Bioaccumulation And Biotransformation of BDE-47 Using Zebrafish Eleutheroembryos (*Danio Rerio*)

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Research Article

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Abstract

Polybrominated diphenyl ethers (PBDEs) industrially used as flame retardants are nowadays considered emerging pollutants as they are endocrine disrupting chemicals (EDCs), persistent in the environment, bioaccumulative and in addition, its hydroxylated (OH-BDEs) and methoxylated (MeO-BDEs) metabolites have similar ecotoxic properties. The aim of this work was to develop an analytical method to be applied in the study of the bioconcentration and biotransformation of BDE-47 due to its bioavailability, toxicity and high persistence and abundance in environmental samples, including humans. So, a dependable ultrasonic extraction process followed to dispersive SPE clean-up step and GC-MS-μECD detection has worked out for the determination of BDE-47 and its main biotransformation products (MeO-BDEs and OH-BDEs), considering the polarity difference. In addition, an alternative method to bioconcentration official guideline OECD 305, developed previously with zebrafish (*Danio rerio*) eleutheroembryos (i.e., hatched but not yet free feeding embryos) is used, reducing dramatically the animal suffering but also time and reagents. Bioconcentration factors (BCF) were calculated using first order one-compartment toxicokinetic model. The profiles found show rapid absorption in the first hours of larval development and great bioaccumulative with capacity, finding bioconcentration factors (BCF) of 6631 and 44210 at nominal concentrations of 10 and 1 $\mu g \cdot L^{-1}$ (< 1% LC50), 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 opens the possibility of metabolic studies with zebrafish eleutheroembryos, which is a very underdeveloped field without official testing or regulation.

Introduction

PBDEs are industrial substances widely used as flame retardants in several commercial products including plastics, textiles, household and electronic equipment because of their efficient performance, good thermal stability and low-cost (Wen et al. 2015). Their dioxin-like properties and structural similarity to endocrine hormones, such as thyroid hormones (THs) and thyroxine (T4) in particular, have warned about neurodevelopmental and endocrine disrupting effects in humans (Butryn et al. 2020). This has led several countries, as in Canada since 2008, the UE since 2012 or the US since 2004, to ban the use of some commercial PBDEs mixtures and the Stockholm Convention on Persistent Organic Pollutants (POPs) decided to include them in the list of POP substances. In contrast to bound flame retardants, for example tetrabromobisphenol A (TBBPA), PBDEs are easily removed from products and enter the environment (Liu et al. 2020). Nowadays, and even though PBDEs are being phased out, as a result of their persistence on the environment, long-term use and recycling processes, these chemicals are still detected in a wide range of biological and environmental samples (Klinčić et al. 2020). Bioaccumulation and biomagnification of PBDEs (Liu et al. 2020) and the consequent consumption through diet, together with inhalation or accidental ingestion of dust (De la Torre et al. 2020) have led to detect potentially dangerous levels in humans (milk, blood plasma and adipose tissue) (Klinčić et al. 2020).

Also the natural metabolites as hydroxylated (OH-BDE) and methoxylated (MeO-BDE) and, recently, diOH-BDEs (Zhang et al. 2020), have been found in a wide diversity of environmental samples (Sun et al. 2020). Several studies have evidenced that metabolites maintain bioactive remains, exhibiting similar or even greater toxicity than the native PBDEs due to their hydrophobic properties (Liu et al., 2011; Usenko et al. 2012) raising the concern about these compounds.

Metabolites are biotransformation products of PBDEs, oxidated by hepatic cytochrome P450 (CYP) enzymes (Li et al. 2010) and have been found in several marine and freshwater animals as mussels, shellfish, fish, clam and whale (Liu et al. 2014; Rotander et al. 2012; Valters et al. 2005). Besides, they are also known to have a natural occurrence in the environment (Malmvärn et al. 2005; Wiseman et al. 2011). However, there are mixed reports depending on the studied organism. Therefore, the bioaccumulation, biotransformation and toxicity in different organisms of these emerging pollutants and their metabolites are still being studied because their behaviour in the environment is unknown or remains unclear.

Bioaccumulation is the process by which a compound is uptake by an organism via any route, including respiration, ingestion, or direct contact. The degree of bioaccumulation is expressed by the bioaccumulation factor (BAF), defined experimentally as the ratio between the concentration of the analyte in the organism and its surrounding medium, such as sediment or soil, and also by the biomagnification factor (BMF), in this case, the concentration of a substance in test organism divided by the concentration in the food. Also for aquatic species the bioconcentration factor (BCF) is used (El-Amrani et al. 2012), which does not take into account the uptake of a substance through the diet and can therefore only be estimated under controlled laboratory conditions (Arnot and Gobas 2006).

The official method for assessing the bioconcentration factors of chemicals is the Organisation for Economic Co-operation and Development (OECD) Test 305 (OECD 2012), which is also proposed by REACH (European Regulation for the Registration, Evaluation and Authorisation of Chemicals) as the guide to evaluate bioconcentration of a substance (REACH 2006). This test evaluates the ratio between the concentration in adult fishes and the surrounding medium after reaching the steady state, requiring many adult fish (up to 104) and long exposure time (up to 60 days), which involves a high cost of the experiments. On the other hand, European REACH and other testing legislation propose wherever possible to change animal experiments with non-animal approaches (Quantitative Structure Activity Relationship (QSAR) approximations, microorganisms, in vitro studies with cell lines, embryos etc.). Therefore, an alternative to the OECD 305 assay has recently been developed with promising results using the eleutheroembryos zebrafish (*Danio rerio*) (Sanz-Landaluze et al. 2015), as European legislation considers eleutheroembryos fish as *in vitro* systems up to the self-feeding stage (2010/63/EU 2010). Due to their high production, low cost, fast embryonic development, fast bioaccumulation kinetics, their transparency and high genomic homology with humans (over 80%), zebrafish in its first developmental stages is gaining interest in ecotoxicology model organism (Lillicrap et al. 2016). In addition, because thyroid hormone regulators are highly conserved among vertebrate species, zebrafish is a useful model to investigate compounds in relation to thyroid hormone (Vancamp, Houbrechts, and Darras 2019) to assess the risk and metabolic pathways of contaminants to animals and humans.

