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In Vitro Metabolism of Photolytic Breakdown Products of Tetradecabromo-1,4-diphenoxybenzene Flame Retardant in Herring Gull and Rat Liver Microsomal Assays

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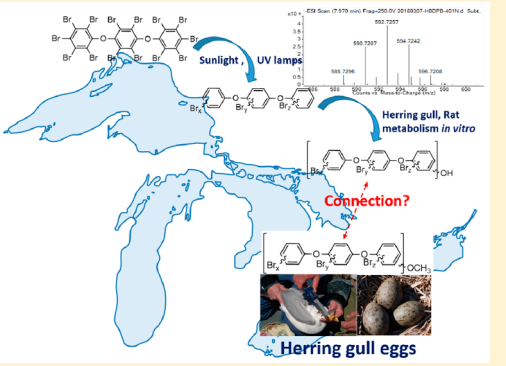
Supporting Information

ABSTRACT: Tetradecabromo-1,4-diphenoxybenzene (TeDB-DiPhOBz) is used as a flame retardant chemical and has been hypothesized to be the precursor of methoxylated polybrominated diphenoxybenzene (MeO-PB-DiPhOBz) contaminants reported in herring gulls from sites across the Laurentian Great Lakes. Here, by irradiating the parent TeDB-DiPhOBz (solution 1) to natural sunlight or UV, we prepared three solutions where solution 2 was dominated by the Br_{8-11}-PB-DiPhOBzs, along with Br_{5-8}-PB-DiPhOBzs (solution 3) and Br_{4-6}-PB-DiPhOBzs (solution 4). The in vitro metabolism of TeDB-DiPhOBz and PB-DiPhOBzs was investigated using harvested wild herring gull (*Larus argentatus*) and adult male Wister-Han rat liver microsomal assays. After a 90 min incubation period of solution 1 in gull or rat microsomal assays, there was no significant ($p > 0.05$) depletion of TeDB-DiPhOBz. OH-PB-DiPhOBz metabolites were detectable after gull and rat microsomal assay incubation with solutions 3 or 4, and showed clear species-specific differences. Also detected were two polybrominated hydroxylated metabolites having polybenzofuran structures. Overall, this study suggested that TeDB-DiPhOBz is slowly metabolized in vitro, and also indicated that if wild herring gulls are exposed (e.g., via the diet) to photolytic products of TeDB-DiPhOBz, OH-PB-DiPhOBz and other metabolites could be formed. OH-PH-DiPhOBz are likely precursors to MeO-PB-DiPhOBz contaminants that we reported previously in eggs of wild Great Lakes herring gulls.



1. INTRODUCTION

Brominated flame retardants (BFRs) are a class of organobromine compounds that have been used over decades to reduce the flammability of a variety of consumer products including textiles, furniture, foams, electronics, and building materials.[1−3] To date, at least 75 different BFRs have been commercially produced.[2] However, studies have been primarily focused on three groups: polybrominated diphenyl ethers and biphenyls (PBDEs and PBBs), hexabromocyclododecane (HBCDD), and tetrabromobisphenol A (TBBPA).[4] Current knowledge of the environmental fate and biotic/abiotic transformation of other novel non-PBDE FRs remains relatively scarce, including the highly brominated tetradecabromo-1,4-diphenoxybenzene (TeDB-DiPhOBz, CAS No: 58965-66-5).[1,4]

TeDB-DiPhOBz is also known as 4′-PeBPO-BDE208 and SAYTEX 120, and a current-use additive flame retardant (FR). TeDB-DiPhOBz is generally used in solid plastic and wire/cable products, and suggested as an alternative FR to BDE-209, which is used in a variety of polymeric applications.[5]

We have previously shown that TeDB-DiPhOBz can undergo rapid photolytic degradation when exposed to artificial UV-A, -B, or -C or to natural sunlight.[6,7] Furthermore, photolytic degradation of TeDB-DiPhOBz in solution by natural sunlight can generate products that affect in vitro expression of genes in a chicken embryonic hepatocyte assay, especially via aryl hydrocarbon receptor (AhR)-mediated *CYP1A4* mRNA expression with an induction change up to thousands of fold.[6,8]

Very recently, our group reported on the identification of a group of novel methoxylated polybrominated diphenoxybenzenes (MeO-PB-DiPhOBzs) in herring gull eggs from the Laurentian Great Lakes of North America, and as of 2010 these newly discovered MeO-PB-DiPhOBz congeners were found to be contaminants in herring gull eggs for the previous 30 years.[9,10] Semiquantitative analysis revealed ΣMeO-PB-DiPhOBz concentrations of up to 36.8 ng/g ww in pooled egg homogenates collected in 2009 from 14 herring gull colony

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sites across the Great Lakes, 2009. For the Channel-Shelter Island site in Saginaw Bay, Lake Huron, in gull egg pools ΣMeO-PB-DiPhOBz concentrations increased from 1982 and peaked around the late 1990s, followed by a general decline until 2010.[9,10] In egg pools from some colonies, ΣMeO-PB-DiPhOBz concentrations were comparable to or somewhat lower than HBCDD and BDE-209, but higher than several newly emerging BFRs, i.e., 1,2-bis(2,4,6-tribromophenoxy)-ethane (BTBPE) and 1,2-dibromo-4-(1,2-dibromoethyl) cyclohexane (TBECH).[10,11] It was hypothesized that these MeO-PB-DiPhOBz contaminants are degradation products that originate from TeDB-DiPhOBz FR.[6,9] However, a very recent study from our lab found that TeDB-DiPhOBz and its debrominated products comprising four homologue groups, Br_{10}- to Br_{13}-PB-DiPhOBz, were not detectable in any surficial sediment samples from several sites in Lakes Huron and Erie. The sampling sites included one near the mouth of the highly FR-contaminated Saginaw River, near the confined disposal facility (CDF) located in Saginaw Bay at Channel-Shelter Island, which receives dredged sediment from the Saginaw River.[12]

A knowledge gap currently exists as to whether maternal herring gulls possess the enzymatic capacity to catalyze the degradation of TeDB-DiPhOBz and/or PB-DiPhOBzs to hydroxylated metabolites, which are highly probable, immediate precursors to the MeO-PB-DiPhOBz contaminants reported in herring gulls. In the present study, we investigated and tested the hypothesis that TeDB-DiPhOBz and/or PB-DiPhOBzs formed photolytically are metabolized in vitro in herring gulls in assays based on isolated microsomal suspensions from enzymatically viable liver tissue harvested from wild female herring gulls from the Great Lakes (Lake Huron) and in comparison to assays based on mammalian model of commercially available liver microsomes of Wister-Han rats.

