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Toxicological effects of PBDEs on *carassius aurats*

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Abstract In this study the effects of polybrominated diphenyl ether compounds (PBDEs), BDE-47 and BDE-99 on *carassius aurats* were discussed. The reproductive capacity viability and proliferation percent decreased with increasing BDE-47and BDE-99 concentrations (p<0.05), presenting a dose-dependent manner. Physiological biomarkers such as cholesterol (CHL), acetylcholinesterase (AChE), ethoxyresorufin-O-deethylase (EROD) and vitamin were studied to reveal the toxicological effects of BDE-47 and BDE-99 exposure. CHL levels generally decrease with increasing BDE-47 exposure. However, there were no significant differences between the samples and the control. As to BDE-99, the CHL level decreased as compared with the control level when the BDE-99 concentrations reached 20 μg/L (p<0.05). The degree of inhibition of the AChE increased with the increase of DE-47and BDE-99 concentrations. The EROD activity during 72 hours' exposure significantly increased to 20 μg/L for BDE-47 (p<0.05), however, EROD activity was not significantly different at different concentrations of BDE-99 (p<0.05). *Carassius aurats* hepatocytes reacted to the BDE-47 exposure with increasing EROD activities in the dose dependent manner. Although at lower concentrations, the induced effects of BDE-47 and BDE-99 were not significantly different, though BDE-47 showed strong induced effects on the EROD activity with increasing concentrations in *carassius aurats* hepatocytes. In contrast, BDE-99 did not influence EROD activity consistently. The V_A level of *carassius aurats* was significantly different from that of the controls for BDE-99 (p<0.001), as far as the V_A level was significantly lower of *carassius aurats* than the controls for BDE-47 (p<0.001). CHL, AChE, EROD, and V_A levels in *carassius aurats* were differently affected by BDE-47 and BDE-99 exposures, therefore, they would be good indicators for exposure.

Key words toxicological effect; *carassius aurats*; PBDE

1 Introduction

The polybrominated diphenyl ethers (PBDEs) represent an important group of flame retardants extensively used in plastics, textiles, furniture and electronic devices (McDonald, 2002). Due to their high lipid solubility, low vapor pressure and resistance to degradation, PBDE congeners can be transported through environmental media and be bioaccumulated in aquatic and wildlife species (Darnerud et al., 2001). During the past two decades, global production has been rising exponentially and the levels detected in biota were found to be doubled every 3–5 years (Meironyté et al., 1999; Ikonomou et al., 2002; Norstrom et al., 2002). While in the aquatic environment the concentrations of important pollutants such as dieldrin and hexachlorobenzene (HCB) have decreased during the last decade. The increasing levels of PBDEs were detected in fish caught in the North Sea over the years from 1977 to 1987 (de Boer, 1989), which were seriously impacted by recent anthropogenic activities (Zhu Jun et al., 2005). Detection in animals living in deep ocean waters indicates a wide distribution of PBDEs in the aquatic environment (de Boer et al., 1998). The wide distribution and relative lack of toxicological information arouses great concern with the environment (Vos et al., 2003). The PBDE congeners, 2, 2', 4, 4'-tetra BDE (BDE-47) and 2, 2'4, 4'5-pentabromodiphenyl ether (BDE-99), have been recently detected at comparable levels with PCB-153 in fish samples from the North Sea (Vos et al., 2003). BDE-47 and BDE-99 are kinds of lower molecular PBDE congeners and are often detected in fish tissues (Darnerud, 2003). Therefore, much attention has been paid to the variation trend of river water quality (Wang Fushun et al., 2007). BDE-47 and BDE-99 have shown rodent developmental neurotoxicant that may also elicit oxidative stress as the potential mechanism of toxicity (Lema et al., 2007). The pur-

pose of this research is to assess the toxicological effects of BDE-47 and BDE-99 on *carassius aurats* after exposure. The effects of BDE-47 and BDE-99 exposure on *carassius aurats* were studied by using two reproductive capacities (viability and proliferation) and four standard physiological biomarkers, i.e., cholesterol (CHL), acetylcholinesterase (AChE), ethoxyresorufin-O-deethylase (EROD) and vitamin A.

