostridiales* were present, but taking into account that *Clostridia* spp
488are anaerobic bacteria, they probably did not grow inside the bioreactors but their spores were
489present in the feed water. In the VHW2 bioreactors, similar bacterial and fungal communities
490were found in I and NI. Many unclassified proteobacteria, *Enterobacteriales sp.*,
491*Burkholderiales sp.* and *Verrucomicrobiales sp.* were detected (Badia-Fabregat et al., 2015b).
492
493*3.2.4. PCA and DCA analysis of PLFA and DGGE results*
494PCA was performed for the PLFA results, and DCA was performed for the DGGE results. The
495PCA of the PLFA profiles showed that the microbial communities of the ROC and VHW
496systems were separated (Fig. 6A), but the DCA graph of the DGGE community profiles
497showed somewhat less separation (Fig. 6D). The different results are due to the two
498community profiling approaches highlighting different aspects: data in the PLFA analysis is a
499quantitative pattern of certain community-level changes of microbes, whereas DGGE data
500give a qualitative estimation of the presence or absence of microbial taxa in the sampled
501systems. Thus, the differences may be in the relative abundances of certain microbial groups
502rather than in the species-level composition of microorganisms.
503

504In the DCA, total pellet samples from one side and influent and non-inoculated ROC samples
505(ROC-In/NI) from another side were separately grouped from the total samples (Fig. 6D), but
506systematic separation between the ROC and VHW samples was not observed. The differences
507in pellet samples are clearly due to the presence of only *T. versicolor*.

508

509For the comparison between VHW experiments (Fig. 6B and 6E), in the PCA graph (Fig 6B),
510only influent wastewater (In) was differentiated from the other samples. Three samples,
511VHW2-I6, VHW1-I23 and VHW1-I30 were seen as outliers: VHW2-I6 due to its high
512bacterial concentration (high correlation with 17:0 bacterial phospholipid) at day 6, when *T.*
513*versicolor* was dying in the VHW2 reactor. No clear grouping of samples from VHW2 was
514found when the N/C ratio was changed. In the DCA, separation between VHW1 and VHW2
515can be observed, indicating that the microbial taxa in the two treatments were mostly
516different. Therefore, there were fewer differences between VHW-I and VHW-NI than between
517VHW1 and VHW2.

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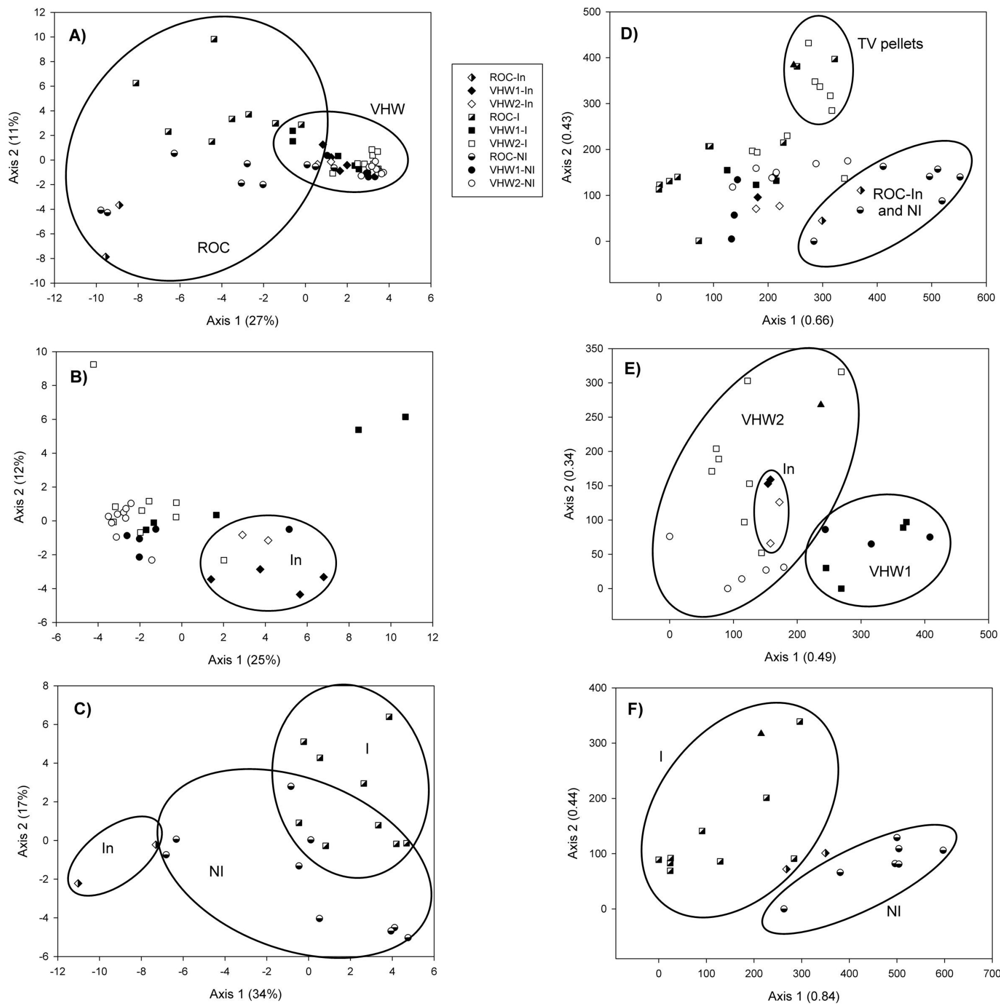

