Evaluation of the bioaccumulation potential of selected alternative brominated flame retardants in marine fish using *in vitro* metabolic transformation rates

Hyun-Jeoung Lee a, Jee-Hyun Jung b, Jung-Hwan Kwon a,*

a Division of Environmental Science and Ecological Engineering, Korea University, 145 Anam-ro, Seongbuk-gu, Seoul 02841, Republic of Korea

b Oil & POPs Research Group, Korea Institute of Ocean Science & Technology, Geoje 53201, Republic of Korea

**HIGHLIGHTS**

- Bioaccumulation potential of 6 novel BFRs was evaluated.
- Biotransformation rates were measured using isolated S9 from marine fish.
- *In vitro* biotransformation rate was extrapolated to BCF using an IVIVE model.
- Less hydrophobic BFRs (6 ≤ logKow ≤ 7) would be classified as bioaccumulative.

**GRAPHICAL ABSTRACT**

**ABSTRACT**

The global consumption of alternative brominated flame retardants (BFRs) has increased with the restriction of the first generation BFRs such as polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecanes (HBCDs). However, many alternative BFRs are suspected to be persistent in the environment and possibly bioaccumulative after their release into the environment because of their chemical properties, which are similar to those of the already banned BFRs. In this study, the bioaccumulation potential of selected alternative BFRs (1,2-bis(2,4,6 tribromophenoxy)ethane (BTBPE), 1,2,3,4,5,6 hexabromobenzene (HBB), pentabromoethylbenzene (PBEB), 2,3,4,5,6 pentabromotoluene (PBT), 2 ethylhexyl-2,3,4,5-tetrabromobenzoate (TBB), and 2,3,4,5 tetrabromo-6-chlorotoluene (TBCT)) was evaluated. The *in vitro* depletion rate constants (k_depl) were measured for the alternative BFRs using liver S9 fractions isolated from five marine fish species (*Epinephelus septemfasciatus*, *Konosirus punctatus*, *Lateolabrax japonicus*, *Mugil cephalus*, and *Sebastes schlegelii*) that inhabit the oceans off the Korean coast. The measured k_depl values were converted to *in vitro* intrinsic clearance rate constants (CL_in vitro) to estimate whole-body metabolic rate constants (k_met) using an *in vitro* to *in vivo* extrapolation (IVIVE) model. Finally, the bioconcentration factors (BCF) were determined using a one-compartment model. The transformation kinetics for obtaining k_depl agreed well with first-order chemical kinetics, regardless of initial BFR concentrations. The values of CL_in vitro were lower in the selected marine fish species than those in freshwater fish species, implying slower metabolic transformation. The derived BCF values based on the total concentration in water (BCF_TOT) ranged from 16 (TBB in *M. cephalus*) to 27,000 (HBB in *K. punctatus*). The BCF values for HBB and PBT were >2000 except for those in *M. cephalus* suggesting further investigation of BCF values of BFRs whose log Kow is between 6 and 7.

© 2018 Elsevier B.V. All rights reserved.

Keywords:
Bioconcentration
Biotransformation
Marine fish
Alternatives
1. Introduction

Flame retardants are added to various products to provide fire resistance. The global consumption of flame retardants has increased with the increasing use of plastic products (Norwegian Institute for Air Research, 2008; Shi et al., 2018; Watanabe and Sakai, 2003). One of the most commonly used groups of flame retardants are organic halogen compounds. Among them, brominated flame retardants (BFRs) have been widely used in Asia (Norwegian Institute for Air Research, 2008). The first-generation BFRs include polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecanes (HBCDs) (de Wit, 2002; Norwegian Institute for Air Research, 2008). Their commercial uses in products are now restricted under the Stockholm Convention on persistent organic pollutants (POPs) because of their environmental persistence, bioaccumulation potential, and toxicity (Brown et al., 2014; Crump et al., 2008; Fernie et al., 2009; Luo et al., 2017; UNEP, 2009a, 2009b, 2013). Selected congeners of PBDEs and HBCDs have been listed for addition to Annex A of the Stockholm Convention in 2009 and 2013, respectively (UNEP, 2009a, 2009b, 2013). With this global regulation, alternative chemicals with similar chemical structures, so-called novel brominated flame retardants (NBFRs), have been introduced to the market, and their production volume has increased (Bergman et al., 2012; Covaci et al., 2011; Nguyen et al., 2017; Norwegian Institute for Air Research, 2008). It is not surprising that the alternatives share similar chemical characteristics compared to those of PBDEs and HBCDs because the bromine radicals produced from bromine-containing aromatic organic molecules are good quenchers of the hydrogen and hydroxyl radicals in flames (Rahman et al., 2001).

Unfortunately, the POP-like properties of NBFRs cannot be separated from their effectiveness as flame retardants. Aromatic bromines are not readily decomposed by aerobic microorganisms (Schwarzenbach et al., 2017), rendering their environmental half-lives sufficiently long to allow for long-range transport. In addition, their limited rate of enzymatic degradation might result in significant bioaccumulation of alternative BFRs, which could be toxic to humans and wildlife, especially via hormonal disruption, as has been demonstrated for many brominated aromatic compounds (Butt et al., 2011; Butt and Stapleton, 2013; Marvin et al., 2011; Palace et al., 2008; Smythe et al., 2017). Although many NBFRs are suspected to have POP-like properties (Wu et al., 2011; Zheng et al., 2018), their persistence, long-range transport potential, bioaccumulation potential, and toxicity have not been sufficiently evaluated to guide regulatory actions.