2 Materials and methods

2.1 Chemicals analysis

2, 2', 4, 4'-tetrabromodiphenyl ether (BDE-47, 100% purity) and 2, 2'4, 4'5-pentabromodiphenyl ether (BDE-99, 98.0% purity) were purchased from Shanghai Anpu Company. The stock solution for dosing was made with 100% acetone which was used as a carrier in all tests. For all treatments the same carrier concentrations (0.1%) were adopted. Analytical grades BDE-47 and BDE-99 in isooctane (100 mg/L) were obtained from iso-octaine solutions. Gas chromatography-mass spectrometry (GC-MS) detection was performed to confirm the nominal concentrations of BDE-47 and BDE-99 stock solutions. Mass spectral data were acquired in the electron impact ionization (GC/EI-MS) mode by using an Agilent 5973GC-MS.

2.2 Samples of carassius aurats

Carassius aurats were obtained from a non-contaminated area on Qingdao Aquafarm. A group of fish, used as feedstuff, ranged in mass from 20 to 40 g, were kept in tanks at 27℃ with freshwater recharged every day. *Carassius aurats* were fed twice every day at a rate of 1%–2% body weight. *Carassius aurats* were treated with BDE-47 and BDE-99 at the concentrations of 2.5, 5.0, 10.0, and 20 μg/L in 72 h, respectively.

2.3 Cell viability change assay

Cell viability was measured by the 3 (4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Li Peng and Peng Ren, 2005).

2.4 Whole-blood cell proliferation assay

Diluted blood was plated in 96-well flat-bottomed culture plates at 100 mL/well and single test BDE-47 and BDE-99 added at a range of final concentrations (2.5, 5.0, 10.0, and 20 μg/L, respectively). Proliferation was induced by Concanavalin A (Con A) at an optimised concentration of 10 mg/mL (data not shown). The concentrations in each well were calculated based on the final volume of 200 mL/well. Cell culture was incubated at 37℃ in a 5% CO_2-humidified air incubator for 72 hours. All assays were performed in triplicate (Luongo et al., 2008).

2.5 Biochemical serum cholesterol measure

Serum cholesterol was determined with an automated procedure (Roche Diagnostic System). Cholesterol esters, in the presence of cholesterol esterase, were hydrolyzed into free fatty acids and cholesterol. The cholesterol was then oxidized by cholesterol oxidase to produce hydrogen peroxide, which was combined with 4-aminoantipyrine and phenol to form a chromophore. The chromophore was read spectrophotometrically at 500 nm, which was proportional to the cholesterol concentrations (Lewis et al., 2001).

2.6 AChE biomarker test

For biomarker tests, separate 72-hour exposures were performed with concentrations based on previous testing. For the AChE biomarker tests, the nominal BDE-47 and BDE-99 concentrations with three replicates were controlled at 2.5, 5.0, 10.0 and 20.0 μg/L, respectively. *Carassius aurats* biomarker tests were run in a 2-L glass container each, in which there were 1 L of test solution and 30 *carassius aurats* in order to obtain sufficient tissue for the assays. After 72 hours, surviving *carassius aurats* were pooled for each replicate, placed in appropriately labeled tubes and frozen until analysis (Peter et al., 2008)

2.7 Effects of BDE-47and BDE-99 on Hepatic enzyme activities

Liver microsomal fractions were prepared as described previously (DeVito et al., 1993). Microsomal protein concentrations were determined by using a protein assay kit (Bio-Rad, Richmond, CA) with BSA as the standard. Hepatic microsomal EROD activity was assayed by using the method of DeVito et al. (1993). All substrate concentrations were 1.5 μM. EROD values were calculated as pmol resorufin per mg protein per min and graphically represented as a percentage of control activity.

