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# Bioaccumulation and Biotransformation of BDE-47 Using Zebrafish Eleutheroembryos (*Danio rerio*)

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Abstract: Polybrominated diphenyl ethers (PBDEs) are well-known endocrine disrupting chemicals identified as organic persistent pollutants. Their metabolites OH-BDE and MeO-BDE have been reported to be potentially more toxic than the postulated precursor PBDEs. One of the most predominant congeners of PBDEs in the environment is BDE-47, due to its high presence in industrially used mixtures. In the present study, the bioaccumulation and biotransformation of BDE-47 into its major metabolites is evaluated using zebrafish (*Danio rerio*) eleutheroembryos adapting a previously developed alternative method to bioconcentration official guideline Organisation for Economic Co-ordination and Development 305, which reduces the animal suffering, time, and cost. For the simultaneous determination of BDE-47 and its metabolites in larvae and exposure medium, and considering the polarity difference of the analytes and the small sample size, the development of a validated analytical method is a step to ensure quality results. In the present study, an ultrasound-assisted extraction followed by a solid phase extraction dispersive clean-up step and gas chromatography-mass spectrometry-microelectron capture detector (GC-MS-μECD) with a previous derivatization process was optimized and validated. Bioconcentration factors (BCFs) were calculated using a first-order one-compartment toxicokinetic model. The profiles found show rapid absorption in the first hours of larval development and great bioaccumulative capacity, finding BCFs of $7294 \pm 899$ and $36\,363 \pm 5702$ at nominal concentrations of 10 and 1 $\mu g\,L^{-1}$, respectively. Metabolization studies show increasing concentrations of the metabolites BDE-28, 2′-OH-BDE-28, and 5-MeO-BDE-47 throughout the exposure time. The results obtained show the feasibility of the method for bioaccumulation and open up the possibility of metabolic studies with zebrafish eleutheroembryos, which is a very underdeveloped field without official testing or regulation. *Environ Toxicol Chem* 2023;00:1–11. © 2023 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

Keywords: Bioconcentration; Biotransformation; BDE-47; GC-MS-μECD; Metabolites; Zebrafish eleutheroembryos

## INTRODUCTION

Polybrominated diphenyl ethers (PBDEs) are industrial substances that have been widely used as flame retardants for decades in commercial products due to their good thermal stability and low cost (Wen et al., 2015). Their proven toxicity (Zhang, Zhao, et al., 2020; Zhang, Zhou, et al., 2020) and structural similarity to thyroid hormones and thyroxine in particular, which induce neurodevelopmental and endocrine disrupting effects in humans (Butryn et al., 2020), have meant that nowadays some commercial PBDEs mixtures are banned, and they have been identified as Persistent Organic Pollutants by the Stockholm Convention since 2009 (United Nations Environmental Program, 2019). However, as a consequence of this persistence in the environment through long-term use and recycling processes, these chemicals are still detected at potentially dangerous levels in humans and in a wide range of biological and environmental samples (De la Torre et al., 2020; Klinčić et al., 2020; Liu et al., 2020).

In addition, hydroxylated (OH-BDE) and methoxylated (MeO-BDE) and, recently, dihydroxylated (diOH-BDE) products (Zhang, Zhou, et al., 2020) have been found in a wide diversity of environmental samples (Liu et al., 2014; Sun et al., 2020). Several studies have evidenced that metabolites maintain bioactive groups, exhibiting similar or even greater toxicity than the native PBDEs (Usenko et al., 2012), increasing the concern about these compounds. Although the mechanism of biotransformation of PBDEs, oxidated by hepatic cytochrome P450 (CYP) enzymes, has been proven (Li et al., 2010), there are contradictory reports

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of PBDE biotransformation depending on the organism studied (Erratico et al., 2013; Liu et al., 2015; Sun et al., 2013) and therefore they need to be further studied. In the present study, the bioaccumulation and biotransformation of 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47) has been evaluated due to its abundance, toxicity (median lethal concentration [LC50] 4.2 mg $L^{-1}$; Usenko et al., 2011), persistence, and bioavailability (log $K_{ow}$ 6.81; Pereira et al., 2016) in the environment.

The degree of bioaccumulation of a compound is expressed by the bioaccumulation factor, defined experimentally as the ratio between the concentration of the analyte in the organism and its surrounding medium. The bioconcentration factor (BCF) is also used for aquatic species (El-Amrani et al., 2012) and estimated under controlled laboratory conditions (Arnot & Gobas, 2006). The official method for assessing the BCF, proposed by the European Regulation for the Registration, Evaluation, and Authorisation of Chemicals (REACH), is guideline 305 of the Organisation for Economic Co-operation and Development (OECD, 2012). This test requires many adult fish (up to 104) and a long exposure time (up to 60 days), which creates a high cost for the experiments. On the other hand, in recent years, these same international organizations have called for a transformation in toxicity testing, seeking greater efficiency and a reduction in animal experimentation. Zebrafish is gaining interest as an ecotoxicology model organism (Lillicrap et al., 2016) because of its fast production, low cost, transparency, and close genomic homology with humans (more than 80%). Furthermore, because thyroid hormone regulators are highly conserved among vertebrate species, zebrafish is a useful model to investigate thyroid hormone-related compounds (Vancamp et al., 2019). In addition, European legislation considers eleutheroembryos as in vitro systems up to the self-feeding stage (2010/63/EU, 2010). In this sense, an alternative method based on the OECD 305 assay, developed in a research group using zebrafish eleutheroembryos (*Danio rerio*; Sanz-Landaluze et al., 2015) for bioaccumulation studies, is used in the present study, drastically reducing the time, cost, and animal suffering. In addition, in our recent studies (Molina-Fernández et al., 2021) it has been observed that eleutheroembryos are able to biotransform the parental compounds into some of their metabolites during the exposure time with this alternative method. This has opened up the possibility for us to continue using it for metabolic studies because this is an underdeveloped field with no official testing or regulation.