2. EXPERIMENTAL SECTION

2.1. Standards and Chemicals. Solid powder of technical SAYTEX-120 (TeDB-DiPhOBz; Lot# 0GN01-$I0) and BDE-206 internal standard were kindly supplied by Wellington Laboratories (Guelph, ON, Canada). Two OH-PB-DiPhOBz standards (4″-hydroxy-2,2′,2″,4-tetrabromodiphenoxybenzene (HBDPB-401N) and 4″-hydroxy-2,2″,3′,4-tetrabromodiphenoxybenzene (HBDPB-402N); purities >99%), which were used for comparison with the formed metabolites from in vitro assays, were synthesized at AccuStandard Inc. (New Haven, CT, U.S.A.). The NADPH regenerating systems were purchased from Corning (Corning, NY, U.S.A.). HPLC grade methanol and diethyl ether were purchased from Caledon Laboratories Ltd. (Georgetown, ON, Canada) and VWR International (Mississauga, ON, Canada), respectively. Ultrapure water was obtained from a Milli-Q system. A suspension of rat liver microsomes (protein content 20 mg/mL) from pooled adult male Wister−Han rats were purchased commercially (Reference number: 452511, Corning Gentest, Woburn, MA, U.S.A.) and used as a model mammalian system to study the in vitro metabolism of TeDB-DiPhOBz and its degradation products. Buffer containing 20 mM KH_2PO_4, and 80 mM Na_2HPO_4 (pH = 7.4) was prepared in our lab (Letcher Lab-Organic Contaminants Research Laboratory (OCRL), National Wildlife Research Center (NWRC), Ottawa, ON, Canada).

2.2. TeDB-DiPhOBz and Photolytic Debromination Products. TeDB-DiPhOBz irradiation by natural sunlight and in combination with UV lamps was carried out to prepare three solutions containing progressively less brominated PB-DiPhOBzs. Preparation of solutions 1 and 2 has been detailed in our previous publication.[6] In brief, technical TeDB-DiPhOBz powder was dissolved in 30% tetrahydrofuran/*n*-hexane solution to achieve a final, nominal concentration of 300 μM with a total volume of 40 mL. Before sunlight or UV irradiation, 20 mL of the resulting solution was transferred into a borosilicate glass tube (16 × 125 mm; Fisher Scientific Inc.; Waltham, MA, U.S.A.), blown down to dryness under a gentle nitrogen flow, redissolved in DMSO, and designated as solution 1 (mainly nonphotodegraded TeDB-DiPhOBz[7]). A second 20 mL volume of the 300 μM TeDB-DiPhOBz solution was exposed for 21 days to natural sunlight irradiation (SI) in an outdoor environment over the period of December 24, 2013 to January 14, 2014 in Ottawa (ON, Canada; latitude 45°40′06″N and longitude 75°74′22″W). On day 21, this sample was brought into the laboratory, blown down to dryness under a gentle nitrogen flow, and redissolved in 2 mL of methanol. Half of this methanol volume (1 mL) was subsequently blown down to dryness under a gentle flow of nitrogen, and redissolved in DMSO, and designated as solution 2 for administration in gull and rat liver miscrosomal assays.

The other half of the previously mentioned methanol solution (1 mL) resulting from 21 days of sunlight irradiation of TeDB-DiPhOBz was used for the preparation of subsequent photolytic solutions 3 and 4. The remaining 1 mL methanol volume was first diluted to a final volume of 20 mL with fresh methanol, and two 10 mL aliquots were transferred into two separate borosilicate glass tubes. These two diluted samples were exposed to constant irradiation by both UV B (wavelength: 302 nm; 8 W) and C (wavelength: 254 nm; 8 W) lamps in a dark UVP cabinet (Upland, CA, U.S.A.). During the irradiation, for every 4 h, 50 μL of the solution was taken out, and analyzed by liquid chromatography-quadrupole-time-of-flight/mass spectrometry (LC-Q-TOF/MS; Agilent Technologies, Mississauga, ON, Canada) to monitor the progressive debromination and formation of increasing lower brominated PB-DiPhOBz products (see details in section 2.6). When these subsamples indicated a dominance of Br_{5-8}-PB-DiPhOBzs (after approximately 20 h of UV irradiation), further irradiation was terminated for one of the two methanol diluted samples. The second methanol diluted sample was irradiated for an additional 20 h of UV to generate even lower brominated PB-DiPhOBzs, and designated as solution 4. Solutions 3 and 4 were both blown down to dryness under a gentle flow of nitrogen and redissolved in DMSO for administration in gull and rat liver microsomal assays.

2.3. Herring Gull Liver Sampling. Detailed information on liver sampling of herring gulls (*Larus argentatus*) is described in full detail elsewhere.[13] In brief, in late April of 2010, $n = 8$ female herring gulls were harvested from Chantry Island, Lake Huron (44°19′N, 81°2′W), and the liver samples were collected within 1 h post mortem using chemically cleaned scalpels and scissors. The collected liver samples were stored in chemically cleaned 2 mL cryovials, and placed immediately on-site into a liquid nitrogen dry shipper. After transport from Chantry Island, the liver tissue was subsequently stored in a −80 °C freezer located in Environment and Climate Change Canada's National Wildlife Specimen Bank located at NWRC, Carleton University (Ottawa, ON, Canada), and until the time of preparation of microsomes.

