# Assessment of the Acute Toxicity, Uptake and Biotransformation Potential of Benzotriazoles in Zebrafish (*Danio rerio*) Larvae Combining HILIC- with RPLC-HRMS for High-Throughput Identification

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Ⓢ *Supporting Information*

**ABSTRACT:** The current study reports on the toxicity, uptake, and biotransformation potential of zebrafish (embryos and larvae) exposed to benzotriazoles (BTs). Acute toxicity assays were conducted. Cardiac function abnormalities (pericardial edema and poor blood circulation) were observed from the phenotypic analysis of early life zebrafish embryos after BTs exposure. For the uptake and biotransformation experiment, extracts of whole body larvae were analyzed using liquid chromatography−high-resolution tandem mass spectrometry (UPLC-Q-TOF-HRMS/MS). The utility of hydrophilic interaction liquid chromatography (HILIC) as complementary technique to reversed phase liquid chromatography (RPLC) in the identification process was investigated. Through HILIC analyses, additional biotransformation products (bio-TPs) were detected, because of the enhanced sensitivity and better separation efficiency of isomers. Therefore, reduction of false negative results was accomplished. Both oxidative (hydroxylation) and conjugative (glucuronidation, sulfation) metabolic reactions were observed, while direct sulfation proved the dominant biotransformation pathway. Overall, 26 bio-TPs were identified through suspect and nontarget screening workflows, 22 of them reported for the first time. 4-Methyl-1-*H*-benzotriazole (4-MeBT) demonstrated the highest toxicity potential and was more extensively biotransformed, compared to 1-*H*-benzotriazole (BT) and 5-methyl-1-*H*-benzotriazole (5-MeBT). The extent of biotransformation proved particularly informative in the current study, to explain and better understand the different toxicity potentials of BTs.
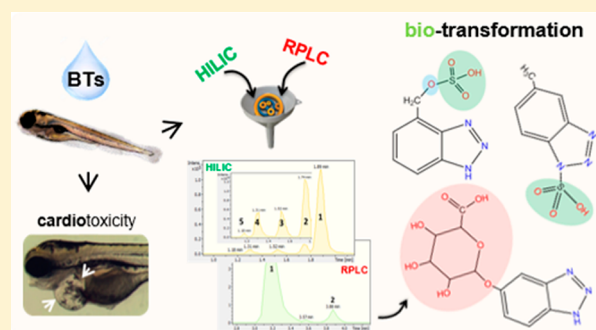
## ■ INTRODUCTION

Benzotriazoles (BTs) constitute a class of high production volume chemicals with a wide applicability domain.[1] They pose low biodegradability and sorption tendency; consequently they are partially or not at all removed from wastewater treatment plants (WWTP), with elimination efficiencies ranging from 0 to 70%[2−5] and an average concentration up to 10 $\mu$g L$^{-1}$ being reported in effluent wastewaters.[1,6] However, in specific cases, BTs were detected at high levels of $\mu$g L$^{-1}$ or even mg L$^{-1}$.[2,7] Thereby, there is clear evidence that BTs persist in aquatic systems, as they are frequently measured in rivers, lakes, and groundwater.[2,5,8] Moreover, BTs (5-methyl-1-*H*-benzotriazole (5-MeBT) and 4-methyl-1-*H*-benzotriazole (4-MeBT)) were detected in whole tissue extracts from fathead minnow (*Pimephales promelas*),[9] proving their potential risk in aquatic organisms, such as fish.

BTs are potentially toxic to water plants and aquatic invertebrates,[10−12] with toxicity end points reported up to 100 mg L$^{-1}$. Differences on toxicity level (EC$_{50}$/LC$_{50}$ values) among substances and variation across species were indicated by toxicity tests performed for several triazoles and benzotriazole (BT) in algae, daphnid, and zebrafish.[13] More recently, studies with benzotriazole ultraviolet stabilizers (BUVSs), highlighted that exposure of zebrafish embryos (ZFE) to BUVSs can alter the expression of the thyroid hormone pathway and decrease the heart rate. The toxicity to fish embryos varied again depending upon the type or structure of BUVSs, even among substances with similar structure.[14,15] Furthermore, Duan et al. (2017)[16] observed that prolong exposure of zebrafish to BT can induce hepatotoxicity,

including changes of liver size and abnormal expression of typical genes and enzymes in liver.

Zebrafish (*Danio rerio*) has emerged as a powerful model organism to study various aspect of developmental and cell biology[17] and physiology, as well as disease modeling and drug discovery.[18,19] Developmental and physiological processes are highly conserved among zebrafish and mammals.[20] Furthermore, in vivo studies have demonstrated the activity of phase I and II metabolizing enzymes of zebrafish during the embryonic[21] and larval[20,22,23] stages. Importantly, the ZFE has been validated, by the Organization for Economic Co-operation and Development (OECD), as an alternative for acute aquatic toxicity testing.[24]

The impact of the toxicants in the aquatic environment is evaluated in more depth when the whole xenometabolome[25,26] (xenobiotics and their metabolites) of aquatic organisms is studied, rather than analyzing only xenobiotics. Al-Salhi et al. (2012)[26] demonstrated that even when some xenobiotics were not detected in fish tissue, their metabolites were markers of their exposure in effluent wastewaters. Biotransformation is known to affect the internal concentration ($C_{int}$) and the uptake of the parent xenobiotics,[27,28] while it constitutes a critical factor for toxic response.[29] Therefore, it becomes necessary to thoroughly study the process of biotransformation of xenobiotics in aquatic organisms.[21] Furthermore, characterizing xenometabolites in depth will lead to a better assessment of their environmental fate after excretion, as they may be similarly or even more persistent compared to parent compounds.[21,30]