2.8 Phsiologcial substance V_A test

V_A was determined with the isocratic HPLC method described by Lee et al. (1992). A 400 μl plasma sample was extracted with 400 μl of butanolethyl acetate (1:1, *v*/*v*) for 1 minute. Aproximately 20 mg of sodium sulphate was added. After vortex-mixing for 1 minute, the sample was allowed to stand at -20℃ for 20 minutes and than was centrifuged at 15000 rpm for 2 minutes. The organic upper

layer was transferred into an Eppendorf tube and stored at -70℃ until analysis. The mobile phase, pumped at 1.5 ml/min, consisted of methanol/butanol/water (75:20, *v*/*v* for V_A). V_A was measured at 290 nm.

2.9 Statistics and analysis

The data were analyzed with ANOVA. Shapiro-Wilks test for normality and test for equal variance were firstly run to determine if the data were parametric and then ANOVA with Dunnett's procedure for comparison was used to determine significant differences from the control response. All statistical analyses were performed by using Origin 7.5.

3 Results

3.1 Effects of BDE-47and BDE-99 on *carassius aurats* viability of liver cells

Figure.1 showed that the effects of BDE-47 and BDE-99 on the viability of liver cells were significantly different under the higher concentrations (p<0.05). The viability percent decreased by 56.0% and 53.6% compared to the control levels when treated with 20.0 μg/L for BDE-47and BDE-99, respectively (p<0.05), and the latter showed significantly higher viability percent, that is, the viability decreased with higher BDE-47 and BDE-99 concentration exposure (p<0.05). It presented a dose-dependent manner, and the toxicological effect of BDE-99 on viability was less than that of BDE-47.

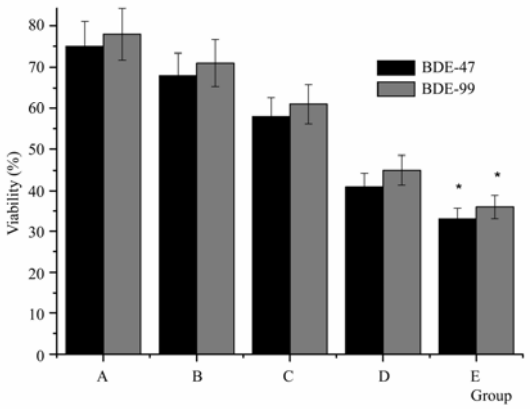

Fig. 1. Effects of BDE-47 and BDE-99 on the viability of liver cells. Data are presented as mean ± SD (n=3). A. Control; B, C, D, and E. indicating the concentrations of BDE-47 and BDE-99 2.5, 5.0, 10.0, and 20.0 μg/L, respectively. * Significant differences as compared with the control levels (p＜0.05).

3.2 Effects of BDE-47 and BDE-99 on *carassius aurats* whole-blood proliferation

In preliminary time-dependent experiments 72 hours were chosen as optimal incubation time for all tested BDE-47 and BDE-99 and it was found that 5 μg Con A/mL was optimal to induce *carassius aurats* whole-blood proliferation. It was found that treatment with increasing concentrations of BDE-47 and BDE-99 would produce significant effects on all cells' proliferation of the whole blood following Con A stimulation (Fig. 2). Furthermore, incubation of cells in the presence of increasing concentrations of BDE-47 and BDE-99 (2.5–20 μg/L) would cause a marked inhibitory effect on the mitogen-induced proliferation, even at very low doses. Similarly, increasing concentrations of the examined BDE-47 and BDE-99 caused the inhibition of cells' proliferation, followed by Con A stimulation. Particularly, BDE-47 concentrations, ranging from 2.5 to 20 μg/L, induced a strong inhibition and BDE-99 induced a lesser inhibitory effect.

