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Fungal treatment for the removal of endocrine disrupting compounds from reverse osmosis concentrate: Identification and monitoring of transformation products of benzotriazoles

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Abstract

The removal of 27 endocrine-disrupting compounds and related compounds (suspect effect) from a reverse osmosis concentrate using an alternative decontamination method based on a fungal treatment involving *Trametes versicolor* was assessed. In addition to chemical analysis, the toxicity of the treated water during the treatment was monitored using a bioluminescence inhibition test and estrogenic and anti-estrogenic tests.

The compounds 1H-benzotriazole (BTZ) and two tolyltriazoles (TTZs), 4-methyl-1H-benzotriazole (4-MBTZ) and 5-methyl-1H-benzotriazole (5-MBTZ), were present in the reverse osmosis concentrate at the highest concentrations (7.4 and 12.8 µg L^{-1}, respectively) and were partially removed by the fungal treatment under sterile conditions (58% for BTZ and 92% for TTZs) and non-sterile conditions, although to lesser extents (32% for BTZ and 50% for TTZs)

Individual biotransformation studies of BTZ and the TTZs by *T. versicolor* in a synthetic medium and further analysis via on-line turbulent flow chromatography coupled to an HRMS-Orbitrap allowed the tentative identification of the transformation products (TPs). Six TPs were postulated for BTZ, two TPs were postulated for 4-MBTZ, and four TPs were postulated for 5-MBTZ. Most of these TPs are suggested to have been generated by conjugation with some sugars and via the methylation of the triazole group. Only TP 148 A, postulated to be derived from the biotransformation of BTZ, was observed in the effluent of the bioreactor treating the reverse osmosis concentrate.

Keywords

Benzotriazoles, reverse osmosis concentrate, *Trametes versicolor*, transformation products, on-line turbulent flow chromatography coupled to HRMS

1. Introduction

Endocrine-disrupting compounds (EDCs) are chemical compounds that alter the hormonal system (USEPA, 2017). EDCs comprise mainly synthetic hormones, such as the synthetic 17α-ethinylestradiol (pharmaceutically active compound (Ingerslev et al., 2003)) but also other xenobiotic compounds, such as bisphenol A (Fent et al., 2006) and benzotriazoles (BTs) (Tangtian et al., 2012). BTs are chemicals with a high production volume that are used in a wide variety of applications, including in dishwasher detergents (Wäschenbach et al., 2001; Janna et al., 2011). Nonetheless, the main applicability of these compounds is in industry due to their high resistance to corrosion (Reemtsma et al., 2010). For example, BTs are employed in cooling and hydraulic fluids, in anti-freezing products, such as aircraft deicing fluids, as an ultraviolet light stabilizer in plastics, as an antifogging agent in photography and airports (Giger et al., 2006; Liu et al., 2011) and as a biocide. BTs are in fact included in Annex I of Council Directive 98/8/EC "Concerning the Placing of Biocidal Products on the Market" as priority contaminants (European Commission, 2007; Reemtsma et al., 2010). The use of BTs has been increasing in recent years, and according to Hart *et al.* (2004), these compounds comprise *c.a.* 0.5–1% of the weight of antifreeze, representing more than 1,000 tonnes per year generated in the United States alone (Hart et al., 2004; Liu et al., 2011). The compounds 1H-benzotriazole (BTZ) and tolyltriazoles (TTZs; 4-methyl-1H-benzotriazole (4-MBTZ) and 5-methyl-1H-benzotriazole (5-MBTZ)) have been detected at relevant concentrations during recent years in surface river waters (*i.e.,* BTZ concentrations between 18 and 275 ng L^{-1} and TTZs concentrations ranging from 29 to 568 ng L^{-1} (Gorga et al., 2013)). The presence of these three BTs in the environment is directly related to their discharge through the effluents from wastewater treatment plants (WWTPs) (Giger et al., 2006) and different authors have therefore studied the removal efficiency during treatments as well as the impact of effluent discharges on the environment (Weiss et al., 2006; Reemtsma et al., 2010; Gorga et al., 2013; Loos et al., 2013; Stasinakis et al., 2013; Mazioti et al., 2015a; Mazioti et al., 2015b). These compounds show high water solubility and high polarity, as well as a moderate persistence against biological and photochemical degradation processes in WWTPs and in the aquatic environment (Loos et al., 2013). Gorga *et al.* (2013) detected BTs at concentrations ranging from 446 to 3,033 ng L^{-1} for BTZ and from 1,169 to 7,588 ng L^{-1} for TTZs in 100% of effluent samples collected from several

WWTPs in Spain. These levels were similar to the concentrations detected in the influent, confirming the low efficiency of WWTPs for the complete removal of these compounds. Loos *et al.* (2013) detected even higher concentrations than those reported by Gorga *et al.* (2013), reaching 221,000 ng L^{-1} for BTZ and 24,300 ng L^{-1} for TTZ in several European WWTP effluents. Stasinakis *et al.* (2013) detected them in raw and treated wastewater and in sludge from a WWTP in Greece at levels ranging from 516 to 15,971 ng L^{-1} for raw water, from 14 to 5,773 ng L^{-1} for treated water and from 72 to 412 µg Kg^{-1} for sludge. Regarding the degradation rates of TTZs, different behaviours have been detected for the two isomers. For example, the study from Weiss *et al.* (2006) comparing the degradation rates of both TTZs in a WWTP using conventional activated sludge (CAS) showed a removal rates of 11% for 5-MBTZ and -6% for 4-MBTZ (mean values). A similar pattern was observed when comparing both isomers in a membrane bioreactor (MBR) treatment, although more efficient removal rates were obtained for this treatment (61% for 5-MBTZ *vs.* 14% for 4-MBTZ) (Weiss et al., 2006).

As a result of their incomplete removal in WWTPs, BTs are discharged into the environment through WWTP effluents, possibly leading to harmful interactions with biota and eventually with humans. Cancilla *et al.* (2003) found that TTZs are lethal to fathead minnows (*Pimephales promelas*) at a median concentration of 22 mg L^{-1}. On the other hand, Harris *et al.* (2007) showed the in vitro anti-estrogenic activity of BTs at different tested concentrations (10 – 1,000 µgL^{-1}), while Tangtian *et al.* (2012) reported hormonal disruption in fishes at concentrations of > 10 µg L^{-1}. Humans can be exposed to these compounds since they have also been detected in drinking waters (Schriks et al., 2010; Janna et al., 2011; Müller et al., 2012) and acceptable daily intake (ADI) levels for humans for BTZ and TTZs have been estimated to be 0.295 and 0.25 mg kg_{bw}^{-1} day, respectively (Schriks et al., 2010). Because of the levels detected in waters and the possible effects that they could have on biota and humans, the three BTs are included in the list of trace pollutants and emerging contaminants to be studied in freshwater environments according to Murray et al. (2010).

Due to the high ubiquity, low degradability and possible health effects of BTs, different researchers have studied the most efficient processes in conventional WWTPs, as well as alternative removal processes. As mentioned before, Weiss *et al.* (2006) showed that MBR (14 – 61% removal) is more efficient than CAS (0 – 11% removal), whereas Liu *et al.* (2011)

showed that the aerobic treatment was more efficient than the anaerobic treatment for BTs, although the half-lives were still high (*i.e.,* 315 days for BTZ). In addition, non-biological processes have been investigated by other authors, such as Müller *et al.* (2012), who investigated the elimination of 4-MBTZ and 5-MBTZ through ozonation as a last step during the water potability process. Another example of alternative technologies is the use of reverse osmosis (RO) filtration as a tertiary treatment in an advanced water recycling plant in Australia, which achieved BTZ and TTZ removals of *c.a.* 70% and 85%, respectively (Loi et al., 2013). Nonetheless, further biological or chemical treatment should be applied to the RO concentrate (ROC). Currently, the main technologies being studied to treat ROC are advanced oxidation processes (AOPs), but they require high chemical dosages and considerable energy consumption (Pérez-González et al., 2012). Therefore, environmentally friendly alternative treatments should be studied.

The main aim of this work was to assess the biodegradation of selected BTs (BTZ, 4-MBTZ and 5-MBTZ) in RO concentrate using an alternative treatment method based on the white-rot fungus *Trametes versicolor.* White-rot fungi are able to degrade a wide range of xenobiotics due to their ability to degrade lignin, the natural polymer most resistant to biodegradation. Fungal enzymes (*e.g.,* laccase and cytochrome P450) are highly unspecific; thus, adaptation is not required, and there is no lag phase in the degradation of xenobiotics (Gago-Ferrero et al., 2012). This type of treatment has been successfully applied for the degradation of emerging ubiquitous pollutants, such as sulfamethazine in sewage sludge from WWTPs (García-Galán et al., 2011), compounds that block or absorb ultraviolet light, such as UV-filters (Gago-Ferrero et al., 2012) and iopromide and ofloxacin in hospital wastewaters (Gros et al., 2014). Fungal bioremediation can thus be a suitable candidate for the treatment of ROC and has already shown optimal degradation results for pharmaceutically active compounds (PhACs) (Badia-Fabregat et al., 2015). The elimination of 27 EDCs and some related compounds (e.g., caffeine) was assessed during the treatment of ROC water with *T. Versicolor*. The generation of any possible TP from the fungal treatment needs to be assessed since these TPs might be more toxic than the target pollutants (García-Galán et al., 2011). In this study, the TPs of three selected BTs (BTZ, 4-MBTZ and 5-MBTZ) were monitored during the fungal treatment using high-resolution mass spectrometry. Although the degradation of BTZ and the generation of TPs

during conventional water treatment have been previously studied by other researchers (Liu et al., 2011; Müller et al., 2012; Huntscha et al., 2014), this is the first time that TPs originating from fungal degradation have been determined. Additionally, acute toxicity and estrogenic and anti-estrogenic activities were evaluated during the treatment using Microtox® and recombinant yeast assay (RYA), respectively.

2. Material and methods

2.1. Chemicals, strains and reverse osmosis concentrate

Standards of target EDCs 5-methyl-1H-benzotriazole (5-MBTZ) and 4-methyl-1H-benzotriazole (4-MBTZ) were purchased from Dr. Ehrenstorfer (Germany). Benzotriazole (BTZ) and labelled benzotriazole (BTZ-d_4) were purchased from Fluka (Buchs, Switzerland). The information regarding the other 25 EDCs analysed within this work, as well as their corresponding labelled compounds, is summarized in S1 of the Supplementary Material. The calibration mixture used in the high-resolution mass spectrometry processes was supplied by Thermo Fisher Scientific (Rockford, IL 61105 USA) (2017a and 2017b).

All the solvents used during the studies were of high purity grade. High-performance liquid chromatography (HPLC)-grade methanol, acetonitrile and water were supplied by Merck (Darmstadt, Germany). Formic acid (98%) was provided by Merck (Darmstadt, Germany). HPLC/high-resolution mass spectrometry (HRMS)-grade acetonitrile and water were supplied by Thermo Fisher Scientific (from Leics, UK and Geel – Belgium, respectively).