519

520**Figure 6.**Principal Component Analysis (PCA) of PLFA results:**A)** Total samples, **B)** VHW 521samples and **C)** ROC samples; and Detrended Correspondence Analysis (DCA) of DGGE 522results: **D)** Total samples, **E)** VHW samples and **F)** ROC samples.Samples can be grouped in: 523influent samples (In), samples from the effluent of the fungal inoculated (I) or the non-524inoculated control (NI) bioreactors, or from whashed pellets of *T. versicolor* from inside the I 525reactor.

526

527In contrast to VHW, in the ROC systems (Fig. 6C and 6F), inoculation with *T. versicolor* 528caused clear separation between the In, NI and I samples. Specifically, the NI and I samples 529evolved differently at both the community and taxa level. Even when *T. versicolor* 530disappeared in ROC-I, the microbial communities were completely different in I and NI.

531

532Environmental and operational parameters such as the pH, COD, conductivity, glucose 533concentration and addition rate, laccase activity, bacterial and fungal concentration, F/B ratio, 534*T. versicolor* concentration and day of operation, were also assessed for their relation with the 535sample distributions in all the analyses. However, low correlations were generally found. 536Only in the DCA analysis of ROC, COD could explain the separation at axis 1, with an r^2 of 5370.47, and laccase activity could explain the separation at axis 2, with an r^2 of 0.35. Moreover, 538time is not an explanatory variable of the sample distribution.

539

540**4. Discussion**

541*4.1. Evaluation of the bioreactor performance: PhACs degradation and monitoring 542parameters*

543A clear effect of inoculation with *T. versicolor* on the degradation of PhAC was observed in 544the ROC and VHW2 experiments. Notably, under non-sterile conditions in the continuously 545fungal-inoculated bioreactor, we achieved similar degradation percentages of global PhAC as 546in a sterile batch bioreactor (Badia-Fabregat et al., 2015a). Therefore, after proper 547optimisation of the process, fungal inoculation might be a suitable treatment for effluents with 548high concentrations of PhAC. However, it should be included only as a pre-treatment process 549because *T. versicolor* cannot remove wastewater COD, as already reported (Anastasi et al., 5502012; Cruz-Morató et al., 2013a). Anastasi et al. (2012) showed that fungi and bacteria can 551work synergically in a two-step treatment, where fungi were used to degrade the most 552recalcitrant compounds (dyes) and activated sludge was used for the removal of COD from 553industrial wastewater.

554

555The results of the analysis of laccase activity in the different treatments confirmed, as reported
556previously (Badia-Fabregat et al., 2015b), that the presence or absence of laccase is not
557directly correlated with the degradative activity of *T. versicolor* and therefore cannot be used
558as a parameter to monitor the survival of the inoculated fungus. Based on the present study,
559we can conclude that traditional methods to monitor inoculated fungi survival and activity,
560such as dry cell weight, laccase activity and visual aspect of the pellets, can only help in
561providing clues about bioreactor performance but cannot be a determinant factor of the
562operation performance evaluation. Complementary tools, such as molecular biology analysis,
563should be used in every experiment for a deeper understanding of the processes taking place
564until optimal conditions for the treatment of non-sterile effluents are achieved.
565
566In our work, the qPCR and PLFA analyses were in complete agreement. Thus, a reduced
567number of analyses might be sufficient because both are suitable for the monitoring some
568parameters (i.e., total fungi) of the bioreactors.
569
570*4.2. Microbial competition with the inoculated fungus*
571Competition of the inoculated fungus with other microorganisms is one of the major
572drawbacks encountered when working with fungal bioreactors at conditions similar to real
573applications. It is difficult to find the balance between nutrient addition and growth
574suppression of indigenous microorganisms. So far, the discussion of microbial competition
575has mainly focused on bacterial growth because bacteria generally grow faster than fungi.
576However, the present work demonstrates that fungi other than the inoculated fungi can
577successfully compete. In our experiments, *Trichoderma asperellum* overtook *T. versicolor*
578between days 15 and 20 in the ROC bioreactor. In a previous VHW batch bioreactor treatment
579(Badia-Fabregat et al., 2015b), *Trichoderma* sp. was also found among other fungi, eventually