Bioaccumulation potential is an important criterion that determines whether chemicals are classified as POPs. To make a regulatory decision on a weight-of-evidence basis, the observed accumulation of chemicals in aquatic and terrestrial food chains is the most important aspect to consider. However, a decision based on strong evidence from field monitoring can be made only after the target chemical has been widely disseminated and globally distributed, failing to satisfy the precautionary principle. The laboratory bioconcentration factor (BCF) is obtained following a standard method, such as the Organization for Economic Cooperation and Development (OECD)'s test guidelines for experiments with fish (OECD, 2012) as a second-tier method. However, the experimental determination of BCF requires many animals and is expensive. In addition, many NBFRs are only sparingly soluble in water, making the experimental determination of fish BCF extremely difficult. Therefore, as an alternative, various bioconcentration/bioaccumulation models may be used (Arnot and Gobas, 2004; Mackay, 1982; Meylan et al., 1999; Nichols et al., 2013; OECD, 2018a, 2018b).

The most sensitive parameter in existing bioconcentration/bioaccumulation models for hydrophobic organic chemicals is the whole-body biotransformation rate constant (Arnot and Gobas, 2004; Nichols et al., 2013; OECD, 2018a, 2018b). In fish, the liver is considered to be the most important organ for clearing xenobiotic chemicals from the body. Liver microsomes and protein fractions such as the S9 fraction have been isolated from various fish species and the in vitro biotransformation rates have been evaluated assuming pseudo-first-order kinetics (Johanning et al., 2012). In addition, standardized methods have been accepted by the OECD (OECD, 2018a, 2018b). To evaluate the whole-body BCF/BAF, extrapolation of the in vitro biotransformation rate to the whole-body biotransformation rate is necessary. Nichols and colleagues proposed an in vitro to in vivo extrapolation (IVIVE) model and evaluated the model performance using rainbow trout as a model fish (Nichols et al., 2013).

Although laboratory testing and models regarding the assessment of bioaccumulation potentials of hydrophobic organic chemicals have been mostly developed with freshwater organisms, bioaccumulation in marine organisms is a significant concern in marine ecosystems (McGeer et al., 2003). In addition, organic chemicals accumulated in marine biota can be biotransferred to humans through the consumption of marine products, such as fish, shellfish, and seaweed (Kim and Kang, 2017). Thus, it is important to evaluate the bioaccumulation potential of POP-like chemicals using marine organism models.

In this study, five marine fish species (Epinephelus septemfasciatus (convict grouper), Konosirus punctatus (dotted gizzard shad), Lateolabrax japonicus (Japanese seabass), Mugil cephalus (flathead grey mullet), and Sebastes schlegeli (Korean rockfish)) that inhabit the coastal oceans of the Korean Peninsula were chosen considering their trophic levels in the ecosystem and propensity for human consumption. Six alternative BFRs (1,2-bis(2,4,6 tribromophenoxy)ethane (TBBPE), 1,2,3,4,5,6 hexabromobenzene (HBB), pentabromoethylenbenzene (PBBE), 2,3,4,5,6 pentabromotoluene (PBT), 2 ethylhexyl-2,3,4,5-tetrabromobenzene (TBB), and 2,3,4,5 tetrabromo-6-chlorotoluene (TBC)) were selected for the evaluation of bioaccumulation potential because previous modeling studies have indicated that they could be as persistent (or more), traveling long distances and bioaccumulative to a similar extent as PBDE congeners already banned under the Stockholm Convention (Jin et al., 2016; Kuramochi et al., 2014; Lee and Kwon, 2016). S9 fractions were isolated from livers of the selected fish species and the in vitro depletion rate constants (kdep) were measured using batch kinetic experiments. The measured values of kdep were converted to whole-body metabolic rate constants (kmet) using an IVIVE model modified with biometric parameters measured for the selected fish species. Finally, the BCF values of the selected alternative BFRs in five fish species were obtained using a one-compartment bioconcentration model to assess bioaccumulation potential.

2. Material and methods

2.1. Chemicals

Trizma hydrochloride (Tris–HCl, ≥99%; CAS Reg. no. 1185-53-1), β-nicotinamide adenine dinucleotide phosphate, reduced tetra (cyclohexylammonium) salt (NADPH, ≥93%; CAS Reg. no. 100929-71-3), glucose-6-phosphate potassium salt (G6P, 98%; CAS Reg. no. 103192-55-8), resorufin (95%; CAS Reg. no. 635-78-9), 7 ethoxoresorufin (≥95%; CAS Reg. no. 5725-91-7), ethyl-3-aminobenzoate methanesulfonate (MS-222, CAS Reg. no. 886-86-2) for anesthetization, and bovine serum albumin (BSA, ≥98%; CAS Reg. no. 9048-46-8) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Six alternative BFRs were selected to evaluate their bioaccumulation potential. Hexabromobenzene (HBB), >98%; CAS Reg. no. 87-82-1 and 2,3,4,5,6 pentabromoethylbenzene (PBBE, 99.8%; CAS Reg. no. 85-22-3) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Two alternative BFRs were selected to evaluate their bioaccumulation potential. Hexabromobenzene (HBB, >98%; CAS Reg. no. 87-82-1 and 2,3,4,5,6 pentabromoethylbenzene (PBBE, 99.8%; CAS Reg. no. 85-22-3) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Six alternative BFRs were selected to evaluate their bioaccumulation potential.
2.2. Animals

The subject fish species were chosen based on those consumed by Koreans considering the trophic levels in the marine food web: *E. septemfasciatus* (convict grouper), *K. punctatus* (dotted gizzard shad), *L. japonicus* (Japanese seabass), *M. cephalus* (flathead grey mullet), and *S. schlegelii* (Korean rockfish). According to the FishBase database (FishBase, 2018), the range of the trophic levels for the five selected fish species are reported to range from 2.5 ± 0.17 (*K. punctatus*) to 4.0 ± 0.66 (*E. septemfasciatus*) and have hepatosomatic index (HSI) values ranging from 0.010 ± 0.0014 to 0.024 ± 0.0036. Physiological parameters such as the body and liver weights of the subject fish were measured and are listed in Table 1. All fish were purchased from the Noryangjin fish market in Seoul as live fish. The fish were caught near the coast of the Korean Peninsula, *i.e.*, Tongyeong (*E. septemfasciatus* and *K. punctatus*, November 2017), Seosan (*L. japonicus*, November 2017), and Boryeong (*S. schlegelii* and *M. cephalus*; June and August 2017) areas. Three samples each of *E. septemfasciatus* and *L. japonicus*, five each of *M. cephalus* and *S. schlegelii*, and eleven *K. punctatus* samples were pooled for the preparation of the liver S9 fraction.