Due to their high use, persistence in the environment, bioavailability (log Kow 6.81) (Pereira et al. 2016) and toxicity (LC50 4.2 $mg{\cdot}L^{-1}$) (Usenko et al. 2011), 2,2',4,4'-tetrabromodiphenyl ether (BDE-47), is one of most investigated PDBEs. 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 scarce and very underdeveloped without official testing or regulation. Therefore, in addition to the bioconcentration study of BDE-47, a metabolic study was also carried out using the most abundant metabolites found in different species in the various studies realized (Lacorte, Ikonomou and Fischer 2010; Liu et al. 2015; Wen et al. 2015; Zhai et al. 2014) which are: 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.

It is important to bear in mind that zebrafish eleutheroembryos samples have a very small size (0.44 mg/larva), a high lipid content (~ 15%) and that exposure concentrations are always below toxicological values (< 1% of LC_{50}). Therefore, the determination methodologies implemented must be extremely sensitive. Conventional Soxhlet has been traditionally the most used extraction method for PBDE from solid samples in a large variety of matrices. Nowadays, there is a trend to use more environmentally friendly methods such as pressurized liquid extraction (PLE) or ultrasound-assisted extraction (UAE) (Lacorte et al. 2010). The cleaning methods used for the analysis of this type of contaminants are usually solid phase extraction (SPE) or gel permeation chromatography (GPC) (Cruz et al. 2017). In this sense, in order to minimize solvent use and sample treatment time, while maintaining an acceptable recovery, the analytical protocol we devised, is based on simultaneous miniaturized extraction with a dispersive SPE clean-up, considering the difference in polarity of the PBDEs and their metabolites (MeO-BDEs and OH-BDEs). GC-MS is the most common routinely technique employed for the determination of PBDEs and MeO-PBDEs (Shelepchikov et al. 2019). However, for determination of OH-BDEs and MeO-BDEs liquid chromatography is often used (Song et al. 2020) due to their relatively high polarity (Zhai et al. 2014). In addition, the official method of the Environmental Protection Agency (EPA) for the detection of PBDEs are based on electron captured detector (ECD), because is the most sensitive detector. For this reason, a simultaneously GC-MS-μECD detection by previous derivatization of OH-BDEs was carried out to determine the BDE-47 and its metabolites under the same instrumental conditions.

Material And Methods

2.1. Reagent and Samples

Individual commercial standards of 1 mL of the PBDEs 28 (150 $mg{\cdot}L^{-1}$), 47 (350 $mg{\cdot}L^{-1}$) and 99 (116 $mg{\cdot}L^{-1}$) in methanol purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany); individual 1 mL standards of the metabolites 6-MeO-BDE-47 (10 $mg{\cdot}L^{-1}$), 5-MeO-BDE-47 (50 $mg{\cdot}L^{-1}$), 3-MeO-BDE-47 (50 $mg{\cdot}L^{-1}$) in methanol, 5-OH-BDE-47 (50 $mg{\cdot}L^{-1}$), 3-OH-BDE-47 (50 $mg{\cdot}L^{-1}$) and 2'-OH-BDE-28 (50 $mg{\cdot}L^{-1}$) in acetonitrile were supplied by AccuStandard Inc. (New Haven, CT, USA). 1,2,3,4-Tetrachloronaphthalene (TCN; Dr. Ehrenstorfer GmbH; 10 $mg{\cdot}L^{-1}$ in isooctane) was used as internal standard (IS) for the PBDE congeners and the methoxylated metabolites (MeO-BDE) and triclosan (TCS; Dr. Ehrenstorfer GmbH; 10

mg·L^{-1} in acetone) 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 are prepared by dilution. The derivatization reagent used for the analysis of the OH-BDE metabolites is N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) + 1% tert-butyl-methylchlorosilane were supplied by Sigma Aldrich (Madrid, Spain). The adsorbents of the "clean-up" stage were C_{18} ODS SPE Buk Sorbent (Agilent Technologies, USA), PSA SPE Bulk Sorbent (Agilent Technologies), SupelTM QuE Z-Sep (Sigma Aldrich) and Florisil (E. Merck, Darmstadt, Germany). Analytical-grade solvents were used; isooctane, n-hexane, dichloromethane (DCM), methyl tert-butyl ether (MTBE) and dimethyl sulfoxide (DMSO) purchased from Sigma Aldrich. The exposed zebrafish (*Danio rerio*) media and eleutheroembryos were provided by AZTI Tecnalia (Bizkaia, Spain).

2.2. Instrument and apparatus

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

Gas chromatographic Agilent Mod. 7890A Series equipped with a HP 7683B Series autoinjector, µECD and HP 5975C VL MSD mass spectrometry detector with ChemStation software (Agilent Technologies, Madrid, Spain) was used for final separation and quantification of the pollutants. Both detectors work 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 x 0.1 mm x 0.1 µm) and ZB-5 (30 m× 0.25 mm I.D., 0.25-µm film thickness) from Phenomenex (Madrid, Spain), using Helium Premier (99.999% purity) X50S Metal Carbide (Barcelona, Spain) as carrier gas.

2.3. 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 the composition of fresh river water (ISO 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. OECD Test 305 states that the exposure concentrations should be 1 % and 0,1 % of the LC_{50} (if detection limits enable it to be determined). (Usenko et al. 2011) show a BDE-47 zebrafish LC_{50} 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 metabolization experiment. Due to the limited solubility of

BDE-47 in water (11 $\mu g \cdot L^{-1}$) (Pereira et al. 2016), it is solved in dimethyl sulfoxide (DMSO) so that the medium finally contains 0.5% DMSO.