Identification of biotransformation products (bio-TPs) is a challenging task and the lack of available reference standards makes it even more difficult.[31] Great effort has been spent for the development of efficient and comprehensive work-flows,[32−37] based on liquid chromatography−high resolution mass spectrometry (LC-HRMS) analysis and postacquisition data processing techniques. Suspect and nontarget screening, introduced by Kraus et al. (2010),[33] are the most commonly applied approaches. Nevertheless, a gap emerges in the literature, indicating the necessity for additional experimental evidence. In particular, the interpretation of the MS/MS spectra, aiming to structure elucidation, may fall short of discriminating isomeric peaks.[31] Thus, a series of compounds may remain as "tentative candidates", due to the lack of additional experimental evidence required to reach the characterization of "probable structure".[38] Helbling et al. (2010)[32] and Jeon et al. (2013)[39] noted the necessity of using a complementary (orthogonal) analytical technique for the confirmation of plausible TPs. Provided that the quantity and purity of the sample does not permit the use of NMR as a confirmatory technique,[32] combined use of hydrophilic interaction liquid chromatography (HILIC) to reversed phase liquid chromatography (RPLC) mode seems to be a powerful alternative tool. To the best of our knowledge, there are only 4 publications described in the literature so far, highlighting the usefulness of adopting HILIC in combination to RPLC.[37,40−42]

Comparative analysis of the acute toxicity induced by BT, 5-MeBT, and 4-MeBT to zebrafish embryos and larvae was performed in this study. The uptake and biotransformation pathways of BTs were also thoroughly studied, as they constitute critical factors for toxic response. Additionally, we aimed to develop a comprehensive analytical strategy (HILIC- and RPLC-HRMS) that would strongly facilitate the identification of bio-TPs. Finally, we investigated if the identification of bio-TPs of BTs and the extent of biotransformation could complement the uptake data, to interpret/explain the observed toxicity of BTs from the acute toxicity test.

## ■ MATERIALS AND METHODS

**Chemicals.** All chemicals were purchased in highest available purity and are listed in detail in the Supporting Information, SI (SI-1).

**Zebrafish Maintenance, Breeding, and BTs Treatment.** ZFE were raised under standard laboratory conditions at 28 °C[43] in the zebrafish breeding establishment of the Biomedical Research Foundation of the Academy of Athens (EL25BIO001). Zebrafish are maintained in accordance with the European Directive 2010/63 for the protection of animals used for scientific purposes and the Recommended Guidelines for Zebrafish Husbandry Conditions.[44] The experimental protocols described in this study were carried out with zebrafish larvae (ZFL) up to 120 h post fertilization (hpf) and therefore are not subject to the regulations of European animal protection guidelines. The test compounds were dissolved in methanol and added to the egg water.

In this study, three different exposure experiments were conducted (SI, SI-2−1). For the calculation of LC$_{50}$ (SI, SI-2−2), 3 hpf embryos were exposed to the tested BTs according to OECD guidelines for Fish Embryo Acute Toxicity Test (TG 236).[24] For the phenotyping evaluation, synchronized 24 hpf embryos (10 embryos/5 mL medium) were exposed to 4-MeBT, 5-MeBT and BT at concentration 10, 50, and 100 μg mL$^{-1}$ with final concentration of methanol (vehicle) up to 0.5% (v/v) in egg water. The chosen concentrations spanned from concentrations where no phenotypical abnormalities were observed, to concentrations close to LC$_{50}$. Development was followed up to 96 hpf and was recorded using a stereomicroscope.

For the uptake and biotransformation experiment, 60 96 hpf embryos per experimental sample were used and exposed under static conditions to 0.1% methanol (control) and 10 μg mL$^{-1}$ 4-MeBT, 5-MeBT, and BT in egg water, for 30 s, 2 h, 4 h, 8 h, and 24 h. At 10 μg mL$^{-1}$, no phenotypic abnormalities were observed. Samples of the exposure medium were also collected at each time interval. To ensure that bio-TPS were exclusively formed through biotransformation processes, abiotic control samples were also prepared and analyzed (SI, SI-2−3). The TG 236 acute toxicity test (LC$_{50}$ calculation and phenotyping) was used as a guideline for selecting the sublethal concentrations used in the uptake and biotransformation experiments. These were performed at the developmental stage of the end point (96 hpf), for a shorter window of time to facilitate pathway analyses. Detailed information on the rationale for the selected developmental stages of the exposure experiments are provided in the SI (SI-2−4)

**Zebrafish Larvae Tissue Extraction.** For extracting tissue samples in a high-throughput manner, we based our sample preparation on a two-step extraction protocol, reported to be the optimum in terms of extraction yield and reproducibility.[45] We sought to cover the widest possible range of polarities.

ZFL were euthanized by MS-222, removed from the exposure medium, rinsed twice with fresh egg water and placed in Eppendorf tubes. The washed larvae were snap-frozen in liquid nitrogen and kept at −80 °C until further preparation. Frozen tissue was homogenized with 0.5 mL of prechilled methanol/water (v:v 1:1) (first extraction step) using an electric homogenizer (Tissue Master 125, OMNI interna-

tional). The mixture was centrifuged (13 000 g, 10 min, 4 °C) (Heraeus Biofuge fresco), the supernatant was collected and then transferred to an Eppendorf tube, constituting the "aqueous" extract. The pellet, retained in the tube from the previous step, was extracted with 0.5 mL prechilled dichloromethane/methanol (v:v 3:1) (second extraction step) using the electric homogenizer. The latter supernatant was afterward centrifuged (13 000 g, 10 min, 4 °C), collected and transferred to an Eppendorf tube, constituting the "organic" extract. All the extracts were stored at −80 °C until analysis. Figure S3 illustrates the sample preparation protocol adopted for the extraction of the zebrafish tissue samples. Recovery rate (aqueous and organic extracts) and matrix effect data are reported in the SI (SI-3). Quantification of the parent BTs and semiquantification of the bio-TPs are also described in the SI (SI-3). The concentrations reported throughout the manuscript are referred to the aqueous extract (SI-3).

