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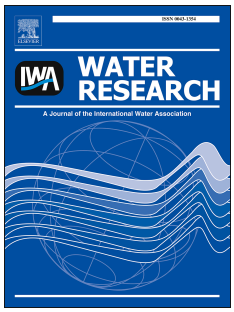

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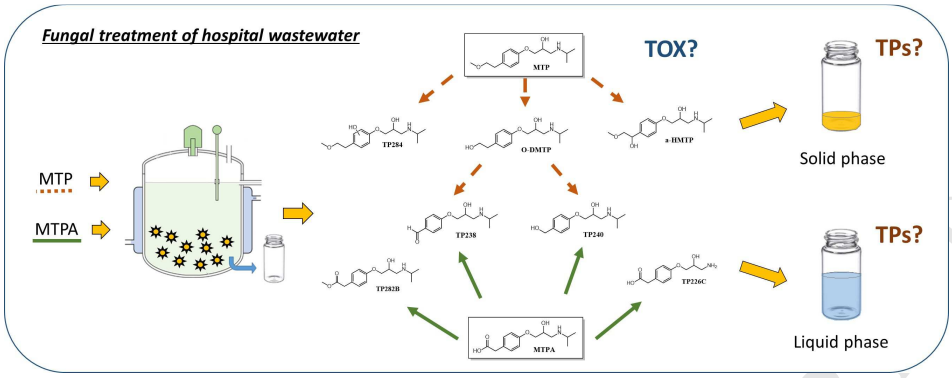

Fungal treatment of metoprolol and its recalcitrant metabolite metoprolol acid in hospital wastewater: biotransformation, sorption and ecotoxicological impact

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ABSTRACT

Hospital wastewater (HWW) effluents represent an important source of contaminants such as pharmaceutical compounds and their human metabolites. To better evaluate dedicated treatment of hospital effluents for pollutant mitigation, not only the parent compounds should be considered but also the intermediates generated during treatment. The metabolite metoprolol acid (MTPA) has been found in urban wastewaters at higher concentration than its parent compound metoprolol (MTP), being more recalcitrant to biodegradation. The aim of this study was to investigate degradation, transformation and sorption of the β-blocker MTP, and its recalcitrant metabolite MTPA, during water treatment based on the fungi *Ganoderma lucidum* (GL), *Trametes Versicolor* (TV) and *Pleurotus ostreatus* (PO). Fourteen intermediates were identified in MTP biotransformation while five of them also attributed to MTPA biodegradation and two to MTPA only. Their identification allowed their correlation in separate biotransformation pathways suggested. The highest degradation rate of metoprolol (up to 51%) and metoprolol acid (almost 77%) was found after 15-days treatment with *Ganoderma lucidum,* with an increase in toxicity up to 29% and 4% respectively. This fungus was further selected for treating real HWW in a batch fluidized bed bioreactor (FBB). Treated wastewater and fungal biomass samples were used to evaluate the distribution of the target compounds and the intermediates identified between solid and liquid phases. While similar elimination capabilities were observed for the removal of metoprolol, and even higher for its persistent metabolite metoprolol acid, the extent on compound transformation diminished considerably compared with the study treating purified water: a high level of the persistent α-HMTP and TP240 were still present in effluent samples (15% and 6% respectively), being both TPs present at high proportion (up to 28%) in fungal biomass. This is the first time that pharmaceutical TPs have been investigated in the fungal biomass.

Keywords: metoprolol, metoprolol acid, fungal water treatment, fungal biomass, suspect screening, transformation products.

1. Introduction

In recent years, the presence of pharmaceuticals (PhACs) in the environment has been recognized as one of the most concerning environmental issues (Verlicchi et al., 2012). Every day, large quantities of wastewaters containing a broad variety of chemicals coming from domestic and industrial uses are discharged into sewage system. Hospital wastewater (HWW) in particular, have been recognized as important source of PhACs, where they can be found at several µg/L (Carraro et al., 2016; Verlicchi et al., 2015, 2010). Since there is not a specific directive or guideline in Europe for treating HWW before its disposal (Rodriguez-Mozaz et al., 2018), these effluents are usually released into municipal sewer system without applying any previous water pretreatment. Their contribution at municipal wastewater treatment plants (WWTPs) range approximately from 0.2% to 2% of total wastewater volume (Carraro et al., 2016). Considering that conventional WWTPs are not designed to completely eliminate these emerging contaminants (Ratola et al., 2012), they can pass through and find their way into the environment. Therefore, the use of alternative on-site wastewater treatments prior to sewer discharge has been highly recommended (Verlicchi et al., 2015), where a decrease of up to 90% on total pharmaceutical load can be achieved (Pauwels and Verstraete, 2006).

Among the different wastewater treatments, activated sludge is currently considered the treatment of choice (Bletsou et al., 2015). However, alternative treatments based on fungi have been reported to be effective in the removal of micropollutants, thanks to its unspecific ligninolytic systems and intracellular enzymatic complexes (Asgher et al., 2008). *Trametes versicolor*, *Ganoderma lucidum* and *Pleurotus ostreatus* (part of the Basidiomycota division and the Agaricomycetes class) have been successfully applied

for the elimination of certain pharmaceuticals (Cruz-Morató et al., 2014; Llorca et al., 2018; Marco-Urrea et al., 2009; Palli et al., 2017) with the overall load elimination of 83% in optimal conditions (Cruz-Morató et al., 2014). Among the extracellular enzymes responsible of pharmaceutical degradation lignin peroxidase, manganese peroxidase and laccase are the most important ones (Asgher et al., 2008). The low specificity of these enzymes make the selected fungi suitable for bioremediation processes. However, while some authors have successfully applied this kind of treatment for pharmaceutical removal (Cruz-Morató et al., 2014; Llorca et al., 2018; Marco-Urrea et al., 2009; Palli et al., 2017), less attention has been paid to the transformation products (TPs) generated, which may sometimes be more persistent or toxic than the parent compound (Escher and Fenner, 2011; Jaén-Gil et al., 2018). Considering that not only PhACs are present in HWW effluents but also their human metabolites, the European Medicines Agency (EMA) has set guidelines on environmental risk assessment indicating that relevant metabolites are those excreted in ≥ 10% of the administered dose (Wharf and Kingdom, 2010). Even so, unknown intermediates from these metabolites can also be generated during wastewater treatment. Therefore, their transformation pathways should also be investigated to better understand pollutant mitigation and properly evaluate wastewater treatment processes.