Bioconcentration and metabolization experiments were carried out according to an alternative method (Sanz-Landaluze et al. 2015). Zebrafish eleutheroembryos were exposed to contaminated solution of BDE-47 at 72 hours 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). 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, 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. At the same time, a control experiment (no addition of the target compound) was also performed. Three replicates of contaminated and blank or control samples were collected for each time evaluated.

2.4. Analytical procedure

2.4.1 Instrumental optimization

Chromatographic separation and detection were carried out using GC-MS-μECD system. The MS was used for the identification of the analytes and the μECD for the quantification of each one of them. Two low-polarity capillary columns: DB-5 and ZB-5MS and different chromatographic parameters were evaluated. The remaining parameters had been optimized previously (El-Amrani et al. 2012; Gonzalo-Lumbreras et al. 2012). The determination of the analytes was carried out by means of a double determination, one before derivatization for the analysis of PBDEs and MeO-BDEs with TCN as IS and another after, for the analysis of OH-BDEs using TCS as IS because PBDEs are not stable to derivatization. The determination of OH-BDEs was carried out by derivatizing with MTBSTFA for 30 minutes to 70˚C to determine them as OH-TMDS-BDE.

Finally, samples (1 μL) were injected in the splitless mode at 280°C. Helium was used as carrier gas at a constant pressure of 28 psi (~ 1.5 $mL \cdot min^{-1}$). 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 minutes. The temperature of the ion source and the transfer line of the mass spectrometer was set at 250 and 270°C, respectively, and 70 eV for the electron beam. 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 NIST databases and by bibliography (Butryn et al. 2015). The μECD temperature was set at 320°C and nitrogen was used as makeup gas (30 mL/min). The flow ratio of the splitter was 1:1 thanks to the additional He 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).

Table 1
Mass spectrometer conditions in SIM mode for the electron impact (EI) ionization.

Analyte	Retention time (min)	m/z	Inicial scanning time (min)
TCN	9.31	266, 264, 268, 194	8 (solvent delay)
BDE-28	12.48	248, 246, 406, 408	11
TMDS-TCS	13.71	290, 288, 218	12.8
BDE-47	16.98	326, 486, 484, 488	15
2'-OH-TMDS-BDE-28	19.69	**423**, **421**, 425, 81	18.5*
6-MeO-BDE-47	20.13	516, **514**, 518, 420	
3-MeO-BDE-47	21.23	356, **51**6, 341, 514	
5-MeO-BDE-47	21.62	516, **356**, 358, 326	
BDE-99	22.98	404, 406, 564, 566	22
5-OH-TMDS-BDE-47	27.07	502, 504, 500, 81	25
3-OH-TMDS-BDE-47	31.72	502, 474, 266, 419	29

* A joint window is established as they elude close retention times, so that the majority m/z of each is monitored (marked in the table)

2.4.2 Sample preparation

To avoid contamination of the sample, plastic materials are not used due to the possible transfer of pollutants to or from the material. Therefore, Hamilton syringes are always used for the addition of the volumes. All reused glass material is washed with acetone, rinsed with deionized water and left overnight in the oven at 250˚C.

Sample preparation consists of an ultrasonic probe-assisted solid-liquid extraction method and a clean-up step (d-SPE). Mixture of extraction solvents n-hexane:DCM (1:1, v/v) and n-hexane: MTBE (1:1, v/v) and several common commercial sorbents (C_{18}, PSA, Z-Sep, Florisil) were evaluated (Cruz et al. 2017). Then, the optimized method was applied to samples exposed to BDE-47 for bioconcentration and metabolization studies. Zebrafish eleutheroembryos in group of 15 were extracted with 500 µL of the n-hexane:MTBE mixture (1:1) and sonicated with an ultrasound probe during 4 min at 40 % of amplitude and a pulse every second (on/off:1s/1s). The internal standards TCN and TCS were previously added so that the extract to be injected contains 20 $\mu g \cdot L^{-1}$ of each. The mixture was centrifuged for 10 min at 10,000 rpm and 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. Then, the extract was centrifuged and evaporated to dryness under a gentle stream of nitrogen and reconstituted in 500, 1000, or 2000 µL of isooctane depends on the accumulation time of each sample.

Extracts from embryo water was prepared as follows: 500 µL of the exposed solution was extracted by 500 µL of the n-hexane:MTBE (1:1) and mixed for 30 s in the vortex. Second step of clean-up of the extracts is not applied in the exposure media because they do 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, concentrated in a gentle stream of nitrogen and reconstituted in 50 µL isooctane.

Finally, 80 and 50 µL of the volume obtained for eleutheroembryos and embryo water, respectively, are derivatised with 20 µL of MTBSTFA to 70˚C for 30 minutes in the oven and injected into the GC-µECD-MS to determine the OH-BDE in the form of OH-TMDS-BDE. PBDEs and MeO-BDEs are determined by direct injection of the remaining volume of isooctane extracts into the GC-µECD-MS.

2.5. Toxicokinetic model equations for bioconcentration factor (BCF)

In this 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 bioconcentration factor (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 (SS) (Eq. (1)):

$$\mathrm{BCF_{ss}} = \frac{\mathrm{C_f(ng \cdot g^{-1})}}{\mathrm{C_W(ng \cdot L^{-1})}} \quad \text{Eq. (1)}$$

Nevertheless, when the SS is not reached, BCF_k can be calculated using a bioconcentration first order kinetic model that describes the chemical uptake and depuration process (Gobas and Zhang 1992; Mackay and Fraser 2000) (Eq. (2)):

$$\frac{\mathrm{dC_f}}{\mathrm{dt}} = \mathrm{k_1 \cdot C_W - k_2 \cdot C_f\ (uptake)\ ;}\quad \frac{\mathrm{dC_f}}{\mathrm{dt}} = \mathrm{-k_2 \cdot C_f\ (depuration)} \quad \text{Eq. (2)}$$

where C_f is expressed in $ng \cdot g^{-1}$, t is the exposure time (h), k_1 is the first-order uptake constant ($L \cdot g^{-1} \cdot h^{-1}$), C_w is expressed in $ng \cdot L^{-1}$, 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 of the in the exposure medium is constant, Eq. (3) is obtained:

$$\mathrm{C_f} = \frac{\mathrm{k_1}}{\mathrm{k_2}} \cdot \mathrm{C_W}\left(1 - \mathrm{e^{-k_2 t}}\right)\ \mathrm{(uptake)\ ;}\ \mathrm{C_f = C_{f,0} \cdot e^{-k_2 t}\ (depuration)} \quad \text{Eq. (3)}$$