2.4. Herring Gull Microsomes, Protein Content, and Enzyme Activity. The protocol for the preparation of the

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present herring gull microsomal suspensions was according to procedures described in previous publications.[14−18] At all times during the procedure, the liver and microsomal samples were kept cool on ice and carried out as rapidly as possible to minimize the degradation of enzyme catalytic activity. In brief, and as fully described in Greaves et al.,[16] approximately 500 mg of minced liver from each individual herring gull was added to 800 μL of cold potassium phosphate buffer (0.1 M; pH = 7.4), which was further homogenized using an UltraTurrax homogenizer (IKA, Wilmington, NC, U.S.A.). The resulting homogenate was centrifuged at 9000*g* for 15 min and maintained constantly at 4 °C (Beckman Optima TLX ultracentrifuge, Beckman-Coulter, Brea, CA, U.S.A.). The supernatant was further centrifuged at 100 000*g* for 60 min and at 4 °C. The remaining pellet was resuspended with 125 μL of cold potassium phosphate buffer (0.1 M; pH = 7.4). The amount of available liver tissue per gulls was limited. Therefore, to maximize the amount of liver microsomes for in vitro metabolism studies, we pooled together the microsomal solutions from the liver samples of all 8 herring gulls. The protein content and CYP1A-catalyzed 7-ethoxyresorufin-*O*-deethylase (EROD) activity of the microsomes were determined simultaneously as we detail elsewhere,[18] and according Kennedy and Jones (1994).[15] Total protein content was quantified against a standard curve containing bovine serum albumin (BSA), while EROD activity was quantified against a standard curve containing resorufin, using a fluorescence plate reader (Cytofluor Model 2350, S/N 932199, Millipore Ltd.). The EROD assay closely followed Kennedy and Jones (1994), but the reaction was terminated after 2 min to ensure resorufin concentrations fell within the calibration curve range. The total mass of the herring gull liver processed into microsomes was 35.7 g, with a total amount of microsomal protein of 630 mg. Thus, the microsomal yield was 1.77%. The CYP1A (EROD) activity of the gull microsomal pool was 86 pmol/mg protein/min, and that of the Wistar-Han rat microsomes was 280 pmol/mg protein/min. With knowledge of the protein content, the herring gull microsomes were then diluted to a final concentration of 20 mg protein/mL, and stored at −80 °C until further use.

2.5. In Vitro Metabolism Assays. Both herring gull and rat microsomal in vitro assays were performed following the optimal assay parameters for CYP and NADPH-dependent enzymes outlined by BD Bioscience with some modification, and also according to the procedures we have used previously.[19] In brief, this assay was conducted in a 15 mL test tube, and the enzymatic reaction system included 887 μL of incubation buffer (i.e., 80 mM KH_2PO_4, 6.0 mM $MgCl_2$, 1.0 mM Na_2-EDTA, pH = 7.4), 50 μL of NADPH-A, 10 μL NADPH-B, 50 μL herring gull or rat microsomal suspension and 3 μL of target chemical solution (in DMSO) or just the vehicle solvent (DMSO) as a control. The incubation time was 90 min. During the experiments, all assay reagents, with the exception of microsomes, were well mixed with vortexing, and preincubated at 37 °C for 5 min (in a water bath). The reactions were initiated by the addition of the 50 μL of the 20 mg/mL of microsomal protein suspension (and thus the addition of 1 mg of protein per assay). The total final volume of incubation solution in each assay was 1 mL. The solution was incubated in a water bath (37 °C) with shaking (120 rpm). For negative control in vitro assays, liver microsomes had previously been heated to 100 °C for 5 min in a water bath to denature and thus deactivate the enzymes. These deactivated microsomes were used in the negative control assays, and included target chemical/mixture substrate and NADPH, to assess for any potential nonenzymatic (or possible "false-positive") target chemical depletion, and/or hydroxylated metabolite formation. Buffer blanks assays did not contain microsomes or chemical substrate, to assess for any background contamination. No background contamination of TeDB-DiPhOBz, PB-DiPhOBzs, or OH-PB-DiPhOBz metabolites was observed in these blank assays.

For each treatment, $n = 6$ replicate samples were assayed, and half of these 6 samples were quenched at the 0 min time point (as controls) by the addition of 0.5 mL of ice-cold MeOH to the tubes. Similarly, the remaining three replicate samples were quenched at the 90 min time point by addition of 0.5 mL of ice-cold MeOH. The quenched samples were acidified with the addition of 10 μL of acetic acid, and 40 ng of the BDE-206 internal standard used for the quantification of TeDB-DiPhOBz. After vortexing well, 2 mL of 80:20 hexane/dichloromethane (DCM) was added, vortexed for 30 s, sonicated for 5 min, and centrifuged at 3500 rpm for 5 min. The (upper) solvent solution was collected in a new tube, and the entire procedure was repeated an additional four times. The collected 10 mL solvent was blown down to dryness under a gentle flow of nitrogen, redissolved in 200 μL of methanol, and was now ready for instrumental analysis. Triplicates of samples spiked with solutions 1, 2, 3, and 4, and OH-PBDE standards were run for quality control purposes, and showed >80% recovery for all of the tested chemicals based on the observed instrumental responses.

2.6. UPLC-TQ-S-MS Analysis. A Waters ACQUITY UPLC I-Class system (UPLC) coupled to Waters Xevo TQ-S mass spectrometer (TQ-S/MS) (Milford, MA, U.S.A.) was used for quantification of TeDB-DiPhOBz. The instrument was equipped with atmospheric pressure photoionization source (APPI) operated in negative mode. LC separation was carried out on a Cortecs UPLC C18 column (2.1 × 50 mm^2, 1.6 μm particle size) (Waters, Mississauga, ON, Canada). The LC mobile phases were water (A) and methanol (B). The mobile phase flow rate was 0.5 mL/min and the gradient was as follows: 0 min, 30% B; 0−1 min, 50% B (linear); 1−15 min, 100% B (linear) and held for 3 min. Toluene was introduced into the TQ-S/MS at a flow rate of 0.02 mL/min by a Series 200 Micro pump (PerkinElmer, Woodbridge, ON, Canada) and via a T connector after the LC system. The capillary voltage was 1.8 kV. The source and probe temperatures were 150 and 300 °C, respectively. The desolvation and cone gas flow rates were 800 and 150 L/h, respectively. Various MS parameters (parent and daughter ions, cone voltage and collision energy) that were tested are listed in Table S1 of the Supporting Information (SI). For each of the target compounds, $[M\text{-}Br+O]^-$ was consistently selected as the parent ions, but the daughter ion varied depending on the specific bromine number. Using the most abundant SRM transitions for each class of homologues, and the optimal mass spectrometer operation parameters, the UPLC-APPI(−)-MS/MS mass chromatograms demonstrated complete chromatographic and mass spectral resolution (Figure S1).