580overcoming *T. versicolor*. However, in the present study, *Trichoderma* spp. were not found
581either VHW1 or VHW2.
582
583*Trichoderma* spp. are filamentous soil fungi used as wood decaying inhibitors (Freitag and
584Morrell, 1992) and as a biocontrol of several plant pathogens (Fernandes Qualhato et al.,
5852013). *T. asperellum* and *T. harzianum* are the most effective antagonists of pathogens in agar
586plate studies through nutrient competition or direct mycoparasitism with antifungal
587metabolites or cell-wall degrading enzymes (Fernandes Qualhato et al., 2013). It has been
588reported that *T. harzianum* consumes glucose at a higher rate than *T. versicolor* (Freitag and
589Morrell, 1992), which gives it a clear competitive advantage in the bioreactor. However,
590*Trichoderma* spp. are more competitive against brown-rot fungi than against white-rot fungi
591(Bruce and Highley, 1991) as overgrowth occurred without lysis of the latter (Baldrian, 2004;
592Bruce and Highley, 1991; Freitag and Morrell, 1992), which is considered the main factor for
593efficient biological control (Fernandes Qualhato et al., 2013). Nonetheless, there is huge
594interspecies and interstrain variability in the level of antagonism of *Trichoderma* spp. (Bruce
595and Highley, 1991; Fernandes Qualhato et al., 2013) and although the mechanism of
596antagonism of *T. asperellum* remains unclear in the present study, it is evident that it was able
597to outcompete and grow on the *T. versicolor* pellets. In a study of Lu et al. (2009),
598*Trichoderma* spp. became the dominant species, together with *Candida* spp., in a microbial
599consortia biofilm reactor. Moreover, *Trichoderma asperellum* was probably responsible for
600the green colour of the biofilm in the bioreactor (Fig. S4).
601
602*Exophiala* spp. were probably the cause of the black colour in the VHW1 bioreactors on the
603final days of operation (Fig. S3). Although the visual appearence was the worst, the effect was
604not as severe as that of *Trichoderma asperellum* in the ROC-I treatment. *Exophiala* spp.

605belong to the so-called black yeasts, which cause superficial mycoses in humans. However, 606the potential biological hazard of dark biofilms in domestic water taps was regarded as low 607because the majority of the species were only opportunistic pathogens in 608immunocompromised humans (Heinrichs et al., 2013). In a study of Isola et al. (2013), some 609*Exophiala* spp., e.g., *E. oligosperma*, produce positive toluene degradation tests, while *E.* 610*equina* did not. For VHW2, the fungi detected in the I and NI bioreactors were similar, mainly 611*Candida spp.* and *Fusarium* spp. (Badia-Fabregat et al., 2015b), indicating that *T. versicolor* 612did not have a major influence on the indigenous fungal community of the wastewater.

613

614*Fusarium* spp. were very ubiquitous as they developed in 4 of the 6 bioreactors. The lack of 615*Fusarium* spp. in the ROC-I and VHW1-I reactors could be explained by the previously 616reported antagonistic behaviour (Ruiz-Dueñas and Martínez, 1996). However, under the 617presence of *T. versicolor*, *Fusarium* spp. were found in VHW2-I and in the fungal batch 618treatment of VHW (Badia-Fabregat et al., 2015b). Therefore, the suggested antagonism may 619be weak or species-specific. *Candida* spp. were present in 3 out of the 4 VHW bioreactors. 620*Candida* spp. were one of the main genera in urban WWTP, together with *Rhodotorula* spp., 621*Trichosporon* spp. and 5 other unidentified genera (Yang et al., 2011). Higher diversity was 622generally found in the samples during the final days of treatment, probably due to the much 623higher HRT than conventional growth rates, which allows proliferation of many types of 624microorganisms.