**LC-HRMS Analysis.** Analyses of larval extracts and exposure media were carried out using a UHPLC/QTOF-MS system (Dionex UltiMate 3000 RSLC, Thermo Fisher Scientific, Dreieich, Germany). LC-HRMS analyses were conducted in both RPLC and HILIC, as well as in both polarities, and are described in detail in the SI (SI, SI-4).

**Identification Procedure.** A suspect and a nontarget screening workflow were established for the identification of candidate bio-TPs. The filtering, the confirmatory as well as the confidence enhancement criteria of both workflows are discussed in detail in the SI (SI-5). The introduced filtering criteria aim to reduce false positive identifications, while, at the same time, minimizing the false negative results. Beyond the cases that identification fails because of reduced ionization efficiency, false negatives arise from the inadequate separation, especially for isomers. By definition, structural isomers have the same molecular formula; hence they cannot be separated in terms of mass spectrometry even with HRMS instruments. Moreover, their retention time in RPLC, in most cases, is very close, if not the same, especially for positional isomers. Thus, their identification remains a challenging task. To address this, we introduced HILIC complementary to RPLC, due to the different selectivity and retention mechanisms, providing orthogonality.

## ■ RESULTS AND DISCUSSION

**Acute Toxicity Analysis of Zebrafish Larvae after BTs Exposure.** $LC_{50}$ values at 96 hpf were 59 $\mu$g mL$^{-1}$ for 4-MeBT, 128 $\mu$g mL$^{-1}$ for 5-MeBT, and 170 $\mu$g mL$^{-1}$ for BT (Figure S2). Consistent to our results, 5-MeBT has been reported to be more toxic compared to BT for some primary producers and daphnia species.[12] However, Pillard et al. (2001)[10] stated higher toxicity for 5-MeBT to fathead minnow and *Ceriodaphnia dubia*, while 4-MeBT and BT shared similar toxicities. Nevertheless, the toxicity results from ZFE correlate well with the uptake and biotransformation data obtained by the biotransformation experiment, as discussed below in detail.

Phenotypic observations at different stages of development and during $LC_{50}$ calculations allow for the assessment of teratogenic effects of chemicals on the development and physiology of the organism during early life development. The concentrations tested here were much higher than the environmentally relevant concentrations, in most cases,[2] to reach the sublethal and lethal concentrations and assess their effects during the first 5 days of zebrafish development. For all three BTs tested, no apparent adverse effects were observed on

larvae exposed to concentration of 10 $\mu$g mL$^{-1}$ (Figure S5A, D, G) when compared to vehicle control (Figure S5C). Therefore, this exposure concentration was used for the biotransformation experiment. At 50 $\mu$g mL$^{-1}$, embryos, treated with 4-MeBT, developed pericardial edema (Figure S5B) and a similar but milder phenotype occurred to embryos exposed to 5-MeBT (Figure S5E). In contrast, embryos treated with 50 $\mu$g mL$^{-1}$ BT showed no phenotype (Figure S5H) and pericardial edema appeared at concentrations higher than 100 $\mu$g mL$^{-1}$ (Figure S5I). For larvae treated with 100 $\mu$g mL$^{-1}$ 4-MeBT, 100% mortality was observed, whereas treatment with 100 $\mu$g mL$^{-1}$ 5-MeBT resulted to larvae with severe abnormalities, including cardiac edema and no blood circulation (Figure S5F). This is the first study reporting on the toxicity of 4- and 5-MeBT in vivo, using ZFE.

**Analytical Strategies for the Identification of Biotransformation Products.** Following the analysis of the ZFL extracts, full scan HRMS data were evaluated, for the identification of potential bio-TPs of BTs formed in the course of the exposure experiment. Figure S6 exemplifies the application of the aforementioned filtering criteria (identification procedure) in the identification of a tentative bio-TP of 4-MeBT. Briefly, mass accuracy and isotopic fit, as well as peak score values, meet the preset criteria.

The presence of a meaningful *time-trend* between time interval samples, was introduced as a *mandatory* criterion in the identification workflow. This additional criterion is based on the fact that the formation of bio-TPs is the outcome of a continuous process, which is the effort of the living organism to detoxify the xenobiotics. Furthermore, because of the xenobiotic origin of the tentative bio-TPs, *they should be absent from the control samples*. Thus, peaks with similar Rt ($\pm0.1$ min) and intensity, present also in the control sample, were rejected and not further processed. This criterion was established due to the high complexity of the samples, which led to a multitude of peaks irrelevant to the exposure (endogenous metabolites), in both treated and control samples. In addition, we introduced *the xenobiotic metabolism relevance of the tentative bio-TP*, as a last filtering step for successful identification in the nontarget screening workflow. In other words, the formation of the tentative bio-TP must be explained by the xenobiotic metabolism rules, in order to be successfully identified. *Retention time plausibility check* was a further criterion in the identification workflow. In the current study it was implemented primarily to provide evidence regarding the elution order of the positional isomers detected, rather than as a strict filtering step for a successful identification.