Among the different PhACs therapeutic families of present in HWWs, β-blockers have been widely detected in such effluents due to the its high consumption for hypertension and cardiovascular diseases (Hughes et al., 2013). Some of them are included into the 20 most commonly encountered pharmaceuticals in European waters (Hughes et al., 2013). For instance, metoprolol (MTP) is largely prescribed in Germany reaching values of almost 100 tons per year (Scheurer et al., 2010) and has been detected in wastewater in the range of 160-2000 ng/L (Maurer et al., 2007; Scheurer et al., 2010), with low elimination rates in conventional WWTPs (usually between 0% and 36%) (Lacey et al., 2012; Rubirola et al., 2014; Scheurer et al., 2010). On the other hand, it is

well-known that MTP is mainly eliminated in human body, up to 85% throughout hepatic oxidative metabolism, and transformed into O-desmethylmetoprolol (O-DMTP), α-hydroxymetoprolol (α-HMTP) and metoprolol acid (MTPA) metabolites. Among them, MTPA is the major compound eliminated via renal excretion around 60-65% (Escher et al., 2006; Kern et al., 2010), while the other metabolites can also be present in urine but at much lower concentration (Godbillon and Duval, 1984). This metabolite has been found *ca.* one order of magnitude higher concentrations than MTP in wastewater (Mamo et al., 2018; Rubirola et al., 2014), and its persistence during biological treatment has been reported in some studies (Radjenović et al., 2008; Rubirola et al., 2014), indicating its potential environmental relevance. Although many studies have focused on the elimination of MTP in wastewater effluents (Benner and Ternes, 2009; Cavalcante et al., 2015; Romero et al., 2016a, 2016b, 2015; Šojić et al., 2012; Wilde et al., 2014), only few data was found concerning its elimination during HWW treatment (Wilde et al., 2014), and even less testing its fungal biotransformation by fungal treatments (Ma et al., 2007). Moreover, none of the studies exploring the intermediates generated after MTP degradation has investigated the biotransformation of the main metabolite MTPA (Benner and Ternes, 2009; Cavalcante et al., 2015; Koba et al., 2016; Ma et al., 2007; Romero et al., 2016b, 2016a, 2015; Rubirola et al., 2014; Slegers et al., 2006; Šojić et al., 2012; Tay et al., 2013; Wilde et al., 2014).

In this study, degradation, transformation and sorption of MTP and its main metabolite MTPA were investigated in batch experiments with three fungi (*Ganoderma lucidum*, *Trametes versicolor* and *Pleurotus ostreatus*) by using liquid chromatography coupled to high resolution mass spectrometry (LC-LTQ-Orbitrap-MS/MS) through a suspect screening methodology. Treated wastewater and fungal biomass samples were used to evaluate the presence the target compounds and their TPs in both compartments. To the authors' knowledge, this is the first time that pharmaceutical TPs have been

investigated in fungal biomass, as well as the first time that biodegradation and biotransformation of MTPA has been studied in wastewater treatment.

2. Materials and Methods

2.1. Chemicals and fungi

Metoprolol tartrate salt (MTP) (Sigma-Aldrich); O-desmethylmetoprolol (O-DMTP), metoprolol acid (MTPA) and α-hydroxymetoprolol (α-HMTP) (Toronto Research Chemicals); and atenolol-d^7 internal standard (CDN isotopes, Quebec, Canada) were purchased at high purity grade (> 98%). Ultra-pure water and acetonitrile LiChrosolv grade were supplied by Merck (Darmstadt, Germany). Working standard solutions were prepared in methanol/water (10:90, v/v). Solid phase extraction (SPE) cartridges Oasis HLB (60 mg, 3 mL) were from Waters Corporation (Milford, MA, USA).

Three different species of fungi from different collections were used: *Ganoderma lucidum* (WRF) FP-58537-Sp strain, United States Department of Agriculture, Madison, Wis. Collection); *Trametes versicolor* (WRF) (American Type Culture Collection #42530 strain); and *Pleurotus ostreatus* was isolated from a fruiting body collected from rotting wood, identified through molecular analysis (Palli et al., 2017). *G. lucidum* and *T. versicolor* were subcultured on 2% malt extract agar petri plates while *P. ostreatus* was maintained on malt extract agar (MEA) plates (ATCC medium 325).

Pellet immobilization was achieved for all the fungi following the same procedure described previously (Blánquez et al., 2004).The pellets obtained by this process were washed with sterile deionized water and kept (if needed) in a 0.8% NaCl solution at 4 ˚C.

2.2. Fungal degradation experiments

Experiments for MTP and MTPA elimination were performed in 250 mL Erlenmeyer flasks for 15 days with *G. lucidum, T. versicolor* and *P. ostreatus* fungi. For each fungus, experiments were carried out in triplicate by spiking selected compounds individually at a concentration of 2.5 mg/L in 100 mL of a defined medium, which consists of 8 g/L of glucose, 3.3 g/L of ammonium tartrate, 1.168 g/L of 2,2-dimethylsuccinate buffer, and 1 and 10 mL of a micronutrient and macronutrient solution from Kirk medium (Kirk et al., 1978). The pH was adjusted to 4.5 before sterilization at 121 °C for 30 min. Flasks were inoculated with pellets equivalent to 3.5 ± 0.8 g/L dry cell weight (DCW). To better assess the different biotransformation regarding the parent compounds selected (MTP and MTPA), the experimental procedure was performed for each compound separately. Additionally, abiotic control (same conditions described above but without biomass), live control (same conditions but without spiking compounds) and killed control experiments (same conditions but with heat-killed biomass) were also performed in triplicate and used to evaluate other potential physicochemical processes affecting pharmaceutical transformation and sorption. All experiments were performed under natural light conditions and temperature maintained at 25 °C. Samples were collected along 15 days and further centrifuged in glass vials to separate fungus from water phase. Then, 100 µL of internal standard were added to achieve a final concentration of 100 µg/L. Finally, samples were directly injected into the LC-LTQ-Orbitrap-MS/MS system (see the following section 2.4.).

2.3. Fluidized bed bioreactor experiments

Biodegradation, biotransformation and sorption of target pollutants and their TPs were investigated along 7 days in a non-sterilized 0.5 L air-pulsed fluidized bed bioreactor (FBB) treating HWW. The HWW was collected directly from the sewer manifold of Sant Joan de Déu Hospital (Barcelona, Catalonia) and pretreated with coagulation-

floculation, which involved the addition of coagulant HyflocAC50 at 43 mg/L during 2 min at 200 rpm and flocculant HimolocDR3000 at 4.8 mg/L for 15 min at 20 rpm (Derypol, Barcelona, Catalonia). Wastewater characteristics were: pH range of 7.8-8.7; chemical oxygen demand (COD) of 633-1012 mg/L O_2; N-NH_4^+ of 9.9-36 mg/L and total suspended solids (TSS) of 193-284 mg/L. Finally, the pH of wastewater was adjusted to 4.5. Concerning bioreactor operation, the FBB experiments were inoculated in duplicate with *G. lucidum* mycelial pellets equivalent to 2.5 ± 0.8 g/L dry cell weight. Electrovalve was set to supply 1 s of air pulse every 2 s and the aeration rate was 0.8 L/min. Glucose and ammonium chloride were supplied at 7.5 C/N molar ratio from concentrated stock solutions in fed-batch operation mode at consumption rate (0.8 g $C_6H_{12}O_6$ g DCW^{-1} and 0.19 g N4Cl g DCW^{-1}). In an attempt to reproduce more realistic conditions, MTP and MTPA were spiked simultaneously at a concentration level of 2.0 ± 0.5 µg/L each. Samples were taken at time 0 and 7 days of operation and further centrifuged in glass vials to separate fungus from water phase. To avoid possible experimental changes during the experiments, *G. lucidum* biomass samples were taken at final experimental time of 7 days only. Then, HWW samples were treated following an SPE methodology described elsewhere (Gros et al., 2012). On the other hand, fungal biomass samples were treated following the solid extraction methodology reported previously (Lucas et al., 2018). Detailed sample preparation procedures are presented in Supplementary Material, S1. Both, water and fungal extracts were reconstituted in 100 µL of methanol/water (10:90, v/v) containing internal standard to a final concentration of 100 µg/L in vial for further injection into LC-LTQ-Orbitrap-MS/MS (see the following section 2.4.)

