



Combining biological processes with UV/H₂O₂ for metoprolol and metoprolol acid removal in hospital wastewater



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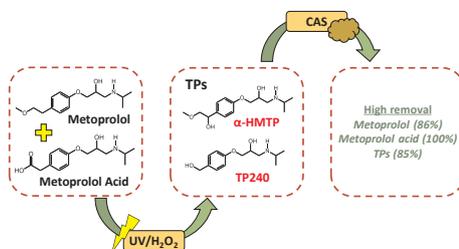
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HIGHLIGHTS

- The transformation of MTP and MTPA was studied in single and combined treatments.
- UV/H₂O₂ single treatments achieved total compound removal in fortified pure water.
- The combination of two treatments was needed to achieve a high removal in HWW.
- UV/H₂O₂ + CAS combination attained the highest removal in fortified HWW.
- The *in vitro* toxicity assays pointed out the presence of some hazardous TPs.

GRAPHICAL ABSTRACT



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ABSTRACT

The transformation products (TPs) of water contaminants generated during wastewater treatment can sometimes be equally or even more hazardous than the parent compounds. Therefore, for a comprehensive assessment of removal efficiency of a water treatment technology, it is mandatory to monitor not only the pollutants but also of their TPs. However, this type of evaluation studies is lacking in the case of water combined treatments. In this study, the elimination of metoprolol (MTP), metoprolol acid (MTPA) and the TPs generated was evaluated in pure water and hospital wastewater (HWW) using UV/H₂O₂ before and after fungal (FG) or conventional activated sludge (CAS). The major transformation pathways were suggested in terms of transformation of the parent compounds through bio-transformation and photo-transformation mechanisms. The results reveal an extended removal of MTP, MTPA and TPs after UV/H₂O₂ single experiment treating spiked pure water at 2.5 mg/L, without increasing the treated effluents toxicity. However, combined treatments were required to achieve similar removal percentages in spiked real HWW at 2.0 µg/L: while AOPs combined with FG exhibited lower removal efficiencies with generation of persistent intermediates (such as α-HMTP and TP240), AOPs combined with CAS attained the higher persistent TPs removal. In particular, AOP + CAS was classified as the most effective combination for HWW with the highest removal of the parent compounds (86% for MTP and 100% for MTPA), of the intermediates generated (up to 85%) and with a low presence of toxic TPs (such as O-

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DMTP). This study demonstrates that comprehensive evaluation of the intermediates generated along water treatment technologies is highly recommended to successfully evaluate their removal efficiencies.

1. Introduction

The occurrence of pharmaceutical active compounds (PhACs) and their metabolites in water bodies has become an imperative concern due to their potential impact on both environment and human health [1–3]. Every day, large quantities of wastewater are discharged into municipal sewer system not only from domestic origin but also from industrial and hospital sources [4]. In particular, hospital wastewater (HWW) has been identified as responsible for introducing high loads of contaminants with potentially toxic effects in aquatic ecosystems [5]. The incomplete elimination of the persistent pollutants in centralized conventional municipal wastewater treatment plants (WWTPs) allows the release of these contaminants into the environment [1,6]. In this sense, dedicated on-site wastewater treatment of HWW has been widely recommended by several authors [5,7]. However, specific directives or guidelines in Europe for the management of hospital effluents are missing and the implementation of full-scale HWW treatment has been introduced only in few cases [5,8]. The use of tailored and dedicated treatment technologies could stir up HWW decentralized treatment.

The use of biological treatments has been widely suggested as a more eco-friendly solution for the removal of organic pollutants from complex wastewater matrices, involving low operational costs and low energy consumption [9–12]. Conventional activated sludge treatments (CAS) cannot always provide satisfactory results in terms of PhACs removal, but they are still the most commonly applied worldwide, and to be considered as a reference to be compared with [13]. Among the different biological based solutions, fungal treatments have been pointed out to provide high removal rates for many PhACs thanks to the generation of unspecific extracellular enzymes able to degrade persistent organic pollutants [14–16]. However, incomplete elimination of non-biodegradable pollutants and bio-recalcitrant intermediates generated in fungal treatments have also been reported [17]. For the remediation of low biodegradable effluents, highly reactive and non-selective advanced oxidation processes (AOPs), such as UV/H₂O₂, have been widely suggested as suitable treatment solutions [18–20]. Despite this, AOPs are characterized by their relatively higher operating costs (compared to biological treatments) [21–23], especially in complex matrices [24]. Therefore, they require larger energy and chemical reagents demand to attain total compound mineralization [13].

Applying AOPs as pre-treatment steps to biological treatments has been suggested to convert the contaminants into more readily biodegradable intermediates and, hence, reducing the total cost of the treatment process [18,25,26]. Among them, solar photo-Fenton, ozonation and UV/H₂O₂ treatments have been applied as tertiary treatments for the remediation of micropollutants present in real municipal WWTP effluents [27,28]. However, the effectivity of combined treatments will always depend on the type of water effluent to be treated [24]. In addition, to properly evaluate the most effective combination for total pollutant mitigation, not only the removal of the parent compounds should be considered but also the presence of the major metabolites and the generated transformation products (TPs) [24,29]. Even though many studies have been focused on the applicability of combined treatments for decontamination of wastewater [13,19,26,30–35], only few of them are related to combined treatment of HWW [36]; and none of them have elucidated tentative transformation pathways of individual PhACs towards a better understanding of the total extent on pollutant removal.

Among the pollutants present in HWW, metoprolol (MTP) has been widely detected in wastewater due to its high consumption for hypertension and cardiovascular diseases [37]. In terms of associated

environmental risk, its presence in natural waters has been related to cardiovascular dysfunctions, such as alteration of the heart rate, in aquatic organisms (e.g. *Daphnia magna*) [38]. Likewise, specific effects on scoliosis and growth retardation were reported in zebrafish embryos when exposed to MTP above 12.6 mg/L for 72 h [39]. Up to now, MTP has been pointed out as a compound of high consumption [40], and has been detected in raw wastewater up to 0.2–2.0 µg/L [41,42]. Removals percentage reported for these compounds in conventional WWTPs are usually low, between 0% and 36% [42–44]. After its consumption, the excretion of MTP as metoprolol acid (MTPA), via renal excretion, constitutes up to 60–65% of the initial MTP dose [45–47]. Therefore MTPA, being MTP major human metabolite, is an additional important pollutant to be studied in wastewater treatment [48]. Up to now, there are no regulations limiting discharges of these PhACs in WWTP effluents for those compounds [3]. In 2020, the Council of the European Union adopted a new regulation for efficient water reuse. The substances of emerging concern, including pharmaceutical active compounds, are mentioned in the risk assessment section but without any threshold value yet [49]. Therefore, the evaluation of PhACs presence, effective removal and potential discharge concentrations into the environment are extremely valuable to provide information for forthcoming studies in the field [50]. While some studies reported MTP and MTPA recalcitrance in conventional activated sludge (CAS) [44,51], others demonstrated its biodegradability in fungal (FG) treatment [17]. However, the complete elimination of their TPs has never been reported by any of the studied treatment.