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 SS conditions, Eq. (1) is simplified to Eq. (4):

$$BCF_K = \frac{C_f}{C_W} = \frac{k_1}{k_2}$$ Eq.(4)

BCF is usually expressed in $L \cdot kg^{-1}$ units. The software OriginPro v.8.5 (OriginLab, Northampton, MA, U.S.A.) and NONLIN 5.1. (Nashville, TN), which is specific for no linear fits were used for kinetic calculations (BCF_k). In this study, as no steady state is reached, a bioconcentration factor at maximum uptake time BCF_{48h} was also calculated for comparison purposes.

Results And Discussion

3.1. Setting the analytical procedure

Based on sensitivity, ZB-5MS was selected for separation despite the increase in retention time with respect to the DB-5 column. MTBSTFA was chosen as a derivatization reagent because it is the most common in phenolic compounds (Schummer et al. 2009). Thus, it avoids the commonly used reagents for methylation of OH-BDEs, diazomethane (DM) and trimethylsilyl diazomethane (TMSDM) (Zhai et al. 2014) which forms MeO-PBDEs derivatized and can be confused with MeO-BDEs metabolites. Although theoretically, PBDEs and MeO-BDEs remain structurally unchanged by the derivatization process with MTBSTFA, a significant loss of these compounds is observed, reducing greatly sensitivity (Fig. S1). Because of this, a double determination was carried out, injecting extracted isooctane directly to GC for determination of PBDEs and MeO-BDEs and with derivatization step for MeO-PBDEs. In addition, three different solvents are tested for derivatization, isooctane, hexane and acetone, with no significant differences (Fig. S1). Therefore, as isooctane is usually used for PBDEs and MeO-BDE, it is the one chosen in the derivatisation process.

The results obtained for sample preparation setting (Fig. 1) were observed with two purposes: clean extract and obtain high recoveries, being the n-hexane:MTBE (1:1,v/v) and Florisil combination meeting both requirements for the majority of analytes (Fig. S2). Good recoveries for PBDEs and MeO-BDEs were obtained using PSA. Nonetheless, the OH-BDEs were completely lost because PSA is usually used to remove polar compounds from a non-polar matrix (Barci et al. 2020). C18 ensures that OH-BDE is not lost in the cleaning stage because eliminate non-polar lipid interferences (Lacorte et al. 2010; Walorczyk, Drozdzyński and Kierzek 2015). However, the recoveries obtained show interferences due to the sample matrix. Supel™ QuE Z-Sep consists of a mixture with ratio 2/5 of ZrO_2/C_{18} (Lozano et al. 2014), where ZrO_2 attracts substances with polar hydroxyl groups (electron-donor group) (Moloney et al. 2018) and C_{18} interacts with non-polar compounds in the lipids. Therefore, favorable recoveries of PBDEs and MeO-BDEs were obtained but the action of ZrO_2 on polar compounds results in the loss of a very important quantity of OH-BDEs. Despite florisil also removed polar compounds (Zhao et al. 2016), it does not affect OH-BDEs as is the case with PSA and Z-Sep. So, the recoveries of PBDEs, MeO-BDEs and OH-BDEs are compensated. On the other hand, with n-hexane:MTBE mixture (1:1), the compounds are extracted from

the matrix better than with the n-hexane:DCM mixture (1:1), as better recovery results are obtained for most analytes. The same behaviour in the exposure media is observed.

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

3.2. Method validation.

The developed method was evaluated in accordance with selectivity, linearity, recovery and detection limits (Table S1). Selectivity was tested using several targets for each sample type. The interference peaks was evaluated for each selected m/z of the analytes. The samples did not show any interference. Only the matrix effect was observed in the zebrafish samples, but it was eliminated by the clean-up step. Reagent and sample blanks were always measured to counteract the background signal to the obtained for the samples. Great linearity was obtained in all analytes ($R^2 > 0.994$) and LODs were evaluated on the basis of external calibration curves and calculating the result of adding three times the standard deviation to the target signal. Method LODs values were between 0.1–0.3 $\mu g \cdot L^{-1}$ and RSD < 12 %. To assess recoveries and as no Certified Reference Material with these compounds and similar matrix is readily available, sample fortification was used.

3.3. 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 Fig. 2, the profiles obtained show a rapid absorption in the first hours of larval development and a high capacity of bioconcentration because the concentrations increase greatly with the time at both exposure levels. As explained in Sect. 2.5, BCFs were calculated according to OECD 305 in two different ways: (i) BCF_{48h} (Eq. (1)) using average BDE-47 concentrations determined experimentally in the exposure media (Table 2 and Fig. S3) and larvae concentration at the maximum time of uptake phase; and (ii) BCF_k by applying Eq. (4), representing the variation of BDE-47 concentration in eleutheroembryos versus time of uptake (Fig. 2) and by fitting data to Eq. (3) using non-linear 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 it 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, European Chemicals Bureau sets a value of BCF > 5000 in substances with log K_{ow} between 5 and 8 (Mhadhbi, Fumega and Beiras 2014), according to the result obtained in this study (BDE-47 log K_{ow} 6.81; (Pereira et al. 2016)).