BDE-47 in zebrafish has been investigated in some studies (Liu et al., 2015; Wen et al., 2015; Zheng et al., 2012), but information on the full spectrum of potential metabolites and the metabolic pathways involved is still unclear. Therefore, the aim of the present study was the evaluation of bioaccumulation and the biotransformation of BDE-47 in the most abundant metabolites founded in the literature, 6-MeO-BDE-47, 5-MeO-BDE-47, 3-MeO-BDE-47, 6-OH-BDE-47, 5-OH-BDE-47, 3-OH-BDE-47, and 2′-OH-BDE-28 (Lacorte et al., 2010; Liu et al., 2015; Wen et al., 2015; Zhai et al., 2014) using zebrafish eleutheroembryos. For this purpose, it was necessary to develop a determination method for BDE-47 and its metabolites, both in the larvae and in the exposure medium. It is important to bear in mind that zebrafish eleutheroembryos samples are very small (0.44 mg/larvae dry wt), have a high lipid content (~15%), and their exposure concentrations are always below toxicological values (<1% of LC50). These sample characteristics make it necessary to implement extremely sensitive determination methodologies, capable of dealing with very small samples. For this reason, conventional Soxhlet, which has traditionally been the most widely used extraction method for PBDE from solid samples in a variety of matrices (Cruz et al., 2017), is not appropriate. To minimize the use of solvents and sample treatment time, the analytical protocol proposed in the present study is based on simultaneous miniaturized ultrasonic probe-assisted extraction with dispersive solid phase extraction (SPE) clean-up. Gas chromatography-mass spectrometry (GC-MS) is the most commonly used technique for the determination of PBDEs and MeO-PBDEs (Shelepchikov et al., 2019) and liquid chromatography for OH-BDEs (Song et al., 2020). However, the official method of the US Environmental Protection Agency (USEPA) for the detection of PBDEs is based on electron captured detection (ECD) because this is the most sensitive detection method. For this reason, simultaneous gas chromatography-mass spectrometry-microelectron capture detector (GC-MS-μECD) analysis by previous derivatization of OH-BDEs was carried out to determine the BDE-47 and its metabolites (MeO-BDEs and OH-BDEs) concentration under the same instrumental conditions.

## MATERIAL AND METHODS

### Reagent and samples

Individual commercial standards of 1 ml of PBDEs 28 (150 mg $L^{-1}$), 47 (350 mg $L^{-1}$), and 99 (116 mg $L^{-1}$) in methanol were purchased from Dr. Ehrenstorfer; individual 1-ml standards of the metabolites 6-MeO-BDE-47 (10 mg $L^{-1}$), 5-MeO-BDE-47 (50 mg $L^{-1}$), 3-MeO-BDE-47 (50 mg $L^{-1}$) in methanol, and 5-OH-BDE-47 (50 mg $L^{-1}$), 3-OH-BDE-47 (50 mg $L^{-1}$), and 2′-OH-BDE-28 (50 mg $L^{-1}$) in acetonitrile were supplied by AccuStandard 1,2,3,4-Tetrachloronaphthalene (TCN; 10 mg $L^{-1}$ in isooctane; Dr. Ehrenstorfer) was used as internal standard for the PBDE congeners and the methoxylated metabolites (MeO-BDE) and triclosan (TCS; 10 mg $L^{-1}$ in acetone; Dr. Ehrenstorfer) for the hydroxylated metabolites (OH-BDE). From these commercial solutions, individual working solutions of 1 mg $L^{-1}$ in the same solvents and a mix of 100 μg $L^{-1}$ of all the analytes to be determined (PBDEs, MeO-BDEs, and OH-BDEs) in isooctane were prepared by dilution. The derivatization reagent used for the analysis of the OH-BDE metabolites was N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) + 1% tert-butyl-methylchlorosilane supplied by Sigma Aldrich. The adsorbents of the "clean-up" stage were $C_{18}$ ODS SPE Buk Sorbent (Agilent Technologies), PSA SPE Bulk Sorbent (Agilent Technologies), SupelTM QuE Z-Sep (Sigma Aldrich), and Florisil (E. Merck). The analytical-grade solvents used (isooctane, n-hexane, dichloromethane, methyl tert-butyl ether [MTBE], and dimethyl sulfoxide [DMSO]) were purchased from Sigma Aldrich. The exposed zebrafish (*Danio rerio*) media and eleutheroembryos were provided by AZTI Tecnalia (Bizkaia, Spain).

## Instrument and apparatus

A Genie-2 vortex mixer from Scientific Industries and a Vibra cell VCx130 ultrasound probe from Sonics & Materials. with a 2-mm diameter titanium microtip and a 130-W high-frequency generator at 20 KHz were used for extraction procedures. An X50S Metal Carbide technical nitrogen stream and an Eppendorf 5415 R microcentrifuge were used for evaporation and centrifugation, respectively.

A gas chromatograph Agilent Mod. 7890 A Series equipped with an HP 7683B Series autoinjector, μECD and HP 5975 C VL MSD MS detector with ChemStation software (Agilent Technologies) was used for final separation and quantification of the PBDEs and their metabolites. The detectors worked simultaneously thanks to the use of a flow splitter, placed at the end of the chromatographic column. The GC system was equipped with two polydimethylsiloxane (95%) capillary cross-linked columns: DB-5 (20 m × 0.1 mm × 0.1 μm) and ZB-5 (30 m × 0.25 mm internal diameter, 0.25-μm film thickness) from Phenomenex, using Helium Premier (99.999% purity) X50S Metal Carbide as the carrier gas.