2.4. Instrumental analysis

Samples collected from flasks experiments and FBB extracts (from wastewater and fungal biomass) were analyzed in a liquid-chromatography system coupled to a hybrid

linear ion trap (LTQ)-Orbitrap mass spectrometer. Detection of MTP and MTPA as well as their tentative TPs was performed via a suspect screening methodology using a ready-made list of accurate masses selected from literature, included prior to sample analysis for MS/MS fragmentation (Table S1). Data was acquired in data dependent acquisition mode (DDA) using collision-induced dissociation (CID) and higher-energy collisional dissociation (HCD) fragmentation energies. For those compounds where reference standards were available (MTP, MTPA, O-DMTP and α-HMTP), verification was performed by comparison with retention times and MS/MS ion fragmentation patterns. When reference standards were not commercially available, confirmation was performed via structural elucidation of MS/MS fragmentation patterns using Mass Frontier 7.0 software (Thermo Scientific). More detailed information of sample analysis is presented in Supplementary Material, S2. After identification, peak area measurement of MTP, MTPA and TPs was performed using the equations presented in Section 2.5. Additionally, accurate quantification of MTP and MTPA in water and biomass of HWW experiments was also performed (see Table S2 for analytical quality parameters).

2.5. Data processing

2.5.1 Elimination of MTP and MTPA in fungal flask experiments

The removal efficiency of MTP and MTPA for the three fungus selected were evaluated along the performed flasks experiments. The contribution of abiotic processes to elimination was calculated using Eq. (1), where A_0 is the area at initial time and A_x^{ac} is the area measured at a particular sampling time in the abiotic control experiments:

$$Abiotic\ degradation\ (\%) = \frac{A_0 - A_x^{ac}}{A_0} \qquad \text{Eq. (1)}$$

227 Elimination by sorption was calculated using Eq. (2), where A_x^{kc} is the area at the same
228 particular sampling time in killed control experiments:

229 $$Sorption\ (\%) = \frac{A_x^{ac} - A_x^{kc}}{A_0} \qquad \text{Eq. (2)}$$

230 Finally, biodegradation was calculated using Eq. (3), where A_x^{de} is the area measured
231 in fungal degradation experiments at the certain experimental time:

232 $$Biodegradation\ (\%) = \frac{A_x^{kc} - A_x^{de}}{A_0} \qquad \text{Eq. (3)}$$

233 **2.5.2 Distribution of pollutants in liquid and biomass solid phases in the fluidized**
234 **bed bioreactors**

235 The distribution of spiked pollutants (MTP and MTPA) in HWW and fungal biomass
236 was calculated using Eq. (4) where A_x^L is the chromatographic area in liquid phase at a
237 specific experimental time, and A_0^L is the area of MTP or MTPA at initial time (all
238 estimated for the total FBB volume of 0.5 L) corrected by the corresponding recovery
239 value in HWW (quality parameters and concentration values are presented in Table
240 S2):

241 $$Presence\ in\ liquid\ phase\ (\%) = \left(\frac{A_x^L}{A_0^L}\right) \qquad \text{Eq. (4)}$$

242 The presence of MTP and MTPA in solid phase was calculated as it can be seen in Eq.
243 (5), where A_x^S is the corresponding area in the solid phase at a certain experimental
244 time (estimated for the total biomass of 2.5 g/L dry weight), and A_0^L is again the spiked
245 area in liquid phase at initial time (estimated for the total FBB volume of 0.5 L). All
246 areas were also corrected by the recovery values calculated in the corresponding liquid
247 and solid phases (quality parameters and concentration values are presented in Table
248 S2):

$$Presence\ in\ solid\ phase\ (\%) = \left(\frac{A_x^S}{A_0^L}\right) \qquad \text{Eq. (5)}$$

Since reference standards for TPs were not available, a proper quantification was not feasible. However, in order to provide tentative values of the presence of TPs in HWW and fungal biomass, Eq. (4) and Eq. (5) were used considering A_0^L as the sum of MTP and MTPA areas corrected by the mean recovery value of these compounds in liquid (91%) and solid biomass (46%) phases.

2.6. Toxicity evaluation

The ISO 11348-3 protocol (ISO, 1998) for testing bacterial bioluminescence was applied to evaluate acute toxicity of samples along the experiments using the Microtox® Model 500 Toxicity Analyzer (Strategic Diagnostics Inc. Newark, DE, US). For this purpose, all flasks and FBB water samples were centrifuged in glass vials to remove any biomass fragments or suspended solids interfering. Then, the percentage of decay on emitted light was measured when samples were in contact with the bioluminescent bacterium *V. fischeri.* The 50% effective concentration (EC_{50}) was measured after 15 min (expressed in dilution percentage). Changes in toxicity (EC_{50}) at a particular experimental time were calculated in percentage as $(EC_{50(initial)} - EC_{50(x)})/EC_{50(initial)}$ adapted from Font et al., 2003.

3. Results and Discussion

3.1 Elimination processes of MTP and MTPA in fungal flasks experiments

Elimination processes such as biodegradation, fungal sorption and other abiotic processes of MTP and its main metabolite MTPA were evaluated in flasks experiments. Fig. 1 summarizes MTP and MTPA presence decay in the different experiments performed as well as the sum of TPs measured for the three-fungal species tested (whose identity is described in Section 3.2 and Supplementary Material, S5). As