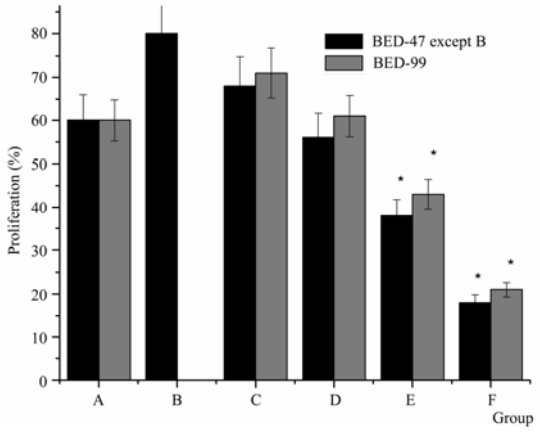

Fig. 2. Effects of BDE-47and BDE-99 on the whole blood proliferation. Data are presented as mean±SD (n=4). A. Control; B. con A; C, D, E, and F. indicating the concentrations of BDE-47 and BDE-99 2.5, 5.0, 10.0, and 20.0 μg/L, respectively. * Significant differences as compared with the control levels (p＜0.05).

3.3 Effects of BDE-47 and BDE-99 on cholesterols

In the BDE-47 exposures, the lowest concentration of 2.5 μg/L showed a significantly higher (p<0.05) CHL level than controls (Fig. 3). After a spike at the 2.5 μg/L exposure, CHL levels generally decreased with the increase of exposure. However, even though the ANOVA showed significant differences (p=0.001), there were no significant differences in the control value by using Dunnett's procedure. As for BDE-99, CHL levels were not significantly different as compared with the control levels under lower concentrations (2.5–10 μg/L) (p<0.05), but the CHL levels decreased as compared with the control levels when the BDE-99 concentrations reached 20 μg/L (p<0.05).

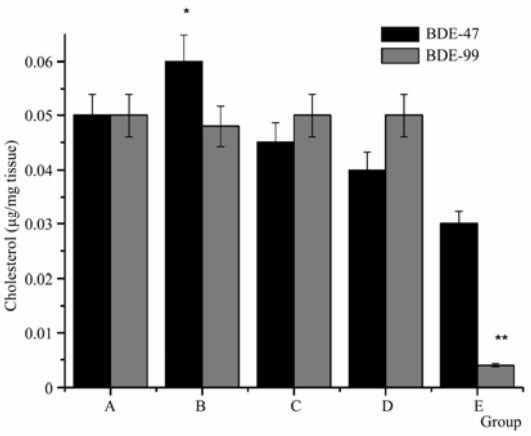

Fig. 3. Effects of BDE-47 and BDE-99 on cholesterols after 72-hour exposure. A. Control; B, C, D, and E. indicating the concentrations of BDE-47 and BDE-99 (2.5, 5.0, 10.0, and 20.0 μg/L, respectively). * Significantly different (p<0.05) from the control value; ** significantly different (p<0.001) from the control value.

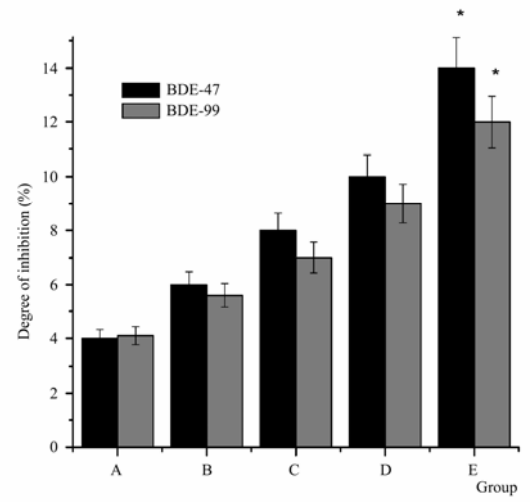

Fig. 4. Degree of inhibition of the AchE in response to BDE-47and BDE-99 concentrations. A. Control; B, C, D, and E. indicating the concentrations of BDE-47 and BDE-99 (2.5, 5.0, 10.0, and 20.0 μg/L, respectively). * Significantly different (p<0.05) from the control value.

