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BDE 49 and developmental toxicity in zebrafish

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Abstract

The polybrominated diphenyl ethers (PBDEs) are a group of brominated flame retardants. Human health concerns of these agents have largely centered upon their potential to elicit reproductive and developmental effects. Of the various congeners, BDE 49 (2,2',4,5'-tetrabromodiphenyl ether) has been poorly studied, despite the fact that it is often detected in the tissues of fish and wildlife species. Furthermore, we have previously shown that BDE 49 is a metabolic debromination product of BDE 99 hepatic metabolism in salmon, carp and trout, underscoring the need for a better understanding of biological effects. In the current study, we investigated the developmental toxicity of BDE 49 using the zebrafish (*Danio rerio*) embryo larval model. Embryo and larval zebrafish were exposed to BDE 49 at either 5 hours post fertilization (hpf) or 24 hpf and monitored for developmental and neurotoxicity. Exposure to BDE 49 at concentrations of 4 μ M–32 μ M caused a dose-dependent loss in survivorship at 6 days post fertilization (dpf). Morphological impairments were observed prior to the onset of mortality, the most striking of which included severe dorsal curvatures of the tail. The incidence of dorsal tail curvatures was dose and time dependent. Exposure to BDE 49 caused cardiac toxicity as evidenced by a significant reduction in zebrafish heart rates at 6 dpf but not earlier, suggesting that cardiac toxicity was non-specific and associated with physiological stress. Neurobehavioral injury from BDE 49 was evidenced by an impairment of touch-escape responses observed at 5 dpf. Our results indicate that BDE 49 is a developmental toxicant in larval zebrafish that can cause morphological abnormalities and adversely affect neurobehavior. The observed toxicities from BDE 49 were similar in scope to those previously reported for the more common tetrabrominated congener, BDE 47, and also for other lower brominated PBDEs, suggest that these compounds may share similarities in risk to aquatic species.

Introduction

Polybrominated diphenyl ethers (PBDEs) are flame retardants that have been incorporated into a wide range of consumer products (Eriksson *et al.*, 2001; McDonald, 2002). Because PBDEs are not chemically bound within these products, they have the ability to leach into the surrounding environment (Eriksson *et al.*, 2001; McDonald, 2002; Sjodin *et al.*, 2003). PBDEs are lipophilic and are generally poorly metabolized in biological systems, and thus

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bioaccumulate in lipid rich tissues in fish and wildlife (Hale *et al.*, 2003; Wolkers *et al.*, 2004; Fangstrom *et al.*, 2005). Due to these physicochemical properties, PBDEs are commonly detected worldwide in aquatic ecosystems, causing the potential for long term exposure and accumulation in aquatic species (Eriksson *et al.*, 2001; Dodder *et al.*, 2002; Hale *et al.*, 2003; Shaw and Kannan, 2009). Although the US production of all commercial PBDE congeners has ceased, with the exception of the fully brominated congener BDE 209 (Ross *et al.*, 2009), the accumulation of lower molecular weight tetra- and penta-brominated congeners is expected to be an environmental reservoir for PBDE exposures over the next decade (Schecter *et al.*, 2005; Shaw *et al.*, 2009; Shaw and Kannan, 2009).

The toxicological concerns of PBDEs center largely upon their potential for neurotoxic and developmental injury. PBDEs have been shown to disrupt thyroid regulation and elicit neurodevelopmental toxicity in several species (Branchi *et al.*, 2003; Fernie *et al.*, 2005; Zhou *et al.*, 2006; Costa and Giordano, 2007). These compounds also have the potential to disrupt biotransformation pathways in vertebrate species, including fish (Hook *et al.*, 2006), humans (Peters *et al.*, 2004), and rodents (Chen *et al.*, 2001). There is particular concern regarding sensitive developmental windows of exposure during pregnancy and to infants due to the presence of PBDE residues in breast milk, associated maternal transfer during pregnancy and impacts on thyroid hormone levels (Mazdai *et al.*, 2003; Schecter *et al.*, 2007; Chevrier *et al.*, 2010; Stapleton *et al.*, 2011), as well as the fact that infants can receive household exposures from numerous sources (Stapleton *et al.*, 2005; Fischer *et al.*, 2006; Stapleton *et al.*, 2008; Wu *et al.*, 2007). Collectively, these studies and others emphasized the need for careful study of the developmental toxicities associated with PBDE congeners in humans as well as in wildlife.