273 expected, MTP, MTPA and TPs were not detected in live (non-spiked) control
274 conditions. Abiotic control experiments showed negligible MTP and MTPA elimination
275 which evidences their high chemical stability. In fungal degradation experiments, partial
276 elimination of MTP was achieved reaching removal values as high as 51%, 49% and
277 17% in water treated with *G. lucidum*, *T. versicolor* and *P. ostreatus* respectively, with
278 high contribution of sorption processes (*ca.* 25 ± 3% of initial compound amount) in all
279 species tested. Only in the experiments with *G. lucidum*, biodegradation is pointed out
280 as the main removal mechanism reaching values up to 28% (Table 1), whereas it was
281 lower in the experiments performed with *T. versicolor* (21%) and not existing in the
282 case of *P. ostreatus*. In any case, overall elimination achieved for MTP by fungi
283 (between 17% and 51%) was lower than that obtained in former activated sludge flasks
284 experiments where MTP was spiked at similar concentration (1 mg/L) and biomass (3
285 gTSS/L), and where total MTP elimination was achieved after 96 hours (Rubirola et al.,
286 2014). Nevertheless, despite from the removal of target pollutants, the generation and
287 elimination of their corresponding TPs should also be considered to properly assess
288 the efficiency of fungal treatment (intermediates are further discussed in Section 3.2
289 and Supplementary Material, S5). To this respect, higher generation of TPs was
290 observed for those experiments exhibiting higher MTP biodegradation rates (Fig. 1). In
291 general, the highest generation of TPs was observed after 7 days of treatment and
292 maintained until the end of the experiments. This fact indicates that, even though MTP
293 was eliminated during the experiments, the elimination of the TPs generated was not
294 accomplished in the same manner.

295 MTPA was more extensively removed than MTP yielding values up to 77%, 54% and
296 35% in water treated with *G. lucidum*, *T. versicolor* and *P. ostreatus* respectively (Fig.
297 1). Nonetheless, results reveal lower contribution to sorption processes compared to
298 those values obtained in MTP experiments; biomass sorption percentages ranged from
299 0% to 11% (Table 1). These levels should be explained by the different partition

coefficients of both compounds. However, the predicted distribution coefficients *logD* values for MTP and MTPA at pH 4.5 were quite similar, indicating their low tendency to be present in solid phase (-1.48 for MTP and -1.27 for MTPA calculated with ChemAxon ("ChemAxon Chemicalize Calculator," 2018)) and without a direct correlation (*logD*) with actual sorption of MTP and MTPA in fungal biomass. Biodegradation was thus pinpointed as the main removal mechanism for MTPA with the three fungi tested (Fig. 1). Among them, *G. lucidum* was pointed out as the most effective fungus reaching biodegradation values around 63%, being 11% accounted as sorption contribution to total removal (Table 1). *T. versicolor* and *P. ostreatus* attained lower biodegradation rates of about 48% and 32% percentages, respectively. In accordance to this, *G. lucidum* was also reported as the most efficient fungus for biodegradation of venlafaxine and O-desmethylvenlafaxine (spiked at 5 mg/L) with total removal values up to 70% and 100%, respectively (Llorca et al., 2018). The optimal removal of MTPA with these fungi needs to be highlighted since it was previously reported as a concerning metabolite, given its high persistence in previous batch activated sludge experiments, generated from the biodegradation of MTP spiked at 1 mg/L (Rubirola et al., 2014), and from atenolol spiked at 10 mg/L (Radjenović et al., 2008). As in the case of MTP experiments, the highest concentrations of MTPA TPs were measured when the highest MTPA biodegradation rates were registered; *i.e.* after 9 and 15 days of treatment with all three fungi tested. Actually, high levels of MTP and MTPA intermediates (between 7% and 31% for MTP degradation and from 51% to 100% for MTPA transformation, Fig. 1) were always detected at the end of corresponding experiments, which underlines the inability of fungal treatments for total compound mineralization, and the generation of a large quantity of new chemical structures. Thus, their identification, toxicity as well as the elucidation of their transformation pathways are necessary to evaluate the performance of a particular water treatment.

3.2 Identification and monitoring of suspected TPs in fungal flasks experiments

A suspect screening methodology for the detection of tentative TPs was applied based on the comparison of accurate masses obtained after compound detection with those gathered from literature. Since multiple peaks can be detected for the same exact mass, comparison with retention times (when reference standards are available) and chemical structure elucidation based on the MS/MS data were performed for confirmation purposes. A summary of accurate masses, elemental composition and tentative chemical structures of TPs detected are presented in Table S3. In accordance to the European Commission Decision 2002/657/EC, measurements were always within mass error of 5 ppm by means of MS^n analysis. This criterion was considered enough to assign the elemental compositions and chemical structures of both parent and fragment ions. Firstly, fragmentation scans were elucidated by using those data acquired in CID fragmentation energy. However, this approach was considered insufficient to discern among similar TP structures. Therefore, HCD fragmentation energy was necessary to obtain complementary small fragments to finally confirm the tentative chemical structures. Once the structures were elucidated (Table S3), proposed degradation pathways were tentatively suggested and presented in Fig. 2. Those compounds with relative abundances higher than 1% were chosen for further consideration.

3.2.1 Metoprolol biotransformation

Fourteen major TPs were tentatively identified along fungi experiments from MTP biodegradation (Fig. 2). Relative TP percentages obtained for the three fungi tested are presented in Fig. 3. Among them, no intermediates were detected in abiotic conditions indicating the absence of any chemical degradation in further MTP elimination. Regarding fungal degradation experiments, the highest number of intermediates was detected after 15 days of treatment, when MTP had already been eliminated in all fungi

353 tested. Among them, TP238, α-HMTP, TP282A, TP284, TP300, TP316 and TP134
354 were classified as the major compounds detected coming from biotransformation
355 mechanisms such as hydroxylation, oxidation and O-dealkylation (Bletsou et al., 2015).
356 Although these TPs were widely detected in water treated with advanced oxidation
357 processes (AOPs) (Cavalcante et al., 2015; Romero et al., 2016a; Wilde et al., 2014),
358 the presence of the cytochrome P450 in fungi species was also suggested to generate
359 them through enzymatic oxidation (Meunier et al., 2004). Moreover, the enzymes
360 known as lignin peroxidases (LiP) and manganese–dependent peroxidases (MnP),
361 also present in these fungal species, allow to carry out oxidative reactions such as
362 carbon–carbon bond cleavages, demethylations, hydroxylations and benzylic alcohol
363 oxidations (Barr and Aust, 1994).

364 In this study, the most significant degradation pathway, with generation of O-DMTP,
365 TP240, TP238 and TP254 (Fig. 3), was identified in all fungi experiments, being
366 especially notorious for those experiments with higher MTP biodegradation rates.
367 Among them, TP238 was identified as the most persistent compound generated at 9%
368 in the experiments with *G. lucidum,* and further transformed into TP254 (at 1%) after 15
369 days of treatment. The formation of TP238 and TP240 were suggested after O-
370 demethylation of MTP and further benzylic hydroxylation through the formation of a
371 radical intermediate (after hydrogen abstraction and stabilized by resonance) of O-
372 DMTP (also a human metabolite), detected at low concentration (up to 1% in
373 *T.versicolor*). The rapid metabolization/biodegradation of O-DMTP in fungal
374 experiments was in agreement with the results obtained in MTP degradation
375 experiments with activated sludge, where the complete elimination of this TP was
376 achieved after 48 h and a maximum concentration observed at 24 h operation
377 (Rubirola et al., 2014). Further TP240 was also classified as a non-recalcitrant
378 compound being detected at < 1%, however, O-DMTP was rapidly transformed into
379 TP238 and TP254 in pure water. This last compound generated from the oxidation of