2.3. Preparation of the liver S9 fractions

Anesthetic (MS-222; 300 mg mL⁻¹) was added to a bucket containing the fish, which were subsequently euthanized. After the fish were euthanized, they were removed from the bucket, and their lengths and weights were measured. The liver was excised from the subject fish and placed in an ice-chilled Petri dish. After measuring the liver weight, the liver tissue was rinsed with 0.15 M KCl solution. The rinsed liver tissue was then transferred to a 0.15 M KCl solution and homogenized using an HS-30E tissue homogenizer (Daian Scientific Co., Seoul, Korea). *E. septemfasciatus*, *L. japonicus*, *M. cephalus*, and *S. schlegelii*, the liver tissue was cut into 2 g pieces and combined for homogenization. Whole livers of *K. punctatus* (approximately 1.1 g per fish) were used for homogenization because of the smaller liver size. One milliliter of the KCI solution was added per gram of homogenate. The minced liver tissue solution was centrifuged at 10,000g at 4 °C for 20 min, and the supernatant (S9 fraction) was carefully taken and stored at −80 °C until use (Lo et al., 2015). The yields of the S9 fraction obtained were between 0.4 (*S. schlegelii*) and 1.2 ml g⁻¹ of liver (*K. punctatus*).

2.4. Protein concentration in the S9 fractions and whole-body lipid contents

The Bradford reagent was diluted 5-fold in distilled water. The BSA stock solution was prepared at a concentration of 1 mg mL⁻¹ and diluted to make standard solutions containing a protein concentration between 0.05 and 0.3 mg mL⁻¹. The protein concentration of the S9 fraction was determined after 4-fold dilution with distilled water. After preparing all solutions and samples, 20 μl of the BSA standard solution and 20 μl of the diluted S9 fraction were added to 1 mL of the Bradford working solution, and the mixed solution was maintained at room temperature for 5 min. The protein concentration was determined using the absorbance at 595 nm using a DR/4000 UV/Vis spectrophotometer (Hach Co., Loveland, CO, USA) (Bradford, 1976).

Whole-body lipid content was measured using the Rose-Gottlieb method (Richardson, 1985) for homogenized tissues after separating bones and heads. Briefly, 6 mL of concentrated hydrochloric acid was added to the homogenized tissues, followed by heating at 70 °C in a water bath for 90 min. After transferring the acid-extracted solution to a Mojonnier tube, deionized water was added to a final sample volume of 20 mL. Then, 7 mL of ethanol and 25 mL of petroleum ether were added, and the tube was shaken vigorously. After the upper ether layer clarified, it was filtered through a filter paper and the mass of dried residue was measured as the dry lipid content.

2.5. Assay conditions for EROD activity analysis

The ethoxyresorufin-o-deethylase (EROD) method was used to determine the activity of CYP1A1, involved in xenobiotic metabolism, as an indicator of S9 activity. The reaction cocktail was comprised of 2 mM NADPH (100 μL; 20% v/v), 4 mM 7-ethoxyresorufin (5 μL; 1% v/v), S9 fraction (100 μL; 20% v/v), and 50 mM Tris buffer containing 10 mM MgCl₂ (295 μL; 50% v/v) (Pikkarainen, 2006; Zamaratskaia and Zlabek, 2009). The reaction was initiated by adding NADPH and 30 min after the initiation of the reaction at 15 °C, 150 μL of the reaction mixture was collected from the experimental vial, and the reaction was terminated by adding the reaction mixture to 500 μL of ice-cold methanol. EROD activity was expressed in the units of picomoles per milligram of protein per minute (pmol mg protein⁻¹ min⁻¹). Resorufin, used for EROD activity, was analyzed using a high-pressure liquid chromatography-fluorescence detector (HPLC-FLD). The HPLC-FLD system was composed of a Waters 515 pump, 717 autosampler, and 2475 multi λ fluorescence detector (Waters, Milford, MA, USA). The samples (10 μL) were injected onto a Fortis C18
column (5 μm, 150 × 4.6 mm, Fortis Technologies Ltd., Cheshire, UK) at a flow rate of 1.0 mL min⁻¹. The mobile phases were 20 mM phosphate buffer (A, pH 6.8) and methanol (B) with a gradient program (Rowland et al., 1973), starting at 30% B, followed by a 3.5 min hold, then an increase to 80% B over 2.5 min, then a 3.1 min hold, a reduction back to 30% B over 5 min, and finally a hold for 3 min.

2.6. Measurement of in vitro intrinsic clearance rate \((CL_{in\,vitro})\)

The assay for determination of in vitro depletion rate constant was performed in 50 mM Tris buffer (pH 7.8) containing 10 mM MgCl₂ at 30 °C, followed by an increase at the rate of 25 °C min⁻¹ to 280 °C, and at 25 °C min⁻¹ to 300 °C, followed by a hold for 6 min. The detector temperature was 240 °C and the detector temperature was 300 °C. The method detection limits for BTBPE, HBB, PBB, PBT, TBB, and TBCT were 8.1, 0.50, 0.38, 1.0, 0.48, and 0.28 nM, respectively. The recoveries at an initial concentration of 1 μM were 88.1–103.3% (BTBPE), 94.8–112.5% (HBB), 89.9–100.6% (PBB), 94.1–98.7% (PBT), 94.0–103.3% (TBB), and 89.9–102.4% (TBCT), respectively (Table S1, Supplementary Material).