Zebrafish have been used as a laboratory model to investigate the reproductive and developmental effects of PBDEs, including those encompassing commercial PBDE mixtures (Kuiper *et al.*, 2008) as well as hydroxylated metabolites (van Boxtel *et al.*, 2008). BDE 47, another pentaBDE congener, has been the primary focus of numerous studies due to the fact that it is typically the most environmentally prevalent congener. BDE 47 has been shown to cause a number of developmental abnormalities in zebrafish, including delayed hatching and reduced growth, abnormal dorsal curvatures of the body, as well as a number of cardiac defects (Lema *et al.*, 2007). In addition to developmental effects, exposure to environmental levels of BDE 47 inhibits zebrafish neurobehavioral function in the form of reduced swimming speed and locomotion (Chou *et al.* 2010). In addition to BDE 47, some other lower brominated PBDE congeners (i.e. BDE 28, 99 and 100) have also been shown to cause developmental abnormalities in zebrafish (Usenko *et al.*, 2011). These studies indicate that zebrafish are a sensitive model for characterizing the adverse responses to PBDE congeners and their metabolites, including the potential for early life stage developmental toxicities.

The present study was initiated to determine the potential for PBDE 49 to cause developmental toxicity in the zebrafish model. Relative to the more widely studied tetrabrominated congener BDE 47 and some of the other common congeners, little is known regarding the toxicity of BDE 49. BDE 49 is commonly detected in the tissues of fish, wildlife and humans, albeit at levels typically lower than those observed for BDE 47 (Mariottini *et al.*, 2008; Roosens *et al.*, 2010; Miller *et al.*, 2009). Studies in our laboratory have shown that BDE 49 is a significant metabolic debromination product resulting from hepatic metabolism of BDE 99 in coho salmon, carp and rainbow trout (Browne *et al.*, 2009; Roberts *et al.*, 2011). BDE 49 can be formed from the debromination of at least one higher molecular weight PBDE congener. This suggests that in addition to environmental pathways for bioaccumulation, enzyme mediated PBDE congener metabolism may also contribute to the presence of BDE 49 in the tissues of aquatic species. Our hypothesis in the present study

was that BDE 49 was a developmental toxicant due to its structural similarities to BDE 47, a more commonly studied tetrabrominated congener.

Materials and Methods

Animals and embryo harvesting

All zebrafish experiments were approved and conducted in accordance with the University of Washington Institutional Animal Care and Use Committee guidelines. Adult AB strain zebrafish (*Danio rerio*) were housed in a single breeding colony in the Department of Environmental and Occupational Health Sciences. The colony was maintained at 28 °C in a self-contained Aquaneering ZTT350 table-top recirculation system on a 14 h light/10 h dark photoperiod. The system was provided with deionized filtered city water treated with Instant Ocean® to obtain a conductivity of 1000 µS/cm ± 100 µS/cm and a pH range of 6.8–7.2 (herein referred to as ‘system water’). Zebrafish embryos were collected within 2 h of first light from breeding groups of 2 males and 4 females. Embryos were lightly rinsed and placed in a 60 mm petri dish of system water, then staged and checked for quality under normal light with a Nikon SMZ800 microscope. Following staging, embryos were placed in a 28 °C incubator until the desired developmental stage for exposures.

PBDE exposures

BDE 49 (2,2',4,5'-tetrabromodiphenyl ether, >99% purity) was obtained from AccuStandard, Inc. (New Haven, CT, USA). A 25 mM stock solution was created by dissolving BDE 49 in DMSO to obtain a nominal stock prior to preparation of working stocks via serial dilutions in 0.2 % DMSO in system water. The concentration of the BDE 49 stock solution was confirmed by analysis using gas chromatography-mass spectrometry operated in electron capture negative ionization mode (GC/ECNI-MS) as previously described (Stapleton *et al.*, 2007).

Zebrafish embryos were assigned to experimental groups at 5 hpf. Two developmental windows were used for exposures based on preliminary range finding studies and logistical considerations, as well as from other literature reports of PBDE developmental injury in zebrafish. Specifically, a series of exposures were initiated at 5 hpf for evaluation of acute toxicity and dorsal curvatures of the spine, whereas a second set of exposures were initiated at 24 hpf for analysis of cardiac heart rate abnormalities and neurobehavioral injury. All exposures occurred in 96 well plates at a density of one embryo per well (see figure legends for numbers of individuals used for each endpoint). All exposures were carried out using 48 h static renewals through 6 dpf at which time significant mortalities were observed and recorded. Two sets of control animals were included in all experimental groups consisting of animals receiving either carrier (0.2 % DMSO) in system water, or system water without carrier. Because no statistically significant vehicle-related effects were observed with regards to any of the experimental endpoints, the data presented for control animals pertains only to those animals receiving system water/0.2 % DMSO.

Determination of acute mortality and tail curvatures

All zebrafish embryos were examined twice daily for survival by light microscopy throughout the course of the experiment. Embryo survival was defined by a lack of movement coupled with loss of detectable heart beat, and mortality data are presented as percent survival in each treatment group over time. Throughout the duration of exposure and beginning at 5 hpf, the effect of BDE 49 on the formation of tail curvatures in live fish was quantified as a discrete variable as the presence of a dorsal curve of the tail, regardless of the severity of the deformity. Larval images were prepared by removing one larva from each exposure group and anesthetizing with MS-222, then mounting in 3 % methylcellulose.