Measuring the unknown intermediates in the evaluation of treatment removal efficiencies is critical due to their potential hazardous effects. Thus, even when complete elimination of the parent compounds is attained, the presence of these TPs should be also considered. To the best of authors' knowledge, this is the first time that four different combinations of treatments (UV/H₂O₂ treatment before/after CAS or FG) were investigated in terms of presence and removal of MTP, MTPA and their TPs in real HWW. The generated intermediates were identified with an automated suspect screening approach which allowed to comprehensively study their presence and transformation pathways along the combined treatments. This study demonstrates that combined treatments are a valuable solution towards a complete removal of MTP, MTPA and their TPs.

2. Methods and materials

2.1. Chemicals and reagents

Metoprolol tartrate salt (MTP) (Sigma-Aldrich), O-desmethylnmetoprolol (O-DMTP), metoprolol acid (MTPA) and α -hydroxymetoprolol (α -HMTP) (Toronto Research Chemicals); and atenolol-d⁷ internal standard (CDN isotopes, Quebec, Canada) were purchased at high purity grade (> 98%). Standard solutions were prepared on a weight basis in methanol (at a concentration of 1000 mg/L) and stored at –20 °C. Ultra-pure water and acetonitrile LiChrosolv grade were supplied by Merck (Darmstadt, Germany). Working standard solutions containing all pharmaceuticals and labeled internal standard were prepared in methanol/water (10:90, v/v). All FG nutrients used were selected regarding the optimum conditions reported previously [17]. For CAS experiments, organic solution (sodium acetate, propionate and yeast extract), phosphate buffer, trace and inorganic solution were added as described elsewhere [52]. For AOP experiments, the titanium (IV) oxysulfate reagent used was 1.9–2.1% from Sigma-Aldrich. The H₂O₂ reagent was 30% w/v 100 vol stabilized PRS from Panreac [53].

2.2. Experimental set-up

UV/H₂O₂ oxidation processes were combined in parallel with FG and CAS treatments as presented in Fig. 1 (each treatment technology is described in detail below). The experimental scheme was first applied treating pure water fortified with MTP and MTPA at initial concentrations of 2.5 mg/L each. Samples were collected at initial experimental time, prior to perform each individual treatment (to ensure reproducibility of samples between treatments) and after each individual treatment to evaluate treatment efficiency. Samples collected were directly injected into the liquid chromatography system coupled to high-resolution mass spectrometry (LC-HRMS) for monitoring of target compounds and TPs. Subsequently, the same experiments were performed in fortified HWW at initial concentration of 2.0 µg/L (to ensure their presence in real wastewater conditions and allow to properly evaluate their elimination) of MTP and MTPA. Samples preparation and analysis are presented in section 2.3 and 2.4, respectively.

2.2.1. Fungal treatment (FG)

Trametes versicolor (ATCC#42530) was maintained on 2% malt agar slants at 25 °C until use. The mycelial suspension of *T. versicolor* and pellets were obtained as previously described [54,55]. Air-fluidized bed bioreactors were operated as a batch per duplicate for 7 days. Fluidized conditions in the reactors were maintained by using 1 s air pulse every 4 s, resulting in an aeration rate of 0.8 L/min. Nutrients for maintenance, namely, glucose and NH₄Cl, were added with a molar C/N ratio of 7.5 at *T. versicolor* consumption rate to both reactors (1.2 g / (g DCW·d)). Temperature was maintained at 25 °C and pH was controlled at 4.5 by HCl 1 M or NaOH 1 M addition. Samples were collected, filtered through 0.45 µm PVDF filters (Millipore, Barcelona, Spain) and frozen in glass containers for pure water experiments and PET containers for HWW experiments (for safety handling in case of breakage). All these parameters were selected based on the optimum conditions reported previously [17].

2.2.2. Activated sludge treatment (CAS)

Activated sludge batch experiments were performed using a 1 L lab-scale Applikon stirred tank reactor coupled with a proportional-integral-derivative (PID) controller for pH, oxygen and temperature. Bioreactors were operated as a batch for 24 h and each experiment was

conducted in duplicate. The activated sludge originated from Celrà WWTP (Catalonia, Spain, 20.000 equivalent inhabitants, 2,100 m³/d), with a hydraulic retention time (HRT) of 48 h and a sludge retention time (SRT) of 20–22 days. The biomass concentration during the experiments was 3 gTSS/L (0.71 ratio VSS/TSS) and aerobic conditions (> 2.5 mg O₂/L) were achieved with continuous air supply. The pH and temperature were maintained at 7.5 and 25 °C, respectively. Activated sludge after treating pure water or HWW was centrifuged 4 min at 8000 RPM (20 °C), prior to perform AOP post-treatment experiments. Mixed liquor samples were filtered (0.45 µm pore size Millex PVDF) and immediately frozen in glass containers for pure water experiments and PET containers for HWW experiments (for safety handling in case of breakage). All these parameters were selected based on the optimum conditions needed for this treatment [44].

2.2.3. UV/H₂O₂ treatment (AOP)

Photo-oxidation treatment processes were performed in duplicate by using an UV Laboratory Reactor System from UV-Consulting Peschl®, an immersion-type photo-reactor of approximately 550 mL. The UV lamp consisted in a 15 W Heraeus Noblelight TNN 15/32 low-pressure mercury vapor lamp emitting at 254 nm. The photo-reactor was mixed with a magnetic stirrer to assure the homogeneity of the solution. Moreover, the photo-reactor was covered with aluminum foil in order to minimize the loss of UV light and avoid any reflections. Potassium ferrioxalate actinometry [56] was used as in previous work in order to characterize the intensity of the light of the UV lamp, resulting in an irradiance of 0.049 W/cm² [53]. The experiments were carried out with 500 mL of wastewater, 15 mg/L of H₂O₂ and a reaction time of 10 min that corresponds to an UV dose or intensity of 29.4 J/cm². The H₂O₂ concentration was analyzed by a spectrophotometric method using titanium (IV) oxysulfate as reported previously [57]. A stoichiometric excess of 20% of sodium thiosulfate was added to stop the oxidation reaction in the collected samples [24]. Then, samples were filtered through 0.45 µm PVDF filters at initial and final time for further sample treatment and analysis. Samples were collected and frozen in glass containers for pure water experiments and PET containers for HWW experiments (for safety handling in case of breakage). All these parameters were selected based on the treatment operative conditions applied previously [24].