## Analytical procedure

***Instrumental determination.*** Chromatographic separation and detection were carried out using a GC-MS-μECD system. The MS was used for the identification of the analytes and the μECD for the quantification. A double determination of the analytes was carried out, one before derivatization for the analysis of PBDEs and MeO-BDEs with TCN as internal standard and another after, for the analysis of OH-BDEs using TCS as internal standard because PBDEs are not stable to derivatization. The determination of OH-BDEs was carried out by derivatizing with MTBSTFA for 30 min to 70 °C to determine them as tert-butyldimethylsilylated derivatives (TBDMS): OH-TBDMS-BDE.

Finally, samples (1 μl) were injected in the splitless mode at 280 °C. Helium was used as the carrier gas at a constant pressure of 28 psi (~1.5 ml $min^{-1}$). The temperature of the column (ZB-5MS) was programmed to increase from 130 °C (2 min) to 230 °C (2 min) at a rate of 25 °C/min and then to 280 °C (2 min) at 2 °C/min. The total time for each chromatogram was 35 min. The temperature of the ion source and the transfer line of the mass spectrometer were set at 250 and 270 °C, respectively, and the electron beam was set at 70 eV. SCAN mode was used to establish the *m/z* values (Table 1) with standards for each analyte, as well as the mass spectra displayed in the libraries of the National Institute of Standards and Technology databases and by bibliography (Butryn et al., 2015). The μECD temperature was set at 320 °C and nitrogen was used as the makeup gas (30 ml $min^{-1}$). The flow ratio of the splitter was 1:1 thanks to the additional helium input at a constant pressure of 15 psi and the restrictors length of each detector calculating with a manual supplied by Agilent (Agilent Technologies, 2011).

***Sample preparation.*** To avoid contamination of the sample, plastic materials were not used due to the possible transfer of pollutants to or from the material. Hamilton syringes were always used for the addition of solutions. All reused glass material was washed with acetone, rinsed with deionized water and left overnight in the oven at 250 °C.

Zebrafish eleutheroembryos in groups of 15 were extracted with 500 μl of the n-hexane:MTBE mixture (1:1) and sonicated with an ultrasound probe for 4 min at 40% of power and a pulse every second (on/off, 1 s/1 s). The internal standards TCN and TCS were previously added so that the extract to be injected contained 20 μg $L^{-1}$ of each. The mixture was centrifuged for 10 min at 10 000 rpm, the organic phase was separated, and the extraction was repeated. The organic phases were purified with 50 mg of florisil through dispersive SPE and mixed for 30 s in the vortex. The extract was then centrifuged and evaporated to dryness under a gentle stream of nitrogen and reconstituted in 500, 1000, or 2000 μl of isooctane depending on the time at which the sample was collected from experiments.

Extracts from embryo water were prepared as follows: 500 μl of the exposed solution was extracted by 500 μl of n-hexane:MTBE (1:1) and mixed for 30 s in the vortex. The second step of extract clean-up was not applied in the exposure media because they did not show any interference or matrix effect due to the lipidic fat. The internal standards TCN and TCS were previously added. The mixture was centrifuged for 10 min at 10 000 rpm, the organic phase was separated, and the extraction was repeated. The organic phases were mixed,

**TABLE 1:** Mass spectrometer conditions in SIM mode for electron impact ionization

| Analyte | Retention time (min) | *m/z* | Initial scanning time (min) |
|---|---|---|---|
| TCN | 9.31 | 266, 264, 268, 194 | 8 (solvent delay) |
| BDE-28 | 12.48 | 248, 246, 406, 408 | 11 |
| TBDMS-TCS | 13.71 | 290, 288, 218 | 12.8 |
| BDE-47 | 16.98 | 326, 486, 484, 488 | 15 |
| 2′-OH-TBDMS-BDE-28 | 19.69 | 423, 421, 425, 81 | 18.5[a] |
| 6-MeO-BDE-47 | 20.13 | 516, 514, 518, 420 | |
| 3-MeO-BDE-47 | 21.23 | 356, 516, 341, 514 | |
| 5-MeO-BDE-47 | 21.62 | 516, 356, 358, 326 | |
| BDE-99 | 22.98 | 404, 406, 564, 566 | 22 |
| 5-OH-TBDMS-BDE-47 | 27.07 | 502, 504, 500, 81 | 25 |
| 3-OH-TBDMS-BDE-47 | 31.72 | 502, 474, 266, 419 | 29 |

[a]A joint window is established because they elude close retention times, so that the majority *m/z* of each is monitored (marked in the table).
TCN = 1,2,3,4-Tetrachloronaphthalene; TBDMS-TCS = tert-butyldimethylsilylated derivative of triclosan.

concentrated in a gentle stream of nitrogen and reconstituted in 50 µl of isooctane.

Finally, 80 and 50 µl of the volume obtained for eleutheroembryos and embryo water, respectively, were derivatised with 20 µl of MTBSTFA to 70 °C for 30 min in the oven and injected into the GC-µECD-MS to determine the OH-BDE in the form of OH-TBDMS-BDE. Polybrominated diphenyl ethers and MeO-BDEs were determined by direct injection of the remaining volume of isooctane extracts into the GC-µECD-MS.

### Exposure of zebrafish eleutheroembryos: Bioconcentration and biotransformation experiments

Zebrafish eleutheroembryos were obtained from adult zebrafish bred, maintained, and exposed in the AZTI Tecnalia (Bizkaia, Spain) under standard conditions (Westerfield, 2007). The OECD Technical Guidance OECD 305 (OECD, 2012) was used to establish the growing conditions (dissolved oxygen ≥60%, 26 ± 2 °C and pH 7.8 ± 0.2) and nominal exposure concentrations.