T. versicolor (ATCC#42530) was obtained from the American Type Culture Collection (CECT, Valencia, Spain) and was maintained by subculturing on petri dishes in malt extract (2%) and agar medium (1.5%) at 25 ºC.

For the Estrogen Receptor Assay (ER-RYA), yeast strain BY4741 (MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0) from EUROSCARF (Frankfurt, Germany), transformed with plasmids pH5HE0 (hER) and pVitBX2 (ERE-LacZ) as described elsewhere (Noguerol et al., 2006), was used.

The bioluminescent bacteria *Vibrio fischeri* and test reagents for Microtox® analyses were supplied by Strategic Diagnostics Inc. (Newark, DE, USA).

ROC effluent was obtained from a pilot plant at the Castell-Platja d'Aro WWTP (Catalonia, north eastern Spain). It consists of a pilot plant with a membrane bioreactor (MBR) treating urban wastewater coupled to a RO unit. More information about the pilot plant can be found elsewhere (Dolar et al., 2012). The ROC for the sterile fungal treatment was autoclaved for 30 min at 121 ºC the same day of sampling then stored at -20 ºC and autoclaved again before the treatment. The ROC for the non-sterile fungal treatment was kept at 4 ºC for one month before the experiment, and the ROC was replaced with a fresh sample every 3-5 d during the treatment in the entrance storage tank.

2.2. Degradation experiments

2.2.1. Degradation of EDCs in a fungal bioreactor treating reverse osmosis concentrate

Two different experiments were performed to evaluate the fungal treatment of ROC: one under sterile conditions and the other one under non-sterile conditions. The treatment under sterile conditions was performed for control purposes in order to determine the capacity of the inoculated fungus to degrade EDCs. The physicochemical characterization of the ROC is shown in Table S1. Two air fluidized bed bioreactors of 1.5 L each were used in parallel for each experiment. In the sterile batch treatment, both bioreactors were inoculated with *T. versicolor* and run under the same conditions (replicates). In the non-sterile continuous experiments, one reactor was inoculated with *T. versicolor* (I) and the other one was not inoculated (NI) to act as a control to assess degradation by indigenous microorganisms. More information on the operational conditions can be found elsewhere (Badia-Fabregat et al., 2015 and 2017). Briefly, the temperature and pH were maintained at 25 ºC and 4.5, respectively. *T. versicolor* was added in the form of pellets at 2-3.6 g dry cell weight (DCW) L^{-1}, and it was maintained inside the bioreactor by means of a mesh in the outlets. In the continuous treatment, 1/3 of the biomass was replaced every 5 days (cellular residence time of 15 days). The hydraulic residence time (HRT) in the continuous treatment was 3 days until day 15 when it changed to 2 days until the end of the experiment (24 days). Nutrients were added in pulses at 0.6 min/h from a concentrated stock at rates of 192-696 mg g_{DCW}^{-1} day of glucose and 0.43-1.57 mg g_{DCW}^{-1} day of ammonia tartrate. Samples were filtered under vacuum conditions with Wathman GF/C

(Sigma Aldrich; Madrid, Spain) filters and 0.45 µm nylon filter (Millipore; Madrid, Spain). In the sterile treatment, samples from both bioreactors were mixed (1:1) to form a composite sample.

2.2.2. Degradation in spiked synthetic medium

The experiments were performed in 125 mL amber serum bottles with cotton plugs in a total reaction volume of 25 mL as described elsewhere (Gago-Ferrero et al., 2012). The composition of synthetic medium for degradation is summarized in Table S2. Fungal pellets were added to the solution to a final concentration of 5 g DCW L^{-1}, and three separate experiments were performed with a corresponding target pollutant: BTZ, 4-MBTZ or 5-MBTZ, each of which was spiked at 10 mg L^{-1}. The experiments were carried out at 25 ºC under orbital agitation of 130 rpm. The treatments were performed in duplicate, and individual bottles were taken at each sampling time. Experimental bottles (EB) consisted of *T. versicolor* pellets with a selected pollutant added to the synthetic medium and an initial glucose concentration of 8 g L^{-1} among other nutrients (Table S2). In the EB+G treatment, additional glucose was added every 3 d to achieve the initial concentration of 8 mg L^{-1}. Uninoculated controls (UNI) with BTZ, 4-MBTZ or 5-MBTZ were monitored in order to take into account any potential abiotic processes. A heat-killed fungal control (HK) with the same amount of fungi as the EB but with the *T. versicolor* having been killed by autoclaving (30 min at 121 ºC) was created to quantify the amount of absorbed/adsorbed contaminant. Finally, blank controls (BC) with fungi and without BTs were included as references in order to detect changes in laccase activity and fungal growth due to the toxicities of the compounds. At each sampling time, the liquid medium was filtered by vacuum with a Whatman GF/C glass fibre filter (Sigma Aldrich; Madrid, Spain) to determine the fungal dry weight and was then filtered with a 0.45 µm nylon filter (Millipore; Madrid, Spain) for the analyses described in the following section.

The fungal degradation of BTZ, 4-MBTZ or 5-MBTZ was determined by comparing their concentrations in the EBs with those in the UNI and HK controls according to the following formulas:

$$Removal\ (\%) = \frac{UNI - EB}{UNI} \cdot 100$$

$$Biotransformation\ (\%) = \frac{HK - EB}{UNI} \cdot 100$$

2.3. Analytical procedures

2.3.1. On-line turbulent flow coupled to UHPLC-MS/MS

In total, 27 EDCs and related compounds were analysed by on-line turbulent flow chromatography coupled to a liquid chromatography tandem mass spectrometry (LC-MS/MS) system using the method developed by Gorga *et al.* (Gorga et al., 2013). The compounds were analysed according to their more efficient ionization mode in the electrospray ionization (ESI) source. Before analysis, samples were centrifuged at 4000 rpm for 20 min at room temperature, and 5 mL of supernatant was spiked with 100 µL of a mixture of labelled internal standards (see section S1 from supplementary material) in methanol for a final concentration of 2 µg L^{-1}.

A Thermo Scientific EQuan MAX Plus chromatographic system (Thermo Fisher Scientific; Industriestrasse, Switzerland) was used for purification and separation purposes. The system was adapted with different turbulent flow chromatographic columns (TFC) for purification purposes (Cyclone P [50 × 0.5 mm, 60 µm particle size, 60 Å pore size] for negative ionization mode compounds and Cyclone MCX [50 × 0.5 mm, 60 µm particle size, 60 Å pore size] for positive ionization mode compounds (Thermo Fisher Scientific, Franklin, MA)). The separation of the target analytes was achieved using a Hypersil GOLD (50 x 2.1; 1.9 µm) analytical column (Thermo Fisher Scientific, Franklin, MA)). The procedure was adapted from a previous method developed using the EQuan on-line sample enrichment system (Gorga et al., 2013). The method consists of a first loading step of the sample into the TFC column and retention of the analytes followed by a transfer step in which the analytes of interest are desorbed from the TFC column onto the analytical column through the same gradient used for analyte separation in the LC system. More detailed information about the on-line chromatographic methods can be seen in Table S3. The injected volume was 250 µL, with a total run time of 13 min for each injection.

The chromatographic system is coupled to a TSQ Vantage triple quadrupole mass spectrometer analyser (Thermo Fisher Scientific, San Jose, USA), equipped with a Turbo Ion Spray source. The ionization of the compounds was performed under positive or negative mode according

their ion affinity (Gorga et al., 2013). The acquisition was performed in selected reaction monitoring mode (SRM) to obtain enough identification points (IP) for confirmation of each analyte according to Commission Decision 2002/657/EC (European_Commission, 2002).

2.3.2. HPLC-DAD

The three selected EDCs (BTZ, 4-MBTZ and 5-MBTZ) were analysed in the samples from experiments performed in a synthetic medium by liquid chromatography coupled to a UV analyser. The chromatograph Ultimate HPLC was equipped with a LiChrospher RP-18 (12.5 cm x 4 mm i.d., 5 µm particle size) column produced by Merck (Madrid, Spain). In this process, 20 µL of sample was injected at a flow rate of 0.6 mL/min with 5% methanol (A) and 95% MiliQ water (B). A chromatographic gradient adapted from Müller *et al.* (Müller et al., 2012) was used for compound separation. The flow rate was maintained at 5% of A for 1 min; then, A increased to 95% over 12 min and was maintained for 5 min. Finally, A returned to the initial conditions (5%) over 0.5 min, and the column was equilibrated for 6.5 min, with a total run time of 24 min per sample. The detection of the analytes was performed in a UV Dionex 300 instrument at 275 nm, as described elsewhere (Hart et al., 2004).

2.3.3. On-line turbulent flow chromatography coupled to UHPLC-Orbitrap-MS

The identification of possible transformation products was conducted by on-line turbulent flow chromatography system coupled to a hybrid linear ion trap – high-resolution mass spectrometer LTQ Orbitrap (TFC-LTQ Orbitrap). An Aria TLX-1 chromatographic system (Thermo Fisher Scientific; Industriestrasse, Switzerland) was used for purification and separation purposes. This system comprised a PAL auto sampler and two mixing quaternary pumps (eluting pump and loading pump). The entire system was controlled via Aria software, version 1.6, under the Xcalibur 2.2 software. The compounds were extracted using on-line turbulent flow chromatography (TFC) based on an earlier published work by Gorga *et al.* (Gorga et al., 2013). The on-line extraction was performed in a Cyclone chromatographic column (50 × 0.5 mm, 60 µm particle size, 60 Å pore size; Thermo Fisher Scientific, Franklin, MA) and the separation of compounds by a Hypersil GOLD analytical column (50 × 2.1; 3 µm; Thermo Fisher Scientific, Franklin, MA). The extraction process was achieved in two main steps using the Focus Mode.

Firstly, 10 µL of sample is introduced into the TFC column at 1.5 mL/min with acidified water (formic acid 0.1%), where the analytes of interest are retained at the active pore sites while the rest of the matrix is discharged to the waste. In the second step, the compounds are desorbed from the TFC column onto the analytical column through the normal LC gradient with water (0.1% formic acid) and acetonitrile. The total run time for each injection is 10 min. The method is detailed in Table 1.