Table 2
BCF values ($BCF_{48\ h}$, BCF_k) and toxicokinetic parameters obtained for BDE-47

Nominal conc. ($\mu g \cdot L^{-1}$)	Found conc. C_w ($\mu g \cdot L^{-1}$)	k_1 ($L \cdot \mu g \cdot h^{-1}$)	k_2 (uptake) ($\mu g \cdot L^{-1}$)	BCF_k (log BCF_k)	BCF_{48h} (log BCF_{48h})
10	4.2	120	0.015	6631 (3.82)	4106 (3.61)
1	0.34	430	0.010	44210 (4.64)	16392 (4.21)

Similar bioaccumulation studies of these compounds in different species can be found in literature. (Gustafsson et al. 1999) and (Vidal-Liñán et al. 2015) calculate BCFs in *Mytilus galloprovincialis* (mussel) obtaining values of 26000 (exposed to 0.31 $ng \cdot L^{-1}$) and 10900 (exposed to 8 $\mu g \cdot L^{-1}$), respectively. (Gu et al. 2017) gives values with a higher degree of magnitude, obtaining 6.44 10^5 in the same species and $2.76 \cdot 10^6$ in oyster exposed to the concentration found in marine environment (0.26 $ng \cdot L^{-1}$). On the other hand, (Lebrun et al. 2014) estimated a lower kinetic BCF in amphipod crustacean *Gammarus pulex* close to 5000 exposed to a nominal BDE-47 concentration of 1 $\mu g \cdot L^{-1}$. The variability of 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) reports a BCF in a young *Psetta maxima* of 24125, 15531 and 33103 at exposure to BDE-47 concentration of 1, 0.1 and 0.001 $\mu g \cdot L^{-1}$ respectively. The degree of magnitude of these BCFs with those obtained in this work match closely. (Liu et al. 2015) showed data on the bioconcentration of the BDE-47 on zebrafish (*Danio rerio*) embryos. BCF values estimated were 26, 68, 489, 750, 2489 and 2430 at 12, 24, 48, 72, 96 and 120 hpf respectively, at nominal exposure concentration of 300 $\mu g \cdot L^{-1}$. These BCFs were calculated directly, without considering the toxicokinetic profile and showing certain variability on the real concentrations found in the water of exposure (decreasing from 300 to 40 $\mu g \cdot L^{-1}$). Even though exposure time is higher in this work (from 4 to 120 hpf) than in ours (72 to 120 hpf) obtained BCF values are much lower. This variability can be explained mainly by different nominal concentration of the BDE-47 used on the experiments, much higher in their case. Theoretically, the accumulation of a compound depends on diffusion and storage in lipids (Beek et al. 2000) and should not depend on concentration. But as can be seen in this and multiple studies (Gustafsson et al. 1999; Sanz-Landaluze et al. 2015; Vidal-Liñán et al. 2015) BCFs find a relationship with concentration, and probably other physiological mechanisms when interiorising compounds, showing saturable kinetics, leading to accumulation. Therefore, the importance of determining the actual concentration of the compounds in the water during the experiment should be emphasised, as most bioconcentration studies calculate with nominal concentrations (Molina-Fernandez et al. 2017; Zheng et al. 2012). Although some authors suggest performing these bioaccumulation studies at concentrations currently found in the environment, there is great diversity of data depending on different media such as river water, effluent or contaminated water. In order to have comparable data, we advocate using the

concentrations suggested in OECD 305 standard guideline (OECD 2012) of 0.1% and 1% of LC_{50} of the compound.

For prediction of bioaccumulation potential of lipophilic compounds water-octanol partition coefficient K_{ow} is the most used parameter, with the general trend that higher hydrophobicity means higher accumulation (Arnot and 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 (ECHA 2003) parabolic relationships are given for substances with a log K_{ow} > 6 and linear equation were proposed by (Arnot and Gobas 2006) and (Petersen and Kristensen 1998) to determine the BCFs in fish and zebrafish larvae, respectively. This estimate is based on the substance's ability to be distributed in the lipid fraction. However, an experimentally obtained BCF is preferable because of the bioconcentration also depends on the physiological responses of each organism, the exposure and the duration of the study, among other factors.

Table 3
BCF values estimated from different QSAR models

Reference	QSAR equation	Life stage	Estimated BCF (log BCF)
(ECHA 2003)	$\log BCF = -0{,}20 \cdot \log K_{ow}^2 + 2{,}74 \cdot \log Kow - 4{,}72$	Aquatic organism	46200 (4.66)
(Arnot & Gobas 2006)	$\log BCF = 0{,}60 \cdot \log Kow - 0{,}23$	Fish	7180 (3.86)
(Petersen & Kristensen 1998)	$\log BCF = 0{,}86 \cdot \log Kow - 0{,}4634$	Zebrafish larvae	247000 (5.39)
EPI Suite v4.1 (US EPA 2012)		Fish	13600 (4.13)

3.4. Biotransformation in zebrafish eleutheroembryos

The TEST software, version 4.1 (EPA 2008) predicts ecotoxicity values using quantitative structure-activity relationships. These values (Table S2), seem to confirm previously reported results describing significant effects of degradation products in certain biological systems.

Zebrafish eleutheroembryos exposed to BDE-47 at 100 $\mu g \cdot L^{-1}$ and 250 $\mu g \cdot L^{-1}$ becomes into their metabolites BDE-28, 2'-OH-BDE-28 and 5-MeO-BDE-47 after 48 hours of exposure (Fig. 3). No quantifiable amounts of the rest of metabolites were detected. The results of control experiment showed that no BDE-47 and none of its metabolites were detected in unexposed zebrafish samples and in their surrounding media. In addition, increasing concentrations of BDE-28, 2'-OH-BDE-28 and 5-MeO-BDE-47 over time were also found in the bioconcentration experiment of BDE-47 (10 $\mu g \cdot L^{-1}$) (Fig. 4). Low amounts of 5-MeO-BDE-47 were detected due to the low exposure concentration as it shows low biotransformation

compared to the other two metabolites. No metabolite was detected in the bioconcentration study of 1 $\mu g \cdot L^{-1}$.