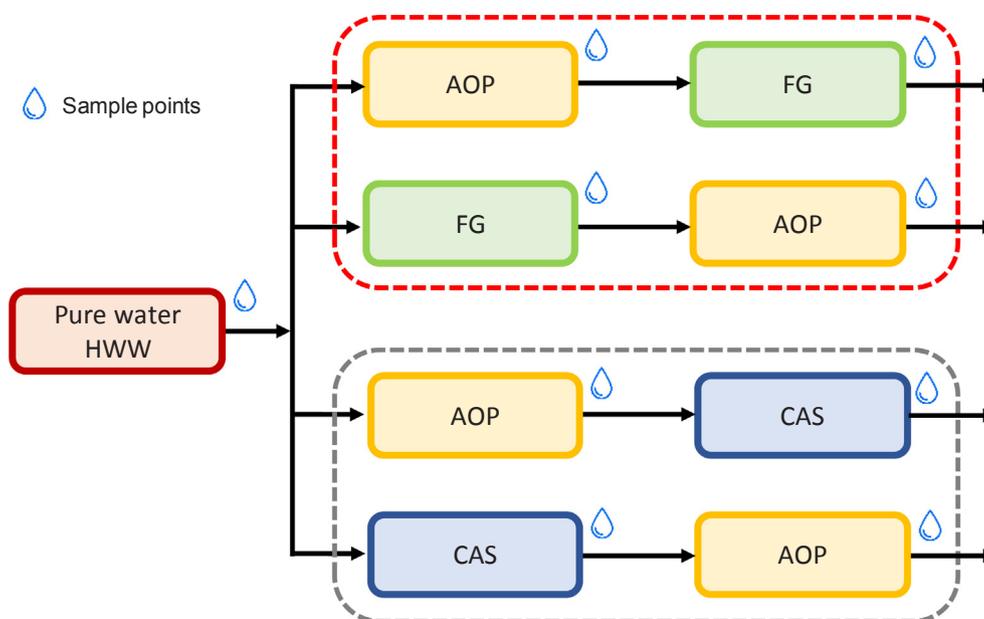


Fig. 1. Scheme of the experimental set-up with UV/H₂O₂ oxidation process (AOP), fungal (FG) and activated sludge (CAS) combined for treating fortified pure water and HWW.

2.3. Hospital wastewater and sample treatment

Hospital wastewater (HWW) was collected directly from the sewer manifold of Sant Joan de Déu Hospital (Barcelona, Catalonia) in the NE of Spain. Fresh samples were collected and pretreated with a coagulation-flocculation process as described previously [58]. The pretreatment used 43 mg/L of coagulant Hyfloc AC50 and 4.8 mg/L of flocculant Himoloc DR3000, both kindly provided by Derypol, S.A. (Barcelona, Spain). Physicochemical conditions of initial HWW were as follows: COD, 210.4 mg/L; TOC, 65.9 mg/L; N-NO₂, 1.6 mg/L; N-NO₃, 5.9 mg/L; P-PO₄, 2.0 mg/L; and N-NH₄, 25.9 mg/L. A volume of 25 mL of raw HWW and 50 mL of treated HWW were pre-concentrated through Solid Phase Extraction in Oasis HLB cartridges (60 mg, 3 mL) (Waters Corp. Mildford, MA, USA) following the methodology previously described elsewhere [59]. The extracts were kept in 1 mL of methanol adding 10 µL of a 1 ng/µL of the isotopically labeled standard. The extracts were further pre-concentrated to facilitate TPs detection through evaporation and reconstitution in 150 µL of methanol:water (10:90, v/v) for LC-LTQ-Orbitrap-MS/MS analysis.

2.4. Instrumental analysis

The detection and identification of the parent compounds and TPs generated in each treatment step were performed with the suspect screening methodology previously described [24]. A liquid

chromatography system coupled to a high-resolution mass spectrometer HPLC-LTQ-Orbitrap Velos™ (Thermo Fisher Scientific) was used for the analysis of the samples. The chromatographic separation was performed using a ZORBAX Eclipse XDB-C18 (150 mm × 4.6 mm, 5 µm) for a total run time of 17 min. The instrument was equipped with a heated electrospray ionization source (HESI-II) and analyses were performed in positive and negative mode. As negative mode showed poor ionization efficiencies, data processing was performed for positive mode only. Samples were acquired in Data Dependent Acquisition mode through full scan from 100 to 1000 mass-to-charge (*m/z*) range at a resolving power of 60,000 FWHM. Selection of the most intense ions (Top 3) for MS/MS full scan fragmentation was performed in a second event and recorded at 30,000 FWHM from 50 to 500 *m/z* range. MS/MS fragmentation modes were investigated by using collision-induced dissociation at 30 eV CE (*Q* = 0.250 and an activation time of 30 ms) in an isolation width of 2 Da. The entire system was controlled via Aria software, version 1.6, under Xcalibur 2.1 software.

The data acquired were processed by an integrated suspect screening methodology using Compound Discoverer 3.0 (Thermo Fisher Scientific). The methodology combines comparison with reference standards, in-house databases, compound prediction tools and literature sources for chemical identification. Detailed workflow regarding the analytical suspect screening strategy applied is presented in Fig. S1. In addition, specific parameters selected to ensure reliability on chemical identification and transformation pathways are presented in