2.8. In vitro to in vivo extrapolation and estimation of BCF

The IVIVE model (Nichols et al., 2013) and a one-compartment BCF model (Arnot and Gobas, 2004) were used to obtain BCF values for the fish. Because these models were originally developed for rainbow trout, fish-specific input parameters were measured and estimated using parameters applied to the selected marine fish species. The input parameters of the IVIVE model are divided into the chemical properties (log \(K_{ow}\)) and biometric parameters (e.g., liver weight as a fraction of whole-body weight \((L_{FPRV})\), fractional whole-body lipid content \((f_{lip})\), blood flow to the liver \((Q_{SH})\), and detailed values for selected marine fish species are shown in Tables S2–S6 (Supplementary Material) with the calculated dependent variables. To extrapolate the \(CL_{in\,vitro}\) value to the \(K_{bc}\) value, a two-step procedure is required. The value of the in vivo intrinsic clearance \((CL_{in\,vivo})\) is obtained using Eq. (1).

\[
CL_{in\,vivo} = CL_{in\,vitro} \times L_{SH} \times HSI \times 24^h
\]

where \(L_{SH}\) and HSI (hepatosomatic index) are the liver S9 protein content \(\text{[mg g}^{-1}\text{]}\) and the liver weight as a fraction of whole-body weight \(\text{[unitless]}\), respectively. The value of HSI was measured when preparing the S9 fraction (Table 1), and the default value of \(L_{SH}\) for rainbow trout (163 mg g⁻¹) (Nichols et al., 2013) was used because the S9 content was not measured for marine fish. Subsequently, the hepatic clearance rate \((CL_{H})\) was calculated using \(CL_{in\,vivo}\), following a well-stirred liver model (Eq. (2)) (Rowland et al., 1973; Wilkinson and Shand, 1975).

\[
CL_{H} = \frac{Q_{SH}}{f_{1d}} \times CL_{in\,vivo}
\]

where \(Q_{SH}\) is the hepatic flow rate \([L \text{ d}^{-1} \text{ kg}_{\text{fish}}^{-1}]\), estimated by multiplying the value of cardiac output \((Q_{C})\) and the liver blood flow as a fraction of cardiac output \((\text{Q}_{\text{FPRV}}))\). The value of \(Q_{\text{FPRV}}\) was estimated to be 0.259 for rainbow trout (Nichols et al., 1990) and used as a default. The \(Q_{C}\) was calculated using an empirical equation by Erickson and McKim, as (Erickson and McKim, 1990)

\[
Q_{C} = \left(0.0237 - 0.78 \times \left(\frac{B_{\text{mg}}}{500}\right)^{-0.1}\right) \times 24
\]

where \(B_{\text{mg}}\) is the weight of the subject fish \([\text{g}]\) and \(T\) is the temperature \([^\circ\text{C}]\). In Eq. (2), \(f_{1d}\) is the hepatic clearance binding term, defined as the

Table 1

<table>
<thead>
<tr>
<th>E. septemberfusatus (n = 3)</th>
<th>K. punctatus (n = 11)</th>
<th>L. japonicas (n = 3)</th>
<th>M. cephalus (n = 5)</th>
<th>S. schlegeli (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trophic level*</td>
<td>4.0 ± 0.66</td>
<td>2.5 ± 0.17</td>
<td>2.9 ± 0.22</td>
<td>3.4 ± 0.43</td>
</tr>
<tr>
<td>Fish weight (g)b</td>
<td>1743.3 ± 58.6</td>
<td>74.2 ± 5.5</td>
<td>1868.0 ± 211.6</td>
<td>1395.0 ± 139.3</td>
</tr>
<tr>
<td>Liver weight (g)b</td>
<td>411 ± 8.5</td>
<td>1.1 ± 0.2</td>
<td>18.0 ± 3.5</td>
<td>24.2 ± 5.5</td>
</tr>
<tr>
<td>Lipid contents (%)b</td>
<td>11 ± 2</td>
<td>19 ± 4</td>
<td>2.1 ± 0.7</td>
<td>2.5 ± 1.0</td>
</tr>
<tr>
<td>Length (cm)b</td>
<td>48.3 ± 1.5</td>
<td>20.9 ± 0.7</td>
<td>62.8 ± 3.0</td>
<td>54.8 ± 2.2</td>
</tr>
<tr>
<td>HSI (L/W/FW)b</td>
<td>0.024 ± 0.005</td>
<td>0.016 ± 0.002</td>
<td>0.010 ± 0.002</td>
<td>0.017 ± 0.002</td>
</tr>
</tbody>
</table>

* FishBase (http://www.fishbase.org).

b Measured value.

c Calculated value using fish weight and liver weight.
ratio of the unbound chemical fraction in the in vitro system (\( f_{U,S9} \)) to that in the blood plasma (\( f_{U,P} \)). The \( f_{U,S9} \) is related to the liver S9 fraction used in the experiment (\( C_{S9} \)) and log \( K_{OW} \). The \( f_{U,P} \) is determined by dividing fractional water content of the blood (\( V_{WBL} \)) by blood-to-water partition coefficient (\( P_{BW} \)) (Nichols et al., 1990; Han et al., 2009).

\[
f_{U,S9} = \frac{1}{C_{S9} \times 10^{(0.694 \times \log K_{OW} - 2.158) + 1}}
\tag{4}
\]

\[
f_{U,P} = \frac{V_{WBL}}{P_{BW}}
\tag{5}
\]

\[
f_{U} = \frac{f_{U,P}}{f_{U,S9}}
\tag{6}
\]