The chromatograph is coupled to a hybrid linear ion trap Fourier Transform Mass Spectrometry Orbitrap analyser (LTQ-OrbitrapVelos^{TM}, Thermo Fisher Scientific; Bremen, Germany) equipped with a diverter valve (used to divert unwanted portions of chromatographic runs to waste) and an Electrospray Ionization (ESI) source. The diverter valve was used with three valve positions: from 0 to 1.5 min, the flow was discharged to the waste; from 1.5 to 7.75 min, the valve was switched to injection mode; and from 7.75 to 10 min, the valve was switched again to the waste. The ionization of the compounds was performed under positive mode after a pre-screening in negative mode without detection of new chromatographic peaks (data not shown). Mass calibration and mass accuracy checks were performed prior to every sample run with LTQ ESI Positive Ion Calibration Solution (Thermo Fisher Scientific; Rockford, IL 61105 USA) and mass accuracy was always within an error of ±2 ppm. The ionization voltage was set at 3.5 kV with the sheath gas flow at 40, auxiliary gas flow at 20, S-Lens RF level at 69%, and the capillary temperature and the source heater temperature at 350 ℃ and 300 ℃, respectively. The samples were acquired using two different acquisition methods in parallel: 1) the first method was triggered through full scan within a mass-to-charge (m/z) range of 100 to 500 m/z at a resolving power of 60,000 FWHM, and 2) the second experiment was performed with data-dependent analysis through the MS fragmentation of the 5th most intense ions obtained in the 1st experiment at each scan time, isolated in the ion trap with a width of 2.0 Da, a collision-induced dissociation activation type (Q = 0.250 and an activation time of 30 ms) and normalized collision energy (35). Xcalibur 2.2 software was used for data interpretation.

Data processing was carried out using the SIEVE 2.0 software (Thermo Scientific; TF, San Jose, USA) in order to perform the chromatographic peak deconvolution.

The detection of the transformation products of BTs (postulated during the previous study, section 2.3.3) was conducted using the TFC-LTQ Orbitrap. The TFC system operated in the same mode and with the same mobile phases as described before. Mass spectrometer acquisition was carried out by SRM: 1) the full scan within a mass-to-charge (m/z) range 100-500 m/z at a resolving power of 60,000 FWHM and 2) the data-dependent analysis at 7,500 FWHM through the MS fragmentation of the ions from a molecular ion list of suspected TPs. The molecular ions were isolated in the ion trap with a width of 2.0 Da, a collision-induced dissociation activation type (Q = 0.250 and an activation time of 30 ms) and normalized collision energy (35). All the processes were controlled by Xcalibur 2.2 software. The data processing was performed using the ExactFinder 2.5 software (Thermo Scientific; TF, San Jose, USA) run with the library built by the authors based on the results from the biotransformation of BTs, and the results were carefully evaluated via manual comparison.

2.3.4. Routine analyses for the evaluation of bioreactor performance

Glucose concentrations were measured with a biochemical analyser YSI 2700 SELECT (Yellow Spring Instruments) within a concentration range of 0 – 20 (±0.04) g L^{-1}.

Laccase activity was measured through the oxidation of 2,6-dimetoxyphenol (DMP) by the laccase enzyme in a modified version of the method for the determination of manganese peroxidase of Kaal et al. (1993). The analysis process is based on the measure of the absorbance variance at λ=468 nm at 30 ºC during 2 min in a Varian Cary 3 UV/Vis spectrophotometer. The reaction was done with 600 µL of sample, 200 µL of 250 mM sodium malonate at pH 4.5 and 50 µL of 20 mM DMP. Activity units per litre (U L^{-1}) are defined as the amount of DMP in micromoles per litre that is oxidized per minute (µmol DMP L^{-1} min). The molar extinction coefficient of DMP was considered to be 24.8 mM^{-1} cm^{-1} (Wariishi et al., 1992).

The amount of biomass was determined as the constant weight at 100 ºC of vacuum-filtered samples in pre-weighted glass-fibre filters (Whatman, Spain).

2.4. Toxicity assessments

2.4.1. Acute toxicity assessment by bioluminescence inhibition test with *Vibrio fischeri*

The bacterial bioluminescence assay was conducted in a Microtox® test instrument following the ISO 11348-3 protocol (ISO, 2007). The effective concentration of toxicants that caused a 50% of inhibition after 15 min of exposure was designed as the EC_{50} value. Toxicity units (TU) were calculated as $100/EC_{50}$.

2.4.2. Estrogenic and anti-estrogenic activity assessment by Estrogenic Recombinant Yeast Assay (ER-RYA)

The yeast-based bioassay, which harbours the human estrogen receptor (hER), is able to monitor and quantify the interactions between the ER and the compounds present in the medium by activation of the LACZ gene. The protocol is described elsewhere (Noguerol et al., 2006). The incubation period was 6 h before adding Y-PER®. For anti-estrogenic activity assessment, a medium with 17β-estradiol at a submaximal concentration (10 nM) was used.

2.5. Calculations and statistical analysis

For removal calculations, the compounds detected below the limit of quantification (LOQ) were considered to have a concentration of ½ the LOQ (Table S4) (EPA, 2000). The mean and standard deviation (SD) were calculated using Microsoft® Excel 2011 functions. One-factor analysis of variance (ANOVA) and t-student tests for statistical analysis were performed with Sigmaplot 11.0; differences were considered significant at $p<0.01$.

3. Results and discussion

3.1 Elimination of EDCs in a sterile *T. versicolor* batch bioreactor treating reverse osmosis concentrate

Fungal batch bioreactor treating ROC under sterile conditions was performed to assess the elimination of EDCs by the ligninolytic fungus *T. versicolor.* The analysis confirmed the presence of TTZ and BTZ, as well as other EDCs (TCEP, OP_2EO, NP and estriol-16-glucuronide) and the human biomarker caffeine, as shown in Figure 1. Other compounds, such as oestradiol-17-glucuronide, estrone, estrone-3-glucuronide, estriol-3-sulfate, TBEP and NP_1EO, were detected in some samples during the treatment without being present at the beginning of the experiment. The two main hypotheses are that i) the compounds are present in

348 the ROC at low concentration levels, near the limit of quantification, which is higher in complex
349 matrices (as is the case at the beginning of the experiment) (Badia-Fabregat et al., 2015) or that
350 ii) conjugation/deconjugation processes occur during the treatment (Badia-Fabregat et al.,
351 2015). The compounds detected at higher concentrations were the BTs (with a maximum of
352 7.40 µg L^{-1} for BTZ and 12.8 µg L^{-1} for TTZs), which were degraded by *T. versicolor* to
353 concentrations <LOQ (Table S4).

354 For calculation of removal percentages, those compounds found at levels below their LOQ were
355 considered have concentrations of LOQ/2 (EPA, 2000) (Table S4 and Figure 1). In this case,
356 removal of 73% of total EDCs was achieved at the end of the treatment (Figure 1A). Similar
357 results were obtained for pharmaceuticals, with a removal of 68% after 6 d treatment, as
358 demonstrated elsewhere (Badia-Fabregat et al., 2015).

359 Monitoring of the target pollutants as performed in the present work is important in order to
360 assess the best treatment for emerging contaminant elimination. Nonetheless, if TPs generated
361 during the treatment are not further degraded, they can also represent an environmental
362 problem because of their possible persistence as well as any possible associated toxicity.
363 Sometimes transformation products might even lead to the generation of the initial compound
364 (*e.g.,* human-generated conjugated metabolites (phase II metabolites) that can be
365 deconjugated), resulting in an increase in the concentration of some compounds after treatment
366 instead of their removal. Therefore, for an exhaustive and complete elimination assessment,
367 parent and transformation products should be included in the analytical methods. Because of
368 this, in the next sections, fungal metabolites of BTZ, 4-MBTZ and 5-MBTZ are tentatively
369 identified during mycoremediation in a synthetic medium and further analysed in a non-sterile
370 ROC treatment in a bioreactor.

371 **3.2. Biotransformation studies of BTZ, 4-MBTZ and 5-MBTZ by *T. versicolor* in a synthetic**
372 **medium**

373 To be able to detect any possible TPs generated during the treatment in real wastewaters,
374 higher concentrations of BTZ and TTZs than those measured in the ROC were used in the

spiked synthetic medium (see section 2.2.2). The results of the biotransformation of these compounds are shown in Figure 2.

The major removal process was attributed to biotransformation since the sorption observed in the HK experiments (difference between removal and biotransformation in Table S5) was almost negligible for the three compounds. The highest removal percentage corresponded to 5-MBTZ, reaching 85% after 14 d of exposure (Figure 2 and Table S5). In contrast, the structural isomer 4-MBTZ showed higher persistence (removal *c.a.* 55%), as was previously observed by Weiss *et al.* (Weiss et al., 2006). However, the most stable compound was BTZ, with an elimination yield of 37% after 14 d of exposure. Similar removals were observed by Liu *et al.* (Liu et al., 2011) during their experiments with bacteria under aerobic conditions.

The fungal dry weight increased 20% between the 1st and the 3rd day in the EB treatment and decreased after depletion of the initial glucose on day 3 (Figure S1). In contrast, when glucose was added periodically to avoid starvation of *T. versicolor*, the fungal dry weight increased between 50 and 70% after 14 d in the EB+G treatment and reached 88% in the BC+G experiment. However, no remarkable differences between removal percentages were observed after the 9th day between the EB and EB+G experiments (Figure 2). This leads us to hypothesize that *T. versicolor* might incorporate BTZ and TTZs in its metabolism as a carbon source despite the 8 g L^{-1} of glucose used in the experiments of EB+G. This effect has been previously reported for other xenobiotics, such as the UV filter benzophenone-3 (Badia-Fabregat et al., 2015). However, further studies with ^{13}C- or ^{14}C-labelled compounds should be performed to confirm this hypothesis. Laccase activity was low in all the spiked experiments, with maximum values of 4.7 U L^{-1} for BC and 3.3 U L^{-1}, 5.9 U L^{-1} and 3.2 U L^{-1} for BTZ, 4-MBTZ and 5-MBTZ, respectively.

3.3. Tentative identification of BT transformation products in a synthetic medium

The samples from experiments with spiked synthetic medium were analysed by on-line TFC-LC-(ESI)-LTQ Orbitrap in order to tentatively identify any possible TPs generated during biological treatment with *T. versicolor*. The acquisition mode involved full scan MS data and, in parallel, the full scan of the MS^2 fragmentation of the 5 most intense ions from the previous MS full scan.

Table 2 shows the results of the proposed elemental composition for the detected TPs. All the m/z values reported in Table 2 were identified in both EB and EB+G experiments, which indicates that the metabolic state of the fungus (related to low or high nutrient availability, respectively) might not be critical in terms of the biotransformation pathway.

3.3.1. 1H-benzotriazole

BTZ was detected at 4.51 min (Figure 3) with a m/z of 120.0556 corresponding to $[M+H]^+$ (error mass: 0.001 ppm). The fragmentation of the molecular ion produced two major fragments (Figure S2) identified as 92 Da (loss of N_2) and 65 Da (C_5H_5; corresponding to the typical fragment of substituted aromatic hydrocarbons (Badertscher et al., 2009)). Six potential TPs were tentatively identified: TP 134, TP 148 A, TP 178 A, TP 178 B, TP 192 and TP 222. Their corresponding extracted ion chromatograms (XICs) can be seen in Figure 3, the proposed pathways are shown in Figure 4 and the spectra of some of them are shown in Figures 4-5 and S3 to S6. The highest chromatographic peak intensities were detected for TP 222 and TP 178 B, corresponding to a generation ratios (calculated as the relative area of the suspected peak in the chromatogram in comparison to BTZ area at t=0) of 11% and 9%, respectively, after 9 d of treatment. After 14 days of treatment, only TP 222 and TP 178 B had generation ratios of 27% and 9%, respectively, whereas BTZ was still present at 63% of its initial concentration (data not shown).