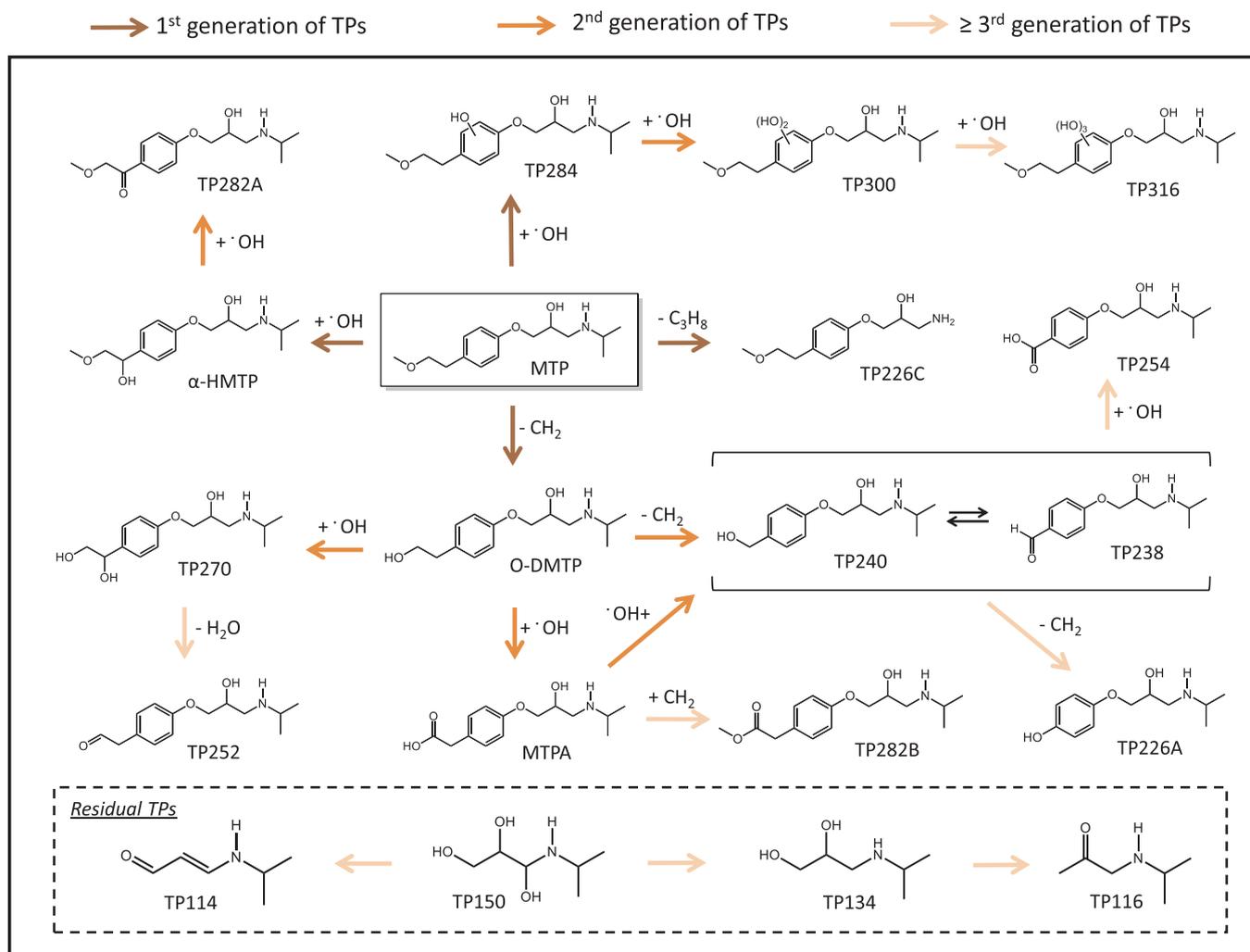


Table S1. The relative presence (%) of the target pollutants MTP and MTPA was calculated as the area of MTP or MTPA (at a given time), relative to the area of MTP or MTPA before any treatment (at initial time):

$$MTP(A)(\%) = \frac{Area_{MTP(A)}^x}{Area_{MTP(A)}^0} 100 \quad (1)$$

The TPs presence was calculated as the sum of the areas of all the detected TPs generated from MTP and MTPA degradation (at a given time), relative to the sum of the areas of spiked compounds (MTP and MTPA) before any treatment (at initial time):

$$TP_{presence}(\%) = \frac{\sum_{i=1}^n Area_{TP_i}^x}{Area_{MTP}^0 + Area_{MTPA}^0} 100 \quad (2)$$

Additionally, the relative distribution of the intermediates

generated was calculated (Eq. (3)) as the area of each TP detected relative to the sum of areas of all detected TPs (at a given time). In this specific case, MTPA was considered as a TP since it can be also generated from the degradation of the parent compound MTP (as a 2nd generation TP).

$$TP_i \text{ distribution}(\%) = \frac{Area_{TP_i}^x}{\sum_{i=1}^n Area_{TP_i}^x} 100 \quad (3)$$

Finally, statistical comparisons between the effluents generated after the four combinations, tested in pure water and HWW, were performed to compare the generated TPs and their distribution in treated samples. In this context, spearman correlations were calculated through the function “cor” (Package “stats”, [60]) and the function “cor.mtest” (Package “corrplot”, [61]). Graphics were generated using the function “corrplot” (Package “corrplot”, [61]).

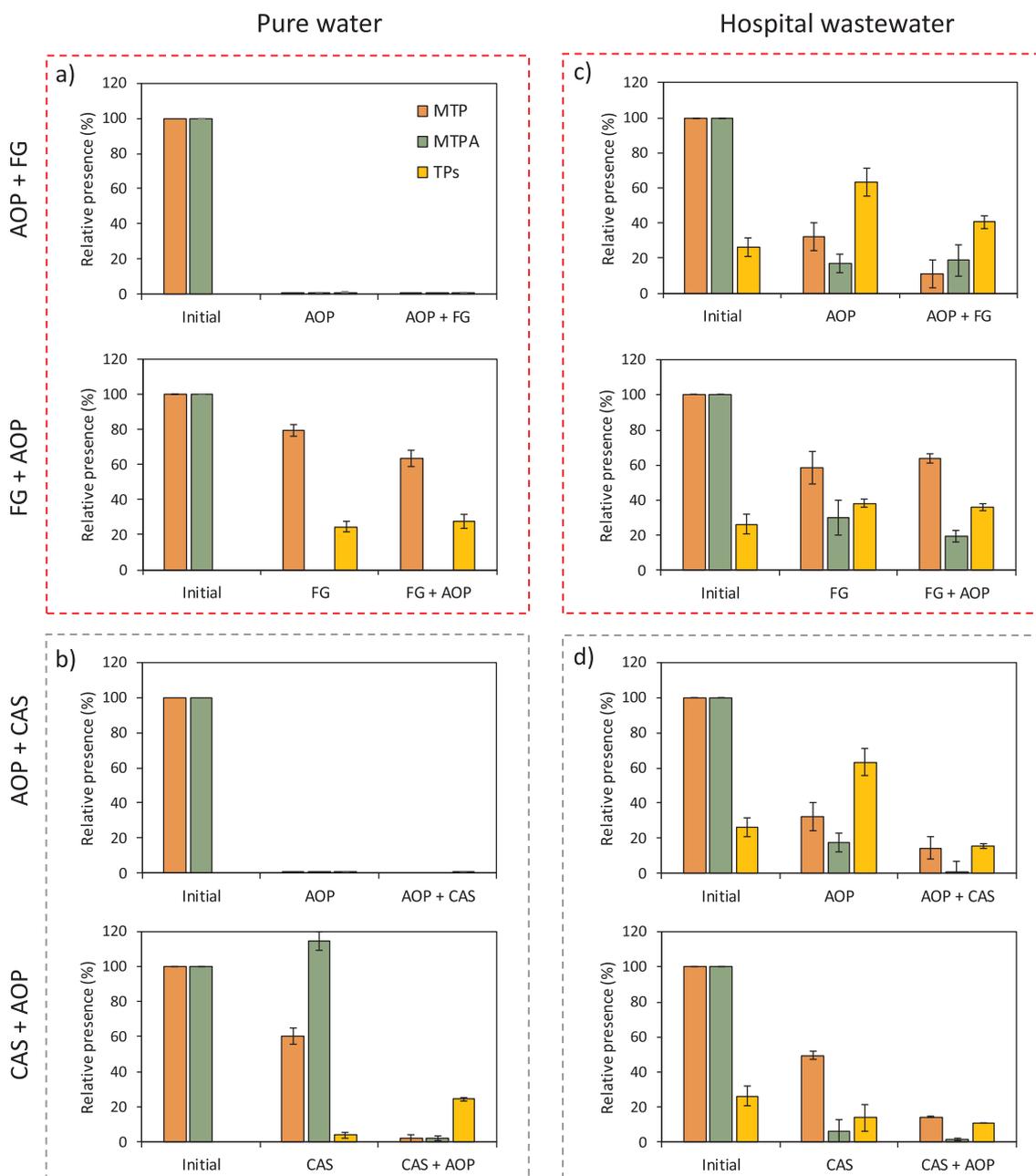


Fig. 3. Relative presence of MTP, MTPA (Eq. (1)) and TPs (Eq. (2)) in combined experiments treating fortified pure water and HWW: a,c) UV/H₂O₂ combined with FG treatment; and b,d) UV/H₂O₂ combined with CAS treatment.