2.7. LC-Q-TOF/MS Analysis. An Agilent 1200 LC system, consisting of a degasser, binary high-pressure gradient pump, and autosampler and coupled to an Agilent 6520A Q-TOF-MS system, was used to characterize the (e.g., PB-DiPhOBz photolytic products) composition of solutions 1−4, and to identify potential in vitro assay metabolites. For the character-

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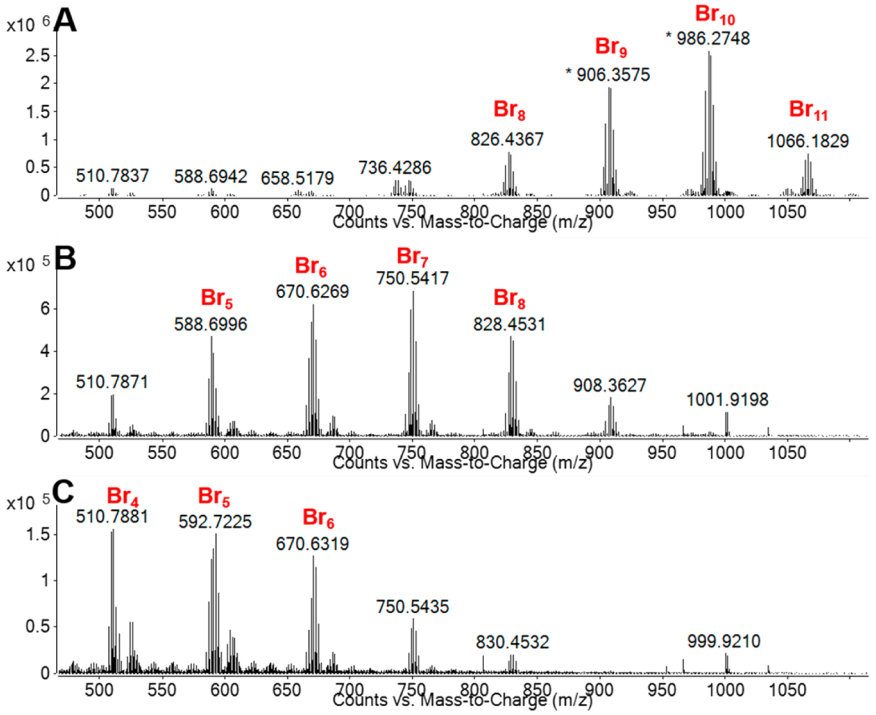

Figure 1. APPI(−)-Q-ToF/MS full scan mass spectra and the characterization of products in the three progressively photodegenerated solutions from tetradecabromo-1,4-diphenoxybenzene (TeDB-DiPhOBz) flame retardant (see Methods). Parts A, B, and C represent solution 2, 3, and 4, respectively. The parent TeDB-DiPhOBz itself was used as solution 1, and its APPI(−)-Q-ToF/MS mass spectra can be found in Figure S1.

ization of the composition of solutions 1−4, the LC-Q-TOF/MS was equipped with an APPI source operated in negative mode. The capillary voltage was 5.0 kV. Nitrogen was used as the drying and nebulizing gas and helium was used as the collision gas. The LC system was equipped with an Xterra Phenyl column (2.1 × 100 mm^2, 3.5 μm particle size) (Waters, Mississauga, ON, Canada). The mobile phase (A, water; B, methanol) flow rate was 0.3 mL/min and the following gradient was employed: 5% B ramped to 100% B in 5 min (linear) and held for 20 min, followed by a change to 5% B and held for 15 min for the next injection. Toluene was introduced into the Q-TOF at a flow rate of 0.02 mL/min by a Series 200 Micro pump (PerkinElmer, Woodbridge, ON, Canada) and via a T connector after the LC system. The Q-TOF instrument was tuned and calibrated with tuning calibration solution (G1969−85000, Agilent Technologies). The TOF-MS was operated at resolution (R) > 20 000 at m/z 601.978977 and within 3 ppm mass error in mass range m/z 50−1700. For each run, 2 μM purine (m/z 119.0363) and 50 nM HP-0921 (m/z 805.9854) were introduced into the Q-TOF with toluene as reference masses.

For the identification of (e.g., OH-PB-DiPhOBz) metabolites, the LC-Q-TOF/MS was equipped with an ESI source operated in negative mode. The Q-TOF instrument parameters were optimized by use of four OH-PBDE standards (6-OH-BDE-85, 6-OH-BDE-90, 2-OH-BDE-123, and 6-OH-BDE-137). The capillary voltage was 4000 V. Nitrogen was used as the drying and nebulizing gas. The gas temperature was 320 °C, dry gas was 5 L/min, and nebulizer gas was 20 psi. Full-scan data acquisition was performed by scanning from m/z 50 to 1700. For each run, TFA anion (m/z 112.9855) and HP-0921 (TFA adduct; m/z 1033.9881) were consistently introduced into the Q-TOF as reference masses.

3. RESULTS AND DISCUSSION

3.1. Characterization of Four Different Photolysis Solutions By LC-APPI(−)-Q-TOF/MS. Before any in vitro depletion experiments with the microsomal assay were performed, the three different solutions prepared by irradiation of parent TeDB-DiPhOBz (as solution 1) under natural sunlight or/and UV, were directly injected for APPI(−)-Q-TOF/MS analysis to determine TeDB-DiPhOBz and debrominated products. The photolytic solution 1 was dominated by TeDB-DiPhOBz.[7] With exception of the $[M\text{-}Br+O]^-$ fragment ion formed in the APPI(−) source, five other fragment ions, $[M\text{-}Br]^-$, $[C_{12}Br_9O_2]^-$, $[C_{12}Br_8O_2]^-$, $[C_{12}Br_7O_2]^-$ and $[C_6Br_5O_1]^-$ were also observed for TeDB-DiPhOBz (Figure S1). These results were highly consistent with the APPI(−)-Q-TOF/MS mass spectrum of TeDB-DiPhOBz presented in our previous study.[7] It is also suggested that under the present APPI(−) parameters the fully brominated TeDB-DiPhOBz results in collision-induced dissociation (CID) product ions where the aromatic carbanions are $[C_{12}Br_9O_2]^-$, $[C_{12}Br_8O_2]^-$, $[C_{12}Br_7O_2]^-$, or $[C_6Br_5O_1]^-$. Cleavage at the ether bond of the [M-Br+O]-fragment ion of TeDB-DiPhOBz was also reported for the structurally analogous and fully brominated BDE-209.[20]

Photolytic solutions 2, 3, and 4 were product mixtures from the irradiation of TeDB-DiPhOBz (Figures 1 and S2). The irradiation conditions to generate photolytic solution 2 were the same as our previous study,[6] where this solution was also dominated by Br_{8-11}-PB-DiPhOBz congeners (Figure 1A). The present solutions 3 and 4 were prepared from solution 2 by additional 20 and 40 h UV B and C irradiation, respectively, and dominated by lower brominated congeners, i.e., Br_{5-8}-PB-DiPhOBz (Figure 1B) and Br_{4-6}-PB-DiPhOBz (Figure 1C), respectively. It is worth noting that, during the whole irradiation process, we did not observe significant signals for PB-DiPhOBz congeners with less than 4 bromine atoms. This