Exposure solutions were prepared with a composition of fresh river water (International Organization for Standardization 7346-3, 1996): 220.5 mg of $CaCl_2$, 63 mg of $NaHCO_3$, 5.5 mg of KCl, and 60.1 mg of $MgSO_4$ per litre of distilled water. The OECD Test 305 states that the exposure concentrations should be 1% and 0.1% of the LC50 (if detection limits enable it to be determined). Usenko et al. (2011) show a BDE-47 zebrafish embryos LC50 value of 4.2 mg $L^{-1}$. Thus, the nominal concentrations chosen to carry out the bioconcentration experiment were 1 and 10 µg $L^{-1}$ for BDE-47, and 100 and 250 µg $L^{-1}$ for the metabolization experiment. Due to the limited solubility of BDE-47 in water (11 µg $L^{-1}$ [Pereira et al., 2016]; 21.24 µg $L^{-1}$ [USEPA Comptox data base]), it is dissolved in DMSO so that the medium finally contains 0.5% DMSO (Hallare et al., 2006).

Bioconcentration and metabolization experiments were carried out according to an alternative method developed previously (Sanz-Landaluze et al., 2015). Zebrafish eleutheroembryos were exposed to contaminated solution of BDE-47 at 72 h post fertilization (hpf) during 48 h (uptake phase). Afterwards, they were exposed for 24 h to the medium without contaminant (depuration phase). Thus, they are not considered laboratory animals because until 144 hpf they do not feed on their own (Sanz-Landaluze et al., 2015). Three groups of zebrafish eleutheroembryos were used (a total of 1050 individuals, 350 in each tank), each in 1-L glass tank, ensuring a density of 2 ml/individual: two tanks included zebrafish contaminated with the studied analyte at mentioned concentrations and the third tank had the nonexposed individuals (control), with only the addition of solvent. The exposure medium was refreshed every 24 h to keep a constant nominal concentration. During exposure, 15 eleutheroembryos and 1.5 ml of medium were pulled out from the tanks at different moments for analysis: 0 (start of the experiment), 17, 24, 41, 48, 65, and 72 h to determine the bioaccumulated concentration of each compound, and 0 and 48 h for the metabolization experiment. Three replicates of exposure and blank or control samples were collected for each time evaluated.

### Toxicokinetic model equations for BCF

In the present study, the quantitative estimation of the degree of bioconcentration is going to be estimated according to the OECD 305 guideline (OECD, 2012). According to this test guideline, the BCF is estimated as the ratio of the concentration of the compound in the fish ($C_f$) to that of the mean calculated in the surrounding medium ($C_W$) at a steady state (Equation 1):

$$BCF_{ss} = \frac{C_f\,(\text{ng kg}^{-1})}{C_W\,(\text{ng L}^{-1})} \quad (1)$$

Nevertheless, when the steady state is not reached, BCF can be calculated using a bioconcentration first-order kinetic model ($BCF_k$) that describes the chemical uptake and depuration process (Gobas & Zhang, 1992; Mackay & Fraser, 2000; Equation 2):

$$\frac{dC_f}{dt} = k_1 \times C_W - k_2 \times C_f\,(\text{uptake}),$$
$$\frac{dC_f}{dt} = -k_2 \times C_f\,(\text{depuration}) \quad (2)$$

where $C_f$ is expressed in ng $kg^{-1}$, $t$ is the exposure time (h), $k_1$ is the first-order uptake constant (L $kg^{-1}$ $h^{-1}$), $C_w$ is expressed in ng $L^{-1}$, and $k_2$ is the first-order elimination rate constant ($h^{-1}$). Assuming that at $t = 0$ the concentration of the analyte in the fish is zero and that the concentration in the exposure medium is constant, Equation (3) is obtained:

$$C_f = \frac{k_1}{k_2} \times C_W(1 - e^{-k_2 t})(\text{uptake}),$$
$$C_f = C_{f,0} \times e^{-k_2 t}(\text{depuration}) \quad (3)$$

where $C_{f,0}$ is the concentration of the analyte in the fish at the beginning of the purification process. By fitting the equations to the experimental data, $k_1$ and $k_2$ can be obtained. Under steady state conditions, Equation (1) is simplified to Equation 4:

$$BCF_k = \frac{C_f}{C_W} = \frac{k_1}{k_2} \quad (4)$$

The BCF is usually expressed in L $kg^{-1}$ units. The software OriginPro Ver. 8.5 (OriginLab) and NONLIN 5.1., which is specific for no-linear fits were used for kinetic calculations ($BCF_k$). In the present study, because no steady state is reached, a BCF at maximum uptake time $BCF_{48h}$ was also calculated for comparison purposes.

## RESULTS AND DISCUSSION

### Setting the analytical procedure

Several parameters for the chromatographic separation and determination (chromatographic column, derivatization reagent and solvent) and sample preparation (extraction

solvents and sorbents for the clean-up step) were optimized (Supporting Information). As a conclusion of the instrumental setting, a double determination was necessary (Supporting Information, Figure S1), injecting extracted isooctane directly to the GC for determination of PBDEs and MeO-BDEs, and with a derivatization step for OH-PBDEs. The results obtained for the sample preparation setting (Supporting Information, Figure S2) were observed with two purposes: to clean the extract and to obtain high recoveries, being the n-hexane:MTBE (1:1,v/v) and Florisil combination meeting both requirements for the majority of analytes (Supporting Information, Figure S3).