2.5. Toxicological assessment

The ISO 11348-3 protocol [62] for testing bacterial bioluminescence (Microtox® bioassay) was used to measure the toxicity only in spiked pure water experiments where MTP, MTPA and their TPs generated were the only potential toxicants in samples (*in vitro* toxicity). This bioassay was not applied to real HWW samples, since other pharmaceuticals (different from MTP and MTPA) and many other compounds are present and potentially contributing to toxicity signal. All the collected samples in pure water experiments were introduced in

glass vials and centrifuged to remove possible interference from biomass fragments or solids in suspension. Then, the decay on emitted light was recorded after 15 min of samples contact with the bacterium *Vibrio fischeri*. The 50% effective concentration (EC₅₀) was expressed in dilution percentage. TU along the combined treatments was calculated as (TU = 100/EC₅₀) [63]. The concentration of sodium thiosulfate added after AOP experiments (to stop the oxidation reaction) was tested and had no toxic effect on luminescent bacteria [24].

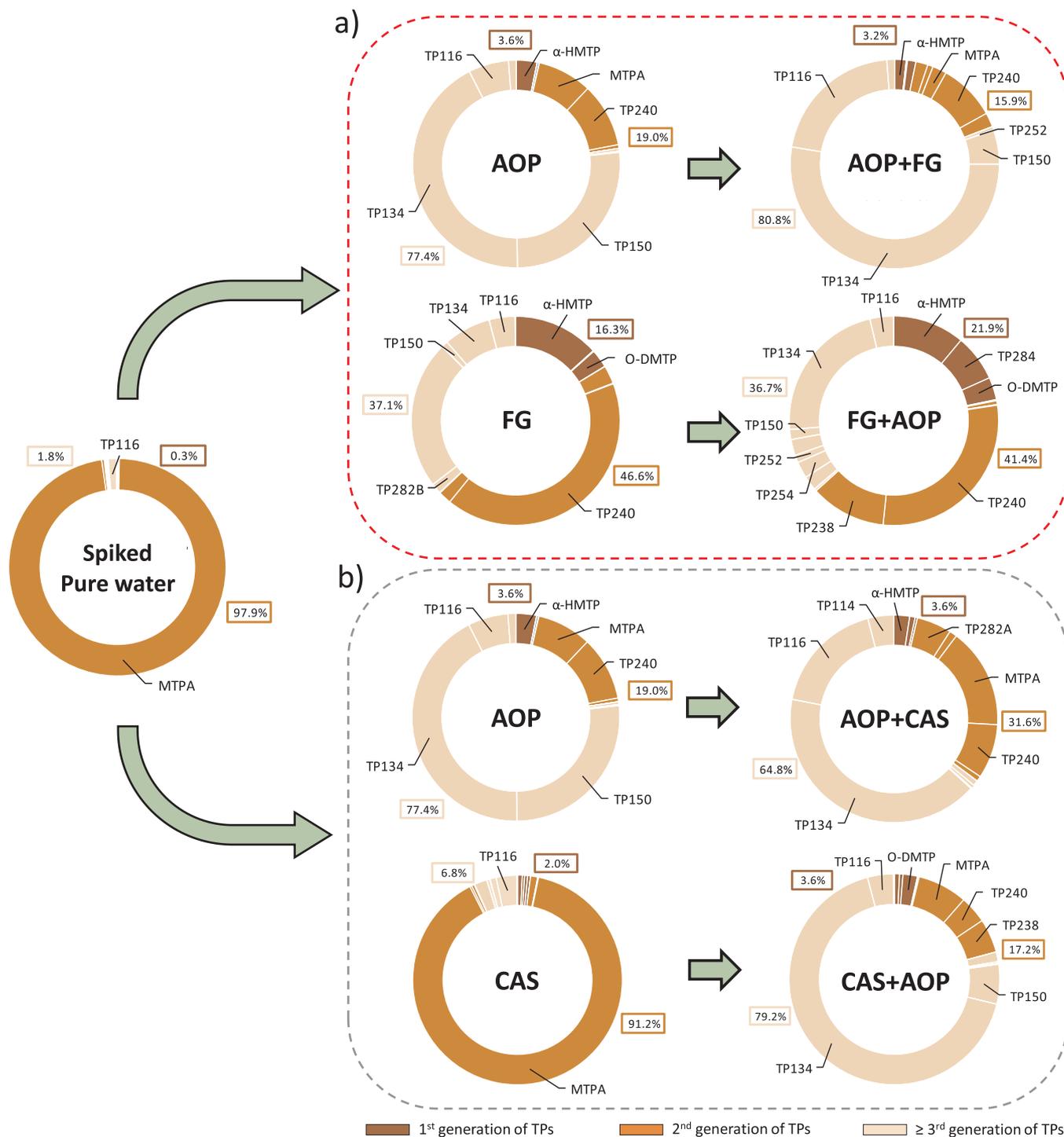


Fig. 4. The circles represent the relative distribution of intermediates in fortified pure water (Eq. (3)), classified regarding their degree on removal (1st, 2nd and ≥ 3rd generation expressed in percentage values, according to Fig. 2) in: a) UV/H₂O₂ combined with FG treatment; b) UV/H₂O₂ combined with CAS treatment.

3. Results and discussion

3.1. Identification of TPs and elucidation of transformation pathways

MTP and MTPA transformation was evaluated for each individual treatment in fortified pure water and HWW. Among the detected compounds, MTP and the human metabolites MTPA, O-DMTP and α -HMTP were confirmed by means of reference standards, retention time, compound exact mass and MS/MS fragmentation spectra. Nineteen intermediates (out of the 29 probable compounds comprised into the in-house database, Table S2), were detected in the samples by comparison of retention time, compound exact mass and MS/MS fragmentation spectra (no reference standard available). No additional TPs were found neither from the list of 356 compounds predicted (Table S3), generated using compound prediction tools of the software Compound Discoverer 3.0. (Table S1), nor from list of 39 compounds collected from literature (Table S4).

