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Comparative antioxidant status in freshwater fish *Carassius auratus* exposed to six current-use brominated flame retardants: A combined experimental and theoretical study

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ABSTRACT

Decabromodiphenyl ether (BDE-209) and several non-polybrominated diphenyl ether (PBDE) brominated flame retardants (BFRs), such as tetrabromobisphenol A (TBBPA), hexabromocyclododecane (HBCD), decabromodiphenyl ethane (DBDPE), hexabromobenzene (HBB) and pentabromotoluene (PBT), are persistent halogenated contaminants ubiquitously detected in aquatic systems. However, data on comparative toxicological effects of these BFRs are lacking for fish. In this study, a combined experimental and theoretical approach was used to compare and analyze the effects of these BFRs on biochemical biomarkers in liver of *Carassius auratus* injected intraperitoneally with different doses (10 and 100 mg/kg) for 7, 14 and 30 days. Oxidative stress was evoked evidently for the prolonged exposure, represented by the significantly altered indices (superoxide dismutase, catalase, glutathione peroxidase, reduced glutathione, and malondialdehyde). The integrated biomarker response (IBR) index ranked biotoxicity as: PBT > HBB > HBCD > TBBPA > BDE-209 > DBDPE. Quantum chemical calculations (electronic parameters, frontier molecular orbitals, and Wiberg bond order) were performed for theoretical analysis. Notably, some descriptors were correlated with the toxicity order, probably implying the existence of a potential structure–activity relationship when more BFRs were included. Besides, theoretical calculations also provided some valuable information regarding the molecular characteristics and metabolic pathways of these current-use BFRs, which may facilitate the understanding on their environmental behavior and fate. Overall, this study adopted a combined experimental and theoretical method for the toxicological determination and analysis of the BFRs, which may also be considered in future ecotoxicological studies.

1. Introduction

Brominated flame retardants (BFRs), such as polybrominated diphenyl ethers (PBDEs), tetrabromobisphenol A (TBBPA), hexabromocyclododecane (HBCD), and novel decabromodiphenyl ethane (DBDPE), hexabromobenzene (HBB) and pentabromotoluene (PBT), have been commonly used in a variety of commercial and consumer products to prevent or suppress the risk of fire initiation and propagation (Harju et al., 2007; Egloff et al., 2011). Over the past few years, the two commercial PBDE formulations known as pentaBDE and octaBDE have been banned or voluntarily withdrawn from use in some regions of the world (Hoh et al., 2005; Betts, 2008) due to growing concerns over their persistence, bioaccumulation and potential toxicity. And the decaBDE mixture, made up mostly of decabromodiphenyl ether (BDE-209), is still used in many countries (Betts, 2008). To date, monitoring data of these BFRs, especially regarding their environmental existence in aquatic ecosystems, have been available (Law et al., 2006; Harrad et al., 2009; Zhang et al., 2010), further highlighting their persistence and bioaccumulation potential. Recently, considerable attentions have been given to evaluate the underlying biological events of these BFRs in aquatic species. For example, BDE-209 can cause thyroid endocrine disruption in zebrafish larvae (Chen et al., 2012). The toxicity test in zebrafish via embryonic exposure indicated that TBBPA can disrupt normal zebrafish development and matrix metalloproteinase expression (McCormick et al., 2010). The toxicity of HBCD was also measured in zebrafish embryos, indicating its capability to induce developmental toxicity and apoptosis (Deng et al., 2009). Furthermore, DBDPE was acutely toxic to water fleas and affected the hatching success of zebrafish eggs (Nakari and Huhtala, 2010). In addition, previous studies have shown that these four BFRs, together with HBB, can change the activities of hepatic enzymes and trigger oxidative stress in different fish species (Ronisz et al., 2004; Shi et al., 2005; Zhang et al., 2008; Zhao et al., 2011). Nevertheless,

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little information is available regarding the toxicity of PBT and the comparative toxicological effects of these current-use BFRs on aquatic organisms.

The production of reactive oxygen species (ROS) can induce oxidative damage and has been regarded as a possible mechanism of toxicity for aquatic organisms exposed to waterborne organic contaminants (Van de Oost et al., 2003; Zhang et al., 2008; Lushchak, 2011). Under normal conditions, these highly reactive substances are continually scavenged by antioxidant defenses consisted of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), as well as low-molecular-mass antioxidants such as reduced glutathione (GSH) (Song et al., 2006; Li et al., 2009). However, oxidative stress occurred when steady-state ROS concentration is transiently or chronically enhanced during xenobiotic metabolism, leading to increased damage to different cellular constituents (Almroth et al., 2008; Lushchak, 2011). Especially, damage to membrane lipids – lipid peroxidation – can result in the formation of secondary products such as malondialdehyde (MDA) and is considered as the greatest cause of cell injury and death (Liu et al., 2007; Modesto and Martinez, 2010). Consequently, many oxidation-related biomarkers, including both enzymatic and molecular parameters, are commonly utilized in aquatic systems to assess the exposure and/or effects of pollution on native populations of fish (Van de Oost et al., 2003).