In this way, a method for the simultaneous extraction and cleaning of PBDEs, MeO-BDEs and OH-BDEs was optimized, reaching a consensus among the analytes of different polarity that resulted in good recoveries, greatly reducing the cost and time of sample treatment. In addition, the combination of both detection systems allows the identification (MS) and quantification (μECD) of trace compounds in complex mixtures with a high degree of selectivity and sensitivity.

## Method validation: QA/QC

The developed method was evaluated in accordance with selectivity, linearity, recovery, and detection limits (Supporting Information, Table S1). Selectivity was tested using several blanks for each sample type. The interference peaks were evaluated for each selected *m/z* of the analytes. Interferences observed in the zebrafish samples were eliminated by the clean-up step. The medium samples did not show any interference peaks. Reagent and sample blanks were always measured to subtract the background signal from that obtained for the samples. Exposure solutions were prepared by dilution from stock solutions with culture water containing 0.5% DMSO. No adverse effects were observed in control samples using 0.5% DMSO. Great linearity was obtained in all analytes ($R^2 > 0.994$) and the limits of detection (LODs) and limits of quantification (LOQ) were evaluated on the basis of external calibration curves and calculating the result of adding three (LOD) or 10 (LOQ) times the standard deviation to the target signal. Method LODs and LOQ values were between 0.10–0.22 and 0.25–0.68 μg $L^{-1}$, respectively, and relative standard deviation was <12%. To assess recoveries and because no Certified Reference Material with these compounds and similar matrix is readily available, sample fortification was used.

## Bioconcentration and toxicokinetics in zebrafish eleutheroembryos

The results of the bioconcentration showed that no BDE-47 was detected in unexposed zebrafish samples (control experiment). In contrast, as illustrated in Figure 1, the profiles obtained show a rapid absorption in the first hours of larval development and a high capacity of bioconcentration because the concentrations of BDE-47 found in larvae increased greatly

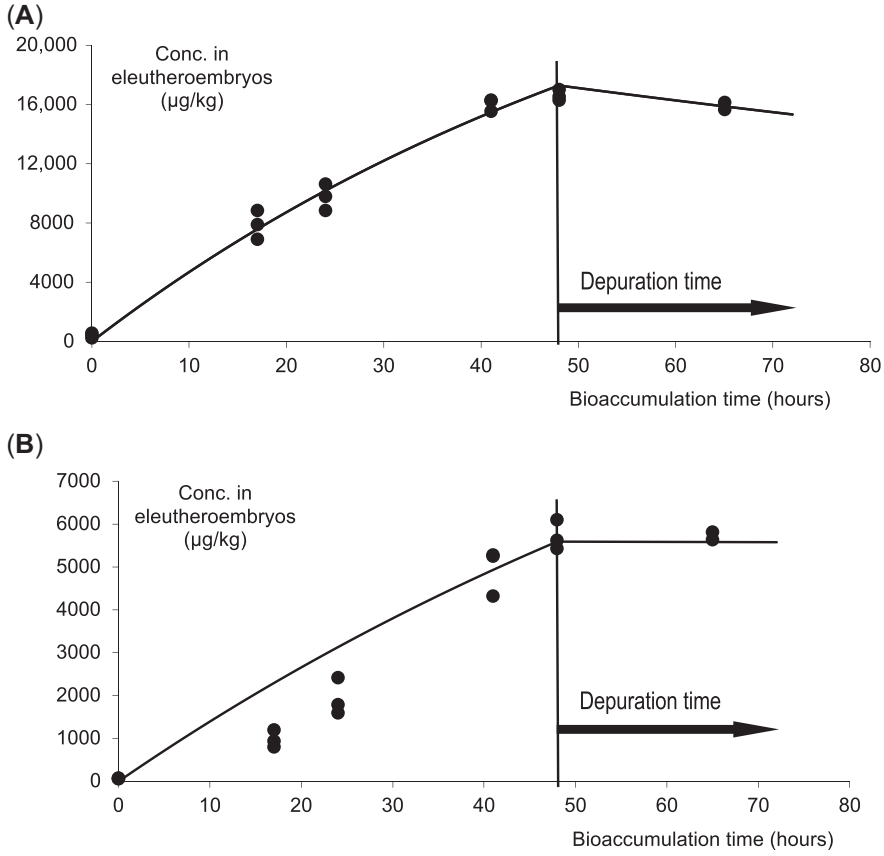


**FIGURE 1:** BDE-47 bioconcentration in zebrafish eleutheroembryos samples exposed to (**A**) 10 μg $L^{-1}$ and (**B**) 1 μg $L^{-1}$.

with time at both exposure levels. As explained in the Toxicokinetic model equations for BCF section, BCFs were calculated according to OECD 305 in two different ways: (i) $BCF_{48h}$ (Equation 1) using average BDE-47 concentrations determined experimentally in the exposure media (Table 2 and Supporting Information, Figure S4) and larvae concentration at the maximum time of uptake phase, and (ii) $BCF_k$ by applying Equation (4), representing the variation of BDE-47 concentration in eleutheroembryos versus time of uptake (Figure 1) and by fitting data to Equation (3) using nonlinear regression fits. The toxicokinetic and BCF values obtained by both calculation procedures are shown in Table 2. The values obtained by both calculation procedures resulted in not matching values of $BCF_k$ and $BCF_{48h}$ because the value does not completely reach the stable state in the absorption phase. According to the OECD 305 consideration, the $BCF_k$ values were preferred for further discussion. In a general view, the European Chemicals Bureau sets a value of BCF > 5000 in substances with log $K_{ow}$ between 5 and 8 (Mhadhbi et al., 2014), according to the result obtained in the present study (BDE-47 log $K_{ow}$ 6.81; Pereira et al., 2016).