The value of \( V_{WBL} \) was assumed to be 0.84 based on previous measurements in rainbow trout (Bertelsen et al., 1998), and the values of \( P_{BW} \) were calculated using the empirical equation proposed by Fitzsimmons et al. (Eq. (7)) (Fitzsimmons et al., 2001).

\[
P_{BW} = \left( 10^{0.73 \times \log K_{OW} - 0.16} \right) + 0.84
\tag{7}
\]

The whole-body first-order metabolic rate constant (\( k_{MET} \)) was obtained by dividing \( C_{LH} \) by the apparent volume of distribution (\( V_{DBL} \)), which was estimated by dividing the partitioning-based bioconcentration factor (\( BCF_p \)), obtained by multiplying \( K_{OW} \) and whole-body lipid content (\( f_{U,P} \)), by \( P_{BW} \).

\[
k_{MET} = \frac{C_{LH}}{V_{DBL}}
\tag{8}
\]

\[
BCF_p = \frac{f_{U,P}}{K_{OW}}
\tag{9}
\]

\[
V_{DBL} = \frac{BCF_p}{P_{BW}}
\tag{10}
\]

Finally, a steady-state one-compartment bioaccumulation model including gill uptake and elimination, fecal egestion, and metabolic transformation (Arnot and Gobas, 2006) was used to estimate whole-body BCF:

\[
d\frac{C_{fish}}{dt} = (k_1 C_{W,FD}) - (k_2 + k_5 + k_{MET}) C_{fish}
\tag{11}
\]

\[
C_{W,TOT} = (1 + \alpha_{DOC} C_{DOC} K_{OW} + \alpha_{POC} C_{POC} K_{OW}) C_{W,FD}
\tag{12}
\]

\[
BCF_{FD} = \frac{C_{fish}}{C_{W,FD}} = \frac{k_1}{(k_2 + k_5 + k_{MET})}
\tag{13}
\]

\[
BCF_{TOT} = \frac{C_{fish}}{C_{W,TOT}}
\tag{14}
\]

where \( C_{W,FD} \) and \( C_{W,TOT} \) are the freely dissolved and total chemical concentrations in the water and \( C_{fish} \) is the chemical concentration in fish, \( k_1 \) and \( k_2 \) are the gill uptake and elimination rate constants, \( k_5 \) is the fecal egestion constant, \( \alpha_{DOC} \) and \( \alpha_{POC} \) are affinity constants for dissolved organic carbon (DOC) and particulate organic carbon (POC), and \( C_{DOC} \) and \( C_{POC} \) are the DOC and POC concentrations in water. Values of \( \alpha_{DOC} \) and \( \alpha_{POC} \) were assumed as 0.08 (Burkhard, 2000) and 0.35 (Seth et al., 1999), respectively. The values of \( C_{DOC} \) and \( C_{POC} \) were assumed to be 2.9 and 0.5 mg L\(^{-1}\) using the default values by the US EPA (US EPA, 2003). The values of \( k_1 \), \( k_2 \), and \( k_5 \) were obtained using empirical equations, and were related to \( B_w g_{s,MT} \), and \( K_{OW} \) (Arnot and Gobas, 2003) and expressed as:

\[
k_1 = \frac{1}{(0.01 + \frac{1}{K_{OW}}) \times B_w g_{s,MT} 0.4}
\tag{15}
\]

\[
k_2 = \frac{k_1}{BCF_p}
\tag{16}
\]

\[
k_5 = 0.125 \times \frac{0.02 \times B_w g_{s,MT} 0.15 \times e^{0.007}}{5.1 \times 10^{-3} K_{OW} + 2}
\tag{17}
\]

### 3. Results and discussion

#### 3.1. Protein content and S9 enzyme activity in S9 fractions

The measured protein concentrations and EROD activities of the isolated S9 fractions are shown in Table 2. The highest protein concentration was measured in the \( L. japonicus \) S9 fraction (5.06 ± 0.06 mg mL\(^{-1}\)), followed by \( M. cephalus \), \( E. septemfasciatus \), and \( K. punctatus \) in order. The EROD activity was measured at 15 °C, which was chosen based on the average temperature of seawater near the Korean Peninsula (Flammarion et al., 1996). The EROD activity was highest in \( L. japonicus \) (3.18 ± 0.04 pmol mg protein\(^{-1}\) min\(^{-1}\)) and the lowest in \( S. schlegelii \) (0.52 ± 0.15 pmol mg protein\(^{-1}\) min\(^{-1}\)).

The EROD activities obtained in this study were slightly lower than those previously reported in S9 fractions of many freshwater and marine fish (Della Torre et al., 2014; Flammarion et al., 1996; Pikkarainen, 2006; Strobel et al., 2015; Webb and Gagnon, 2002). We could only find EROD activities for the fish species belonging to the same orders as \( M. cephalus \) and \( L. japonicus \) in the literature (Della Torre et al., 2014; Pikkarainen, 2006; Webb and Gagnon, 2002). The reported EROD activities were 2.7 ± 3.9 pmol mg protein\(^{-1}\) min\(^{-1}\) (Baltic sea perch, \( Perca fluviatilis \)) (Pikkarainen, 2006), 7 to 15 pmol mg protein\(^{-1}\) min\(^{-1}\) (sea bass, \( Dicentrarchus labrax \)) (Della Torre et al., 2014), and 9.2 ± 1.2 pmol mg protein\(^{-1}\) min\(^{-1}\) (sea mullet, \( Mugil cephalus \)) (Webb and Gagnon, 2002). These values were higher than those of \( M. cephalus \) and \( L. japonicus \) (2.61 ± 0.19 and 3.18 ± 0.04 pmol mg protein\(^{-1}\) min\(^{-1}\) , respectively) obtained in this study by a factor of 2.2–4.7. The EROD activity is related to the sexual maturity and environmental temperature. It has been acknowledged that the EROD activity is higher in immature fish and at lower temperature (Sleiderink et al., 1995). Higher EROD activities in the earlier studies (Della Torre et al., 2014; Pikkarainen, 2006; Webb and Gagnon, 2002) were obtained for immature fish, whereas adult fish were used in this study. It is also known that EROD activities are greater at lower temperature (Ferreira et al., 2006). Because \( M. cephalus \) were caught in August when seawater temperature was warmer, the EROD activity may be underestimated.