Therefore, 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 was most likely to occur at the ortho position of biphenyl (Sun et al. 2013). The formation of 2'-OH-BDE-28 could be caused by hydroxylation of BDE-28 or by the OH radicals attacking the bromine atom in the ortho position of BDE-47 under the catalysis of oxygenase. (Yamazoe, Yagi, and Oyaizu 2004). Although the formation of the OH-BDE-47 from the BDE-47 is carried out in the same way as the formation of the 2'-OH-BDE-28. However, 2'-OH-BDE-28 may be predominant because it has fewer bromine atoms, suffering reduction and oxidation reactions more readily, and is therefore more likely to be attacked by reducing H and OH radicals (Erratico, Szeitz and Bandiera 2013; Tang et al. 2021).

Exploring the literature, (Tang et al. 2021) shown an 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. (Erratico et al. 2013) found the biotransformation of BDE-47 to 6-OH-BDE-47, 5-OH-BDE-47, 3-OH-BDE-47, 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 orthologues with humans, including enzymes of the cytochrome P450 (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). In this sense, (Yang, Zhao and Chan 2017) observed a CYP1A induction *in vivo* at BDE-47 exposure, belonging to the phase I liver enzymes metabolizing by in zebrafish larvae at 120 hpf. (Yang and Chan 2015) observed in the zebrafish liver cell line, a slight increase in transthyretin (TTR) at 96 h and an effect on transcription of the enzymes UGT2A1, SULT1. This suggests that the chemical BDE-47 could be hydroxylated as it has binding affinity for TTR..

In contrast, (Liu et al. 2015; Wen et al. 2015; Zheng et al. 2012) studied the biotransformation *in vivo* of BDE-47 in zebrafish embryos and neither of them found hydroxylated and methoxylated metabolites. They found a small amount of 2'-OH-BDE-28, BDE-28, BDE-85 and BDE- 99 but they did not take these quantities as transformation of the BDE-47. However, they use zebrafish from 4 (embryos) to 120 hpf (larvae) and the biotransformation capacity has been shown to vary dynamically throughout embryonic development of zebrafish and proved immature in early life-stage (Zindler et al. 2020). Our eleutheroembryos began the exposure with a higher metabolic capacity (72 hpf) when the zebrafish CYP1A expression increases 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 experimental design and when contrasting the results.

The results obtained demonstrate the ability of the zebrafish eleutheroembryos to metabolize the compounds after 72 hpf, which can open the way to the use of these experiments to replace the experimentation with adult fish. At present there are no official guidelines to carry out metabolization studies in higher organisms, and following the line marked by the recent approval of two new OECD test guidelines (TG 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. Further underpinned by the claim for further research involving continued “step-wise comparisons” of in vitro rates and increased levels of biological organization carried out by authors actually working in the extrapolation between data obtained in this *in vitro* models and *in vivo* ones (Laue et al. 2020).

Conclusions

A miniaturized analytical method has been developed to determine simultaneously the BDE-47 and their metabolites (MeO-BDEs and OH-BDEs) with different polarity in aqueous and fish samples by GC-MS-μECD. Moreover, the use of both detectors at the same time, thanks to the splitter, achieves an 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 the that BDE-47 fulfils the very bioaccumulative criterion (vB-) according to European regulations (REACH) (BCF > 5000) (REACH 2006) with a 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 the possibility of metabolic studies with zebrafish eleutheroembryos, which is a very underdeveloped field without official testing or regulation.

The proposed alternative method, based on the official OECD guideline 305, provides comparable data and can therefore be used as a substitute for experimental work on adult fish, reducing the amount of reagents, time and suffering of the animals during experiments.

Declarations

Competing interests

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

Consent for publication. Consent to Participate

Not applicable

Authors Contributions

P. De Oro-Carretero: Data curation; Formal analysis; Investigation; Methodology; Software; Supervision; Validation; Visualization; Roles/Writing - original draft;

J. Sanz-Landaluze: Conceptualization; Data curation; Funding acquisition; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Writing - review & editing.

Ethics approval. Availability of data and materials

Zebrafish eleutheroembryos were cultured from wild type adult zebrafish bred and maintained in AZTI Zebrafish Facility (REGA ES489010006105). All experiments with eleutheroembryos were conducted according to the principles of the OECD 305 and the European legislation EU Directive 2010/63/EU.