Fragmentation pathway of TP 222 suggests the presence of a $C_4H_7O_3$ group (RDB=1) attached to N. One of the most likely groups could have come from the tetrose sugar and could be attached through *N*-Glycoside bond (Table 2; proposed fragmentation pathway in Figure 5B). This hypothesis is based on the enzymatic activity of glycosyltransferases present in the fungus system (Bowman and Free, 2006), which catalyses the transfer of saccharide moieties from an activated nucleotide sugar (glycosyl donor) to a nucleophilic glycosyl acceptor (N-based in this case) (Williams and Thorson, 2009) (Figure 4). However, to the authors' knowledge, this is the first report of tetrose conjugated metabolites during mycoremediation. In contrast, it was not possible to suggest a plausible molecular structure for TP 178 B since no MS^2 spectra for structural elucidation was available (Table 2). With a hypothetical molecular formula of $C_9H_{12}ON_3$ corresponding to its $[M+H]^+$ and an RDB equal to 5.5, our hypothesis is that the

aliphatic chain contains an alcohol because of the loss of an H_2O molecule based on its MS spectra (Figure S5). For this proposed TP, a structural isomer was detected at a different retention time (TP 178 A at 4.7 min; Table 2). In this case, we assume that both isomers differ in the position of the hydroxyl group. According to the calculated log K_{ow} by the ALOGPS 2.1 program (Tetko et al., 2005; VCCLAB, 2005), the molecule with the secondary alcohol is more lipophilic than the molecule with the primary alcohol. Based on this, the TP 178 B (with a secondary alcohol in the aliphatic group) would be the one detected at t_r=5.0 min, and TP 178 A (with a primary alcohol) would be the one detected at t_r=4.7 min. Finally, the structure of TP 178 A could be confirmed based on its MS^2 spectra (Figure 5A). The main hypothesis for the formation of these compounds is the reaction with prenyltransferases and/or terpene synthases (Wessjohann et al., 2013) (similar to reactions performed in fungal secondary metabolism (Keller et al., 2005)), leading to the corresponding alkylated compound and, subsequently, hydroxylation in the optimum position to generate TP 178 A and TP 178 B (Figure 4) due to lignin peroxidase (LiP), manganese peroxidase (MnP) and/or cytochrome P450 enzymes (Barr and Aust, 1994).

Two other minor TPs were postulated: TP 192 (percentage formation of 1 and 2% after 9 d and 14 d, respectively) and TP 148 A (percentage formation of 1 and 3% after 9 d and 14 d, respectively). TP 192 was detected at t_r = 5.06 min (Table 2) with a $[M+H]^+$ corresponding to $C_9H_{10}O_2N_3$ as the most accurate formula and RDB = 6.5. The proposed structure and fragmentation pattern can be seen in Table 2 and Figure S6, respectively. The fragmentation pattern suggests the presence of a propanoic acid attached to a triazole ring, which is confirmed by the loss of the carboxylic acid as shown in Figure S6. This compound could be generated from the oxidation of the primary alcohol of TP 178 A onto a carboxylic acid by LiP, MnP and/or cytochrome P450 enzymes (Barr and Aust, 1994) (Figure 4). A similar compound was proposed by Huntscha *et al.* (Huntscha et al., 2014), although the authors suggest a position inside the phenyl ring attached to the alkyl acid. Comparing MS^2 fragments generated in both works, the same pattern is obtained, and none of them are conclusive enough to elucidate the position of the propanoic acid. Further studies based on ^{1}H-NMR and ^{13}C-NMR would be necessary to identify the position of the acid. In the case of TP 148 A, this structure was detected during the analysis of the samples using the SIEVE software and identified in the ChemSpider database.

462 According to the structure given in the ChemSpider database, the tentative structural elucidation
463 is shown in Figure S4. A similar biotransformation product was postulated by Liu *et al.* (2011)
464 during aerobic degradation of 5-MBTZ but not during the degradation of BTZ.

465 Finally, TP 134 was detected as a minor TP (0.23 and 0.25% after 9 d and 12 d, respectively).
466 The proposed structure is based on the methylation of the N. This compound was postulated
467 according to its exact mass with an error below 0.3 ppm and an RDB of 5.5 at a retention time
468 of 5.2 min since no MS^2 was available (Table 2 and Figure S3). The fragmentation of the
469 ionized parent compound ($[M+H]^+$) did not generate any stable ions. However, the methylation
470 (Figure 4) could be mediated by the methyltransferases enzymes present in fungi (Wessjohann
471 et al., 2013). In addition, the same structure has been proposed by Liu *et al.* (2011) and
472 Huntscha *et al.* (2014) during the biodegradation of BTZ under aerobic conditions with sewage
473 sludge.

474 3.3.2. 4-methyl-1H-benzotriazole

475 The confirmation of this compound was based on the comparison with pure standard and,
476 additionally, by its typical fragmentation pattern: the loss of 28 Da corresponding to N_2 followed
477 by the loss of CNH group (27 Da) (Figure S7). The degradation of this compound detected at
478 4.9 min (Table 2) suggested two suspected TPs (see the XICs for both of them in Figure 6): TP
479 236 and TP 148 B, with percentage formations between 0.1 – 0.3% in association with a 55%
480 disappearance of 4-MBTZ (data not shown). Thus, in this case, further metabolism could occur.

481 Concerning TP 148 B, the tentative structure was based on the methylation of the N group
482 (Table 2). Its fragmentation pathway suggests the typical fragmentation of triazoles by the loss
483 of N_2 (28 Da) to generate the ion $C_8H_{10}N^+$ followed by the loss of C_2H_3N to form the ion $C_6H_7^+$,
484 which corresponds to the typical fragmentation of substituted aromatic hydrocarbons (2009)
485 (Figure S8). Biotransformation compounds with the same elemental composition were detected
486 by Huntscha *et al.* (Huntscha et al., 2014). The authors proposed the methylation of the phenyl
487 ring in different positions. Nonetheless, in this study, we linked the methyl group to N based on
488 the most plausible reaction during mycoremediation and according to our MS^2 data (Figure S8
489 and Figure 4). However, methyltransferase enzymes (Wessjohann et al., 2013) would be able to

490 methylate the phenyl ring as well as the N group and further studies based on ^{1}H-NMR and ^{13}C-
491 NMR are necessary in order to identify the correct position.

492 In contrast, the RDB and exact mass of TP 236 indicates the presence of a $C_4H_7O_3$ group with
493 an RDB equal to 1 and attached to N by an *N*-Glycoside bond as in the case of TP 222 from
494 BTZ. This group could be, again, the tetrose sugar attached due to the activity of
495 glycosyltransferases present in the fungus system (Bowman and Free, 2006; Williams and
496 Thorson, 2009) (Figure 4). However, the generation of the MS^2 spectra was not possible due to
497 the low intensity of the signal, and the TP is postulated based only on MS spectra.

498 3.3.3. 5-methyl-1H-benzotriazole

499 5-MBTZ (detected at tr = 5 min; Figure 6 and Table 2) was the most efficiently degraded. Its
500 identification, as in the case of 4-MBTZ, was based on the common loss of N_2 for aromatic
501 azides and the consequent loss of a CNH group to generate the ion $C_6H_7^+$ (79 Da) (Figure S9),
502 as well as by comparison with the pure standard. Four different chromatographic peaks were
503 identified as potential TPs (Figure 6 B): TP 148 C, TP 164, TP 206 and TP 236. At the end of
504 the experiment (14 d), 5-MBTZ was at 15% of its initial concentration because of mineralization
505 or transformation into other compounds, as described above. TP 236 was the one with the
506 highest percentage (12.44%) followed by TP 148 C (*c.a.* 4%).

507 The fragmentation, RDB and exact mass of TP 236 suggests the presence of the group $C_4H_7O_3$
508 (RDB=1) attached to N, as in the case of TP 222 of BTZ and TP 236 of 4-MBTZ (Table 2). This
509 group could be linked due to the activity of glycosyltransferases (Bowman and Free, 2006;
510 Williams and Thorson, 2009). On the other hand, the proposed structure for TP 206 is also
511 based on the first alkylation due to prenyltransferases and/or terpene synthases (Wessjohann et
512 al., 2013) followed by oxidation as a result of the activity of LiP, MnP and/or cytochrome P450
513 enzymes (Barr and Aust, 1994) leading to carboxylic acid, as observed for TP 192 of BTZ
514 (Figures 3 and 7A for TP 206 and Figures 3 and S6 for TP 192).

515 In contrast, for the other major TP, TP 148 C, the most likely molecular formula of the ionized
516 compound ($C_8H_{10}N_3$) indicates the presence of an extra methyl group. The methylation of the N
517 group of 5-MBTZ (Figure 4) is the most probable reaction under aerobic conditions, as has been

postulated in this work for the biotransformation of BTZ and 4-MBTZ. The tentative elucidation of this TP (Figure S10) was based on the loss of N_2 and the subsequent loss of CH_3N to generate the ion $C_7H_7^{\cdot+}$ (corresponding to the typical fragment of substituted aromatic hydrocarbons (Badertscher et al., 2009)). However, again, since this process is regulated by methyltransferases enzymes (Wessjohann et al., 2013), the methylation could be done in the phenyl group. Huntscha *et al.* (2014) suggested the methylation of the phenyl ring as the most probable position due to the match of retention times of the TPs with the same m/z and molecular formula generated from 4- and 5-MBTZ. These factors did not occur in our study since TP 148 B (coming from 4-MBTZ) and TP 148 C (coming from 5-MBTZ) have different chromatographic retention times (see Table 2).

Finally, the structure of TP 164 has been proposed based on oxidation into a primary alcohol in position 5 and further oxidation to a carboxylic acid due to LiP, MnP and/or cytochrome P450 activity (Barr and Aust, 1994) (Figure 4). Figure 7B shows the tentative fragmentation pattern of this structure. This product was previously confirmed by Huntscha *et al.* (2014) during the biodegradation of 5-MBTZ by comparison with pure standard and by Müller *et al.* (2012) during the ozonation of 5-MBTZ.

Nevertheless, the authors want to remark that any proposed enzymatic pathway for the biotransformation of the three selected benzotriazoles is based on hypotheses according to the available literature. Therefore, enzymatic assays would be necessary to finally confirm these pathways.

3.4. Biodegradation of EDCs, including BTZ, 4-MBTZ, 5-MBTZ and their TPs, in a non-sterile continuous fungal treatment of reverse osmosis concentrate

The concentration of EDCs, including BTs and their previously identified TPs, was evaluated in a non-sterile continuous bioreactor treating ROC effluent. The 27 EDCs and related compounds in the treated ROC water were analysed via the on-line LC-MS/MS method (results are shown in Table 3), and the postulated TPs were screened by the on-line TFC-HRMS method. In terms of the total amounts of detected EDCs, fewer compounds were detected in the non-sterile ROC than in the previous sampling campaign for the sterile batch treatment: BTZ and TTZs were detected at 1.57 µg L^{-1} and 1.60 µg L^{-1}, respectively (section 3.1).