625

626Regarding the bacterial community, mainly *Enterobacteriales sp.* and *Burkholderia sp.* were 627detected in the bioreactors. *Enterobacteriales sp.*, which are common in urban wastewater, 628were present in each bioreactor, independently of the wastewater origin and whether it was 629inoculated by *T. versicolor*. Moreover, *Enterobacteriales sp.* could be responsible for the pink

630colour of the biofilm in ROC-I (Fig. S4) (Deorukhkar et al., 2007). *Burkholderia spp.* were
631present in 3 of the 4 VHW bioreactors and in the batch bioreactor (Badia-Fabregat et al.,
6322015b). *Burkholderia* are β-proteobacteria, which are a major population in environmental
633samples and aerobic MBRs and RO systems (Ayache et al., 2013). Thus, bacteria from those
634genera were probably responsible for the lysis of *T. versicolor* in VHW2-I around day 6
635(Badia-Fabregat et al., 2015b).

636

637*4.3. Control of indigenous microbial growth*

638The effect of the C/N ratio on fungal and bacterial populations is controversial because some
639studies reported no correlation (Wymore et al., 2013), whereas other studies found that N
640stimulated fungal growth and decreased bacterial growth (Rousk and Bååth, 2007). Zhang and
641Geissen (2012) found that the addition of glucose was not sufficient to recover *Phanerochaete*
642*chrysosporium* activity after fungal lysis, and carbamazepine degradation was achieved only
643after N addition. In our experiments, we found a recovery of *T. versicolor,* an increase in the
644F/B ratio and a subsequent increase in the PhAC degradation percentage in VHW2-I when the
645C/N ratio of the added nutrients was decreased. Nevertheless, further experiments altering the
646C/N ratio should be performed to confirm the fungal-favouring conditions.

647

648On the other hand, we found that some degradation of micropollutants can be achieved by the
649indigenous fungi in conditions favouring fungi instead of bacteria. For instance, in the control
650reactor of the ROC treatment (ROC-NI), a *Penicillium* sp. with laccase activity was detected
651when PhAC removal reached 38%. *Penicillium* spp. are known for their ability to degrade
652some PAHs and for the production of extracellular enzymes, such as laccases (Rodríguez et
653al., 1996). *Trichoderma* spp. and *Fusarium* spp. also degrade organic pollutants (Cobas et al.,
6542013; Machín-Ramírez et al., 2010; Rafin et al., 2000). Thus, the degradation of PhAC

655achieved in ROC-NI and ROC-I at HRT 2 d (when *T. versicolor* was not active) might be
656assigned to those three fungi.
657
658Based on the PLFA results, the concentration of microorganisms in the wastewater feed
659determines their concentration inside the bioreactor throughout treatment. Even so, the total
660concentration of microorganisms is largely independent of *T. versicolor* survival, which was
661overtaken in the bioreactor with a lower microbial load (ROC). Thus, the key factor to address
662is not the total quantity but the specific microorganisms developed in each treatment. In the
663same line, DCA and PCA statistical analysis showed that the feed water and/or the different
664operational conditions applied to each experiment were more important to the community that
665developed in the bioreactors than inoculation with the fungus (i.e., inoculation of *T. versicolor*
666did not strongly affect the development of microbial communities). Yang et al. (2011) also
667found that the different origin of wastewater was determinant for the development of yeasts in
668biological treatments.
669

670**5. Conclusions**

671The present work demonstrates the importance of monitoring the microbial community
672growing in non-sterile fungal reactors. The bacteria and fungi that developed inside the
673bioreactors were quantified and identified, indicating that the failure of some fungal-assisted
674wastewater treatment processes might be due to the competition exerted by indigenous fungi
675rather than bacteria, in contrast to the results reported in the literature. The high correlation
676found between the qPCR and PLFA results demonstrates the suitability of both methods for
677the monitoring the total bacteria and fungi in that type of treatment. Further experiments, i.e.,
678at different nutrient (C/N) ratios and including molecular tools to identify the microbial

679community, are needed to fully understand the behaviour of the inoculated fungus and to 680optimise the operational conditions.

681

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696

697**References**

698Anastasi, A., Spina, F., Prigione, V., Tigini, V., Giansanti, P., Varese, G.C., 2010. Scale-up of a
699 bioprocess for textile wastewater treatment using Bjerkandera adusta. Bioresour.
700 Technol. 101, 3067–3075.

701Anastasi, A., Spina, F., Romagnolo, A., Tigini, V., Prigione, V., Varese, G.C., 2012. Integrated
702 fungal biomass and activated sludge treatment for textile wastewaters bioremediation.
703 Bioresour. Technol. 123, 106–111.

704APHA-AWWA-WEF, 1995. Standard Methods for the Examination of Water and Wastewater,
705 19th ed. American Public Association/AmericaWaterWorks Association/Water
706 Environment Federation, Washington, DC.

707Ayache, C., Manes, C., Pidou, M., Croué, J.P., Gernjak, W., 2013. Microbial community
708 analysis of fouled reverse osmosis membranes used in water recycling. Water Res. 47,

709 3291–9.