3.4 Effects of BDE-47 and BDE-99 on acetylcholinesterase

There were statistically significant differences in AChE levels in *carassius aurats* (p=0.001). The evaluation of the BDE-47 and BDE-99 inhibitory action involved the establishment of decreasing acetylcholinesterase activity, which was expressed as the current decrease of the constant substrate concentrations. The experimental data presented in Fig. 4 demonstrate that the degree of inhibition of the acetylcholinesterase increases with the increase of the BDE-47 and BDE-99 concentrations, and the inhibition effect of BDE-47 is bigger than that of BDE-99. AchE acted as a key enzyme of nerve conduction, which suffered from disturbance of heavy metal, aether and so on, and resulted in the incapable stop of receptor function of Ach and nerve membranous posterior, making organisms at excitement status for a long-term and physiological process maladjustment as well as death finally.

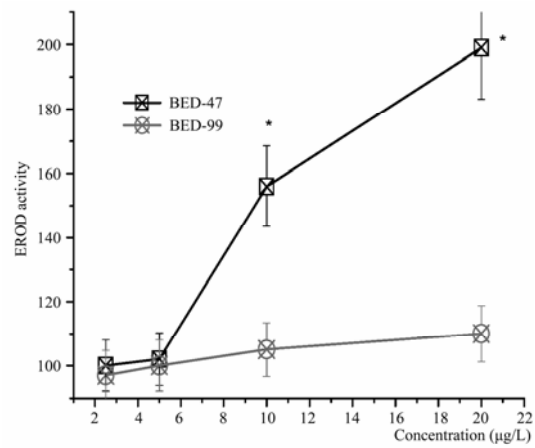

Fig. 5. Effects of BDE-47 and BDE-99 on EROD, presented as a percentage of control activity±SEM. * Significantly different from the control value (p<0.05).

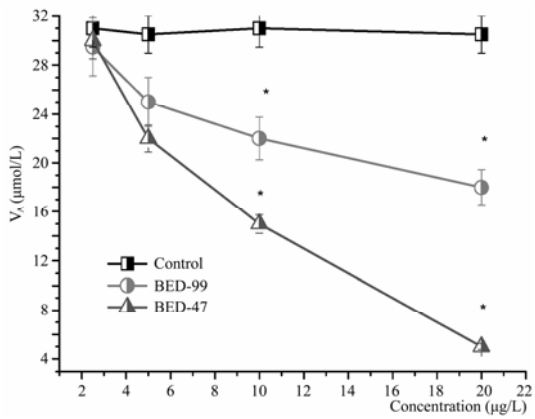

Fig. 6. Effects of BDE-47 and BDE-99 on plasma V_A, presented as a percentage of control activity±SEM. * Significantly different from the control value (p<0.05).

3.5 Effects of BDE-47 and BDE-99 on hepatic enzyme activities

EROD (ethoxy-resorufin-O-deethylase) activity following the 72-hour exposures significantly increased to 20 μg/L for BDE-47 (p<0.05) (Fig. 5), however, EROD activity was not significantly different at the lower concentrations of BDE-99 (p<0.05). *Carassius aurats* hepatocytes reacted with BDE-47 exposure alone with increasing EROD activity in a dose dependent manner. Although at lower concentrations, the induced effects of BDE-47 and BDE-99 were not significantly different. However, BDE-47 showed strong induced effects on EROD activity with

increasing concentrations in *carassius aurats* hepatocytes. In contrast, BDE-99 did not influence EROD activity consistently.

3.6 Effects of BDE-47 and BDE-99 on plasma V_A

All data on vitamin A in this study are shown in Fig. 6, which presents the mean level of V_A in *carassius aurats* and controls. The plasma V_A level of *carassius aurats* was significantly different from that of controls for BDE-99 ($p<0.001$). VA levels displayed a similar trend in both groups ($p>0.05$). However, V_A level was significantly lower in *carassius aurats* than in controls for BDE-47 ($p<0.001$). VA is an important physiological substance related with growth tardiness, abnormality and fecundity decrease. PBDE exposure might disturb V_A dynamic and result in storage function being out of tune.