Nowadays, theoretical calculation, as a supplement to experimental determination, has been widely employed to elucidate the molecular characteristics (Jarmelo et al., 2012), transformation pathways (Wang et al., 2012), and toxicological predictions (Karelson et al., 1996; Wang et al., 2005; Gu et al., 2010) of organic compounds. To our knowledge, theoretical estimations based on the highly precise density function theory (DFT) method for BFRs toxicity were primarily reported for PBDEs (Wang et al., 2005; Harju et al., 2007; Gu et al., 2010), whereas limited data are available for the non-PBDE BFRs. Therefore, a further theoretical investigation was needed to better understand the molecular properties and poisoning mechanisms of other BFRs.

In the present study, a combined experimental and theoretical approach was used to evaluate and interpret the comparative antioxidant responses in fish chronically exposed to six current-use BFRs. The liver was chosen for analytical procedures due to its importance on the metabolism and storage of toxicants (Song et al., 2006). A panel of frequently used oxidative stress biomarkers (SOD, CAT, GPx, GSH, and MDA) was selected for biochemical assays, and the integrated biomarker response (IBR) index was applied to improve the discriminatory power of these indices, while electronic-related properties and Wiberg bond order were calculated for theoretical analysis. Notably, as for HBCD molecule, three dominated diastereoisomers (α-, β-, and γ-HBCD) were included for the computations given that structural dissimilarities raise substantial differences in physicochemical, biological and toxicological profiles, which ultimately lead to the distinctive environmental fate (Janak et al., 2005). Thus, the goals of this study were to: (1) assess the potential risk of PBT on aquatic species; (2) characterize and compare the oxidative stress-inducing capability of these BFRs in fish liver; (3) make a comparison between chemical properties and oxidative effect induced by the BFRs.

2. Materials and methods

2.1. Chemicals

BDE-209, TBBPA, HBCD, DBDPE, HBB, and PBT (see Fig. 1 for chemical structures) were purchased and utilized for antioxidant status evaluations in fish. They were dissolved in corn oil to obtain different experimental concentrations. Kits for biochemical assays were acquired from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other chemicals used were of analytical grade and obtained from commercial sources.

2.2. Animals and experimental exposure

Freshwater goldfish *Carassius auratus*, commonly found in China and widely used in aquatic toxicology, was chosen as the test species. The goldfish (body weight: 26.83 ± 3.25 g) were purchased from a local aquatic breeding base. They were initially acclimatized in aquaria containing 150 L dechlorinated and aerated freshwater at least for 10 days before the experiment, with the total mortality near zero. During acclimatization, fish were fed daily with commercial fish pellets and food residue was removed. The fish were starved for 24 h prior to experiment and dissection. Throughout the experimental period, the water conditions were as follows: temperature, 24 ± 2 °C; pH, 7.8 ± 0.5 units; dissolved oxygen, 6.4 ± 0.4 mg L^{-1}; conductivity, 0.252 ± 0.006 mS/cm; total hardness, 194.3 ± 13.0 mg L^{-1} $CaCO_3$; photoperiod, 12-h light/12-h darkness. The experiment was conducted in accordance with national and institutional guidelines for animal welfare.

Fourteen glass aquaria were used to conduct the experiment. After acclimatization, 15 fish were randomly selected and placed in each aquaria. Regarding the BFRs treatments, fish in per dose group were treated individually with six BFRs at different doses (10 and 100 mg/kg) via single intraperitoneal injection, an exposure method which has been previously utilized for oxidative stress evaluations of TBBPA and HBCD in different fish species (Ronisz et al., 2004; Shi et al., 2005). Two other groups were chosen as contrast groups, an aqueous control group exposed to clean freshwater and a solvent control group receiving an equal volume of corn oil (injection volume = 0.2 mL). Fish were exposed to these conditions for 7, 14 and 30 days. Experimental aquaria were continuously aerated and water were refreshed every 24 h to minimize the contamination from metabolic wastes.

2.3. Sample preparation

On completion of each exposure interval, 5 fish in each treatment were killed by a sharp blow on the head, and their livers were quickly dissected out on an ice-cold plate, cleaned of extraneous tissues in physiological saline solution (0.9% NaCl), and stored at −80 °C until assay.

Tissues were homogenized (1:10, w/v) using an Ultra Turrax homogenizer (Ika, Germany) in chilled physiological saline solution (0.9% NaCl). The homogenates were centrifuged (Eppendorf, Germany) at 4000 × *g* for 15 min at 4 °C, and the supernatants were collected for biochemical determination.

2.4. Biochemical analysis

The supernatants were assayed for biochemical parameters using the Diagnostic Reagent Kits according to the manufacturer's instructions. SOD (EC 1.15.1.1) activity was examined by the inhibition of cytochrome c reduction in the presence of hypoxanthine/xanthine oxidase $O_2^{-\bullet}$ generator system (McCord and Fridovich, 1969). CAT (EC 1.11.1.6) activity, determined by H_2O_2 breakdown, was estimated following the method of Claiborne (1985). GPx (EC 1.11.1.9) activity was evaluated based on the rate of NADPH oxidation by the coupled reaction with glutathione reductase (Lawrence and Burk, 1976). GSH level was determined according to the method of Jollow et al. (1974). MDA content, as a biomarker for lipid peroxidation, was measured using the method described by Jain et al. (1989) based on 2-thiobarbituric acid (2,6-dihydroxypyrimidine-2-thiol; TBA) reactivity. Specific activity of