710Bååth, E., Anderson, T.-H., 2003. Comparison of soil fungal/bacterial ratios in a pH gradient
711 using physiological and PLFA-based techniques. Soil Biol. Biochem. 35, 955–963.

712Badia-Fabregat, M., Lucas, D., Gros, M., Rodríguez-Mozaz, S., Barceló, D., Caminal, G.,
713 Vicent, T., 2015a. Identification of some factors affecting pharmaceutical active
714 compounds (PhACs) removal in real wastewater. Case study of fungal treatment of
715 reverse osmosis concentrate. J. Hazard. Mater. 283, 663–671.

716Badia-Fabregat, M., Lucas, D., Pereira, M.A., Alves, M., Pennanen, T., Fritze, H., Rodríguez-
717 Mozaz, S., Barceló, D., Vicent, T., Caminal, G., 2015b. Continuous fungal treatment of
718 non-sterile veterinary hospital effluent: pharmaceuticals removal and microbial
719 community assessment. Appl. Microbiol. Biotechnol. DOI: 10.1007/s00253–015–7105–
720 0.

721Baldrian, P., 2004. Increase of laccase activity during interspecific interactions of white-rot
722 fungi. FEMS Microbiol. Ecol. 50, 245–53.

723Blánquez, P., Casas, N., Font, X., Gabarrell, X., Sarrà, M., Caminal, G., Vicent, T., 2004.
724 Mechanism of textile metal dye biotransformation by Trametes versicolor. Water Res. 38,
725 2166–72.

726Blánquez, P., Sarrà, M., Vicent, M.T., 2006. Study of the cellular retention time and the partial
727 biomass renovation in a fungal decolourisation continuous process. Water Res. 40, 1650–
728 6.

729Blánquez, P., Sarrà, M., Vicent, T., 2008. Development of a continuous process to adapt the
730 textile wastewater treatment by fungi to industrial conditions. Process Biochem. 43, 1–7.

731Bruce, A., Highley, T.L., 1991. Control of growth of wood decay Basidiomycetes by
732 Trichoderma spp . and other potentially antagonistic fungi. For. Prod. J. 41, 63–67.

733Casas, N., Blánquez, P., Vicent, T., Sarrà, M., 2013. Laccase production by Trametes
734 versicolor under limited-growth conditions using dyes as inducers. Environ. Technol. 34,
735 113–119.

736Cheng, Z., Xiang-hua, W., Ping, N., 2013. Continuous Acid Blue 45 decolorization by using a
737 novel open fungal reactor system with ozone as the bactericide. Biochem. Eng. J. 79,
738 246–252.

739Cobas, M., Ferreira, L., Tavares, T., Sanromán, M.A., Pazos, M., 2013. Development of
740 permeable reactive biobarrier for the removal of PAHs by Trichoderma longibrachiatum.
741 Chemosphere 91, 711–6.

742Cruz-Morató, C., Ferrando-Climent, L., Rodriguez-Mozaz, S., Barceló, D., Marco-Urrea, E.,
743 Vicent, T., Sarrà, M., 2013a. Degradation of pharmaceuticals in non-sterile urban
744 wastewater by Trametes versicolor in a fluidized bed bioreactor. Water Res. 47, 5200–10.

745Cruz-Morató, C., Lucas, D., Llorca, M., Rodriguez-Mozaz, S., Gorga, M., Petrovic, M.,
746 Barceló, D., Vicent, T., Sarrà, M., Marco-Urrea, E., 2014. Hospital wastewater treatment
747 by fungal bioreactor: Removal efficiency for pharmaceuticals and endocrine disruptor
748 compounds. Sci. Total Environ. 493, 365–376.

749Cruz-Morató, C., Rodríguez-Rodríguez, C.E., Marco-Urrea, E., Sarrà, M., Caminal, G.,
750 Vicent, T., Jelic, A., García-Galán, M.J., Pérez, S., Díaz-Cruz, M.S., Petrovic, M.,
751 Barceló, D., 2013b. Biodegradation of Pharmaceuticals by Fungi and Metabolites
752 Identification, in: Vicent, T., Caminal, G., Eljarrat, E., Barceló, D. (Eds.), Emerging
753 Organic Contaminants in Sludges. Springer, pp. 165–213.

754Daughton, C.G., Ternes, T.A., 1999. Pharmaceuticals and personal care products in the
755 environment: agents of subtle change? Environ. Health Perspect. 107, 907–38.