Further confirmation of the tentatively identified bio-TPs was based on MS/MS spectra interpretation. In particular, we relied on the similarity of the MS/MS spectra of the BTs and their respective bio-TPs, as well as on diagnostic neutral losses observed for the conjugated bio-TPs (glucuronic acid and sulfate moieties). Hydroxylated bio-TPs shared similar fragmentation patterns with the parent compounds (Figure S7). Regarding N-conjugated glucuronides, all BTs shared similar fragmentation patterns. Confirmation was based on the diagnostic elimination of a glucuronide conjugate of 176.0314 Da ($C_6H_8O_6$), resulting in the corresponding parent compound (Figure S8). The mass shift of 79.9568 Da corresponding to the diagnostic elimination of $-SO_3$ was common in both O-conjugated and N-conjugated sulfate bio-TPs, resulting in the formation of the hydroxylated bio-TP and the parent BT, respectively (Figure S9). The same characteristic elimination of
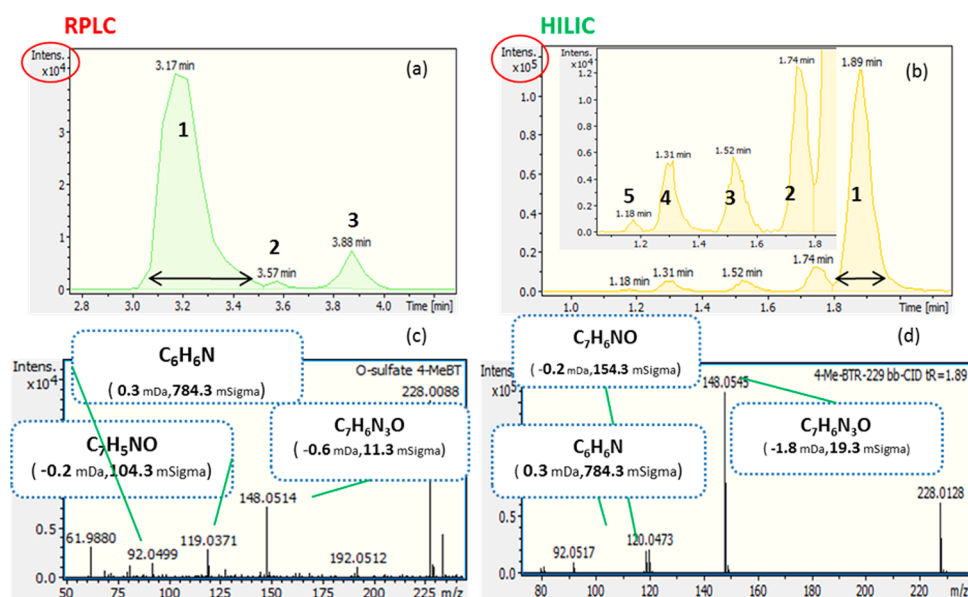
**Figure 1.** Benefits of HILIC compared to RPLC: separation of isomers, narrow peak width and higher sensitivity. Identification of O-S-4-MeBT in negative ionization and in both RPLC and HILIC. (a) EIC of O-S-4-MeBT in RPLC, (b) EIC of O-S-4-MeBT in HILIC, (c) MS/MS spectra of the dominant peak of O-S-4-MeBT in RPLC, and (d) MS/MS spectra of the dominant peak of O-S-4-MeBT in HILIC.

the $-SO_3$ moiety was also observed in the MS spectra, through in-source fragmentation.

Apart from the criteria that were mandatory for the successful identification, the detection in both RPLC and HILIC and ESI polarities was also evaluated, to provide additional experimental evidence. Identification of O-sulfate conjugate of 4-MeBT (O-S-4-MeBT) exemplifies the applicability of the aforementioned criteria (Figure S10). O-S-4-MeBT was detected in both chromatographic systems and polarities, with improved detection sensitivity in negative ionization mode. The complementarity of HILIC to RPLC is described in detail in the following section.

**HILIC Complementary to RPLC: A Comprehensive Approach in the Identification Task.** The need for additional separation mechanisms for the identification of positional isomers, as well as for additional experimental evidence, led us to introduce HILIC as complementary technique to RPLC. HILIC provided better results in specific cases, as shown in the next paragraphs.

The improved separation efficiency and chromatographic performance (smaller peak width and better peak shape) of the specific HILIC separation is highlighted. Identification of O-S-4-MeBT exemplified the utility of 1.7 $\mu$m HILIC column, which resulted in a symmetrical peak with less than the half peak width compared to RPLC (Figure S10a, b). Similar results were obtained from the identification of N-conjugated glucuronide of 5-MeBT (N-G-5-MeBT) (Figure S11). Consequently, the majority of bio-TPs identified in HILIC mode, were characterized by sharp peaks well separated from each other. Worth noting is that although the bio-TPs of 4-Me-BT were eluted within a minute (1.1−2.1 min) in HILIC, they were well separated (Figure S12). Thus, even for poorly retained compounds good separation is achieved.

Another key feature of HILIC analysis is that the mobile phase composition is fully compatible with ESI. The increased organic solvent content enhances the ionization efficiency and thus the detection sensitivity. This becomes clear in the identification of O-S-4-Me-BT, obtaining a 10-fold higher signal

in HILIC than in RPLC (Figure S10a, b). In this way, MS/MS spectra, which were not obtained by RPLC, were clearly recorded in HILIC (Figure S13), providing additional experimental evidence for the identification. Nevertheless, this was not always the rule; in case of O-glucuronide-conjugates higher signal was obtained in RPLC mode. Because of the different retention characteristics and selectivity provided by RPLC and HILIC, reversed elution order was observed in many cases (Figures 1 and S11), enhancing the identification (orthogonality). An additional confirmatory factor was the presence of common MS and MS/MS spectra between the two chromatographic modes (Figure 1c, d).