the aldehyde intermediate onto a carboxylic acid (in TP254) could be related to lignin peroxidases (LiP), manganese-dependent peroxidases (MnP) and/or cytochrome P450 enzymes (Barr and Aust, 1994). A secondary degradation pathway was suggested with generation of α-HMTP (another human metabolite generated after pharmaceutical consumption) followed by TP282A and TP298. α-HMTP was found up to 5% in the experiments with *G. lucidum* and *T. versicolor,* where higher MTP biodegradation rates were observed. Further oxidation to TP282A and hydroxylation to TP298 was found with gradually lower occurrence comparing to α-HMTP, which indicates the great persistence of α-HMTP in fungal treatments, as well as in treatments performed with activated sludge (Rubirola et al., 2014). The last degradation pathway was characterized by the multiple oxidations of aromatic ring with formation of the intermediates TP284, TP300 and TP316, especially notorious throughout *T. versicolor* biodegradation. As it can be seen, the TP284 was generated and rapidly transformed to the subsequent TP300. The same profile was identified for this last TP being practically degraded at 15-days treatment to further generate TP316 up to 6%. These compounds could be generated from the unspecific and aromatic peroxygenase (UPO) also secreted by fungi, able to catalyse the hydroxylation of aromatic rings and alkyl chains (Hofrichter et al., 2010). Finally, other TPs worth to mention are TP134, formed from the transformation of those TPs with secondary amine structure (Fig. 2). Since it can be designed as a residual TP, its formation might be considered as an indicator to evaluate the extent of mineralization through O-dealkylation catalysed by cytochrome P450 monooxygenases (Urlacher and Girhard, 2012). In fact, the presence of TP134 increased at the same time as biodegradation values of the parent compound (Table 1). Another remarkable aspect to consider in fungal degradation experiments is that MTPA is not generated from MTP biodegradation whereas in activated sludge experiments was identified as the major TP, with levels up to 40% of initial MTP concentration after 96 h treatment (Rubirola et al., 2014); and when atenolol was

spiked at 10 mg/L in 26 days of treatment reaching values up to 60% (Radjenović et al., 2008). Likewise, MTP biotransformation into MTPA metabolite achieved conversion values of 59% in experiments performed with fungus *Cunninghamella blakesleeana* (Ma et al., 2007). Since the presence of TP226C and TP282B were also negligible in MTP fungal experiments, it seems that this transformation pathway does not take place along fungal water treatments with GL, TV and PO. In fact, the high relative percentages of TP238 compared to the other TPs formed denoted a significant prioritization of its transformation pathway instead of the metabolic pathway that favours the generation of MTPA metabolite. However, the rapid degradation rate of MTPA intermediates prior to sampling at 3 days of treatment cannot be discarded. This difference on metabolite formation depending on the treatment used was also observed in man, dogs and rats where the same MTP metabolites were recovered but in different relative proportions (Borg et al., 1975). Therefore, the presence of such recalcitrant MTPA in biological based treatment technologies was thus depending on the organisms used for water treatment.

3.2.2 Metoprolol acid biotransformation

Seven major TPs were tentatively identified during MTPA biodegradation experiments (Fig. 2). Relative TP percentages obtained for the three fungi tested are presented in Fig. 3. Also in this case, no intermediates were detected in abiotic conditions indicating the absence of factors involved in MTPA transformation. Among them TP238, TP240, TP254 and TP134 were classified as the major compounds detected in fungal degradation experiments. As expected, the highest presence of TPs was found after 15 days of treatment when the maximum concentration of MTPA had already been eliminated. In contrast to MTP biodegradation experiments, only three biodegradation pathways were suggested. However, the presence of O-DMTP was not detected while the generation of TP240 and TP238 were much higher reaching values up to 60%.

Their formation might be also related to a benzylic hydroxylation through the formation of the radical intermediate after hydrogen abstraction (Barr and Aust, 1994). Such high levels allowed the further generation of TP254 up to 15% whereas this compound was only detected at 1% in MTP degradation experiments. The higher biodegradation of MTPA and the reduced number of transformation pathways compared to MTP might explain the higher amount of the TPs detected in MTPA experiments. On the other hand, the generation of TP282B and TP226C was only detected when treating MTPA in fungal experiments, but at low concentration levels. This fact indicates that the transformation pathway involving the generation of TP238 was also prioritized when treating MTPA in single experiments, as observed in MTP fungal biodegradation. In this case, the methylation of MTPA to TP282B could be mediated by the methyltransferases enzymes present in fungi (Wessjohann et al., 2013) while N-dealkylation of TP226C could be catalyzed by cytochrome P450 monooxygenases (Urlacher and Girhard, 2012). Otherwise, the high levels of TP134 (more than 2.5 times higher than in MTP experiments), previously suggested as an indicator of mineralization, pointing out the more extended progress in the transformation pathway in fungi experiments but still the incomplete elimination of MTPA TPs.

3.3 Toxicity tests in flasks experiments

Toxicity was monitored in water samples to detect potential toxic TPs generated along the fungal flask experiments. A slightly increase on toxicity values along MTP experiments was observed in all fungi tested (29% in *G. lucidum*, 15% in *T. versicolor* and 24% in *P. ostreatus*, Table S4). In the case of MTPA experiments, a slight increase on toxicity at the end of the experiment was also observed (4%, 11% and 29% for GL, TV and PO respectively). These results are slightly higher than those reported in batch experiments using activated sludge at 1 mg/L of MTP and 3 gTSS/L during 72 h, where no significant differences among toxic units were observed (Rubirola et al., 2014). In

459 the later study, the metabolite O-DMTP from MTP elimination was reported to be the
460 most toxic compound detected (EC_{50} of 18 mg/L). However, in the present study this
461 TP was always below than 1.5% of the MTP and MTPA initial concentration (2.5 mg/L),
462 probably not enough concentration to elicit any toxicity on *V.fischeri*.