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Table 1. LC-ESI(−)-Q-TOF-MS Analysis and the Theoretical and Observed Molecular Masses of Hydroxylated-Polybrominated Diphenyl Ether (OH-BDE) Standards and Hydroxylated Metabolites of Photolytic Breakdown Products (PB-DiPhOBzs) of Tetradecabromo-1,4-diphenoxy Benzene (TeDB-DiPhOBz)

	chemicals	observed ion mass	theoretical	molecular formula	mass defect (ppm)
four OH-BDE standards					
standards for LC-ESI(−)-Q-ToF/MS optimization	6-OH-BDE-85	580.6052	580.6072	$C_{12}O_2Br_5H_5$	3.4
	6-OH-BDE-90	580.6049	580.6072	$C_{12}O_2Br_5H_5$	4.0
	2-OH-BDE-123	580.6045	580.6072	$C_{12}O_2Br_5H_5$	4.7
	6-OH-BDE-137	658.5143	658.5177	$C_{12}O_2Br_6H_4$	5.2
OH-PB-DiPhOBz metabolites					
herring gull microsome with solution 1	none				
herring gull microsome with solution 2	none				
herring gull microsome with solution 3	metabolite 1 (RT = 8.40 min)	672.6331	672.6329	$C_{18}O_3Br_5H_9$	0.3
herring gull microsome with solution 4	metabolite 1 (RT = 8.27 min)	592.7254	592.7244	$C_{18}O_3Br_4H_{10}$	1.7
	metabolite 2 (RT = 8.40 min)	672.6332	672.6329	$C_{18}O_3Br_5H_9$	0.4
	metabolite 3 (RT = 8.70 min)	672.6324	672.6329	$C_{18}O_3Br_5H_9$	0.7
rat microsome with solution 1	none				
rat microsome with solution 2	none				
rat microsome with solution 3	metabolite 1 (RT = 8.20 min)	592.7246	592.7244	$C_{18}O_3Br_4H_{10}$	0.3
	metabolite 2 (RT = 8.30 min)	672.6342	672.6329	$C_{18}O_3Br_5H_9$	1.9
rat microsome with solution 4	metabolite 1 (RT = 8.00 min)	514.8153	514.8139	$C_{18}O_3Br_3H_{11}$	2.7
	metabolite 2 (RT = 8.23 min)	592.7255	592.7244	$C_{18}O_3Br_4H_{10}$	1.8
	metabolite 3 (RT = 8.30 min)	672.6315	672.6329	$C_{18}O_3Br_5H_9$	2.1
OH-polybenzofuran metabolites					
herring gull microsome with solution 1	none				
herring gull microsome with solution 2	metabolite 1 (RT = 8.12 min)	430.8768	430.8767	$C_{18}O_3Br_2H_8$	0.2
	metabolite 2 (RT = 8.38 min)	510.7837	510.7832	$C_{18}O_3Br_3H_7$	1.0
herring gull microsome with solution 3	metabolite 1 (RT = 8.12 min)	430.8765	430.8767	$C_{18}O_3Br_2H_8$	0.5
	metabolite 2 (RT = 8.38 min)	510.7829	510.7832	$C_{18}O_3Br_3H_7$	0.6
herring gull microsome with solution 4	metabolite 1 (RT = 8.12 min)	430.8766	430.8767	$C_{18}O_3Br_2H_8$	0.2
	metabolite 2 (RT = 8.38 min)	510.7857	510.7832	$C_{18}O_3Br_3H_7$	4.9
rat microsome with solution 1	none				
rat microsome with solution 2	metabolite 1 (RT = 8.12 min)	430.8774	430.8767	$C_{18}O_3Br_2H_8$	1.6
rat microsome with solution 3	metabolite 1 (RT = 8.12 min)	430.8764	430.8767	$C_{18}O_3Br_2H_8$	0.7
rat microsome with solution 4	metabolite 1 (RT = 8.12 min)	430.8757	430.8767	$C_{18}O_3Br_2H_8$	2.3

finding suggested that PB-DiPhOBz congeners exhibit a similar intensity profile as structurally analogous PBDEs after injection into APPI(−) source (Figure 1 and Figure S2). Previous studies demonstrated that in the LC-APPI(−)-Q-TOF/MS analysis of a broad range of PBDE congeners there was a lack of $[M\text{-}Br+O]^-$ ion formation for mono-, di- or tri-BDE congeners that have similar base structures as for dibromo- or tribromo-DiPhOBzs.[21] In our recent studies, when 19 individual standards of PBDEs, with congeners ranging from 1 to 10 bromine atoms, were separately analyzed by LC-APPI(−)-Q-TOF-MS, there were no LC-APPI(−)-Q-TOF-MS responses for the PBDE congeners with less than 3 bromine atoms.[8] Overall, our present results suggest there might be PB-DiPhOBz congeners with less than 4 bromine atoms in solutions 3 or 4, but were not detectable by LC-APPI(−)-Q-TOF/MS analysis. However, several OH-Br_2-PB-DiPhOBz and OH-Br_3-PB-DiPhOBz congeners were detected by LC-ESI(−)-Q-TOF-MS analysis of photolytic solutions 3 or 4, which had been incubated in gull or rat microsomal assays (see Section 3.3).

3.2. In Vitro Metabolism of Non-Irradiated TeDB-DiPhOBz. After incubation with either the herring gull or rat microsomes for 90 min, no significant (*t*-test, $p > 0.05$) differences were observed between nonirradiated (solution 1) TeDB-DiPhOBz concentrations at the time points of 0 min and maximum incubation time of 90 min ($n = 3$ replicates for each time point). No other lower brominated PB-DiPhOBz congeners were detected, and thus enzyme-mediated debromination of TeDB-DiPhOBz was either too slow within the time frame of the assay or microsomal enzymes are not present or active in either the rat or herring gull microsomal suspensions.