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Figures

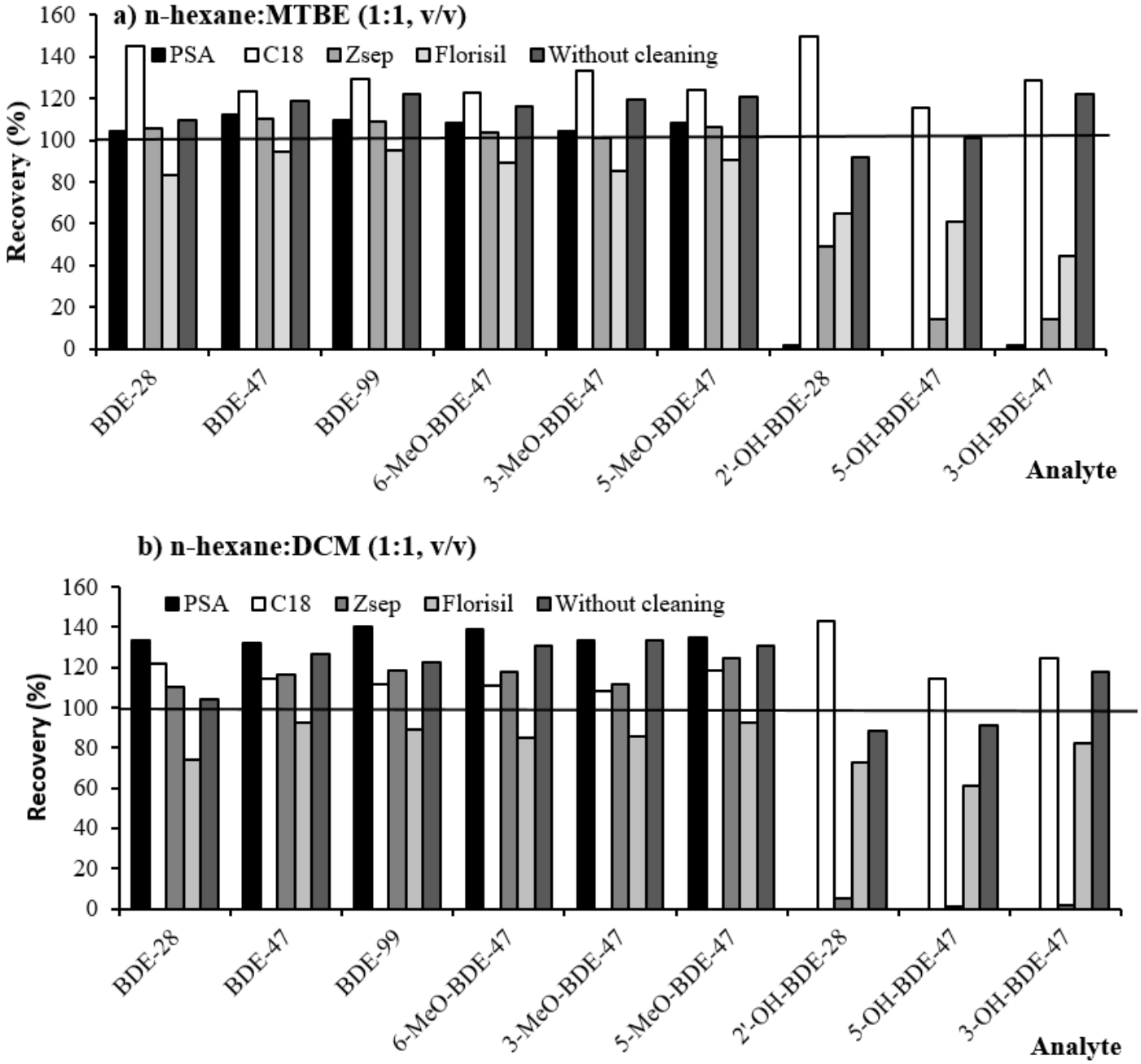

Figure 1

Recoveries obtained with the different adsorbents by extraction with a) n-hexane:MTBE (1:1, v/v) and b) n-hexane:DCM (1:1, v/v) in zebrafish eleutheroembryos of known concentration (10 µg·L-1)

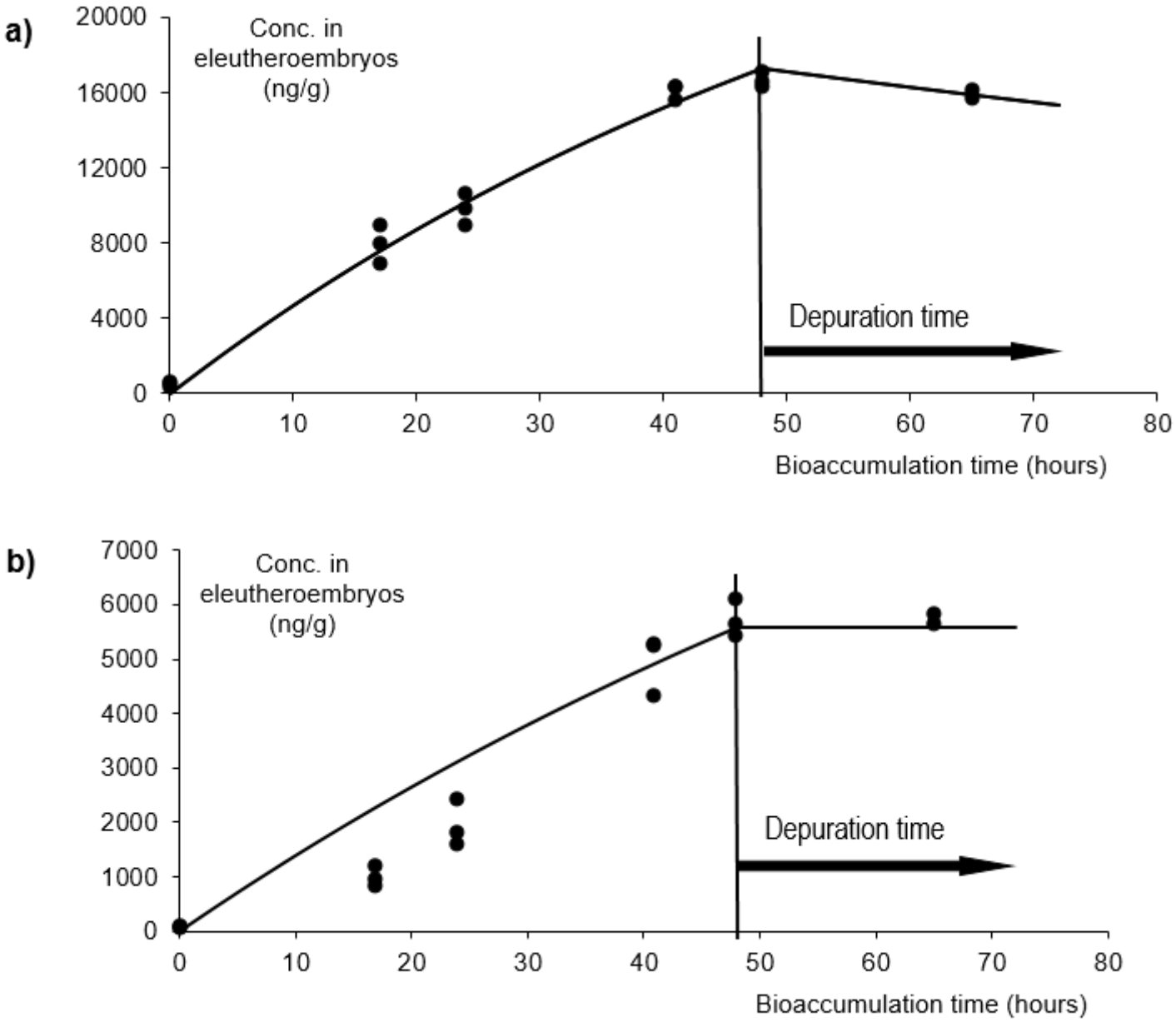

Figure 2

BDE-47 bioconcentration in zebrafish eleutheroembryos samples exposed to a) 10 µg·L-1 and b) 1 µg·L-1