463 **3.4 Monitoring of MTP, MTPA and TPs in HWW treated in a FBB bioreactor**

464 HWW was spiked with both MTP and MTPA at 2 µg/L each in order to be able to follow
465 the fate and transformation of both compounds in a fungal fluidized bed bioreactor
466 using *G. lucidum* in realistic conditions (Maurer et al., 2007; Scheurer et al., 2010). This
467 fungus was selected due to the optimal elimination percentages observed for MTP and
468 MTPA in the flask experiments compared to the other fungi tested. Fig. 4 shows the
469 presence of MTP and MTPA as well as the intermediates present in both liquid and
470 solid phases at initial time and after 7 days of treatment. In contrast to the previous
471 batch experiments under sterile conditions, in the bioreactor the fungus was competing
472 against bacteria for nutrients. In addition, the presence of other contaminants (including
473 pharmaceuticals) in the real HWW could affect fungus metabolism and growth.
474 However, *G. lucidum* treatment was successfully implemented with real HWW and the
475 elimination rates of MTP were rather similar: 33% of MTP elimination in the FBB
476 bioreactor compared to the 35% obtained in flask experiments for the same period of
477 time (7 days). Therefore, other factors involved (*e.g.* organic matter, bacteria, pollutant
478 concentration among others) thus seemed not to interfere excessively in MTP
479 elimination. In fact, MTPA removals in bioreactor were even higher than in batch
480 experiments: 64% of MTPA elimination compared to the 46% obtained in flasks
481 experiments. Although this extent on degradation of MTP was less than those values
482 obtained in CAS experiments (Rubirola et al., 2014), the recalcitrant metabolite MTPA
483 observed was successfully eliminated in fungal experiments. Likewise, direct sorption
484 measures into biomass were also similar to those calculated in the previous flasks

485 experiments, up to 13% and 4% for MTP and MTPA respectively. These values are in
486 accordance with those measured in the previous study reporting the greater sorption
487 capabilities of *G. lucidum* than *T. versicolor* for pharmaceutical elimination in spiked
488 synthetic medium (Lucas et al., 2018). In the present study, and for the first time, not
489 only the target pollutants were investigated in solid phase biomass, but also the
490 sorption of the different intermediates generated along FBB batch experiments.

491 Eleven out of sixteen intermediates detected in flasks experiments were also found in
492 water and biomass samples from *G. lucidum* FBB experiments (Fig. 4). Most of them
493 (O-DMTP, TP238, TP282A, TP298, TP300, TP316, TP226C, TP282B and TP134)
494 were detected in water at low percentage values (< 5%) comparing to those values
495 obtained in flasks experiments, except α-HMTP at 15% from MTP degradation and
496 TP240 at 6% also generated from MTPA elimination. After 7 days of treatment, most of
497 the TP300 was detected in the biomass solid phase (11%) while α-HMTP (28%) and
498 TP240 (25%) were retained in less proportion in comparison to their presence in HWW
499 liquid phase. These high levels may be related to the sorption of these TPs from liquid
500 phase, but also to the transformation of MTP and MTPA occurring directly in the
501 biomass phase. Regarding the transformation pathway, the extent on MTP and MTPA
502 transformation did not go as far as in flask experiments: TP240 and α-HMTP were still
503 present at high level in FBB effluents (at 6% and 15%, respectively), while their further
504 intermediates (TP254, TP282A and TP298; generated up to 15% in flasks experiments
505 after 7 days of treatment, Fig. 3) were not detected in the same real effluents. Likewise,
506 the relative presence of the residual TP134 in *G. lucidum* FBB experiments attained a
507 percentage < 1%, lower than those obtained in pure water flasks experiments (4% and
508 7% from MTP and MTPA degradation, respectively). This lower extent on TP
509 transformation might be related to the presence of other contaminants competing on
510 fungal degradation capacity, as well as natural organic matter. Otherwise, a slight
511 increase on toxicity values about 36% (initial EC_{50} of 64% and final EC_{50} of 41%,

512 expressed in dilution percentage) after wastewater treatment was also observed. This
513 might be associated to the transformation products of other contaminants present in
514 HWW.

515 **4. Conclusions**

516 Degradation, transformation and sorption capabilities of *Ganoderma lucidum*, *Trametes*
517 *versicolor* and *Pleurotus ostreatus* fungi were investigated to evaluate the elimination of
518 metoprolol and its recalcitrant metabolite metoprolol acid from water. Fourteen
519 transformation products were detected as generated from MTP biodegradation and
520 within them, five were identified as generated also from MTPA biotransformation. In
521 addition, two TPs were specifically generated from MTPA biodegradation. Results
522 revealed an increase on toxic effects along the fungal treatment of both MTP and
523 MTPA, attributed to the TPs generated from their biodegradation. The maximum
524 efficiency was achieved through *G. lucidum* with removals up to 51% and 77% for MTP
525 and MTPA respectively (at 15 days of treatment) and therefore, this fungus was further
526 selected for treating HWW in an aerobic fluidized bed bioreactor. Even though
527 degradation rates achieved for MTP were quite similar to those obtained in Erlenmeyer
528 flasks experiments, MTPA removals obtained were even better (64% at 7 days of
529 treatment). However, the extent on compound transformation decreased, with the
530 presence of less transformed and persistent intermediates such as TP240 and α-
531 HMTP, detected and highly eliminated through their generation and/or sorption into
532 solid biomass phase. This is the first time that pharmaceutical TPs have been
533 investigated in the biomass from fungal treatment. A slight increase on toxicity along
534 water treatment was also observed in the experiments with real water, though, in this
535 case, it is not easy to correlate with MTP and MTPA TPs formation, since many other
536 TPs originated form the degradation of other contaminants can also be generated.

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Table 1. Abiotic degradation, sorption and biodegradation percentages of MTP and MTPA along *G. lucidum* (GL), *T. versicolor* (TV) and *P. ostreatus* (PO) experiments along 15 days of treatment. Calculations were performed using Eq. (1), (2) and (3).

Degradation mechanism	Fungi	MTP				MTPA			
		0d	3d	10d	15d	0d	3d	10d	15d
Abiotic degradation (%)	-	0	0	0	0	0	1	4	3
Sorption (%)	GL	0	9	13	23	0	1	8	11
	TV	0	6	29	28	0	0	6	3
	PO	0	9	20	25	0	0	0	0
Biodegradation (%)	GL	0	6	34	28	0	19	50	63
	TV	0	5	11	21	0	9	39	48
	PO	0	10	6	0	0	20	21	32