Isomers were detected for all bio-TP classes, except for S−N-conjugates, which complicated the identification task. The most representative example in our study, highlighting the benefits arising from the complementary use of HILIC, is the identification of O−S-conjugates. The application of HILIC revealed the presence of more peaks than those detected in RPLC (Figure 1a, b). Particularly, we observed five well separated peaks by the application of HILIC, while three were detected by RPLC, reducing this way the false negative results. This may be attributed to the additional retention mechanisms taking place in HILIC (distribution, hydrogen bonding, dipole−dipole interactions) providing structural selectivity for some moieties, as well as to the improved separation efficiency provided by a sub-2-$\mu$m HILIC column.

Our results demonstrated that the adoption of HILIC provided additional experimental evidence for the identification. Reduction of false negative results was also observed. On the other hand, there are cases that HILIC was deficient compared to RPLC, such as the identification of hydroxylated-bio-TPs, where more peaks were detected in RPLC mode. Thereby, we conclude that the optimum results are obtained by the complementary use of both RPLC and HILIC. The benefits emerged, suggest their combined use as a powerful tool for comprehensive and high-throughput identification of bio-TPs. We strongly believe that this approach could benefit the field more broadly than just studies of this class of compounds
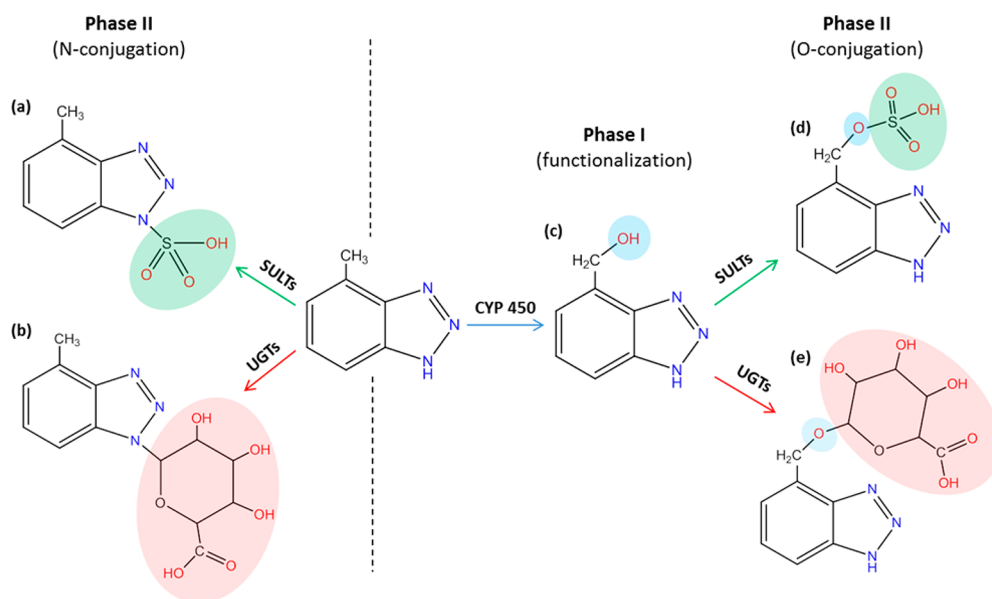
**Figure 2.** Biotransformation pathway of 4-MeBT: (a) *N*-sulfate-4-MeBT; (b) *N*-glucuronide-4-MeBT; (c) hydroxy-4-MeBT; (d) *O*-sulfate-4-MeBT; (e) *O*-glucuronide-4-MeBT (**UGTs** = uridine 5′-diphospho-glucuronosyltransferases; **SULTs** = sulfotransferases).

(BTs), bringing to light bio-TPs which would otherwise remain unexploited.

**Uptake of Benzotriazoles by Zebrafish Larvae.** The process of uptake is a key factor for the bioaccumulation of a xenobiotic by a living organism.[27] Thus, we tried to assess the uptake of BTs by ZFL evaluating the time courses of their $C_{int}$. Figure S14 illustrates the time course of the $C_{int}$ of BTs throughout the exposure experiment. Given that exposure medium concentration was the same (10 $\mu$g mL$^{-1}$) for all BTs, a differentiation in the time profile and the $C_{int}$ level in steady state is observed for BT treated samples ($C_{int} \approx$ 23 $\mu$g g$^{-1}$), compared to 4-MeBT and 5-MeBT treated samples ($C_{int} \approx$ 46 $\mu$g g$^{-1}$). The uptake of polar compounds by zebrafish is influenced by their physicochemical properties.[28] Consequently, the lower lipophilicity of BT compared to 4- and 5-MeBT may result in less retention in zebrafish tissue, therefore in a lower bioaccumulation potential. Furthermore, BT may be more readily excreted by zebrafish. Regarding the time profiles of BTs $C_{int}$, we note that 4- and 5-MeBT treated samples reached maximum $C_{int}$ at 4 h post exposure (hpe), while BT treated samples at 8 hpe.

The difference observed in the uptake rate could influence the extent of their biotransformation by zebrafish, which was confirmed by the biotransformation experiment. It could also indicate differences to the $C_{int}$, reaching the target site, which is the biologically effective dose[46] and may lead to differing toxicity end points (e.g LC$_{50}$).[47] LC$_{50}$ data obtained by the acute toxicity test (Figure S2) confirmed this hypothesis. The lowest toxicity of BT, is consistent with the lower $C_{int}$ observed. However, $C_{int}$ of the parent compound alone, was not sufficient to explain the observed higher toxicity of 4-MeBT compared to 5-MeBT. Thus, detailed assessment of the biotransformation profiles of BTs by ZFL was conducted. The higher toxicity of 4-MeBT compared to 5-MeBT and BT correlated well with its higher biotransformation rate, as it is presented below.