Two HRT (3 and 2 days) were applied during the experiment (Badia-Fabregat et al., 2017). However, no statistically significant differences were found in the global removal of EDCs, which were 74% and 65%, respectively (Table 3). Moreover, removal percentages were not significantly different than those obtained from the non-inoculated control bioreactor (NI). These trends are similar for TTZ and BTZ, with removal percentages between 35 and 68% (Table S5). However, both compounds were detected below MLQ in most of the samples collected along the treatment, even in the uninoculated control. For 5-MBTZ, these removal percentages were lower than those observed in the synthetic medium and in the sterile ROC batch treatment. Competition with indigenous microorganisms probably decreases the capacity of *T. versicolor* to degrade these compounds (Badia-Fabregat et al., 2017).

Finally, the screening for determining the presence of any TP was conducted via the SRM method (see section 3.2.3) with the molecular ion list built according to the results obtained from the synthetic medium (Table 2). After data processing, only one TP was identified as a potential TP generated during the treatment of ROC by fungi: TP 148 A, postulated to be a TP of BTZ (Table 2). However, we think that the presence of this compound could be linked to the degradation of other compounds since its response in the instrument is much higher than the one observed during the experiments with synthetic water (data not shown). In addition, the identity and the concentration of this compound could only be confirmed by comparison with a standard compound using a SRM method in a more sensitive instrument (*i.e.,* LC-MS/MS used for the quantification of 27 EDCs and related compounds in this work).

3.5 Evaluation of the toxicity

Three tests were considered in order to monitor the toxicity along the experiments in synthetic medium as well as in bioreactor experiments: acute toxicity by the bioluminescence assay (assessed with *V. fischeri*) and estrogenic and anti-estrogenic activities (assessed by RYA). Toxicity assays need to be performed because the generation of any possible TP from the fungal treatment might be more toxic than the target pollutants (García-Galán et al., 2011).

In the first place, the EC_{50} values after 15 min for *V. fischeri* (Microtox® assay) were determined to be 25, 26 and 4 mg L^{-1} for BTZ, 4-MBTZ and 5-MBTZ (Table S6), respectively, and are in

accordance with the results obtained by Pillard *et al.* (2001). Different toxicities for the structural isomers of methyl BTs are observed: 5-MBTZ is one order of magnitude more toxic than its structural isomer 4-MBTZ. Additionally, no estrogenic activity was detected for concentrations of up to 5300 mg L^{-1} for BTZ and 2400 mg L^{-1} for 4-MBTZ and 5-MBTZ (Table S6). However, anti-estrogenic activity was found for the three compounds at concentrations above 150 mg L^{-1} for both TTZs and above 340 mg L^{-1} for BTZ (Figure S11 and Table S6). Nevertheless, the anti-estrogenic response of BTZ was 10 times lower than the values reported by Harris *et al.* (2007) in a previous work.

The EC_{50} values detected in our experiments were notably higher than the concentrations in the environment. This is a common aspect in toxicological studies for contaminants of emerging concern (Fent et al. 2006b). It should be highlighted that the toxicity can be detected using *in vivo* or *in vitro* assays. *In vivo* assays (e.g., monitoring of fish or zooplankton development) are more tedious and time-consuming but the results are usually more reliable. On the other hand, *in vitro* assays, such as RYA, are faster and cheaper but might lead to the omission of some effects due to the specificity of the targets monitored in those assays. Therefore, chronic toxicity can be analysed through *in vivo* assays that can detect EC_{50} values at much lower concentrations (at environmentally relevant concentrations) than those assessed by acute toxicity tests based on *in vitro* assays for many emerging contaminants (Fent et al. 2006b). For example, Tangtian *et al.* (2012) reported the hormonal disruption by BTZ in fishes at concentrations of > 10 µg L^{-1}. Therefore, the endocrine-disrupting activity of BTs can neither be confirmed nor denied based on the results of the toxicological assays of this study. In the case of our study, the RYA assays were performed with the sole purpose of general toxicity monitoring during the experiments.

In this context, only the anti-estrogenicity and acute toxicity based on bioluminescence assays were monitored during the fungal biodegradation of the pollutants in the synthetic medium. The initial toxicity units (TU) for acute toxicity during the bioluminescence assays in the experiments were below 4% and not significantly different than the blank control. These values did not change during the treatments, with the exception of the samples with spiked 4-MBTZ, which after 14 d exhibited a significant increase in toxicity (TU = 11 ± 3). A hypothesis is that the

generated TPs, i.e., TP 148 B and TP 236 (Table 2), with formation ratios of 0.1 and 0.3%, respectively (*vs.* 55% of disappearance of 4-MBTZ) as well as other potential non-identified TP compounds, are more toxic than the parent compound. In contrast, no anti-estrogenic activity was detected in any of the samples, probably due to the low concentrations of the target pollutants. The concentrations during the experiments (max. 10 mg L^{-1}) were 2 orders of magnitude below the effective anti-estrogenic concentrations, and in this case, the generated TPs were apparently less toxic.

Finally, in the case of bioreactor experiments with ROC, no estrogenic activity was detected in any of the samples analysed; however, possible anti-estrogenic activity was found in samples from the non-inoculated continuous bioreactor. Moreover, an increase in the acute toxicity (5 TU) was found in the non-inoculated treatment (Badia-Fabregat et al, in preparation). However, values were always below 25 TU (threshold value for an effluent to be considered toxic according Decret 130/2003 from Generalitat de Catalunya, 2003). This could indicate that, even though bacteria can degrade target EDCs, the compounds generated as transformation products from these and other pollutants can be more anti-estrogenic than those generated by fungal treatment.

4. Conclusions

The biodegradation by *T. versicolor* in sterile batch mode allowed removing up to 73% of EDCs from RO brine. BTZ (7.4 µg L^{-1}) and TTZs (12.8 µg L^{-1}), the compounds detected at the highest concentrations, were removed 58 and 92%, respectively. Those removal percentages were maintained in a continuous process treating non-sterile ROC in fungal bioreactor.

Based on fungal biodegradation in synthetic medium, BTZ was the most recalcitrant compound, with a removal of less than 40%. In addition, differences in removal yields between the isomers 4-MBTZ (55% biodegradation) and 5-MBTZ (85% biodegradation) were observed, in accordance with previously published works. BTZ and the two TTZs led to the formation of different TPs after treatment with *T. versicolor*. The major products were postulated to form through the methylation of the triazole ring as well as from the formation of *N*-glycosidic bonds with some sugars present in the medium.

BTZ, TTZs and the tentatively identified TPs were monitored along the treatment of ROC in a fungal bioreactor run in continuous mode under non-sterile conditions. BTZ and TTZs were removed both in the fungal bioreactor and in the non-inoculated control bioreactor, indicating that microorganisms present in the ROC were able to degrade the selected compounds without the need for the addition of fungi, although this leads to more toxic compounds. TP 148, a presumably non-toxic TP generated from the biotransformation of BTZ, was detected in the fungal treatment but not in the control (non-inoculated) bioreactor.

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Conflict of interests

The authors declare that there are no conflicts of interest.

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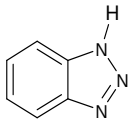
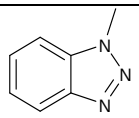
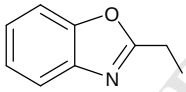
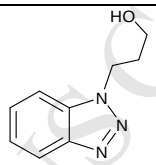
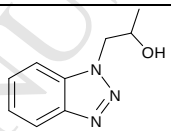
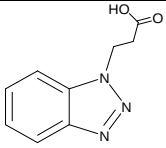
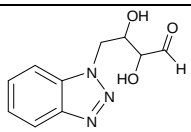
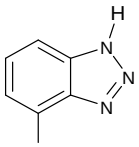
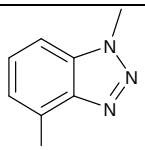
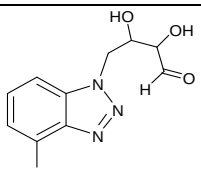
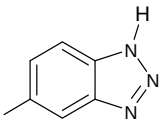
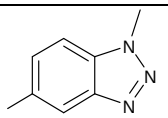
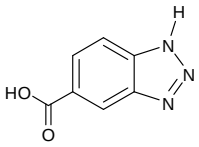
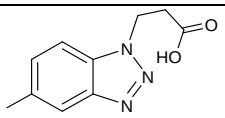
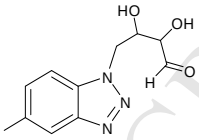
787 **Table 1**: Chromatographic parameters for the direct analysis of BTZ, 4-MBTZ, 5-MBTZ and
788 related products by TFC-LTQ-Orbitrap
789

Time (min)	On-line TFC			Step	TFC and LC connection	LC		
	Flow (mL/min)	A%	B%			Flow (mL/min)	A%	B%
0.00	1.50	0	100	Loading	Out	0.50	5	95
1.50	0.50	5	95	TFC elution and LC separation	In	0.00	5	95
1.75	0.50	100	0		In	0.00	100	0
6.75	0.50	100	0		In	0.00	100	0
7.75	0.25	0	100	Return to initial conditions	Out	0.25	5	95
8.25	0.50	0	100	Return to initial conditions	Out	0.50	5	95
10.0	0.50	0	100	Return to initial conditions	Out	0.50	5	95

790 **A**: acetonitrile
791 **B**: water (0.1% of formic acid)

792 **Table 2:** List of proposed transformation products identified during analysis by TFC-LTQ-
793 Orbitrap for samples after 9 d and 14 d.

	tr (min)	Proposed $[M+H]^+$	RDB	m/z	Theoretical m/z	Error (ppm)	Proposed Structure	Log Kow (Tetko et al., 2005; VCCLAB, 2005)
BTZ	4.5	$C_6H_6N_3$	5.5	120.0556	120.0556	0.001		1.43
TP 134	5.2	$C_7H_8N_3$	5.5	134.0713	134.0713	0.149		1.64
TP 148 A	5.11	$C_9H_{10}NO$	5.5	148.0756	148.0757	-0.520		2.28
TP 178 A	4.7	$C_9H_{12}ON_3$	5.5	178.0975	178.0974	0.561		0.93
TP 178 B	5.0	$C_9H_{12}ON_3$	5.5	178.0975	178.0974	0.561		1.04
TP 192	5.06	$C_9H_{10}O_2N_3$	6.5	192.0767	192.0768	-0.567		0.85
TP 222	4.62	$C_{10}H_{12}O_3N_3$	6.5	222.0873	222.0873	0.001		0.34
4-MBTZ	4.9	$C_7H_8N_3$	5.5	134.0715	134.0713	1.492		1.92
TP 148 B	5.57	$C_8H_{10}N_3$	5.5	148.0868	148.0869	-0.675		2.13
TP 236	4.93	$C_{11}H_{14}O_3N_3$	6.5	236.1031	236.1030	0.424		0.82
5-MBTZ	5.0	$C_7H_8N_3$	5.5	134.0715	134.0713	1.492		1.92

TP 148 C	5.61	$C_8H_{10}N_3$	5.5	148.0868	148.0869	-0.675		2.13
TP 164	4.4	$C_7H_6O_2N_3$	6.5	164.0454	164.0453	0.610		1.04
TP 206	5.12	$C_{10}H_{12}O_2N_3$	6.5	206.0923	206.0924	-0.485		1.33
TP 236	4.95	$C_{11}H_{14}O_3N_3$	6.5	236.1031	236.1030	0.424		0.82

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795

796 **Table 3**: Initial concentration of EDCs and related compounds, as well as removal percentages,
797 during the non-sterile continuous experiment. The treatments included a bioreactor inoculated
798 with *T. versicolor* (fungal treatment) and a non-inoculated control bioreactor. Removal
799 percentages for an HRT of 3 days are the mean of samples taken on days 12, 13, 14 and 15,
800 and the percentages for an HRT of 2 days are the mean of samples taken on days 21, 22, 23
801 and 24.