The EROD activities in the S9 fraction of rainbow trout, a representative model freshwater fish species, were found to be between 11 and 13 pmol mg protein\(^{-1}\) min\(^{-1}\).
129 pmol mg protein$^{-1}$ min$^{-1}$ (Connors et al., 2013; Flammarion et al., 1996; Smith and Wilson, 2010; Strobel et al., 2015). Although the EROD activities depend on age and sex, the approximately one order-of-magnitude lower values imply that CYP1A-related metabolic activity would be lower in these selected marine fish species than in rainbow trout. These results suggest that the CYP-related xenobiotic transformation rates of hydrophobic organic chemicals in marine fish species might be low, leading to higher bioaccumulation factors when biotransformation is dominated by CYP-related enzymatic processes.

3.2. Determination of in vitro intrinsic clearance

The metabolic transformation rate is often described by Michaelis–Menten kinetics, in which the apparent reaction rate is described by pseudo-first-order reaction kinetics at sufficiently low substrate concentrations. Because the concentration of hydrophobic organic contaminants, such as BFRs in this study, is much lower than the typical half-saturation constant ($K_M$) in Michaelis–Menten kinetics, $k_{depl}$ should be determined in the range where the Michaelis–Menten kinetics is approximated to first-order transformation kinetics to accurately reflect the environmental levels of BFRs. Because it is difficult to determine Michaelis–Menten parameters for the alternative BFRs for which major transformation products have not been identified and the solubility in water is very low, $k_{depl}$ values were measured at two different initial BFR concentrations to ensure that the obtained $k_{depl}$ is independent of initial concentration. Fig. 2 shows representative examples determining $k_{depl}$ for TBB and TBCT in E. septemfasciatus and K. punctatus. All experimental results are shown in Figs. S1 to S5 (Supplementary Material). As shown in Figs. 2, S1–S5 and Table S7 (Supplementary Material), the measured values of $k_{depl}$ at two different initial concentrations did not significantly differ from each other. Paired $t$-test for $k_{depl}$ values at two different initial concentrations showed that all $p$ values were $>0.05$ except for TBB in Epinephelus septemfasciatus ($p = 0.022$). However, $k_{depl}$ values at two different initial concentration did not differ much (0.26 and 0.27 h$^{-1}$; Table S7, Supplementary Material). In many cases, the remaining concentration after 2 h was not significantly different ($p = 0.05$) from the initial concentration at 0 h. In addition, $k_{depl}$ values between 0 and 6 h were not significantly different from those between 0 and 2 h for most cases at $p = 0.05$. Thus, the slopes of the entire time range (0–6 h) were used to obtain $k_{depl}$ although it is expected that some enzymatic activity would be lost with time (Johanning et al., 2012). Furthermore, no concentration changes of all BFRs were observed using the heat-denatured S9 fraction for five marine fish species, suggesting that the depletion of BFRs obtained using the activated S9 fraction is the result of biotransformation.

The values of $C_{Lin\text{ vitro}}$ at the two different initial BFR concentrations are shown in Table 3. The obtained values were within a factor of 17, ranging from 0.034 to 0.57 mL h$^{-1}$ mg$^{-1}$ protein, with chemical-to-chemical and species-to-species variations in $C_{Lin\text{ vitro}}$. The $C_{Lin\text{ vitro}}$ of TBB was the highest of all the tested compounds in the five fish species, except for that of the M. cephalus S9 fraction. TBCT was the most rapidly depleted BFR in the M. cephalus S9 fraction. The $C_{Lin\text{ vitro}}$ values for all

![Fig. 2. Decline of the natural logarithm of the concentration of selected alternative BFRs: (a) 2-ethylhexyl-2,3,4,5 tetrabromobenzoate (TBB) in Epinephelus septemfasciatus, (b) 2,3,4,5 tetrabromo-6-chlorotoluene (TBCT) in Epinephelus septemfasciatus, (c) TBB in Konosirus punctatus, and (d) TBCT in Konosirus punctatus. Open squares and triangles represent results obtained at initial concentrations of 0.1 and 1 μM, respectively. Closed circles present concentration changes using denatured S9 fractions at 1 μM.](image-url)
BFRs were greatest in the *M. cephalus* S9 fraction. For the other fish species, no trend in metabolic capacity was observed.

The measured \( CL_{\text{in vitro}} \) values did not show a clear correlation with EROD activity (Fig. S6, Supplementary Material). BFRs are thought to be metabolized via several pathways including oxidative debromination, reductive debromination, and oxidative CYP enzyme-mediated pathways (phase I and II) (Hakk and Letcher, 2003). Because the experimental conditions of the *in vitro* biotransformation favor oxidative pathways, reductive debromination and phase II biotransformation would not be represented in the incubation conditions of the present study. The structural similarity of alternative BFRs in this study with polybrominated benzenes (PBBs) and PBDEs implies that other oxidative enzymes could be also important. Cytochrome enzymes such as CYP2B and CYP4A also play significant roles in transforming PBBs and PBDEs (Hakk and Letcher, 2003). Activities of other oxidative enzymes are not well represented by the EROD activity used for estimating CYP1A activity. It needs further investigations to know the major biotransformation pathways.