Similar bioaccumulation studies of these compounds in different species can be found in the literature. Gustafsson et al. (1999) and Vidal-Liñán et al. (2015) calculated BCFs in *Mytilus galloprovincialis* (mussel), obtaining values of 26 000 L $kg^{-1}$ (exposed to 0.31 ng $L^{-1}$) and 10 900 L $kg^{-1}$ (exposed to 8 µg $L^{-1}$), respectively. Gu et al. (2017) gives values with a higher degree of magnitude, obtaining $6.44 \times 10^5$ L $kg^{-1}$ in the same species and $2.76 \times 10^6$ L $kg^{-1}$ in oyster exposed to the concentration found in a marine environment (0.26 ng $L^{-1}$). On the other hand, Lebrun et al. (2014) estimated a lower kinetic BCF in amphipod crustacean *Gammarus pulex* of close to 5000 when exposed to a nominal BDE-47 concentration of 1 µg $L^{-1}$. The variability in the results is mainly due to the natural dispersion due to the different species used in the experiments.

Related to fish, Mhadhbi et al. (2014) reported BCF values of BDE-47 in a young *Psetta maxima* of 24 125, 15 531, and 33 103 L $kg^{-1}$ at exposure to BDE-47 concentrations of 1, 0.1, and 0.001 µg $L^{-1}$, respectively. The order of magnitude of Mhadhbi et al. (2014) BCFs with those obtained in the present study match closely. Liu et al. (2015) presented data on the bioconcentration of BDE-47 on zebrafish (*Danio rerio*) embryos. The BCF values estimated were 26, 68, 489, 750, 2489, and 2430 at 12, 24, 48, 72, 96, and 120 hpf, respectively, at a nominal exposure concentration of 300 µg $L^{-1}$. These BCFs were calculated directly using Equation (1) at each sample time and show certain variability in the real concentrations found in the exposure water (decreasing from 300 to 40 µg $L^{-1}$). Even though exposure time is higher in the present study (from 4 to 120 hpf) than in ours (72 to 120 hpf) and considering that between 96 and 120 hpf a steady state is reached, the obtained BCF values are lower than found in the present study. This lower value at higher concentrations is found in this and multiple studies (Gustafsson et al., 1999; McGeer et al., 2003; Sanz-Landaluze et al., 2015; Vidal-Liñán et al., 2015). Theoretically, the accumulation of a compound depends only on diffusion and storage in lipids (Beek et al., 2000) and should not depend on concentration, but BCFs have a relationship with concentration, probably because other physiological mechanisms when interiorising compounds, showing saturable kinetics (McGeer et al., 2003). Therefore, the importance of determining the actual concentration of the compounds in the water during the experiment should be emphasized because most bioconcentration studies calculate BCFs with nominal concentrations (Molina-Fernandez et al., 2017; Zheng et al., 2012).

For prediction of the bioaccumulation potential of lipophilic compounds, the water–octanol partition coefficient $K_{ow}$ is the parameter used most often, with the general trend that higher hydrophobicity means higher accumulation (Arnot & Gobas, 2006). Based on quantitative models of structure–activity relationships (QSAR), equations (Table 3) have been developed that can predict the value of BCF in relation to the octanol–water partition coefficient, log $K_{ow}$ (BDE-47 log $K_{ow}$ 6.81; Pereira et al., 2016). In European Chemicals Agency (2003), parabolic relationships are given for substances with a log $K_{ow} > 6$ and linear equations were proposed by Arnot and Gobas (2006) and Petersen and Kristensen (1998) to determine the BCFs in fish and zebrafish larvae, respectively. This estimation of BCF is based on the substance's ability to be distributed in the lipid fraction. However, an experimentally obtained BCF is preferable to these QSAR methods because the bioconcentration also depends on the physiological responses of each organism, and the exposure and duration of the study, among other factors, and although the estimation models are improving, there are still many of these processes that are not perfectly modelled.

## Biotransformation in zebrafish eleutheroembryos

The Toxicity Estimation Software Tool (TEST; USEPA) software, Ver. 4.1, predicts ecotoxicity values using quantitative structure–activity relationships. These values (Supporting Information, Table S2) seem to confirm previously reported results describing similar toxicological effects of biotransformation

**TABLE 2:** Bioconcentration factor values ($BCF_{48h}$, $BCF_k$) and toxicokinetic parameters obtained for BDE-47

| Nominal conc. (µg $L^{-1}$) | Measured conc., $C_w$ (µg $L^{-1}$) | $k_1$ (L µg $h^{-1}$) | $k_{2(uptake)}$ (µg $L^{-1}$) | $BCF_k$ (log $BCF_k$) (L $kg^{-1}$) | $BCF_{48h}$ (log $BCF_{48h}$) (L $kg^{-1}$) |
|---|---|---|---|---|---|
| 10 | 4.2 | 124 ± 8 | 0.017 ± 0.001 | 7294 ± 899 (3.86) | 4106 (3.61) |
| 1 | 0.34 | 430 ± 30 | 0.0101 ± 0.0009 | 36363 ± 5702 (4.56) | 16392 (4.21) |

**TABLE 3:** BDE-47 Bioconcentration factor values estimated from different QSAR models

| Reference | QSAR equation | Life stage | Estimated BCF (log BCF) |
|---|---|---|---|
| European Chemicals Agency (2003) | $\log BCF = -0.20 \times \log K_{ow}^2 + 2{,}74 \times \log K_{ow} - 4{,}72$ | Aquatic organism | 46200 (4.66) |
| Arnot and Gobas (2006) | $\log BCF = 0.60 \times \log K_{ow} - 0{,}23$ | Fish | 7180 (3.86) |
| Petersen and Kristensen (1998) | $\log BCF = 0.86 \times \log K_{ow} - 0{,}4634$ | Zebrafish larvae | 247000 (5.39) |
| EPI Suite v4.1 (US EPA, 2012) | | Fish | 13600 (4.13) |

BCF = bioconcentration factor; QSAR = quantitative model of structure–activity relationship.

products to their parental compounds in certain biological systems.