	Initial time (µg L^{-1})	SD	Fungal inoculated bioreactor		Non-inoculated control bioreactor	
			Rem. HRT 3 d (%)	Rem. HRT 2 d (%)	Rem. HRT 3 d (%)	Rem. HRT 2 d (%)
TCPP	5.44	0.006	99*	99*	99*	99*
Tolyltriazole (5MBTZ)	1.60	0.23	55*	45*	49*	57*
Benzotriazole	1.57	0.49	35	68*	68*	68*
TCEP	<LOQ		0	0	0	0
Methylparaben	<LOQ		50	-5	100*	100*
TBEP	<LOQ		0	0	-320	-364
Estriol-16-glucuronide	0		n.a.	n.a.	increase	increase
Estrone-3-sulfate	0		n.a.	increase	n.a.	n.a.
Estrone-3-glucuronide	0		n.a.	increase	n.a.	n.a.
Estrone	0		n.a.	increase	n.a.	n.a.
Ethynilestradiol	0		n.a.	increase	n.a.	n.a.
Diethylstillbestrol	0		n.a.	increase	n.a.	n.a.
TOTAL	9.39	0.56	74*	65*	75*	79*

802 BQL: Below Quantification Limit; n.a.: not applied; * Statistically significant removal;

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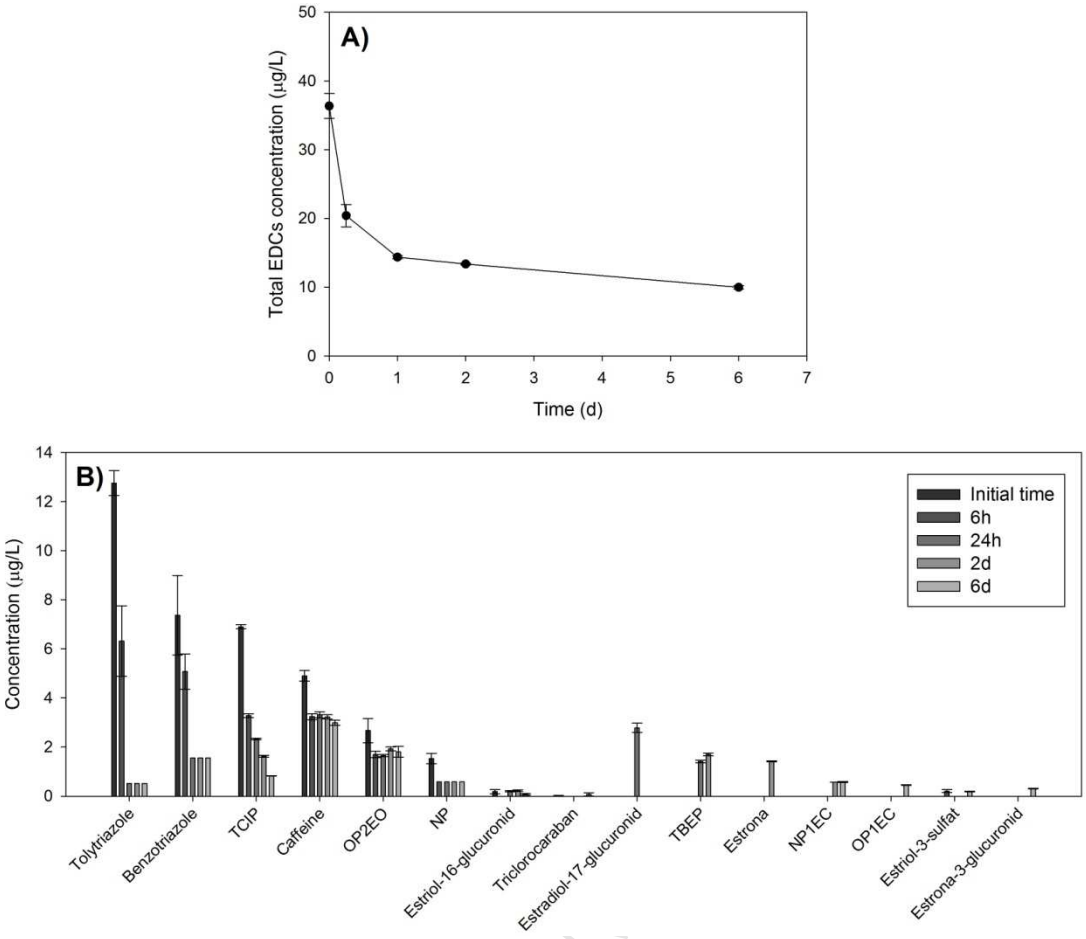

804

805 **Figure 1:** Evolution of **A)** total EDCs and related compounds concentration and **B)** individual
806 compounds in the sterile batch bioreactor.

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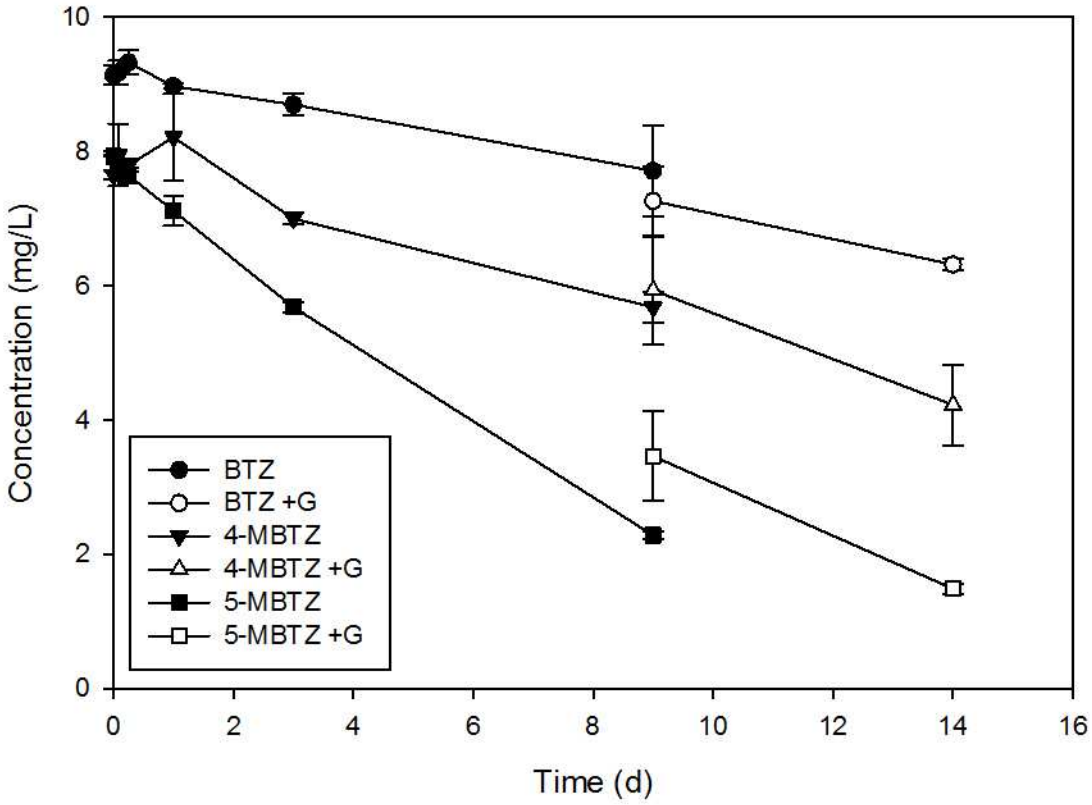

810

811 **Figure 2:** Concentrations of BTZ, 4-MBTZ and 5-MBTZ in the EB and EB+G treatments in the
812 spiked experiments in synthetic media. The results are the means of duplicates.