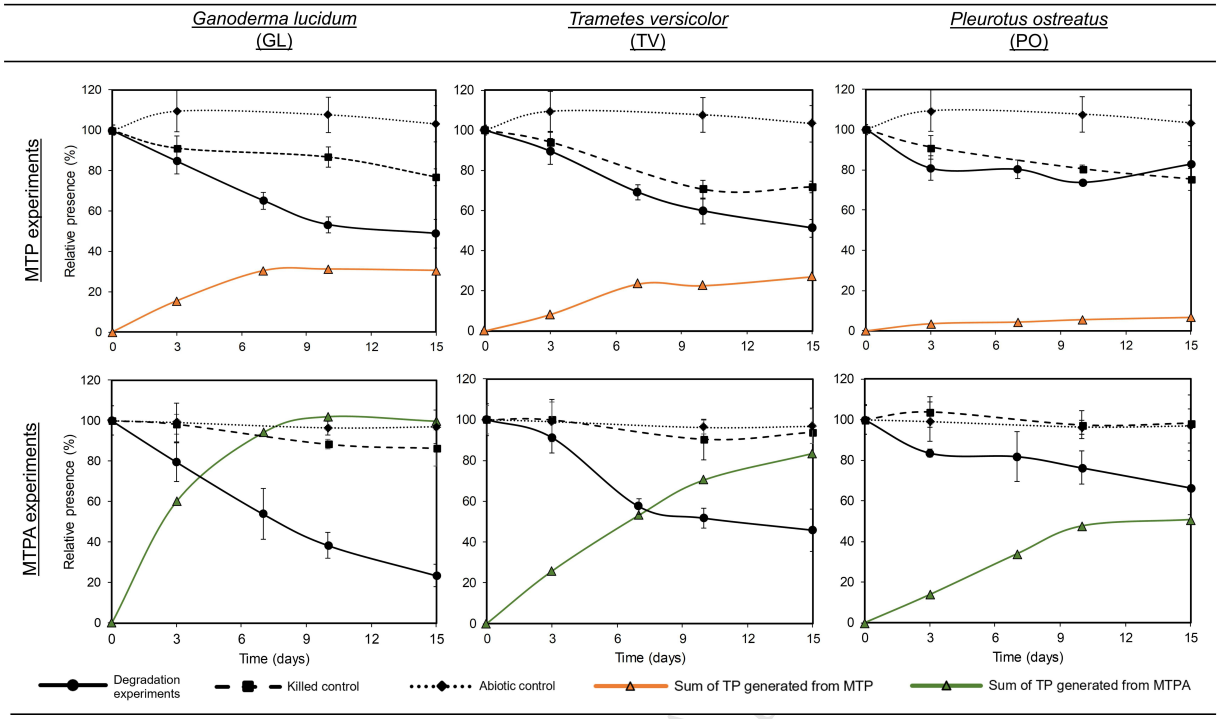

Fig. 1. MTP, MTPA and TP relative presence (A/A_0)-(%) in water samples along the time from abiotic control and fungal conditions, both heat-killed control and fungal degradation experiments with *G. lucidum*, *T. versicolor* and *P. ostreatus*. Colored lines indicate the sum of TPs generated in fungal degradation experiments.

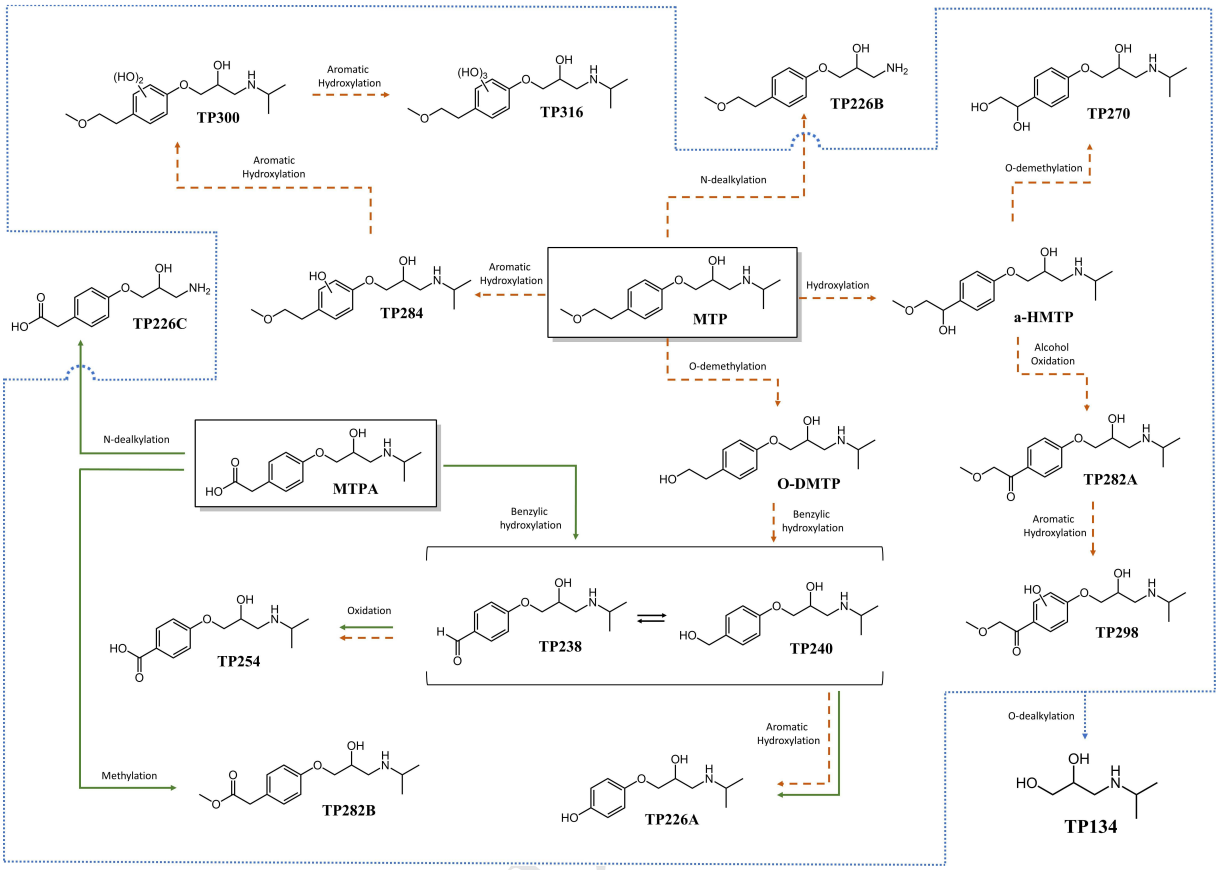

Fig. 2. Transformation pathways suggested of MTP (dotted orange lines) and its main metabolite MTPA (solid green lines) elucidated from *G. lucidum*, *T. Versicolor* and *P. ostreatus* fungal degradation experiments. MTP, MTPA and all intermediates identified except TP226B and TP226C may generate TP134.