The major transformation pathways were suggested from successive hydroxylation, oxidation and O-dealkylation of MTP and MTPA chemical structures (Fig. 2). Among them, the main transformation pathway detected in this study was related to the formation of the intermediates TP238 and TP240 after rapid O-demethylation of MTP structure and benzylic hydroxylation, through the formation of a radical intermediate of O-DMTP in biological treatments [64,65]. These intermediates were also observed in physico-chemical treatments through oxidative reactions involving the attack of highly reactive radicals on the ether side chain of the parent compound [66]. TP238 and TP240 formation was reported not only from the parent compound MTP but also from its main human metabolite MTPA [17]. In this last study, TP238 and TP240 were mainly transformed from MTPA fungal biodegradation into TP254, through the oxidation of the primary alcohol and the aldehyde intermediate onto a carboxylic acid, and detected at high concentration [17]. In the case of CAS, MTP biodegradation resulted in the generation of MTPA, which was the major generated intermediate, and in some cases classified as persistent [44,51]. α -HMTP was both reported as human metabolite, generated after pharmaceutical consumption, and TP in biological treatments [44,51]. α -HMTP is usually persistent, it was detected in influent WWTPs (at 36 ng/L), and consequently it may be classified of important concern [17,44]. Further oxidation of α -HMTP to TP282A was reported at much lower concentration. Alternatively, the binding of the hydroxyl radical in the MTP aromatic ring may lead to the formation of TP284, which is further oxidized to TP300 and TP316 [66]. These TPs were especially relevant in treatments with plausible generation of hydroxyl radicals (e.g. fungi and AOPs, [17,66,67]). It is important to mention that TP284, TP300 and TP316 were generated only from MTP degradation and not from its main metabolite MTPA [17,66,67]. Finally, another worth mentioning intermediate is TP134, generated from the O-dealkylation of the TPs maintaining the secondary amine on their chemical structure. TP134 is a residual TP and it may be considered as an indicator of the removal extent of the generated TPs [17,24].

All detected TPs were classified based on the number of transformations undertaken from the parent compound MTP (1st, 2nd or \geq 3rd generation of TPs). Only those TPs with relative distributions \geq 1% were further considered for discussion. Detailed information of TPs distribution percentages is presented in Table S5–S12.

3.2. Combined treatments of fortified pure water

3.2.1. AOP + FG/FG + AOP treatments of fortified pure water

Overall results obtained from the combined experiments in fortified pure water for AOP + FG and FG + AOP treatments are presented in Fig. 3a and Fig. 4a. The combination strategy where biological FG treatment was placed after AOP treatment (AOP + FG) was very effective for the elimination of the parent compounds (Fig. 3a). Almost complete elimination (99.9%) of MTP and MTPA was achieved,

although most of the elimination was obtained by AOP alone as a first step (99.6%). In terms of relative presence of all intermediates generated, low values were observed, with a percentage of 0.8% and 0.6% accounted after AOP and AOP + FG, respectively. These results demonstrate the high capability of AOP treatment to achieve high MTP and MTPA removal and almost extended TP removal in pure water matrix. In fact, a high relative distribution percentage of the \geq 3rd generation TPs (Fig. 4a) was observed after both AOP + FG experiment (80.8%) and AOP single treatment (77.4%). Among them, the most abundant intermediates (TP150, TP134 and TP116) can be classified as residual chemical structures, near to total compound removal (Fig. 2). TP150 was previously classified as a mutagenic compound and TP116 as a persistent compound using *in silico* estimations (due to the aliphatic secondary amines in molecular structure), both likely to increase the hazards on treated water [24]. However, no acute toxicity values were observed using *in vitro* experiments after the treatment(s), probably due to their low TP presence in treated effluents (Fig. 3a).

The combination FG + AOP was much less effective than AOP + FG for MTP removal (from 20.4% with FG alone up to 36.4% with FG + AOP, Fig. 3a) whereas MTPA was completely removed after FG treatment alone. In terms of relative presence of TPs, the percentage value after FG treatment (24.6%) was very similar to the values obtained after FG + AOP (27.6%). Altogether, these values were much higher than in AOP + FG combination (Fig. 3a). Moreover, there were less \geq 3rd generation intermediates (36.7%) and more 1st and 2nd generation TPs (21.9% and 41.4%, respectively, Fig. 4a). The overall low efficiency of AOP treatment in the configuration FG + AOP might be attributed to the polysaccharide mucus secreted by fungi during fungal treatment, which can affect AOP oxidation afterwards. Considering the generated intermediates, the high contribution of TP240 (41.6%) and its oxidized compound TP254 (23.0%) in FG experiments was previously reported in *Trametes Versicolor* [17]: TP240 was mostly generated from MTPA biotransformation while α -HMTP from MTP only [17]. The slight toxicity measured using *in vitro* experiments, from the initial time (0.0 TU) to FG treated effluents (3.2 TU) and after FG + AOP experiments (4.3 TU), might be explained by the presence of O-DMTP after FG treatment alone (2.8%) and after FG + AOP experiments (3.5%). Actually, O-DMTP was previously described to be 3.6 times more toxic than the parent compound MTP in *vibrio fischeri* bioassays [44]. However, the generation of unknown toxic metabolites from fungi (non-related to MTP and MTPA degradation) cannot be discarded.

3.2.2. AOP + CAS/CAS + AOP treatments of fortified pure water

Overall results obtained from AOP + CAS and CAS + AOP combined experiments in fortified pure water are presented in Fig. 3b and Fig. 4b. AOP + CAS allowed complete elimination of MTP and MTPA (Fig. 3b). In comparison to AOP + FG treatment, this combination slightly reduced the proportion of intermediates in treated effluents from 0.8% after AOP to 0.4% after AOP + CAS treatment. In terms of relative distribution of generated intermediates (Fig. 4b), 2nd generation of TPs increased from 19.0% after AOP to 31.6% after AOP + CAS (15.7% more than in AOP + FG), suggesting the generation of some MTP persistent intermediates after CAS treatment. Indeed, relative MTPA contribution increased 7.1% moving from AOP to AOP + CAS. This is in agreement with some authors indicating the recalcitrant presence of MTPA after CAS experiments along with its generation during MTP degradation, up to 40% of initial MTP concentration (1 mg/L) after 48 h [44,51]. It is important to highlight that the presence of intermediates after AOP + CAS treating fortified pure water treatment was small (0.4%) compared with the spiked parent compounds at 2.5 mg/L, highlights the effectiveness of AOP + CAS combination. Although almost a complete removal of MTP and MTPA and TPs was already achieved by only AOP treatment, CAS as a post-treatment step additionally provided an extended transformation of TP150 into TP116 through the intermediate TP134 (Fig. 2, Fig. 4b), as

also observed after AOP + FG combination (Fig. 4a). As in AOP + FG, no toxic effects were observed after *in vitro* experiments in AOP + CAS effluents.

Considering the last coupling CAS + AOP, MTP and MTPA removal efficiency was high (97.8% and 97.7%, respectively) but not complete (Fig. 3b). In terms of TP presence, the relative amount after CAS + AOP treatment (24.3%) was similar to that after FG + AOP treatment (27.6%), though very different TP distribution was observed (Fig. 4b): the presence of intermediates from ≥ 3rd generation after CAS + AOP treatment was higher (79.2%) than after FG + AOP (36.7%). Those differences between FG + AOP and CAS + AOP might be related to the minor complexity of the matrix after CAS (no mucus generated like it is

with FG), allowing a better performance of CAS + AOP. It is also important to highlight how the presence of MTPA even increased after CAS alone, reaching a relative percentage values of 114.4%. MTPA has been described as a major 2nd generation TP in CAS treatment in previous studies [44,51]. However, MTPA was easily removed when coupling CAS + AOP. In addition, no toxic effects were observed after CAS + AOP experiments using the *in vitro* bioassays.