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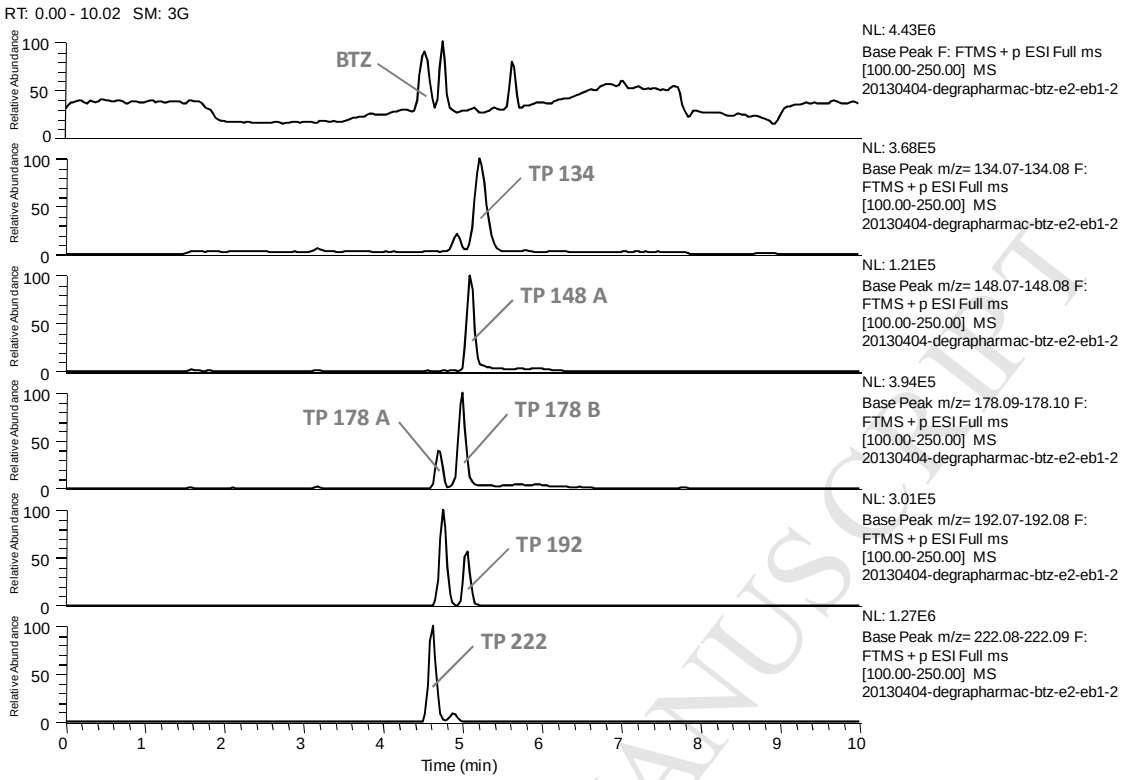
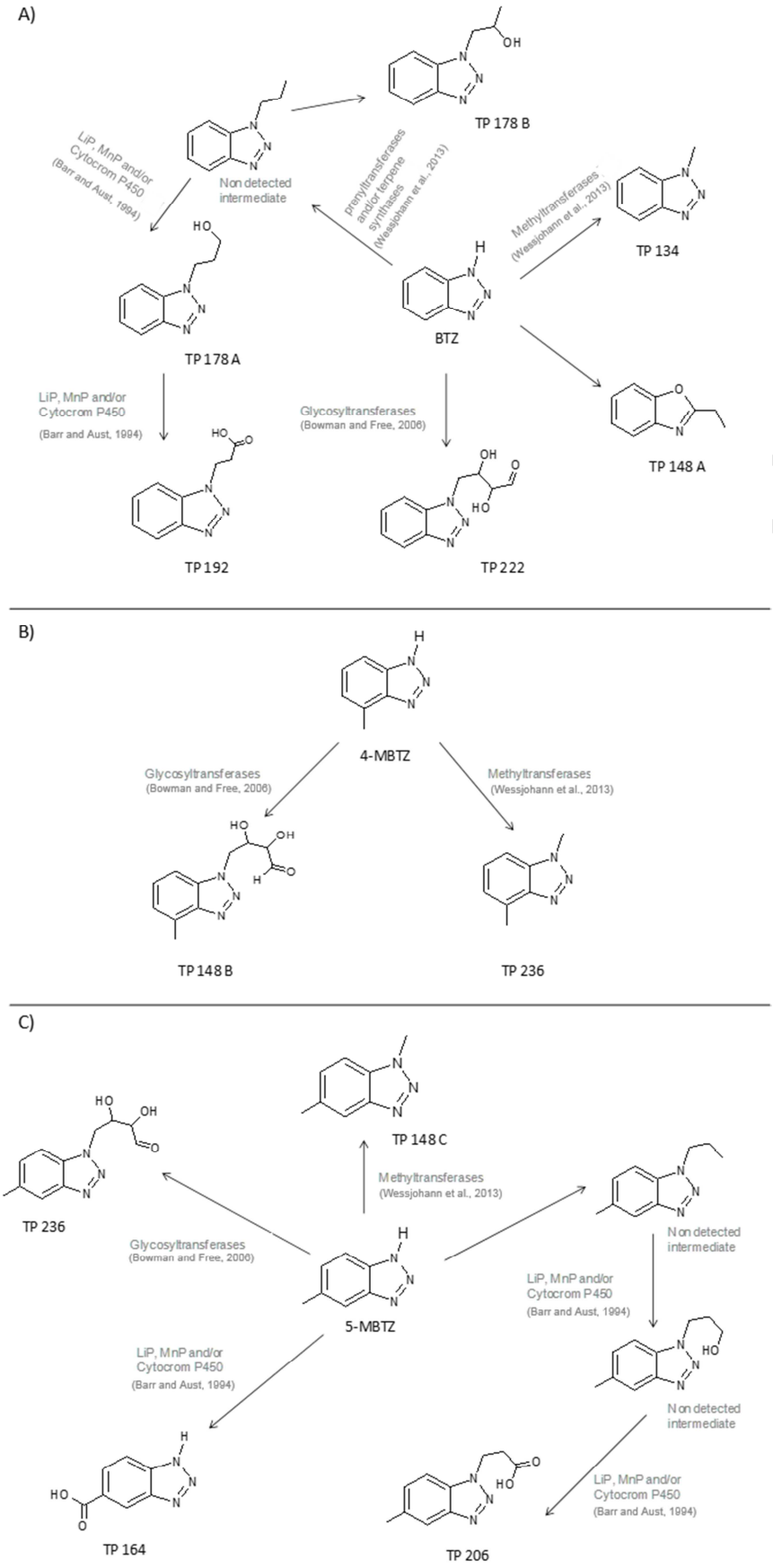
814



815

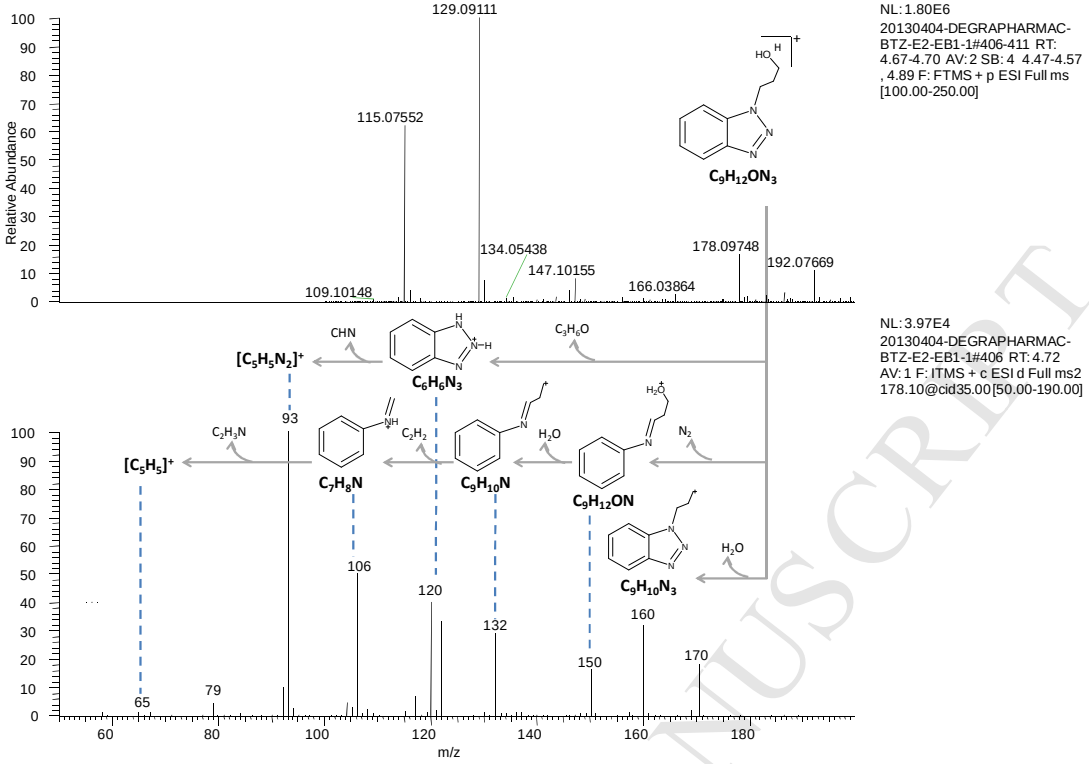
816 **Figure 3:** Full scan chromatogram (m/z=100-250 Da) of 10 mg L^{-1} of BTZ treated with *T.*
817 *versicolor* after 9 days with the presence of glucose and extracted ion chromatograms of the
818 postulated TPs.



819

820 **Figure 4:** Proposed pathways during mycoremediation with *T. versicolor* for BTZ (**A**), 4-MBTZ
821 (**B**) and 5-MBTZ (**C**).

(A)



(B)

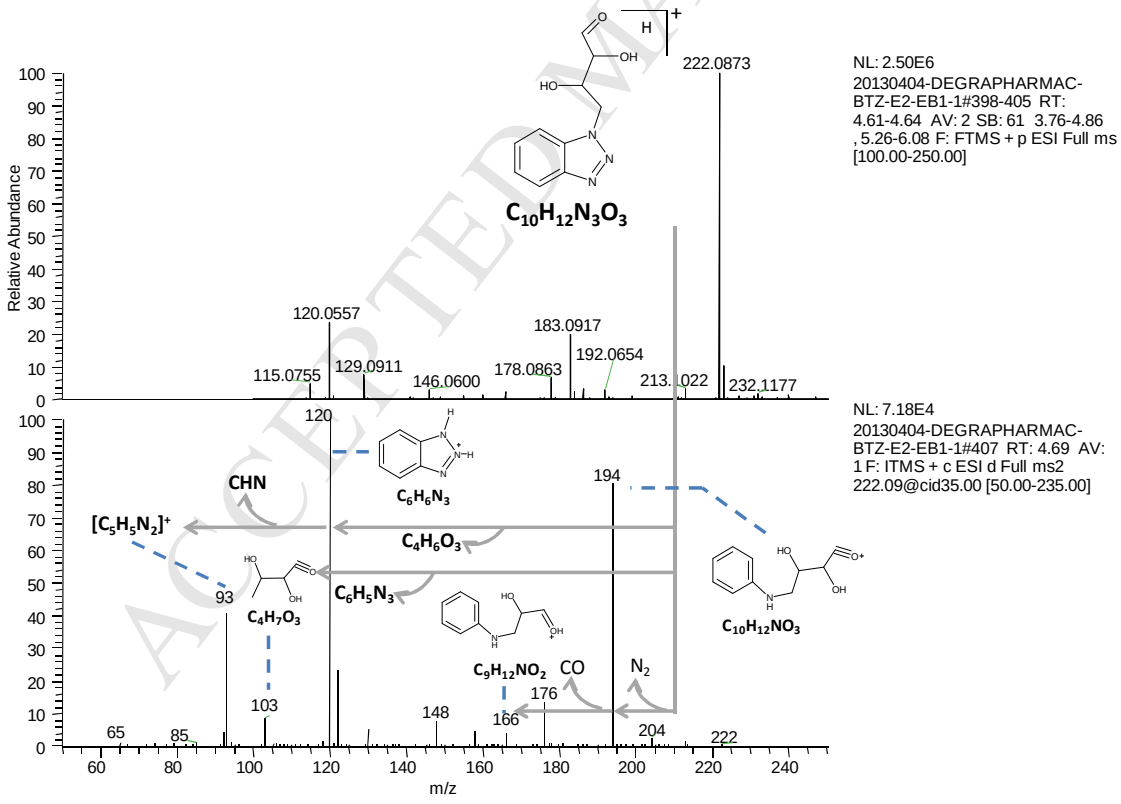


Figure 5: (A) Mass spectra of TP 178 A from BTZ (via HRMS) and the proposed structural fragmentation based on MS^2 experiments (via LTQ). **(B)** Mass spectra of TP 222 from BTZ (via HRMS) and the proposed structural fragmentation based on MS^2 experiments (via LTQ).