Zebrafish eleutheroembryos exposed to BDE-47 at 100 and 250 μg $L^{-1}$ was trasnformated to their metabolites BDE-28, 2′-OH-BDE-28, and 5-MeO-BDE-47 after 48 h of exposure (Figure 2). No quantifiable amounts of the other metabolites tested (3- and 6-MeO-BDE-47, 3- and 5-OH-BDE-47) were detected. In addition, increasing concentrations of BDE-28, 2′-OH-BDE-28, and 5-MeO-BDE-47 over time were found in the BDE-47 bioconcentration experiment (10 μg $L^{-1}$; Figure 3). No metabolite was detected in the bioconcentration study of 1 μg $L^{-1}$. The ratios between metabolites and BDE-47 concentration found in larvae at different exposure times in both metabolization and bioaccumulation experiment were also determined (Supporting Information, Tables S2 and S4), showing that the biotransformation contributed less than 1% of the parental BDE 47 bioaccumulated in all experiments (maximum value of 0.75% for BDE-28, 0.25% for 2′-OH-BDE-28, and 0.13% for 5-MeO-BDE-47). Wiseman et al. (2011) suggested the contamination of BDE standards with a small amount of MeO/OH-BDEs and also that MeO-BDEs can be biotransformed to OH-BDEs. The metabolism of MeO-BDEs is said to contribute greater amounts of OH-BDEs than the parent PBDE. However, none of the metabolites they studied were detected in quantifiable amounts in the surrounding media at initial exposure ($t = 0$ h; Supporting Information, Table S5 and Figure S5) and the concentration of 5-MeO-BDE-47 found in eleutheroembryos is much smaller than that of OH metabolites.

The main metabolite formed is BDE-28, through the elimination of a bromine atom. Previous studies have also shown that halogen removal from PBDEs during metabolic degradation can form lower brominated BDE congeners within fish tissues (Stapleton & Baker, 2003) and this is most likely to occur at the ortho position of the biphenyl (Sun et al., 2013). The formation of 2′-OH-BDE-28 could be caused by hydroxylation of BDE-28 or by OH radicals attacking the bromine atom in the ortho position of BDE-47 under oxygenase catalysis (Yamazoe et al., 2004). Formation of OH-BDE-47 from BDE-47 is carried out in the same way as the formation of 2′-OH-BDE-28. However, 2′-OH-BDE-28 may be predominant because it has fewer bromine atoms, suffers reduction and oxidation reactions more readily, and is therefore more likely to be attacked by reducing H and OH radicals (Erratico et al., 2013; Tang et al., 2021).

Exploring the literature, Tang et al. (2021) have shown aerobic degradation of BDE-47 to debrominated (BDE-28 and BDE-7) and hydroxylated (6-OH-BDE-47, 5-OH-BDE-47, 2′-OH-BDE-28, and 4′-OH-BDE-17) metabolites by *Pseudomonas aeruginosa* YH through the biological action of P450 enzyme and dioxygenase. In another study, four species of microalgae (*Isochrysis galbana*, *Prorocentrum minimum*, *Skeletonema grethae*, and *Thalassiosira pseudonana*) were exposed to 20 μg $L^{-1}$ of BDE-47 and debromination to BDE-28 occurred in all species with concentration ratios of 0.36%, 0.45%, 0.87%, and 0.04%, respectively, on the sixth day of exposure. However, biotransformation to OH-BDEs was only found in *I. galbana* on exposure to high concentration (2000 μg $L^{-1}$; Po et al., 2017). On the other hand, Deng & Tam (2016) found ratios of less than 0.3% of BDE-28 biodegradation in the freshwater microalgae *Chlorella isolate* and the metabolites 6-OH- (0.4%–3%), 5-OH- (3%–15%), and 6-MeO-BDE-47 (<3%), from the hydroxylation and methoxylation of BDE-47 (10 μg $L^{-1}$). The congeners BDE-28, 6-OH-BDE-47, 5-OH-BDE-47 and a methoxylation product (4-MeO-BDE-42) were observed in young whole pumpkin plants with concentration ratios of 0.7%, 0.013%, 0.023%, and 0.24%, respectively (Sun et al., 2013). Methoxylation products were also detected in rainbow trout after exposure to deca-BDEs (Feng et al., 2010). Erratico et al. (2013) reported the biotransformation of BDE-47 to 6-OH-BDE-47, 5-OH-BDE-47, 3-OH-BDE-47, 2,4-dibromophenol (2,4-DBP), and 2′-OH-BDE-28 metabolites by human liver microsomes through P450 2B6 enzymes. Zebrafish have enzymes that are highly conserved in mammals and have