To our knowledge, prior to the present study, there have been no reports of any kind examining the in vitro or in vivo metabolism of TeDB-DiPhOBz. The low biotransformation potential of TeDB-DiPhOBz shown in the present in vitro study with microsomes from wild Great Lakes herring gulls might be due to its high log octanol−water partition coefficient (i.e., > 10), and is consistent with previous reports of another highly brominated flame retardant, BDE-209. Using an in vitro assay based on liver microsomes of wild ring-billed gulls (*Larus delawarerensis*) harvested from sites in the Montreal (QC, Canada) area, no significant depletion of BDE-209 or the formation of lower brominated products was observed.[22] In contrast to in vitro metabolism studies in birds, avian in vivo metabolism studies with BDE-209 have shown debromination of BDE-209. Using subcutaneous silastic implants to administer BDE-209, European starlings (*Sturnus vulgaris*) accumulated BDE-209 in muscle and liver tissues, and well as the presence of debrominated product congeners down to hexa-BDEs.[23] Between the control and exposed groups of starlings, there were also much more pronounced octa- (BDE-196 and -197) and nona-BDEs (BDE-206, -207, and -208) in the exposed

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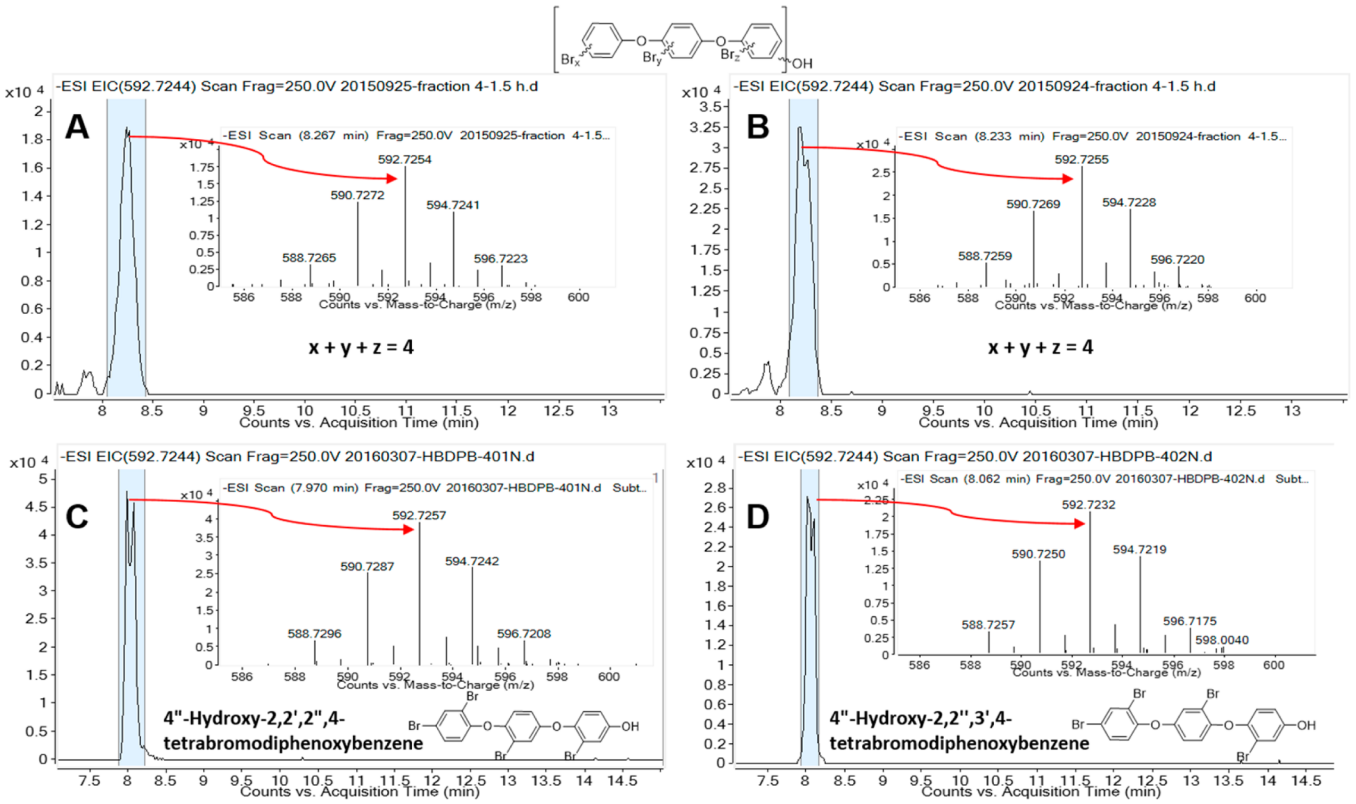

Figure 2. LC-ESI(−)-Q-TOF-MS extracted ion mass chromatograms showing the detected hydroxylated polybrominated diphenoxybenzene (OH-Br_3-PB-DiPhOBz) congener (formula: $C_{18}O_3Br_4H_{11}$; theoretical mass: 592.7244; see other OH-PB-DiPhOBz metabolites in Figures S3, S4, and Table 1) in the irradiation time solutions 4 and after administration to the in vitro herring gull (A) or rat (B) microsomal assay, and comparisons with two OH-Br_3-PB-DiPhOBz standards, 4″-hydroxy-2,2′,2″,4-tetrabromodiphenoxybenzene (C) and 4″-hydroxy-2,2″,3′,4-tetrabromodiphenoxybenzene (D, 5 ppm for each).

group. In vivo studies of diet-exposed BDE-209 male American kestrels (*Falco sparverius*) reported quantifiable BDE-209 in all plasma, liver and fat samples in the exposed birds as well as lower brominated PBDE congeners, mainly *meta*- and *para*-debromination products of BDE-209 in plasma, liver and/or fat.[24] In vitro or in vivo studies have also been conducted on the metabolism of BDE-209 based on species other than birds. For example, Stapleton et al. exposed juvenile carp to BDE-209 amended food for 60 days, followed by a 40-day depuration period. The researchers did not observe the net accumulation of BDE-209 throughout the experiment despite an exposure concentration of 940 ng/day/fish, which clearly indicated evidence of limited BDE-209 bioavailability and uptake from food.[25] In follow-up studies, the same researchers incubated common carp and rainbow trout microsomal solutions with BDE-209 for 24 h, observing 22% and 65% depletion of BDE-209 by rainbow trout and common carp, respectively.[26]