Therefore, UV/H₂O₂ can be considered as the treatment of choice when treating simple matrices, such as fortified pure water. Nevertheless, the implementation of an additional CAS treatment (both before or after AOP treatment) allowed similar extent of pollutant elimination (MTP, MTPA and TPs) without adding any toxic effect in

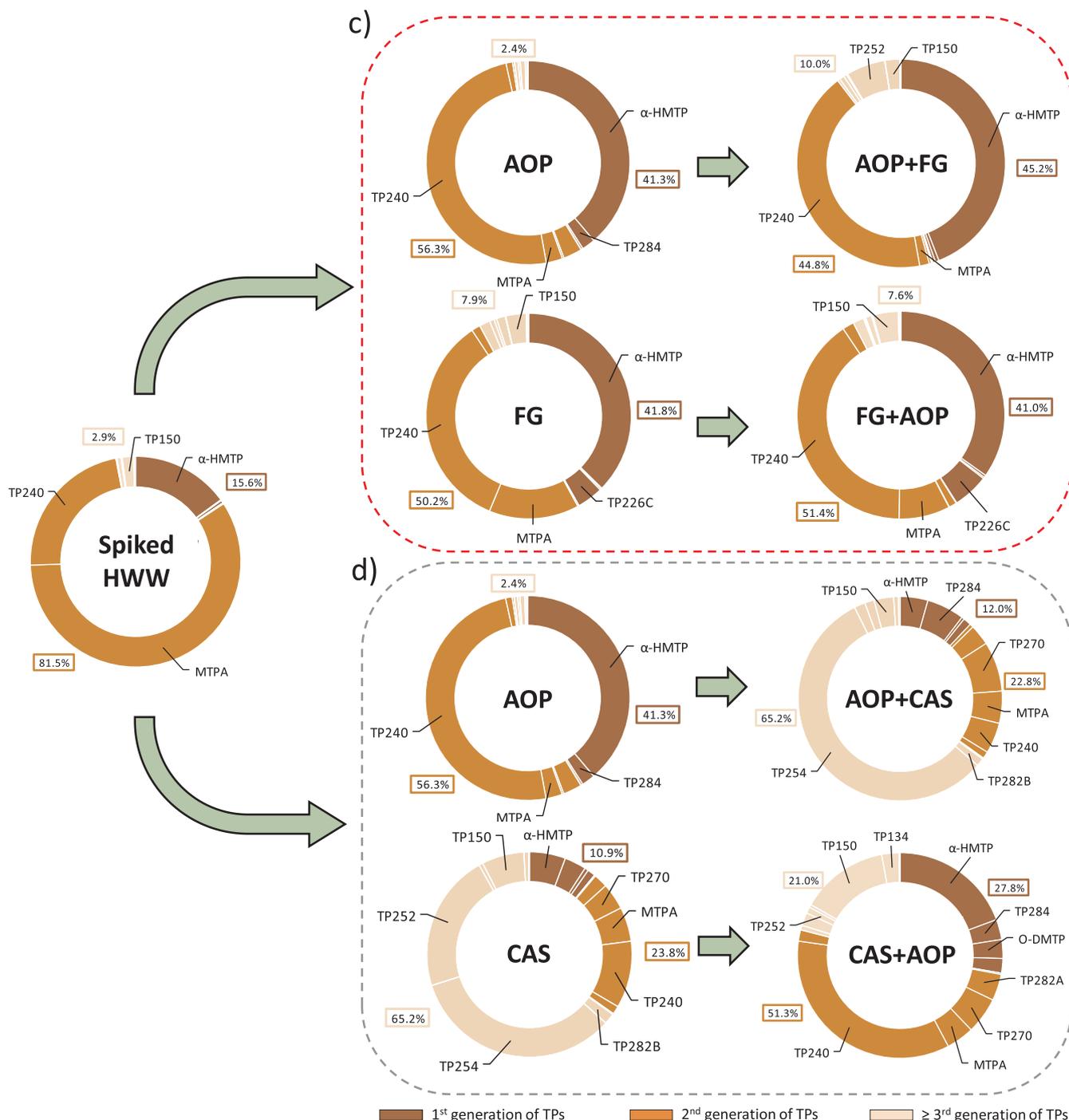


Fig. 5. The circles represent the relative distribution of intermediates in fortified HWW (Eq. (3)), classified regarding their degree on removal (1st, 2nd and ≥ 3rd generation expressed in percentage values, according to Fig. 2) in: c) UV/H₂O₂ combined with FG treatment; d) UV/H₂O₂ combined with CAS treatment.

treated effluents. However, an increase in the *in vitro* toxicity was measured along the FG + AOP experiments due to the tentative presence of the O-DMTP intermediate. Moreover, the generation of unknown toxic metabolites from fungi (non-related to MTP and MTPA degradation) cannot be discarded.

3.3. Combined treatments of fortified hospital wastewater

The same experimental set-up performed with fortified pure water (Fig. 1) was applied to a real-case scenario to treat real HWW fortified with 2.0 µg/L of MTP and MTPA. Their removal as well as the relative distribution of the generated TPs are presented in Fig. 3c, 3d and Fig. 5. Since these experiments were performed with real HWW, 26.4% TPs were already detected without applying any treatment (α -HMTP and TP240, mainly). Other related pharmaceuticals such as atenolol, present in HWW at an initial concentration of 0.5 µg/L, may also have transformed into MTPA (also named atenolol acid) and generate some of these intermediates after degradation [44,51]. As previously reported, it is important to mention that MTP and MTPA removal efficiency can be altered by many other factors including the presence of organic matter, bacteria and pollutant concentration among others [17,24,44]. Finally, since many more unknown chemicals (different from MTP and MTPA) may contribute to the overall toxicity on treated effluent, *in vitro* measurements were not performed.

3.3.1. AOP + FG/FG + AOP treatments of fortified HWW

Overall results obtained from single and combined treatment experiments in fortified HWW for AOP + FG and FG + AOP are presented in Fig. 3c and Fig. 5c. High removal of MTP and MTPA were observed after AOP alone (67.8% and 82.8%, respectively) though still less effective than in fortified pure water (with removals higher than 99.6% for both MTP and MTPA), due most likely to the matrix complexity. While MTP increased its removal to 88.9% after AOP + FG, this combination did not increase MTPA removal (81.2%). The relative presence of intermediates was higher after AOP (63.2%) than after AOP + FG (40.6%). In comparison with fortified pure water AOP + FG experiments, the contribution of \geq 3rd generation TPs was lower (10.0%, Fig. 5c) compared with 80.8% observed in pure water (Fig. 4a). These values indicate the low degradation extent of TPs. Among the intermediates detected, TP240 (2nd generation) and α -HMTP (1st generation) were classified as the most persistent compounds, as it was also observed in previous fungal treatment of MTP and MTPA with *Ganoderma lucidum* [17]. These recalcitrant intermediates should be considered of important concern since they were found at a relative distribution of 42.5% and 44.1%, respectively (Fig. 5c). Therefore, further improvements and/or adjustments of the technologies included in this combination may be required to avoid the discharge of these compounds into the environment.