756Deorukhkar, A.A., Chander, R., Ghosh, S.B., Sainis, K.B., 2007. Identification of a red-
757 pigmented bacterium producing a potent anti-tumor N-alkylated prodigiosin as Serratia
758 marcescens. Res. Microbiol. 158, 399–404.

759Dhouib, A., Aloui, F., Hamad, N., Sayadi, S., 2006. Pilot-plant treatment of olive mill
760 wastewaters by Phanerochaete chrysosporium coupled to anaerobic digestion and
761 ultrafiltration. Process Biochem. 41, 159–167.

762Dolar, D., Gros, M., Rodriguez-Mozaz, S., Moreno, J., Comas, J., Rodriguez-Roda, I.,
763 Barceló, D., 2012. Removal of emerging contaminants from municipal wastewater with
764 an integrated membrane system, MBR-RO. J. Hazard. Mater. 239-240, 64–9.

765Eikenes, M., Hietala, A.M., Alfredsen, G., Gunnar Fossdal, C., Solheim, H., 2005.
766 Comparison of quantitative real-time PCR, chitin and ergosterol assays for monitoring
767 colonization of Trametes versicolor in birch wood. Holzforschung 59, 568.

768EPA, 2000. Assigning values to non-detected/non-quantified pesticide residues in human
769 health food exposure assessments. Washington, DC.

770Fernandes Qualhato, T., Cardoso Lopes, F.A., Stecca Steindorff, A., Silva Brandão, R.,
771 Amorim Jesuino, R.S., Ulhoa, C.J., 2013. Mycoparasitism studies of Trichoderma
772 species against three phytopathogenic fungi: evaluation of antagonism and hydrolytic
773 enzyme production. Biotechnol. Lett. 35, 1461–8.

774Freitag, M., Morrell, J.J., 1992. Changes in selected enzyme activities during growth of pure
775 and mixed cultures of the white-rot decay fungus Trametes versicolor and the potential
776 biocontrol fungus Trichoderma harzianum. Can. J. Microbiol. 38, 317–23.

777Frostegård, A., Bååth, E., 1996. The use of phospholipid fatty acid analysis to estimate
778 bacterial and fungal biomass in soil. Biol. Fertil. Soils 22, 59–65.

779Frostegård, Å., Bååth, E., Tunlio, A., 1993. Shifts in the structure of soil microbial
780 communities in limed forests as revealed by phospholipid fatty acid analysis. Soil Biol.
781 Biochem. 25, 723–730.

782Gardes, M., Bruns, T.D., 1993. ITS primers with enhanced specificity for basidiomycetes--
783 application to the identification of mycorrhizae and rusts. Mol. Ecol. 2, 113–8.

784Generalitat de Catalunya, 2003. DECRET 130/2003, de 13 de maig, pel qual s’aprova el
785 Reglament dels serveis públics de sanejament. DOGC.

786Gros, M., Rodríguez-Mozaz, S., Barceló, D., 2012. Fast and comprehensive multi-residue
787 analysis of a broad range of human and veterinary pharmaceuticals and some of their
788 metabolites in surface and treated waters by ultra-high-performance liquid
789 chromatography coupled to quadrupole-linear ion trap tandem. J. Chromatogr. A 1248,
790 104–121.

791Hai, F.I., Yamamoto, K., Nakajima, F., Fukushi, K., 2008. Factors governing performance of
792 continuous fungal reactor during non-sterile operation – The case of a membrane
793 bioreactor treating textile wastewater Tap water. Chemosphere 74, 810–817.

794Heinrichs, G., Hübner, I., Schmidt, C.K., de Hoog, G.S., Haase, G., 2013. Analysis of black
795 fungal biofilms occurring at domestic water taps. I: compositional analysis using Tag-
796 Encoded FLX Amplicon Pyrosequencing. Mycopathologia 175, 387–97.

797Isola, D., Selbmann, L., de Hoog, G.S., Fenice, M., Onofri, S., Prenafeta-Boldú, F.X.,
798 Zucconi, L., 2013. Isolation and screening of black fungi as degraders of volatile

799 aromatic hydrocarbons. Mycopathologia 175, 369–79.

800Kaal, E.E.J., de Jong, E., Field, J.A., 1993. Stimulation of ligninolytic peroxidase activity by
801 nitrogen nutrients in the white rot fungus Bjerkandera sp. Strain BOS55. Appl. Environ.
802 Microbiol. 59, 4031–4036.

803Klamer, M., Bååth, E., 2004. Estimation of conversion factors for fungal biomass
804 determination in compost using ergosterol and PLFA 18:2ω6,9. Soil Biol. Biochem. 36,
805 57–65.