No studies have reported the *in vitro* intrinsic clearance rates using fish S9 fractions for the alternative BFRs chosen in this study. Only one study has reported the *in vitro* clearance rate constants for TBB using rat and human liver microsomes and cytosols (Roberts et al., 2012). The *in vitro* clearance rate constants in the rat liver cytosol and microsomes are higher than those in human liver cytosol and microsomes. The *in vitro* clearance rate constant of TBB in human liver cytosol and microsomes were 12.42 ± 0.020 and 15.54 ± 0.033 \( \mu \text{M} \cdot \text{h}^{-1} \cdot \text{mg}_{\text{protein}}^{-1} \) and those in rat liver cytosol and microsomes were 25.32 ± 0.093 and 375 ± 0.058 \( \mu \text{M} \cdot \text{h}^{-1} \cdot \text{mg}_{\text{protein}}^{-1} \) (Roberts et al., 2012). The corresponding rate constants for TBB at an initial concentration of 1 \( \mu \text{M} \) in the S9 fractions of the five marine fish species (0.19 ± 0.010 to 0.45 ± 0.001 \( \mu \text{M} \cdot \text{h}^{-1} \cdot \text{mg}_{\text{protein}}^{-1} \), Table 3) are orders of magnitude lower than those in rat and human liver cytosols and microsomes. Although further investigations are needed, these results suggest slower metabolic transformation rates in marine fish compared to those in mammals, implying a higher bioaccumulation potential of alternative BFRs for these marine fish.

### 3.3. Extrapolation to the whole-body metabolic rate constant (\( k_{\text{MET}} \))

As shown in Table 3, the values of \( CL_{\text{in vitro}} \) did not depend on the initial concentration of the BFR alternatives. Thus, the results obtained for the initial concentration of 1 \( \mu \text{M} \) were used to derive the whole-body metabolic rate constants (\( k_{\text{MET}} \); \( \text{d}^{-1} \)), which are listed in Table 4. The \( k_{\text{MET}} \) values in *E. septemfasciatus*, *K. punctatus*, and *L. japonicus* S9 fractions were the lowest for BTBPE and the highest for HBB. For S9 fractions from *S. schlegelii*, PBT had the greatest \( k_{\text{MET}} \) values, followed by HBB, PBEB, TBCT, TBB, and BTBPE in decreasing order. The values of \( k_{\text{MET}} \) among different fish species decreased in the order *M. cephalus* > *E. septemfasciatus* > *L. japonicus* > *S. schlegelii* = *K. punctatus*, except for those for PBEB and PBT.

The \( k_{\text{MET}} \) value for a given compound not only depends on the metabolic capability of the fish species, represented by \( k_{\text{detox}} \) or \( CL_{\text{in vitro}} \), but also are affected by bioavailability of the compound denoted by \( f_{\text{dL}} \), as demonstrated in Eqs. (4)–(6). Higher protein contents lower the bioavailability of hydrophobic BFRs, resulting in decreased \( k_{\text{MET}} \) when other conditions are identical. Thus, the \( k_{\text{MET}} \) values in Table 4 do not coincide with the \( CL_{\text{in vitro}} \) values in the same table. In contrast, the \( CL_{\text{in vitro}} \) for BTBPE was greatest under *in vitro* conditions, and \( k_{\text{MET}} \) was calculated to be smaller than those of the other BFRs. This is because BTBPE has the highest log \( K_{\text{ow}} \) value (9.15) of the alternative BFRs tested, leading to the lowest unbound fraction in the liver (\( f_{\text{dL}} \)) and lowest \( k_{\text{MET}} \) values.

### 3.4. Evaluation of bioaccumulation potential using a one-compartment fish model

The calculated BCF values based on the total concentration in water (BCF\(_{\text{Wt}}\)), freely dissolved concentration (BCF\(_{\text{Fd}}\)), and assuming no metabolism (BCF\(_{\text{Kmet} = 0}\)) are summarized in Table 5. The range of BCF\(_{\text{Kmet} = 0}\) values ranged between 1800 (BTBPE in *E. septemfasciatus*) and 34,000 (HBB in *K. punctatus*) and values in *K. punctatus* were greater than those of the other four fish species. The BCF\(_{\text{Kmet} = 0}\) values ranged from 710 (TBCT in *M. cephalus*) to 570,000 (BTBPE in *K. punctatus*). Except

---

Table 3

Calculated *in vitro* intrinsic clearance rate (\( CL_{\text{in vitro}} \), \( \text{mL} \cdot \text{h}^{-1} \cdot \text{mg}_{\text{protein}}^{-1} \)) for the BFR alternatives.

<table>
<thead>
<tr>
<th>BFR abbreviation</th>
<th>BFR concentration (( \mu \text{M} ))</th>
<th>E. septemfasciatus</th>
<th>K. punctatus</th>
<th>L. japonicus</th>
<th>M. cephalus</th>
<th>S. schlegelii</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTBPE</td>
<td>1</td>
<td>0.16 ± 0.011</td>
<td>0.16 ± 0.008</td>
<td>0.13 ± 0.02</td>
<td>0.20 ± 0.01</td>
<td>0.18 ± 0.02</td>
</tr>
<tr>
<td>HBB</td>
<td>0.1</td>
<td>0.15 ± 0.010</td>
<td>0.16 ± 0.003</td>
<td>0.13 ± 0.02</td>
<td>0.19 ± 0.01</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>PBEB</td>
<td>0.09 ± 0.008</td>
<td>0.061 ± 0.004</td>
<td>0.058 ± 0.015</td>
<td>0.13 ± 0.01</td>
<td>0.048 ± 0.009</td>
<td></td>
</tr>
<tr>
<td>PBT</td>
<td>0.1</td>
<td>0.087 ± 0.005</td>
<td>0.063 ± 0.003</td>
<td>0.061 ± 0.005</td>
<td>0.12 ± 0.01</td>
<td>0.052 ± 0.004</td>
</tr>
<tr>
<td>TBB</td>
<td>1</td>
<td>0.11 ± 0.01</td>
<td>0.052 ± 0.006</td>
<td>0.14 ± 0.02</td>
<td>0.40 ± 0.01</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>TBCT</td>
<td>0.1</td>
<td>0.10 ± 0.01</td>
<td>0.052 ± 0.006</td>
<td>0.13 ± 0.01</td>
<td>0.40 ± 0.02</td>
<td>0.13 ± 0.01</td>
</tr>
</tbody>
</table>