4 Discussion

The effects of BDE-47 and BDE-99 exposure on *carassius aurats* were studied by using two reproductive capacities (viability and proliferation) and four standard physiological biomarkers, i.e., cholesterol (CHL), acetylcholinesterase (AChE) ethoxyresorufin-O-deethylase (EROD) and vitamin A. The viability percentages decreased by 56.0% and 53.6% as compared with control levels when treated with 20.0 μg/L of BDE-47 and BDE-99, respectively ($p<0.05$). It presented a dose-dependent manner. The MTT assay generally indicates a negative exposure effect on cell viability at BDE-47 and BDE-99 concentrations used in this study. In this study, the effects of BDE-47 and BDE-99 on *carassius aurats* growth were determined by using a whole-blood proliferation test. The use of the whole-blood proliferation was reported by Bloemena et al. (1989) and by Fasanmade and Jusko (1995) in rat. This approach offers a series of advantages over the analysis of single cell populations, such as the reduction in the time of assay as well as the generation of data resembling responses *in vivo* (Yancy et al., 2001; Thies et al., 1999; Edfors-Lilja et al., 1998). Incubation of cells in the presence of increasing concentrations of BDE-47 and BDE-99 (2.5–20 μg/L) caused a marked inhibitory effect on the proliferation, even at very low doses. BDE-47 induced a strong inhibition which became significant and dose-dependent at the lowest concentration. and that BDE-99 induced a lesser inhibitory effect than BDE-47. There have been no specific biomarkers developed for PBDE exposure in fish. A problem of how to deal with most environmental contaminants is the lack of suitable biomarker to distinguish the actions of chemicals (Peter and Katy W. Chung, 2008). The biomarkers used in this study were chosen to determine if those known biological parameters for *carassius aurats* would be affected by BDE-47 and BDE-99 exposure. CHL, AChE, EROD, and VA levels in *carassius aurats* were differently affected by BDE-47 and BDE-99 exposures and this would be a good indicator of exposure.

CHL comes from the diet and is stored and processed in hepatopancreas (Anger, 2001), it is not able to produce CHL itself. Whereas, PBDE can influence CHL with an increase in exposure. As for BDE-99, the CHL level decreased as compared with the control level when the BDE-99 concentrations reached 20 μg/L ($p<0.05$). AchE serves as a key enzyme for nerve conduction, which is suffered by heavy metals (Peter and Katy, 2008). It is clear that the degree of inhibition of acetylcholinesterase increased with increasing concentrations of BDE-47 and BDE-99, and that the inhibition effect of BDE-47 is bigger than that of BDE-99.

Kuiper et al. (2004) had exposed freshly isolated carp hepatocytes for 5 days to the individual PBDE congeners at the concentrations of 0, 2.5, and 7.5 μM, respectively. No cytotoxicity was observed from BDE-47 and BDE-100; BDE-99 and BDE-153 showed no cytotoxicty but became cytotoxic at 7.5 μM. In this study, EROD activity after 72-hour exposure significantly increased at 20 μg/L for BDE-47 ($p<0.05$), and BDE-47 showed strong induced effects on EROD activity with its increasing concentrations in *carassius aurats* hepatocytes. In contrast, BDE-99 did not influence EROD activity consistently. On account of the similar constructions of PBDEs and PCBs, Besselink et al. (1998) reported inhibition of EROD activity by PCBs at IC_{50} concentrations ranging from 0.24 μM (PCB-126) to 31.88 μM (PCB-153). Thus, our results indicate inhibitory potential similar to that of PCBs for some widely distributed PBDEs, including the predominantly detected congeners, BDE-47 and BDE-99.

V_A serves as an important physiological substance related to growth tardiness, abnormality and fecundity decrease (Selhub et al., 1993). In this study, the V_A level of *carassius aurats* was significantly different from that of controls for BDE-99 ($p<0.001$). However, the V_A level was significantly lower in *carassius aurats* than in controls for BDE-47 ($p<0.001$).

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