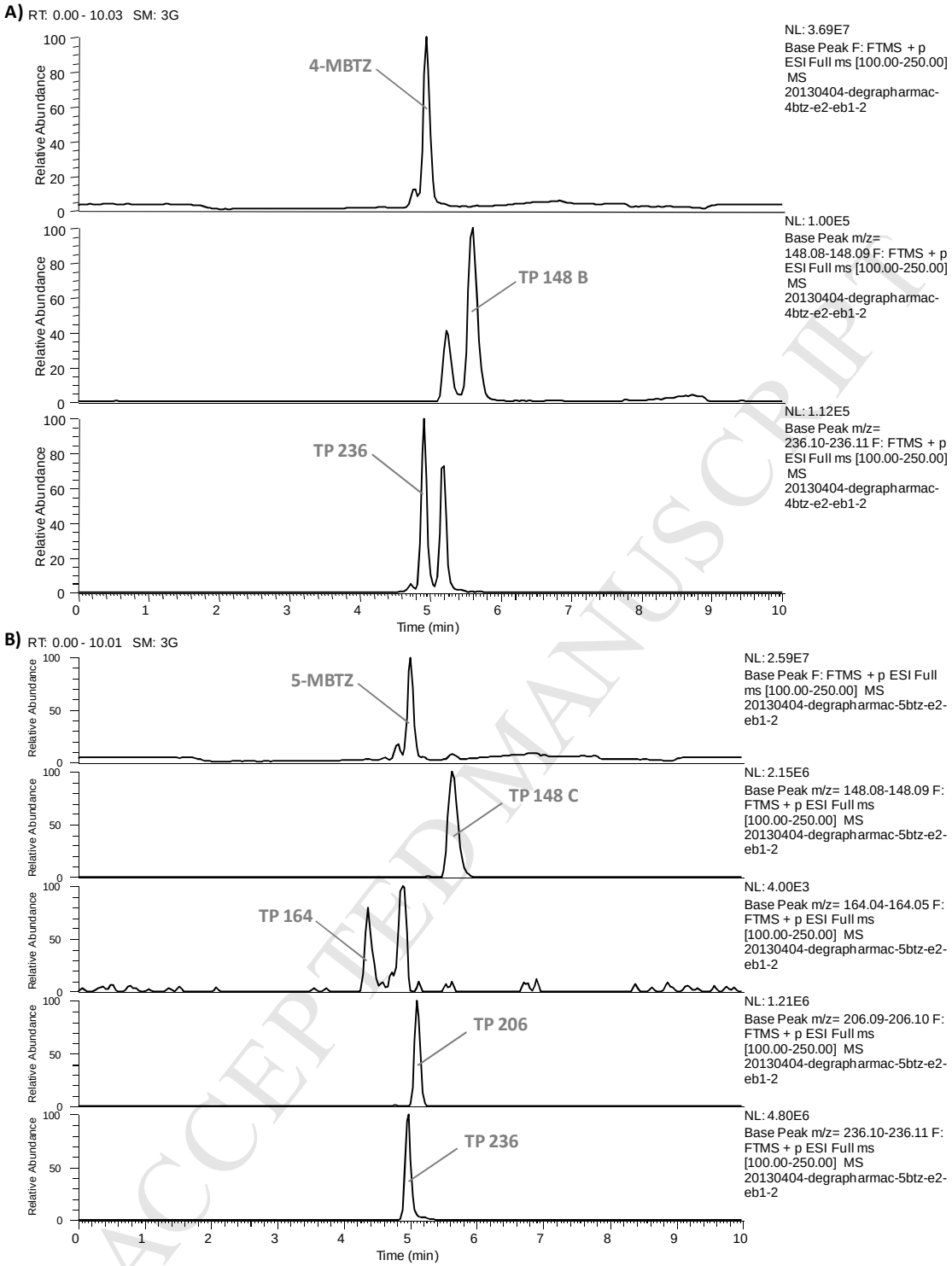
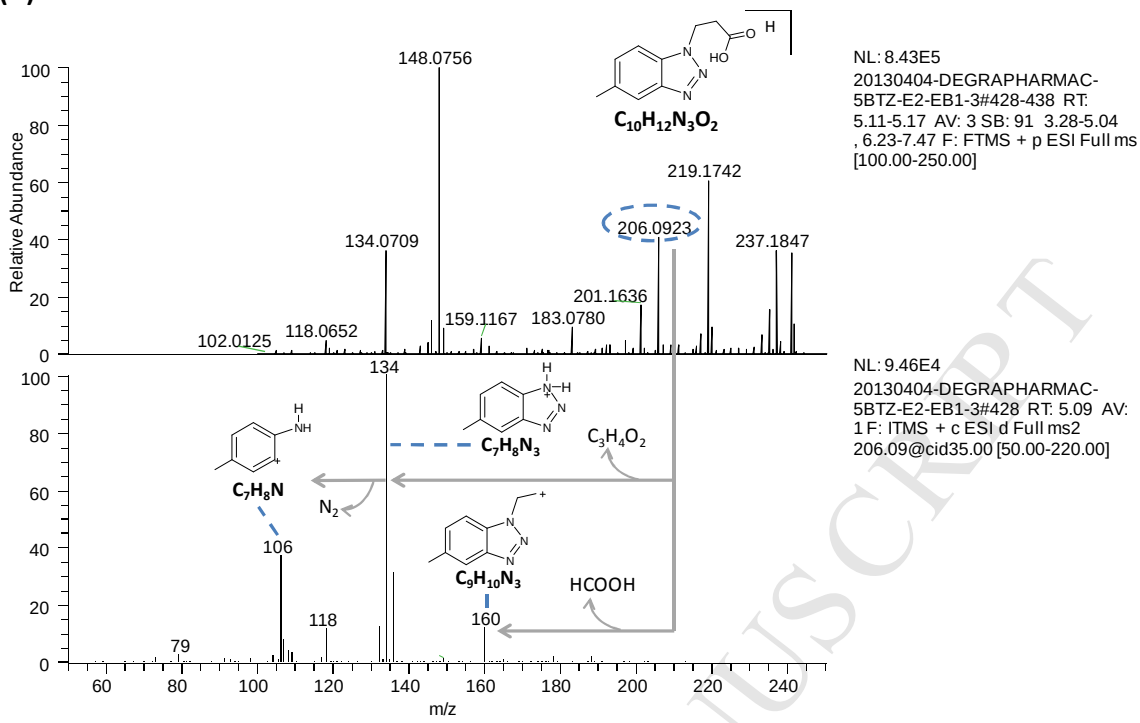


Figure 6: Full scan chromatograms (m/z=100-250 Da) of 10 mg L^{-1} of A) 4-MBTZ and B) 5-MBTZ treated with *T. versicolor* after 9 days with the presence of glucose and extracted ion chromatograms of the postulated TPs (A for TPs coming from 4-MBTZ and B for those coming from 5-MBTZ).

(A)



(B)

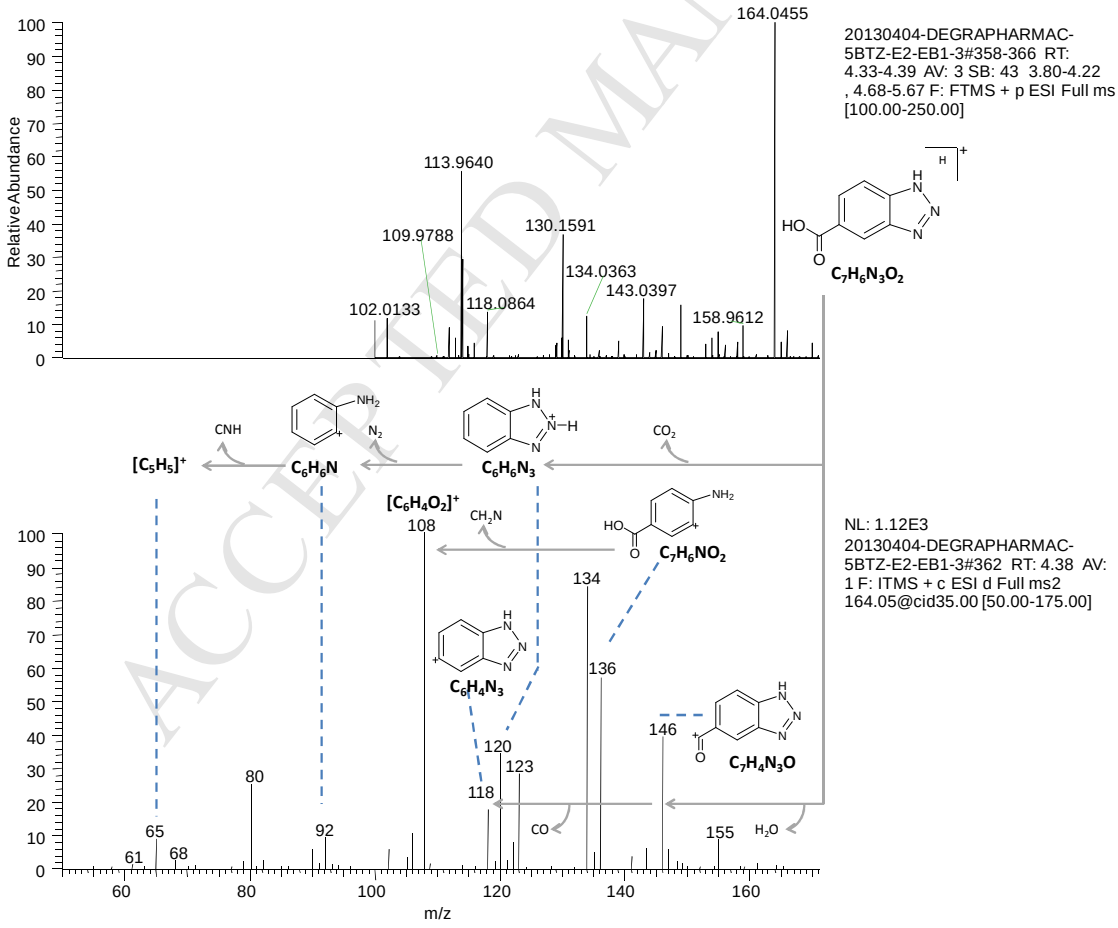


Figure 7: (A) Mass spectra of TP 206 from 5-MBTZ (via HRMS) and the proposed structural fragmentation based on MS^2 experiments (via LTQ). **(B)** Mass spectra of TP 164 from 5-MBTZ (via HRMS) and the proposed structural fragmentation based on MS^2 experiments (via LTQ).

Highlights

- Bioremediation of wastewaters by fungi treatment with *Trametes versicolor*
- Removal of endocrine disrupting compounds
- Study of degradation of benzotriazoles and transformation products generated
- Liquid chromatography coupled to a hybrid linear ion trap - high resolution mass spectrometer (Orbitrap)