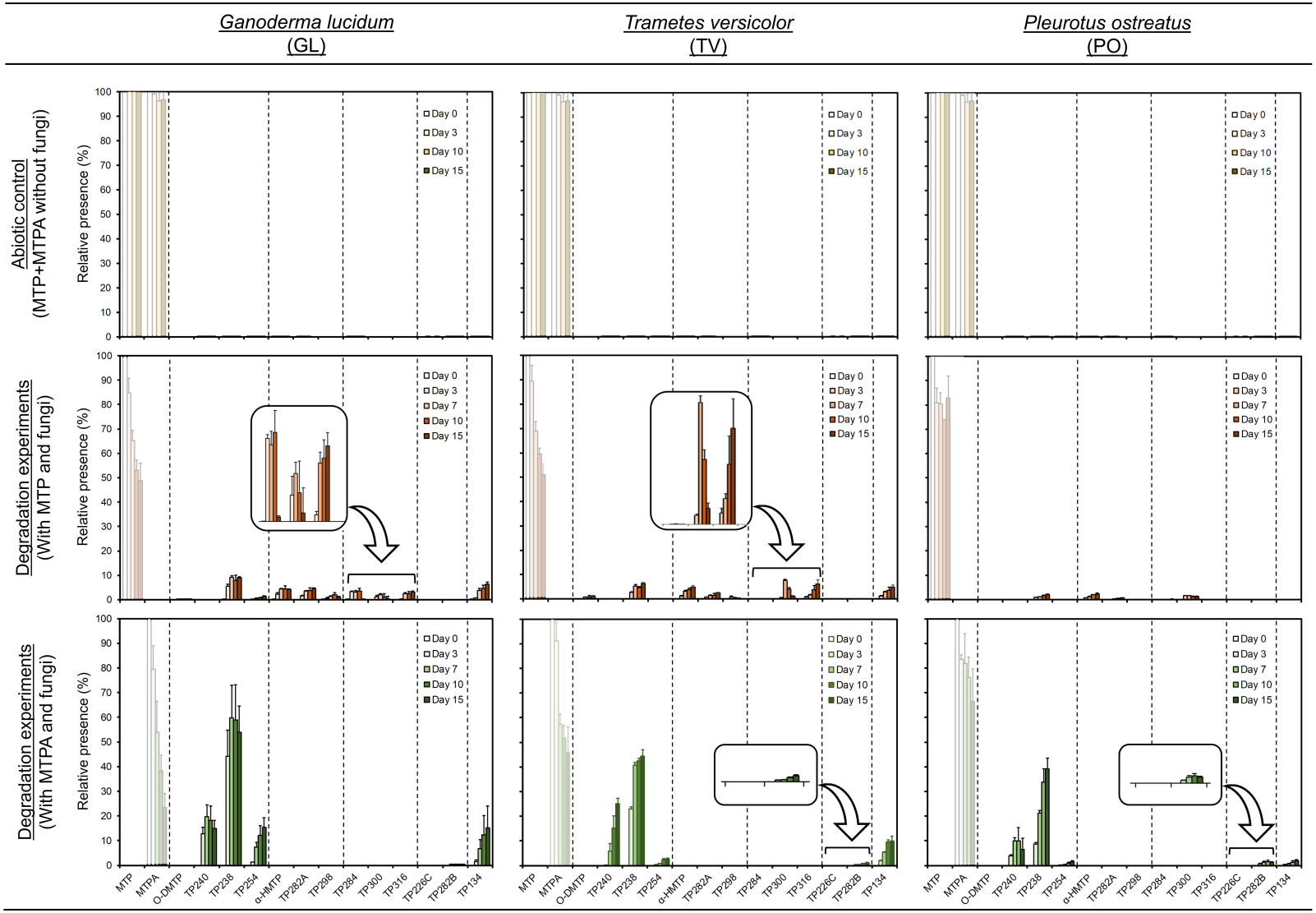

Fig. 3. MTP, MTPA and TP relative presence (A/A_0)-(%) in abiotic control and fungal degradation experiments with *G. lucidum*, *T. versicolor* and *P. ostreatus* along 15 days of treatment. TPs are grouped based on their direct connection in degradation pathways.

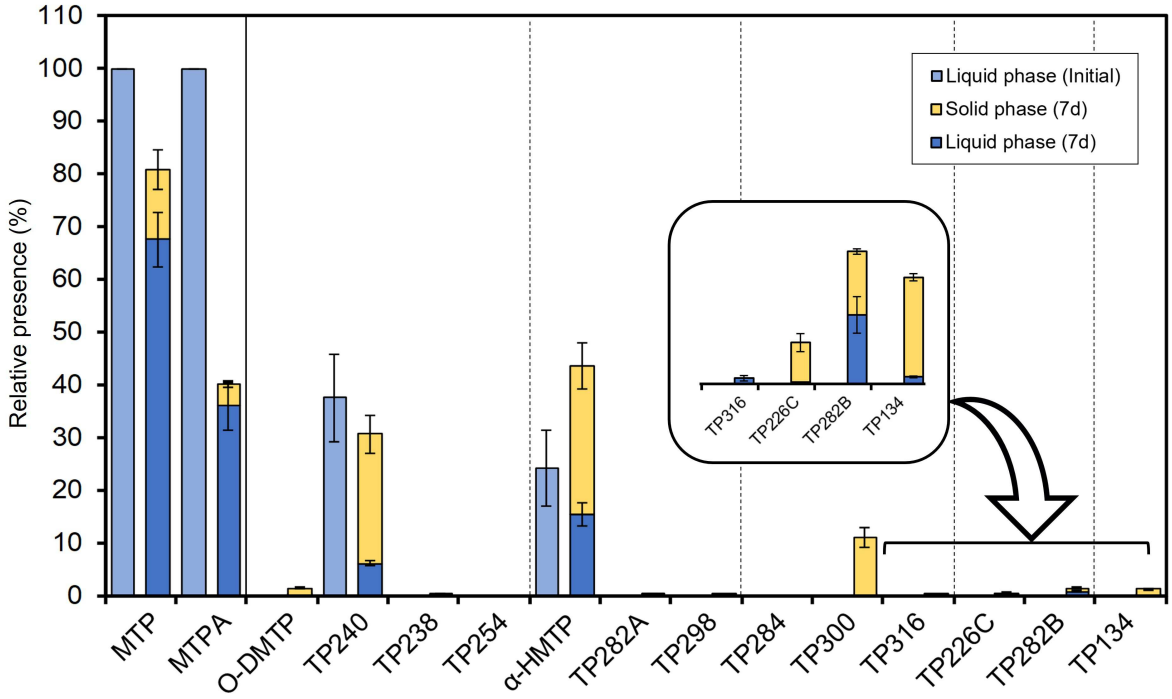

Fig. 4. MTP, MTPA and TP relative presence in water and biomass at 0 and 7 days treating HWW in a FBB bioreactor. Calculations were performed using Eq.(4) and (5). TPs are grouped based on their direct connection in degradation pathways.

Highlights:

- Degradation, transformation and sorption of MTP and MTPA in fungal treatment.
- Satisfactory elimination of the recalcitrant metabolite MTPA, up to 77%.
- Tentative identification of 16 TPs from MTP and MTPA biotransformation.
- *G. lucidum* treating hospital wastewater in an aerobic fluidized bed bioreactor.
- Distribution of TPs between hospital wastewater and fungal biomass phases.

Author's agreement

We the undersigned declare that the manuscript entitled "Fungal treatment of metoprolol and its recalcitrant metabolite metoprolol acid in hospital wastewater: biotransformation, sorption and ecotoxicological impact" is original has not been full or partially published before, and is not currently being considered for publication elsewhere.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship that are not listed. We further confirm that the order of authors listed in the manuscript has been approved by the undersigned.

Signature of all authors:

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Author's contribution

A.J.G., S.R.M., F.C.R., M.L. and M.S. designed the experiment; F.C.R. carried out the fungal bioreactors; A.J.G. performed the sample treatment, chromatographic analysis and data processing; M.V. performed the bioassays; A.J.G. wrote the manuscript. All authors reviewed the manuscript and agree on the content.