**Biotransformation of BTs by Zebrafish Larvae.** This is the first study reporting on the biotransformation of BTs in zebrafish, and in aquatic organisms in general. There is one study relevant with the in vivo biotransformation of BTs.[48]

Specifically, *N*-glucuronide of BT was stated to be one of the major metabolites, excreted in the urine of male rats, after the oral administration of 1-amino-benzotriazole. Nevertheless, environmental transformation of BTs under different experimental conditions[49−52] is well documented and discussed in detail in the SI (SI-10−1).

Consistent to the literature,[21,22,28,53] both oxidative and conjugative metabolic reactions of ZFL were observed. Recently published data, highlight zebrafish to be able to biotransform a range of xenobiotics, through both phase I and II reactions, during early life development (even before 24 hpf). For instance, biotransformation of benzocaine, valproic acid, and phenacetin was initiated even before 28 hpf.[28] Interestingly, sulfate containing TPs of clofibric acid were detected already at 3 hpe (7hpf), showing the high metabolic activity of ZFE.[21]

Overall, 26 bio-TPs were tentatively identified, by means of the applied analytical procedure, while 22 are reported for the first time. The extent of analytical evidence supporting each tentative bio-TP was variable, therefore different identification confidence levels (ICL) were assigned. Table S1 summarizes all the detected bio-TPs, encompassing information for the total number of isomers, as well as the ICL. Extensive analytical information (detection in RPLC or HILIC in positive and/or negative ionization, number of isomers etc.) regarding the identified bio-TPs is reported in the SI (SI-10−2).

The metabolic pathways involved in the biotransformation of BTs by ZFL are summarized in Figure 2. Hydroxylated (Figure 2c), sulfate conjugated (Figure 2a, d) and glucuronic acid conjugated (Figure 2b, e) bio-TPs were detected. An additional bio-TP was detected through the nontarget screening workflow, only for 4-MeBT treated samples. Although it meets all the filtering criteria we have set, we could not conclude to a probable molecular formula and/or structure that is also explained by the xenobiotic metabolism rules. The experimental evidence for this unidentified bio-TP, is provided in the SI (Figure S19). The detection of *O*-sulfate and *O*-glucuronide conjugates was not unexpected, as they are frequently reported for a plethora of xenobiotics,[20−22,28] and the same applies for the hydroxylated bio-TPs. It is noteworthy, that glutathione
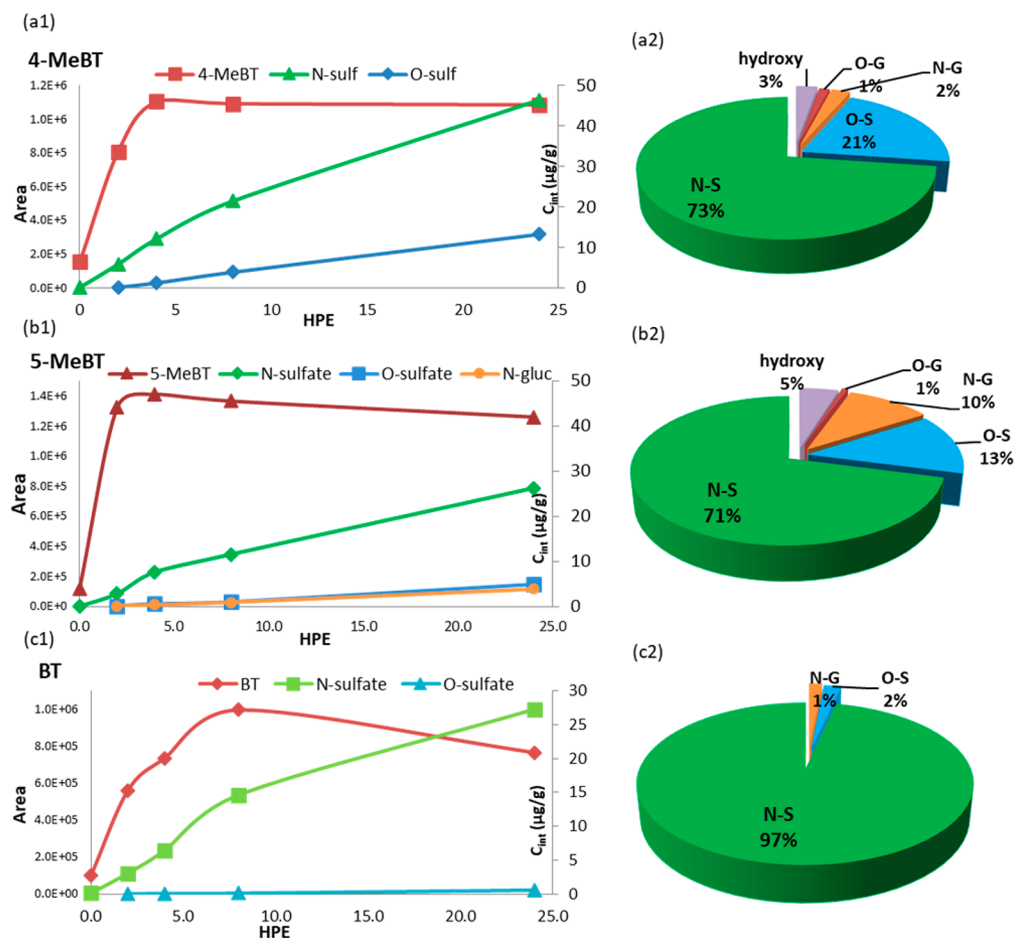
**Figure 3.** (a1, b1, c1) Time profiles for 4-MeBT, 5-MeBT, BT, and their major bio-TPs respectively. (a2, b2, c2) Contribution of each metabolic reaction to the overall biotransformation for 4-MeBT, 5-MeBT, BT, respectively (HPE = hours post exposure, N−S = N-sulfate, O−S = O-sulfate, O-G = O-glucuronide, O-G = O-glucuronide).