806Landeweert, R., Veenman, C., Kuyper, T.W., Fritze, H., Wernars, K., Smit, E., Horton, T.R.,
807 Bruns, T.D., Agerer, R., Chen, D.M., Cairney, J.W.G., Dickie, I.A., Xu, B., Koide, R.T.,
808 Landeweert, R., Leeflang, P., Kuyper, T.W., Hoffland, E., Rosling, A., Wernars, K., Smit,
809 E., Klamer, M., Roberts, M.S., Levine, L.H., Drake, B.G., Garland, J.L., Guidot, A.,
810 Debaud, J.C., Marmeisse, R., Wallander, H., Massicotte, H.B., Nylund, J.E., Ekblad, A.,
811 Wallander, H., Nasholm, T., Olsson, P.A., Bidartondo, M.I., Ek, H., Wallander, H.,
812 Söderström, B., Ek, H., Wallander, H., Nilsson, L.O., Hagerberg, D., Bååth, E., Cullen,
813 D.W., Lees, A.K., Toth, I.K., Duncan, J.M., Heuser, T., Zimmer, W., Marx, D.H.,
814 Ingestad, T., Kähr, M., Colpaert, J.V., Tichelen, K.K. van, Assche, J.A. van, Laere, A.
815 Van, Colpaert, J.V., Laere, A. Van, Assche, J.A. Van, Bloem, J., Bolhuis, P.R., Veninga,
816 M.R., Wieringa, J., Paul, E.A., Harris, D., Klug, M., Ruess, R., Pennanen, T., Liski, J.,
817 Bååth, E., Kitunen, V., Uotila, J., Westman, C.J., Fritze, H., Smalla, K., Cresswell, N.,
818 Mendonca-Hagler, L.C., Wolters, A., Elsas, J.D. van, Gardes, M., Bruns, T.D., Fromin,
819 N., Hamelin, J., Tarnawski, S., Roesti, D., Jourdain-Miserez, K., Forestier, N., Teyssier-
820 Cuvelle, S., Gillet, F., Aragno, M., Rossi, P., Brüggemann, J., Stephen, J.R., Chang, Y.J.,
821 Macnaughton, S.J., Kowalchuk, G.A., Kline, E., White, D.C., Wintzingerode, F. von,
822 Gobel, U.B., Stackebrandt, E., Borneman, J., Hartin, R.J., Fritze, H., Pietikainen, J.,
823 Pennanen, T., Hedrick, D.B., Peacock, A., Stephen, J.R., Macnaughton, S.J.,
824 Brüggemann, J., White, D.C., 2003. Quantification of ectomycorrhizal mycelium in soil
825 by real-time PCR compared to conventional quantification techniques. FEMS Microbiol.
826 Ecol. 45, 1855–1871.

827Libra, J.A., Borchert, M., Banit, S., 2003. Competition strategies for the decolorization of a
828 textile-reactive dye with the white-rot fungi Trametes versicolor under non-sterile
829 conditions. Biotechnol. Bioeng. 82, 736–744.

830Lu, Z., Sun, X., Yang, Q., Li, H., Li, C., 2009. Persistence and functions of a decolorizing
831 fungal consortium in a non-sterile biofilm reactor. Biochem. Eng. J. 46, 73–78.

832Machín-Ramírez, C., Morales, D., Martínez-Morales, F., Okoh, a. I., Trejo-Hernández, M.R.,
833 2010. Benzo[a]pyrene removal by axenic- and co-cultures of some bacterial and fungal
834 strains. Int. Biodeterior. Biodegradation 64, 538–544.

835Marco-Urrea, E., Pérez-Trujillo, M., Vicent, T., Caminal, G., 2009. Ability of white-rot fungi
836 to remove selected pharmaceuticals and identification of degradation products of
837 ibuprofen by Trametes versicolor. Chemosphere 74, 765–772.

838Mikesková, H., Novotný, C., Svobodová, K., 2012. Interspecific interactions in mixed
839 microbial cultures in a biodegradation perspective. Appl. Microbiol. Biotechnol. 95,
840 861–70.

841Nübel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R.I., Ludwig, W.,
842 Backhaus, H., 1996. Sequence heterogeneities of genes encoding 16S rRNAs in
843 Paenibacillus polymyxa detected by temperature gradient gel electrophoresis. J.
844 Bacteriol. 178, 5636–43.

845Pennanen, T., Frostegard, A., Fritze, H., Baath, E., Pennanen, T., Frostegård, Å.S.A., Fritze,

846 H., 1996. Phospholipid Fatty Acid Composition and Heavy Metal Tolerance of Soil
847 Microbial Communities along Two Heavy Metal-Polluted Gradients in Coniferous
848 Forests . Phospholipid Fatty Acid Composition and Heavy Metal Tolerance of Soil
849 Microbial Communities along .