The opposite treatment combination FG + AOP was much less effective for MTP removal (36.1%) than AOP + FG (88.9%). The elimination of MTPA was only slightly lower (80.6%) compared to AOP + FG (81.2%), (Fig. 3c). Moreover, MTPA removal after FG + AOP increased only 10.7% compared to FG alone (69.9%) while no substantial changes were observed on MTP elimination. A similar pattern was observed in terms of relative presence of all generated intermediates (38.2% after FG and 36.2% after FG + AOP). These results demonstrate that FG + AOP was less effective than AOP + FG treating complex matrices. As in AOP + FG treatment, TP240 and α -HMTP were also classified as the most recalcitrant TPs after FG experiments, unable to be eliminated with this combined treatment configuration (Fig. 5c). Altogether, it can be suggested that FG + AOP did not provide any additional advantage compared with AOP + FG.

3.3.2. AOP + CAS/CAS + AOP treatments of fortified HWW

Overall results obtained from the combined experiments in fortified HWW for AOP + CAS and CAS + AOP are presented in Fig. 3d and

Fig. 5d. AOP + CAS combination was quite effective in terms of removal of MTP (85.6%) and MTPA (99.5%), as shown in Fig. 3d. The relative percentage of TPs decreased dramatically from 63.2% after AOP treatment to 15.4% after AOP + CAS, much lower than in AOP + FG (40.6%). Moreover, the distribution of \geq 3rd generation TPs after AOP + CAS treatment increased considerably up to 65.2% compared to those present after AOP alone (2.4%), Fig. 5d. The recalcitrant TP240 and α -HMTP generated after AOP were successfully reduced after CAS post-treatment with the generation of the \geq 3rd generation intermediate TP254.

In CAS + AOP similar values were obtained in terms of removal of MTP (85.7%) and MTPA (98.5%). In contrast to FG + AOP, this combination lead to a decrease in the relative TP presence: from 13.8% after CAS pre-treatment to 11.0% after CAS + AOP. However, even though TPs presence was slightly lower, their distribution was very different, compared to AOP + CAS: \geq 3rd generation TPs decreased drastically from CAS (65.2%) to CAS + AOP (21.0%) while 1st and 2nd generation TPs increased up to 27.8% and 51.3%, respectively (Fig. 5d). This was attributed to the formation of the characteristic persistent compounds TP240 and α -HMTP after AOP post-treatment. Otherwise, these persistent compounds were easily eliminated, or not generated extensively, by applying CAS as a post-treatment in AOP + CAS combination (Fig. 5d). This fact confirms that the generation and the elimination of intermediates were dependent also on the chosen sequence of applied treatments. Additionally, and in contrast with CAS treatment of fortified pure water, high reduction of MTPA (93.9%), without any further generation, was observed in CAS treating HWW (Fig. 3d). This can be related to the different matrix conditions, affecting MTP and MTPA degradation pathways. Finally, it is important to remark that the presence of the identified toxic intermediate O-DMTP was observed at a very low concentration < 1%. These results confirm that the elimination of the intermediates generated is directly dependent on the chosen sequence of applied treatments.

3.4. Evaluation of combined treatments and statistical analysis

Different combined treatment strategies were compared in the present study to achieve not only the highest elimination of the parent compounds but also of the generated intermediates. Additionally, the toxicity was evaluated along pure water experiments using *in vitro* measurements.

The experiments performed in fortified pure water demonstrated that the AOP treatment was the most effective treatment, out of the three single treatments tested (AOP, FG and CAS). AOP allowed the complete removal of MTP, MTPA and their intermediates without a toxicity increase. In CAS experiments, MTP was mainly transformed into the recalcitrant metabolite MTPA up to 114.4% whereas MTP was only removed 20.4% in FG experiments. In the latest case, acute toxicity increased from 0.0 TU up to 3.2 TU. The application of an AOP post-treatment was justified in both cases (FG and CAS) in order to reduce the presence of the parent compounds, the intermediates generated and the observed acute toxicity. The CAS + AOP combination, allowed the elimination of the recalcitrant metabolite MTPA up to almost 100% with no toxicity measured in effluents. Spearman correlation (Fig. S2) between AOP + CAS and CAS + AOP combinations, in terms of TP distribution, was classified as moderate ($r_s = 0.47$). On the other hand, MTP was only eliminated up to 36.4% after FG + AOP and increased *in vitro* toxicity due to the presence of the metabolite O-DMTP up to 4.3 TU. Spearman correlation (Fig. S2) between AOP + FG and FG + AOP combinations, in terms of TP distribution, was classified as non-significant ($p > 0.05$) but similar to CAS + AOP ($r_s = 0.65$ and 0.52 , respectively). In this context, FG + AOP was considered as the least effective combination in terms of removal of MTP, MTPA and generated intermediates in pure water.

The experiments performed with fortified HWW showed that the complete removal of the parent compounds (MTP and MTPA) and their

TPs was not fully accomplished by any of the evaluated single treatments studied. The combined treatments based on CAS and UV/H₂O₂ showed the best efficiency in terms of complete removal. The highest removal degree of target contaminants was observed in AOP + CAS with the largest contribution of \geq 3rd generation TPs. In CAS + AOP combination, the recalcitrant intermediates α -HMTP and TP240 were generated after the AOP post-treatment from MTP and MTPA removal. These two combinations showed a moderate correlation ($r_s = 0.53$) among them in terms of TP distribution (Fig. S3). However, non-significant correlation ($p > 0.05$) was found between the best treatment AOP + CAS and the less efficient combinations AOP + FG and FG + AOP. On the other hand, treated effluents from AOP + FG and FG + AOP showed a strong correlation among them ($r_s = 0.68$), but quite similar to the combination CAS + AOP ($r_s = 0.61$ and $r_s = 0.62$, respectively). As a conclusion, AOP + CAS was significantly the most successful combined treatment in comparison with the other tested combinations. Despite this, a detailed evaluation of the combined technologies would be required (in terms of operating conditions of each of the technologies involved) before scale-up and full-scale application [12,55].

4. Conclusions

A comprehensive overview of MTP and MTPA degradation and transformation was performed in experiments where fortified pure water and real HWW was treated with UV/H₂O₂ combined with FG or CAS biological processes. Major transformation pathways were suggested regarding the transformation of the parent compounds through bio-transformation and photo-transformation mechanisms. This comprehensive study allowed to characterize MTP and MTPA removal/transformation and to identify the most persistent and toxic intermediates. While AOP single treatment was enough to achieve almost total compound removal in spiked pure water experiments, combined treatments were required for hospital wastewater: among the studied combinations, AOP + CAS attained the highest removal rates not only for MTP but also for its recalcitrant metabolite MTPA and the generated intermediates. This study demonstrates that combined treatments may represent a solution when applied to complex wastewater matrices for the extended elimination of the TPs generated. On the other hand, this study demonstrates that target analysis of parent compounds along the water treatment does not provide enough information about the treatment performance. Comprehensive studies of the generated TPs combined with toxicity estimation are highly recommended.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cej.2020.126482>.

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