conjugates were not detected, while it is a commonly reported biotransformation reaction for ZFE.[21,54] Amino acid conjugation, reported by Brox et al. (2016),[21] was neither observed. We hypothesize that this may be attributed to the different physicochemical properties among the xenobiotics that may influence the biotransformation reactions that take place.

Interestingly, as regards conjugative metabolic reactions, conjugation of the endogenous moiety was observed not only after the hydroxylation (O-conjugates) (Figure 2d, e), but also directly to the nitrogen of BTs (N-conjugates) (Figure 2a, b). Respectively, in a recent study, ZFE metabolized clofibric acid through both direct (conjugation with aminomethanesulfonate, carnitine, etc.) and indirect (hydroxylation followed by sulfation) conjugation reactions.[21] In general, direct conjugation is feasible for compounds that bear an exposed functional group a priori. Thus, valproic acid, bearing a free hydroxyl group, was also directly biotransformed by ZFE to its taurine and glucuronide conjugates.[28] On the contrary, metabolism of phenacetin required phase I functionalization (hydroxylation) prior to conjugation with sulfate and glucuronide moieties.[28]

Although both O- and N-conjugated bio-TPs were detected, no bio-TP that has undergone both reactions was observed. However, the phenomenon of subsequent phase II reactions (diconjugation) has been reported for zebrafish liver cells and microsomes[29] as well as for ZFE and adults exposed to bisphenol-S and benzophenone-2.[55] We hypothesize that in our study after conjugation, the monoconjugated bio-TPs may be

sufficiently polar to undergo excretion processes, or to be transported to "compartments" (of zebrafish) that are incapable of biotransformation, and therefore, no subsequent conjugation step is implemented.

**Comparative Analysis of Biotransformation Profiles.** Biotransformation of BTs by ZFL, involves the direct conjugation of the parent compound to either glucuronic acid or sulfate moieties, as well as hydroxylation followed by further conjugation with charged species. Comparative analysis of the bio-TPs among the BTs was performed, to search for potential differences in the extent of biotransformation and/or biotransformation profile of BTs.

Hydroxylated bio-TPs were identified for 4-MeBT and 5-MeBT treated samples, while for BT no hydroxylated bio-TPs were detected, probably due to low concentration. Nevertheless, we assume that they were formed, as they are intermediates of the detected O-Sulfate bio-TPs of BT, as described subsequently. A hypothesis is that phase I functionalization of BT takes place at a lesser extent comparing to the other BTs, due to the lower $C_{int}$ (Figure S14) and potency (Figures S2 and S5) of BT. In other words, BT constitutes a less potent stressor for ZFL, leading to less extensive biotransformation. Thus, direct phase II conjugation may be sufficient to meet the needs for the most part of biotransformation of BT.

Similarly, O-G-bio-TPs were detected for both 4- and 5-MeBT but not for BT. This correlates well with the relative

intensity of hydroxylation, since the formation rate of the hydroxylated intermediates is determinant for the subsequent conjugation (Figure 2c, e). Contrary, N-G-bio-TPs were detected for all the BTs, as they are independent to hydroxylation (Figure 2b). However, BT treated samples provided the lowest $C_{int}$ for this bio-TP class, probably due to the lower bioaccumulation and toxicity potential of the parent compound (BT).

Figure 3 summarizes the time profiles of the parent BTs and their main bio-TPs, as well as the share of each metabolic pathway to the overall biotransformation. O-S-bio-TPs were detected in all exposure experiments. $C_{int}$ of O-S-bio-TPs of BT ($\sim$0.6 $\mu$g g$^{-1}$) was 1 order of magnitude lower compared to the respective of 5-MeBT ($\sim$5 $\mu$g g$^{-1}$) and 4-MeBT ($\sim$14 $\mu$g g$^{-1}$) (Figure 3**b1, c1, d1**). This may be again attributed to the lower formation rate of their intermediates (hydroxylated bio-TPs), as well as to the lower uptake and the lower potency of the parent BT. $C_{int}$ of O-S-4-MeBT was about 3-fold higher compared to O-S-5-MeBT, indicating that 4-MeBT biotransformed in a greater extent. Regarding O-S-4-MeBT, conjugation of SO4H seems to take place mainly in the benzene ring, while for O-S-5-MeBT in the triazole ring (SI-10−2, Figure S21). Contrary to O-S-bio-TPs, $C_{int}$ of N-S-bio-TPs was in the same order of magnitude for all the BTs, which is reasonable, as this pathway is independent to hydroxylation (Figure 2a). Nevertheless, $C_{int}$ of N-S-4-MeBT ($\sim$47 $\mu$g g$^{-1}$) was almost twice to the respective bio-TPs of 5-MeBT and BT ($\sim$27 $\mu$g g$^{-1}$) (Figure 3b1, c1, d1), highlighting the more extensive biotransformation of 4-MeBT. The excretion of 4- and 5-MeBT's bio-TPs (mainly sulfate conjugates) in the exposure medium demonstrated a more extensive metabolism of the methylated BTs compared to BT. The detection of bio-TPs in the exposure medium revealed the high metabolic potential of ZFL, consistent to the literature.[21] Furthermore, it highlighted the relevance and usefulness of measuring bio-TPs of xenobiotics in real water samples.