3.3. In Vitro Metabolism of Photolytic PB-DiPhOBz Products. Given that lower brominated PB-DiPhOBz congeners were found in the photolytic product mixtures of solutions 2, 3 and 4, we tested the hypothesis that the in vitro microsomal metabolism of PB-DiPhOBzs generates hydroxylated (OH-) PB-DiPhOBz products via phase I of metabolism of detoxification.[27] Phenolic metabolic products have been shown previously for the in vitro biotransformation of, e.g., PBDE congeners[17] and the organophosphate ester flame retardant, triphenyl phosphate (TPHP),[28,29] using a microsomal assay based on mammalian liver and chicken embryonic hepatocytes, respectively. In the present study, identification of OH-PB-DiPhOBz metabolites after incubation in gull and rat microsomes of photolytic solutions 2, 3, and 4, was conducted by use of LC-ESI(−)-Q-TOF/MS, which underwent parameter optimization based on four structure-similar chemicals to OH-PB-DiPhOBzs, i.e. 6-OH-BDE-85, 6-OH-BDE-90, 2-OH-BDE-123, and 6-OH-BDE-137. In fact, all three sources, APPI, ESI, and atmospheric pressure chemical ionization (APCI), showed great signal intensity for these four tested OH-PBDEs. Here, we selected ESI source for identification of OH-PB-DiPhOBzs due to its two clear advantages: (1) ESI source is a relatively "soft ionization" technique, and this can be advantageous in the sense that the molecular ion is always observed with very little fragmentation and (2) ESI source is specifically sensitive for OH-PB-DiPhOBZs,[8] and this would be another advantage to avoid unnecessary disturbances from parent PB-DiPhOBz congeners in microsomal assay extracts. In our initial experiments, we also tried to screen the possible methxylated (MeO-) PB-DiPhOBz products, but detect none of them. Nondetection of MeO-PB-DiPhOBz in the microsomal assays is reasonable, because methylation normally happens in subsequent phase II reactions of detoxification.[30]

As shown in Table 1 and Figures S3 and S4, at least one (retention time (RT) = 8.40 min; observed molecular weight: 672.6331 Da) and three (RT = 8.27, 8.40, and 8.70 min; observed molecular weight: 592.7254, 672.6332, and 672.6324 Da, respectively) OH-PB-DiPhOBz congener metabolites were detected from herring gull microsomes incubated with solutions 3 and 4, respectively (Figure S3). Similarly, at least two (RT = 8.20 and 8.30 min; observed molecular weight:

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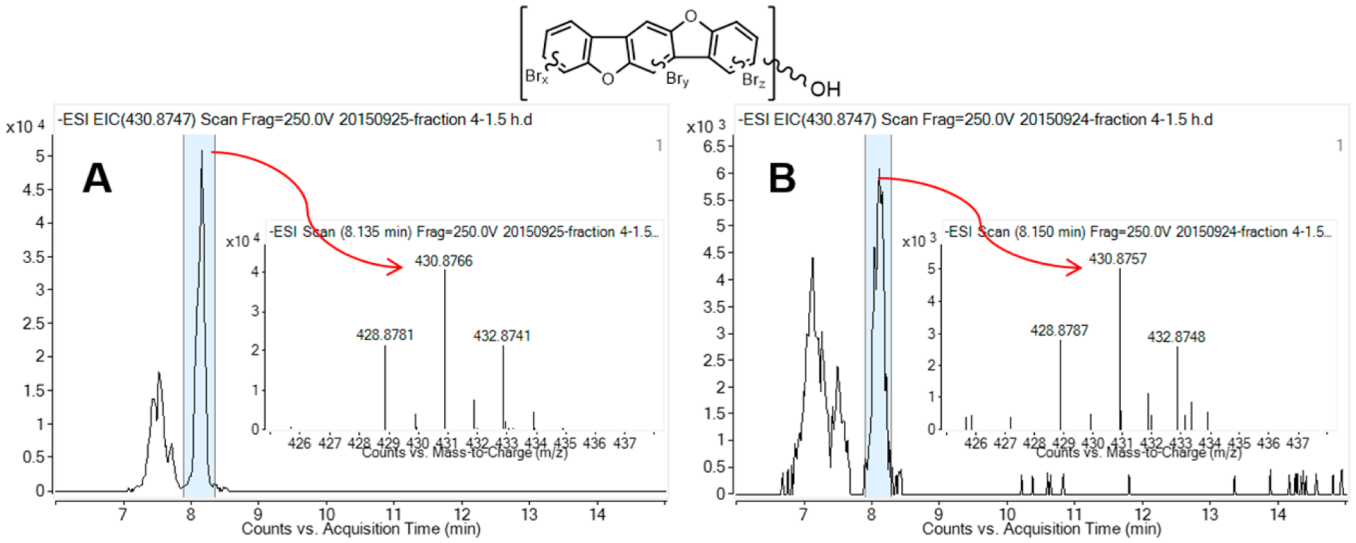

Figure 3. LC-ESI(−)-Q-TOF-MS extracted ion mass chromatograms showing the detected hydroxylated metabolites of dibrominated dibenzofuran congener (formula: $C_{18}O_3Br_2H_8$; theoretical mass: 430.8747; see other furan metabolites in Figures S5, S6 and Table 1) in the irradiation time solutions 4 after administration to the in vitro herring gull (A) or rat (B) microsomal assay.

592.7246 and 672.6342 Da, respectively) and three (RT = 8.00, 8.23, and 8.30 min; observed molecular weight: 514.8154, 592.7255, and 672.6315 Da, respectively) OH-PB-DiPhOBz metabolites were detected from rat microsome incubation with solutions 3 and 4, respectively (Figure S4 and Table 1). No OH-PB-DiPhOBz metabolites were observed from gull or rat microsome incubation with solution 2. These results indicate that enzyme-mediated hydroxylation occurs at a measurable rate within the 90 min microsomal incubation period, for PB-DiPhOBz enzyme substrates with 8 bromine atoms or less, as indicated by solutions 3 and 4 where we characterized Br_{5-8}-PB-DiPhOBz and Br_{4-6}-PB-DiPhOBz photolytic congeners, respectively.