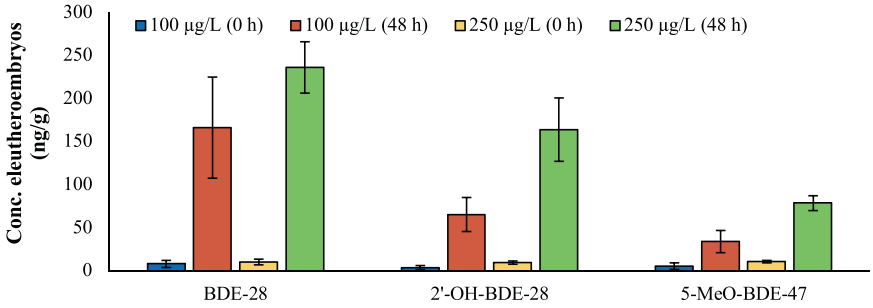


**FIGURE 2:** Transformation of BDE-47 (100 and 250 μg $L^{-1}$) by zebrafish eleutheroembryos after 48 h of exposure.

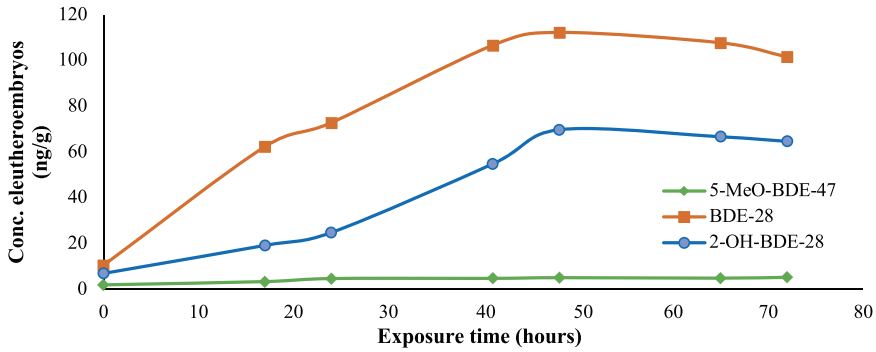


**FIGURE 3:** Metabolites of BDE-47 (10 μg $L^{-1}$) observed by transformation in zebrafish eleutheroembryos over time.

orthologues with humans, including enzymes of the CYP family, glucuronosyltransferases (UGTs), and sulfotransferases (SULTs), therefore zebrafish can perform phase I metabolism reactions, such as oxidation, N-demethylation, O-demethylation, and N-dealkylation, and phase II metabolism reactions, such as sulphation and glucuronidation (De Souza Anselmo et al., 2018). Yang et al. (2017) observed a CYP1A induction in vivo at BDE-47 exposure belonging to the phase I liver enzymes metabolizing in zebrafish larvae at 120 hpf. Yang and Chan (2015) observed a slight increase in transthyretin at 96 h and an effect on transcription of the enzymes UGT2A1 and SULT1 in the zebrafish liver cell line. This suggests that the chemical BDE-47 could be hydroxylated because it has binding affinity for transthyretin.

In contrast, Liu et al. (2015), Wen et al. (2015), and Zheng et al. (2012) studied the biotransformation in vivo of BDE-47 in zebrafish embryos and did not find hydroxylated and methoxylated metabolites. They found a small amount of 2′-OH-BDE-28, BDE-28, BDE-85, and BDE- 99 but did not take these quantities as transformation of the BDE-47. However, they used zebrafish from 4 (embryos) to 120 hpf (larvae) and the biotransformation capacity has been shown to vary dynamically throughout the embryonic development of zebrafish and proved immature in early life stages (Zindler et al., 2020). Our eleutheroembryos began exposure with a higher metabolic capacity (72 hpf) when the zebrafish CYP1A expression increased dramatically (Saad et al., 2016), which may have been a key factor in finding significant concentrations of BDE-28 and 2′-OH-BDE-28. Therefore, the age of the zebrafish should be considered during the experimental design and when contrasting the results.

The results obtained demonstrate the ability of zebrafish eleutheroembryos to metabolize compounds after 72 hpf, which can open the way to the use of these experiments to replace experimentation with adult fish. At present there are no official guidelines to carry out metabolization studies in higher organisms. Hence, in line with the recent approval of two new OECD test guidelines (319A and 319B) to determine biotransformation rates but using in vitro assays by primary hepatocytes (RT-HEP) or liver S9 subcellular fractions (RT-S9) from rainbow trout, metabolization by zebrafish eleutheroembryos could be considered as the leap to higher organisms, as request by other authors asking for further research involving continued "step-wise comparisons" of in vitro rates and increased levels of biological organization (Laue et al., 2020).

## CONCLUSIONS

A scaled-down analytical method has been developed to determine simultaneously the BDE-47 and their metabolites (MeO-BDEs and OH-BDEs) with different polarities in aqueous and larvae samples by GC-MS-μECD. Moreover, the use of both detectors at the same time, thanks to the splitter, achieves unambiguous identification and a high-sensitivity quantification suitable for trace and metabolites determination is achieved thanks to the advantages of each detector.

The experimental BCF values estimated in our study showed that BDE-47 fulfils the bioaccumulative criterion (vB-) according to European regulations (REACH; BCF > 5000; REACH, 2006) with satisfactory agreement with the results of the literature for other aquatic organisms and those predicted by QSAR models. Therefore, the results obtained show the feasibility of the method for bioconcentration and opens up the possibility of metabolic studies with zebrafish eleutheroembryos, which is a very underdeveloped field without official testing or regulation. Although these results are promising, more studies are needed to generate a large amount of data that will allow us to compare the benefits offered by this methodology with the other methods that are being proposed as alternatives for the study of the bioaccumulation and biotransformation of substances.

*Supporting Information*—The Supporting Information are available on the Wiley Online Library at https://doi.org/10.1002/etc.5569.

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could have appeared to influence the work reported in the present study.

*Author Contributions Statement*—**Paloma De Oro-Carretero**: Data curation; Formal analysis; Investigation; Methodology; Software; Supervision; Validation; Visualization; Roles/Writing—original draft. **Jon Sanz-Landaluze**: Conceptualization; Data curation; Funding acquisition; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Writing—review & editing.

*Data Availability Statement*—Authors confirm that the data supporting the findings of the present study are available within the article and/or its supplementary information materials.

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