We assume that biotransformation relies mostly on direct sulfation (N-S-bio-TPs), which contributed more than 70% to the overall biotransformation for all BTs (Figure 3a2, b2, c2). In case where this is not sufficient (mainly for 4- and 5-MeBT, with higher $C_{int}$ and potency), phase I functionalization (hydroxylated-bio-TPs) is implemented aiming to activate BTs for further conjugation with charged species, mainly sulfate moieties. In this way, O-S-bio-TPs are formed alongside with N-S-bio-TPs assisting the biotransformation process. The low signals observed for O-G-bio-TPs, in general, indicate that hydroxylation followed by glucuronic acid conjugation is not the preferred detoxification/biotransformation pathway for BTs (Figure 3b2, c2, d2). BT exhibits almost exclusively N-sulfate conjugation (Figure 3c2), which seems to meet the needs of biotransformation alone. Although sulfation predominates over glucuronidation, the latter appeared to be functional, regarding the metabolism of BTs.

It is indicated by our results that ZFL produced mostly conjugative metabolites (Figure 3a2, b2, c2). This agrees with data reported in the literature for zebrafish liver cells and microsomes, stating the predominance of conjugative pathways.[29] In addition, exclusive metabolism through phase II pathways of ZFL and adults was reported for bisphenol-S and benzophenone-2.[55] Exclusive phase II biotransformation was also observed for valproic acid.[28] In the same study, biotransformation of phenacetin and benzocaine was implemented mainly through phase I reactions in the first 48 hpe,

while afterward phase II reactions were dominant.[28] Metabolism of clofibric acid was implemented mainly through conjugative reactions, while a wide variety of phase II metabolites were identified.[21]

Given that 4- and 5-MeBT are isomers and have similar physicochemical properties, we expected their uptake rates in zebrafish to be similar,[28] which was confirmed by our results (Figure S14). Nevertheless, although the $C_{int}$ of parent 4- and 5-MeBT was similar, 4-MeBT was more extensively biotransformed (Figure 3a1, b1). This shows that 4-MeBT may induce more chemical stress to zebrafish, as more "biotransformation effort" is being put (by zebrafish) for the same amount ($C_{int}$) of parent xenobiotic. The indication for higher potency from the biotransformation experiment, is in accordance with the results from the acute toxicity test.

The toxicity of the identified bio-TPs was not investigated experimentally, as they were not commercially available. Especially N-sulfate and O-sulfate conjugates that constitute the major bio-TPs of BTs are reported here for the first time. However, assessment of their relative toxicity compared to their parent BTs was performed applying in-house developed QSAR toxicity prediction models (ToxTrAMS).[56] These models were further developed from toxicity data reported in the literature for fathead minnow. The fathead minnow is a freshwater fish that belongs to the minnow family (*Cyprinidae*), just like the zebrafish, therefore it constitutes a relevant toxicity prediction model for our study. Predicted acute toxicity data for the detected bio-TPs are presented in the SI (Table S2). It is obvious, according to the predicted toxicity results, that both N- and O-sulfates (the major bio-TPs) are less potent compared to the parent BTs. Consequently, we believe that the higher toxicity observed for 4-MeBT compared to 5-MeBT (Figures S2 and S5) is caused by the higher potency of the parent compound, rather than by one of its bio-TPs. Nevertheless, the potential toxicity of bio-TPs needs to be further studied experimentally, since bioactivation has been reported for a number of compounds, so far.[57,58]

Our findings reinforce the statement that comparing toxicokinetics across chemicals without explicit consideration of biotransformation has limited explanatory power.[27] Additionally, bioaccumulation and biotransformation constitute key processes that can modify the toxicity of chemicals.[28] The extent of biotransformation proved particularly informative in the current study to explain and better understand the different toxicity potentials of BTs. It should be mentioned that bio-TPs constitute an amount of the parent xenobiotic that is omitted, if we search only for the parent compound, which may lead to erroneous conclusions for the overall $C_{int}$ of the xenobiotic. Interestingly, the comparison of bioaccumulation ($C_{int}$ of BTs) and biotransformation ($C_{int}$ of bio-TPs) data revealed which xenobiotic poses the highest potency to zebrafish, reinforcing the observations from the in vivo acute toxicity test. Thus, we suggest that detailed assessment of biotransformation should be included in toxicity studies. Therefore, the combined use of HILIC and RPLC is recommended, as it proved a powerful tool for comprehensive and high-throughput identification of new bio-TPs.

**Environmental Implications.** The environmental levels of the tested BTs are much lower, in most cases,[1,6] than effect concentrations in our study. Consequently, we conclude that the effects observed here are probably only relevant at high concentrations.[2,7] However, chronic effects may occur after a longer period of exposure. We also have to keep in mind that in

the aquatic environment there is a multitude of coexisting xenobiotics (chemical stressors). Thus, mixture toxicity effects may be more severe for lower concentration of BTs.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.8b01327.

Chemicals; $LC_{50}$ calculation experiment and results; sample preparation; instrumental analysis parameters; suspect workflow; nontarget workflow implementation and analytical results; complementarity of HILIC and RPLC; MS/MS spectra of bio-TPS; detailed bio-TPs identification results; retention time prediction results; and acute toxicity prediction results (PDF)

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### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

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