Both herring gull and rat microsomes were able to metabolize PB-DiPhOBz, but there were clear species-specific differences in the ability to metabolize individual congeners. Specifically, the incubation of PB-DiPhOBz in herring gull microsomes resulted in two OH-Br_5-PB-DiPhOBzs (RT = 8.40 and 8.70 min; theoretical molecular weight: 672.6329 Da) and one OH-Br_4-PB-DiPhOBz (RT = 8.30 min, theoretical molecular weight: 592.7244 Da). However, metabolites from the coincubation of rat microsomes and PB-DiPhOBz included one OH-Br_3-PB-DiPhOBz (RT = 8.00 min, theoretical molecular weight: 514.8139 Da), one OH-Br_4-PB-DiPhOBz (RT = 8.20 min, theoretical molecular weight: 592.7244 Da) and one OH-Br_5-PB-DiPhOBz (RT = 8.30 min, theoretical molecular weight: 672.6329 Da). This likely reflects the differences in the isoforms and catalytic activities of cytochrome P450 isozymes present in the rat versus herring gull microsomal suspensions.[31,32]

To further investigate the specific structure identity of the OH-PB-DiPhOBz metabolites formed in vitro, we synthesized two OH-Br_4-PB-DiPhOBz standards, 4″-hydroxy-2,2′,2″,4-tetrabromodiphenoxybenzene (HBDPB-401N, Figure 2C) and 4″-hydroxy-2,2″,3′,4- tetrabromodiphenoxybenzene (HBDPB-402N, Figure 2D), and analyzed the two standards by LC-ESI(−)-Q-TOF-MS for comparisons with the OH-PB-DiPhOBz congeners formed from herring gull or rat microsomes exposed to PB-DiPhOBz congeners. As shown in Figure 2, the OH-Br_4-PB-DiPhOBz congeners formed in vitro from herring gull or rat microsomes exhibited extremely similar RT in the LC column (all were eluted at approximate 8 min) and mass characteristics in the ESI(−)-Q-TOF with the synthesized HBDPB-401N and HBDPB-402N standards. These findings further confirm that these detected ions were indeed for OH-PB-DiPhOBzs. However, full identification (i.e., specific positions of OH- or Br-) of other formed OH-PB-DiPhOBz products in vitro was not possible due to the lack of other synthesized OH-PB-DiPhOBz standards.

3.4. Hydroxylated Polybrominated Di-Benzofuran Products Formed In Vitro. In a study using a chicken embryonic heptatocyte (CEH) assay that was administered the product mixture characterized by the present photolytic solution 2, we recently reported that there was a large expression change in aryl hydrocarbon receptor (AhR)-related *CYP1A4* mRNA levels with induction up to 5200-fold.[6] In contrast, there was no mRNA expression change in comparable CEH assay assessments with photolytic solution 1. In a follow-up study on TeDB-DiPhOBz photolysis, LC-APPI(−)-Q-TOF-MS analysis of photolytic solution 2 revealed the presence of numerous brominated homologue groups of polybenzofurans.[8] Similarly in the present study, several molecular mass ions were observed that share exactly the same molecular masses with hydroxylated metabolites of polybenzofurans (Figures S5, S6, and 3). Specifically, one OH-Br_2-polybenzofuran (RT = 8.12 min, theoretical molecular weight: 430.8767 Da) and one OH-Br_3-polybenzofuran (RT = 8.38 min, theoretical molecular weight: 510.7832 Da) were detected as a result of herring gull microsomal assay incubation with photolytic solutions 2, 3, or 4. One OH-Br_2-polybenzofuran (RT = 8.12 min, theoretical molecular weight: 430.8767 Da) was detected as a result of rat microsomal assay incubation with photolytic solutions 2, 3, or 4. Benzofurans and their analogues constitute a major group of naturally occurring compounds, where polybrominated dibenzofurans (PBDFs) are of particular interest because of their ability to bind to the AhR, activate AhR-mediated signaling pathways, and elicit typical dioxin-like toxic and biological effects.[33] To our knowledge, until the present study, enzyme-mediated hydroxylation of the present polybrominated dibenzofurans (formed photolytically) was not known.

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3.5. Environmental Implications. We have previously reported on three major Br_4-to Br_6-MeO-PB-DiPhOBz congeners in the eggs of Great Lakes herring gulls.[9,10] We had hypothesized that TeDB-DiPhOBz is the fundamental precursor and source to these MeO-PB-DiPhOBz contaminants. In the present study, we demonstrated that TeDB-DiPhOBz can photolytically degrade in a progressive fashion to lower brominated PB-DiPhOBz products, with Br_4- to Br_7-PB-DiPhOBz products being the most frequently observed and estimated to be most concentrated. Furthermore, we showed that enzyme-mediated hydroxylation occurs in vitro for tri-, tetra-, and penta-PB-DiPhOBzs administered to assays based on herring gull (and rat) liver microsomes. It is well established that for suitable xenobiotic substrates, metabolism can be enzyme-mediated via Phase I pathways to generate OH-containing metabolites. These Phase I metabolites can subsequently undergo Phase II conjugation processes including methyltranferase-mediation of OH-containing substrates to MeO-containing metabolites. It is therefore plausible that the present OH-PB-DiPhOBz metabolites formed from PB-DiPhOBz photolytic products as a result of enzyme-mediated pathways in herring gulls, and could be further metabolized to MeO-PB-DiPhOBzs by herring gulls.

The in vitro metabolism of PB-DiPhOBz to OH-PB-DiPhOBz metabolites observed in this study has also been shown for other flame retardant chemicals. For example, HBCDD was reported to be metabolized to OH-HBCDD metabolites in in vitro experiments and in environmental samples.[34] Both α- and β-1,2-dibromo-4-(1,2- dibromoethyl)-cyclohexane (DBE-DBCH) can be metabolized into measurable metabolites, especially OH-DBE-DBCH and $(OH)_2$-DBE-DBCH using the same model rat microsomal assay.[19] Several in vivo and in vitro studies have also observed formation of OH-BDEs from various PBDE congeners.[35−37] Specifically, both mono- and di-OH-BDEs were detected in tissues of BDE-99 or -100 exposed rats and mice,[35,36] and BDE-153 was reported to be biotransformed into six mono-OH-PBDEs in female mice.[37] A recent study also identified hydrolyxated triphenyl phosphate (OH-TPHP) isomers in TPHP-exposed CEH.[28,29] Most importantly, certain hydroxylated metabolites, i.e., OH-BDEs or OH-TPHP, are more potent for some toxicological end points than the postulated precursor FRs.[38−40] Given the similar structures between OH-PBDEs and OH-PB-DiPhOBz, assessment of toxicological activities should be conducted on these novel hydroxylated chemicals.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b02172.

Further details are given on the UPLC-APPI(−)-TQ-S/MS parameters and mass chromatograms for TeDB-DiPhOBz and PB-DiPhOBz, APPI(−)-Q-ToF/MS full scan mass spectra of solution 1, and mass spectra of all OH-PB-DiPhOBz metabolites resulting from herring gull or rat microsomal assay (PDF)

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Notes

The authors declare no competing financial interest.

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