850Pointing, S.B., 2001. Feasibility of bioremediation by white-rot fungi. Appl. Microbiol.
851 Biotechnol. 57, 20–33.

852Rafin, C., Potin, O., Veignie, E., Lounes-Hadj Sahraoui, A., Sancholle, M., 2000. Degradation
853 of benzo[a]pyrene as sole carbon source by a non white rot fungus, Fusarium solani.
854 Polycycl. Aromat. Compd. 21, 311–329.

855Rajala, T., Velmala, S.M., Tuomivirta, T., Haapanen, M., Müller, M., Pennanen, T., 2013.
856 Endophyte communities vary in the needles of Norway spruce clones. Fungal Biol. 117,
857 182–190.

858Rodríguez, A., Falcón, M.A., Carnicero, A., Perestelo, F., De la Fuente, G., Trojanowski, J.,
859 1996. Laccase activities of Penicillium chrysogenum in relation to lignin degradation.
860 Appl. Microbiol. Biotechnol. 45, 399–403.

861Rousk, J., Bååth, E., 2007. Fungal and bacterial growth in soil with plant materials of
862 different C/N ratios. FEMS Microbiol. Ecol. 62, 258–67.

863Ruiz-Dueñas, F.J., Martínez, M.J., 1996. Enzymatic Activities of Trametes versicolor and
864 Pleurotus eryngii Implicated in Biocontrol of Fusarium oxysporum f. sp. lycopersici.
865 Curr. Microbiol. 32, 151–155.

866Song, H., 1999. Comparison of pyrene biodegradation by white rot fungi. World J. Microbiol.
867 Biotechnol. 15, 669–672.

868Spina, F., Cordero, C., Schilirò, T., Sgorbini, B., Pignata, C., Gilli, G., Bicchi, C., Varese,
869 G.C., 2015. Removal of micropollutants by fungal laccases in model solution and
870 municipal wastewater: Evaluation of estrogenic activity and ecotoxicity. J. Clean. Prod.
871 100, 185–194.

872Temp, U., Eggert, C., 1999. Novel Interaction between Laccase and Cellobiose
873 Dehydrogenase during Pigment Synthesis in the White Rot Fungus Pycnoporus
874 cinnabarinus. Appl. Environ. Microbiol. 65, 389–395.

875Tigini, V., Prigione, V., Varese, G.C., 2014. Mycological and ecotoxicological characterisation
876 of landfill leachate before and after traditional treatments. Sci. Total Environ. 487, 335–
877 341.

878Verlicchi, P., Al Aukidy, M., Zambello, E., 2012. Occurrence of pharmaceutical compounds in
879 urban wastewater: removal, mass load and environmental risk after a secondary
880 treatment--a review. Sci. Total Environ. 429, 123–55.

881Wariishi, H., Valli, K., Gold, M.H., 1992. Manganese(II) oxidation by manganese peroxidase
882 from the basidiomycete Phanerochaete chrysosporium. Kinetic mechanism and role of
883 chelators. J. Biol. Chem. 267, 23688–95.

884Weber, S.D., Ludwig, W., Schleifer, K.-H., Fried, J., 2007. Microbial composition and
885 structure of aerobic granular sewage biofilms. Appl. Environ. Microbiol. 73, 6233–40.

886White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal
887 ribosomal RNA genes for phylogenetics, in: Innins, M.A., Gelfand, D.H., Sninsky, J.J.,
888 White, T.J. (Eds.), PCR Protocols. Academic Press, San Diego, p. 315.

889Wymore, A.S., Compson, Z.G., Liu, C.M., Price, L.B., Whitham, T.G., Keim, P., Marks, J.C.,
890 2013. Contrasting rRNA gene abundance patterns for aquatic fungi and bacteria in

891 response to leaf-litter chemistry. Freshw. Sci. 32, 663–672.

892 Yang, Q., Angly, F.E., Wang, Z., Zhang, H., 2011. Wastewater treatment systems harbor
893 specific and diverse yeast communities. Biochem. Eng. J. 58-59, 168–176.

894 Yang, S., Hai, F.I., Nghiem, L.D., Nguyen, L.N., Roddick, F., Price, W.E., 2013. Removal of
895 bisphenol A and diclofenac by a novel fungal membrane bioreactor operated under non-
896 sterile conditions. Int. Biodeterior. Biodegradation 85, 483–490.

897 Zhang, Y., Geissen, S.U., 2012. Elimination of carbamazepine in a non-sterile fungal
898 bioreactor. Bioresour. Technol. 112, 221–7.

899