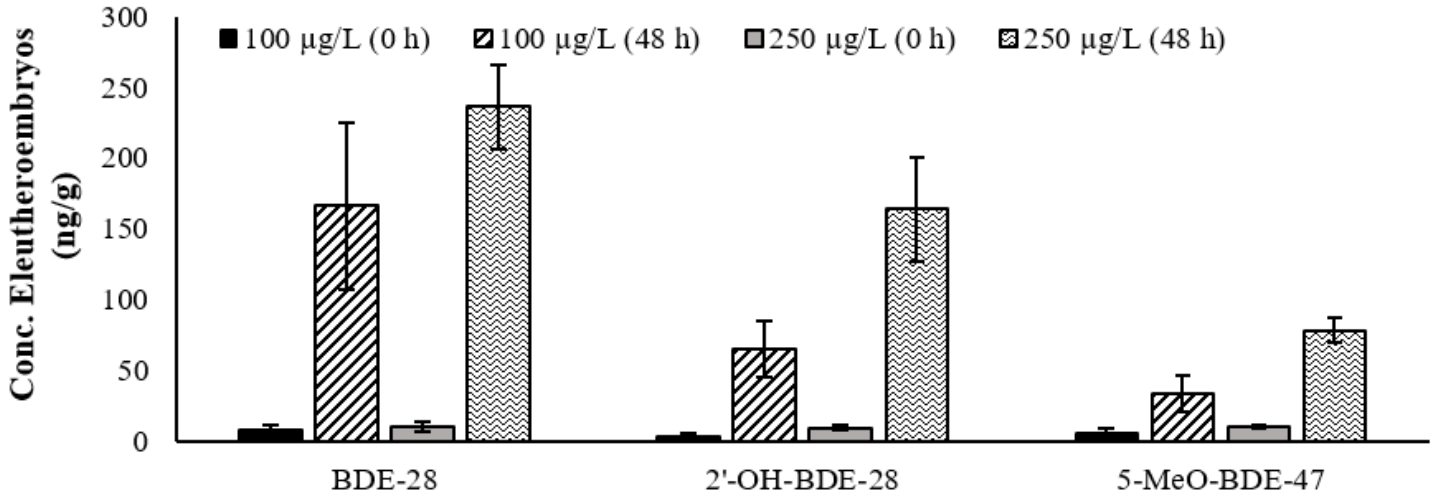

Figure 3

Transformation of BDE-47 (100 µg·L-1 and 250 µg·L-1) by zebrafish eleutheroembryos after 48 hours of exposure

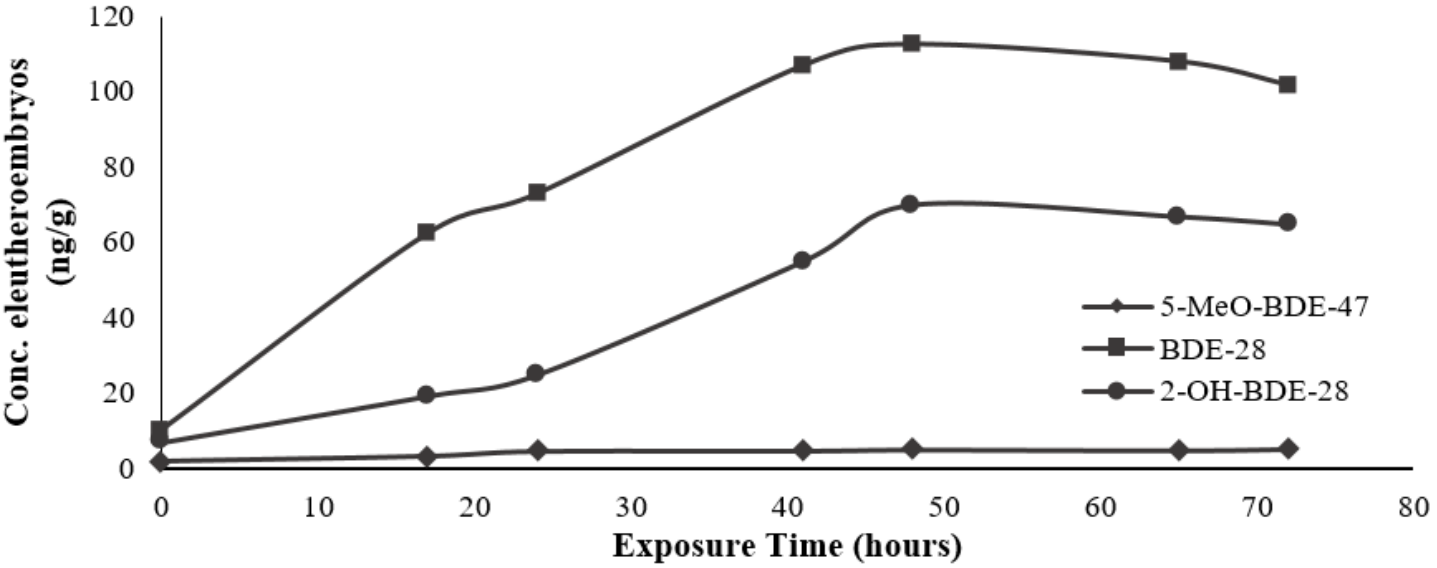

Figure 4

Metabolites of BDE-47 (10 µg·L-1) observed by transformation in zebrafish eleutheroembryos over time

Supplementary Files

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