### Table 4

Calculated whole-body metabolism rate constant (\( k_{\text{MET}} \); \( \text{d}^{-1} \)) for the BFR alternatives.

<table>
<thead>
<tr>
<th>Species</th>
<th>Abbreviation of BFRs</th>
<th>BTBPE</th>
<th>HBB</th>
<th>PBEB</th>
<th>PBT</th>
<th>TBB</th>
<th>TBCT</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. septemfasciatus</em></td>
<td>0.0012 ± 0.0004</td>
<td>0.0061 ± 0.0019</td>
<td>0.0027 ± 0.0008</td>
<td>0.0048 ± 0.0015</td>
<td>0.0026 ± 0.0008</td>
<td>0.0032 ± 0.0010</td>
<td></td>
</tr>
<tr>
<td><em>K. punctatus</em></td>
<td>0.00037 ± 0.00012</td>
<td>0.0013 ± 0.0004</td>
<td>0.0004 ± 0.000013</td>
<td>0.00054 ± 0.00017</td>
<td>0.00067 ± 0.00022</td>
<td>0.00045 ± 0.00020</td>
<td></td>
</tr>
<tr>
<td><em>L. japonicus</em></td>
<td>0.0011 ± 0.0006</td>
<td>0.0045 ± 0.0027</td>
<td>0.0040 ± 0.0022</td>
<td>0.0024 ± 0.0013</td>
<td>0.0022 ± 0.0012</td>
<td>0.0025 ± 0.0014</td>
<td></td>
</tr>
<tr>
<td><em>M. cephalus</em></td>
<td>0.0075 ± 0.0044</td>
<td>0.0041 ± 0.0024</td>
<td>0.0046 ± 0.0027</td>
<td>0.0047 ± 0.0027</td>
<td>0.0024 ± 0.0014</td>
<td>0.12 ± 0.07</td>
<td></td>
</tr>
<tr>
<td><em>S. schlegelii</em></td>
<td>0.0021 ± 0.0011</td>
<td>0.0053 ± 0.0028</td>
<td>0.0048 ± 0.0024</td>
<td>0.0062 ± 0.0031</td>
<td>0.0033 ± 0.0018</td>
<td>0.0044 ± 0.0027</td>
<td></td>
</tr>
</tbody>
</table>

Values of \( k_{\text{MET}} \) at initial concentration of 1 \( \mu \text{M} \) were used to obtain \( k_{\text{MET}} \) values. Errors were calculated using error propagation of standard deviations of experimental values used for calculation of \( k_{\text{MET}} \).
for M. cephalus, BTBPE and HBB had the highest and lowest BCF
values, respectively, in the marine fish species. The calculated BCF values of HBB and PBT for four fish species (except for M. cephalus) were ≤20,000, the regulatory limit for bioaccumulation (TSCA, 2017). The lowest value was 16 (TBB in M. cephalus) and the highest value was 27,000 (HBB in K. punctatus) and the BCF values for HBB were highest for all marine fish species. For all BFRs, the lowest BCF values were estimated for M. cephalus, mostly because of the highest clearance for the individual BFRs. Because metabolic transformation is regarded as the key process determining the bioaccumulation potential (Arnot and Gobas, 2003), the calculated BCFTOT values of BTBPE in in vitro experiments showed a maximum value of 5700 at log OW (dBCF/dkMET). On the other hand, the calculated BCFTOT of BTBPE in E. septemfasciatus decreased from 110 to 52 when the log KOW value increased by 0.50 log units (from 9.15 to 9.65). On the other hand, BCF values in marine fish for the tested BFRs showed a maximum value of 5700 at log KOW of 6.07. The sensitivity indices, defined as the increase in the calculated BCF with respect to the increase in KOW (dBCF/dKOW at the KOW value used), were negative for the more hydrophobic BFRs, whereas they were close to zero for HBB, implying that the greatest BCF value is likely for chemicals with a lower log KOW of approximately 6. Although there are other factors to be considered such as bioavailability, the very low biotransformation rates of the studied BFRs in the liver S9 fraction warrant further study to evaluate their bioaccumulation potential.

### Acknowledgments

We appreciate anonymous reviewers for constructive comments and suggestions. This research was partly supported by the Korea Ministry of Environment (MOE) as “Technology Program for establishing biocide safety management” (2018002490001) and by a Korea University grant.

### Appendix A. Supplementary data

Further recovery is shown in Table S1. Chemical- and fish-specific input parameters for the in vitro to in vivo extrapolation (IVIEV) model and one-compartment BCF model for all fish species are presented in Tables S2–S6. Values of Kow are shown in Table S7. Sensitivity analyses are presented in Tables S8 and S9. Experimental determination of Kow is described in Figs. S1–S5. A plot of the Clow versus the EROD activity is presented in Fig. S6. Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.10.432.

### References


Zamaraetskaia, G., Zlabek, V., 2009. EROD and MROD as markers of cytochrome P450 1A activities in hepatic microsomes form entire and castrated male pigs. Sensors (Basel) 9, 2134–2147.