Images were captured using a Digital Sight DS-2Mv NIKON camera mounted on a SMZ800 NIKON light microscope. After imaging, each larva was euthanized and removed from treatment pool for the duration of the experiment.

Effects of BDE 49 on cardiac function

To determine if BDE 49 exposures negatively affected cardiac function, two time points were analyzed during the developmental exposures (3 and 6 dpf). The earlier time point (3 dpf) was not associated with the onset of mortality, or with clear developmental toxicities as analyzed by other endpoints. For analysis of cardiac function, zebrafish embryos in each treatment group (n=4) were anesthetized in MS-222 and mounted in methylcellulose prior to a three minute recovery period before recording cardiac function. Heart beat rates for each larvae for both the atrium and ventricle were quantified using a Nikon SMZ800 light microscope recorded manually for a period of 1 minute. All larvae used for cardiac function analysis were not used for further studies following collection of heart rate data.

Analysis of neurobehavior by touch-escape responses

To determine whether exposure to BDE 49 negatively affects zebrafish neurobehavioral function, we tested the touch-escape response of larval zebrafish exposed to BDE 49 at 2 points during development (5 and 6 dpf) (Hill *et al.*, 2009). In these experiments, 4 embryos were removed from their respective exposure wells and placed in a petri dish containing system water that was marked with a 1 mm X 1 mm grid. Once transferred, the larva was swirled to the center of the plate and allowed a one min recovery (resting) period. After resting, each larva was lightly touched dorsally near the tail fin area with a blunt metal probe, and then the distance traveled was recorded. Larvae were allowed 15 s of rest between touches (trials) and the experiment was terminated at the end of data collection. Results were compiled from data comprising 3 different behavioral trials repeated with a minimum of three larvae from the same set for each exposure concentration.

Statistical Analysis

Prior to statistical analysis, the Shapiro–Wilk test was used to determine if the experimental data conformed to the assumptions of normality. The effects of BDE 49 exposure on atrial and ventricular heart rates were assessed by using the (Wilcoxon) Mann-Whitney U test. The Kruskal–Wallis nonparametric one-way analysis of variance by ranks and the Dunn's multiple comparisons post-hoc test were used to determine the significance of the effects of BDE 49 exposures on larval touch-escape responses. GraphPad Prism Software version 5.0 for Windows (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses. Treatment-related effects were considered significant at $p < 0.05$.

Results and discussion

Effects of BDE 49 on zebrafish survival

Exposure to BDE 49 caused a decrease in zebrafish larval survival (Fig. 2). No treatment-related mortalities were observed until 6 dpf, at which time survival had decreased from 64% in the low BDE 49 dose (4 μ M), to 11% survivorship at the highest dose tested (32 μ M, Fig. 2). The percent survivorship was dose-dependent, and once initial mortalities were observed, the slope of the dose response curve was relatively steep. Although not a focus of the study, we monitored hatching success of zebrafish embryos from 24–72 hpf, and by 72 hpf all of the embryos in all treatment groups had successfully hatched (data not shown). These data are similar to those reported in zebrafish after 72 hr exposures to BDE 47 (96.9–97.9 % hatching success, Lema *et al.*, 2007) suggesting a relatively similar effect of the two congeners with regards to hatching rates.

Effects of BDE 49 on tail curvatures

Embryonic exposure to BDE 49 caused a clearly observable increase in the number of animals exhibiting dorsal tail curvatures, with tail curvatures occurring prior to the onset of mortality (Fig. 3). The initial appearance of embryos with noticeable tail curvatures occurred at 4 dpf in the highest exposure group (32 μ M), with 44% of the larvae within this treatment group experiencing readily observable tail curvatures (Fig. 3). The incidence of embryos displaying tail curvatures increased with higher exposure concentrations of BDE 49 and with time. By 5 dpf, some zebrafish within the exposure groups showed tail curvatures (40% incidence of tail curvatures at 4 μ M, 60% incidence at 8 μ M, 80% incidence at 16 μ M, and 100% incidence at 32 μ M, Fig. 3). By 6 dpf, all of the surviving zebrafish receiving BDE 49 exposures exhibited some severity of tail curvatures. By contrast, control animals not receiving BDE 49 exhibited normal morphology. The potency of BDE 49 induced abnormal dorsal curvatures of the trunk and tail regions were generally similar to those observed in zebrafish exposed to BDE 47 (Lema *et al.*, 2007). For example, many BDE 49 exposed larvae did not fully develop functioning swim bladders (data not shown).

Although the mechanism of the dorsal tail curvatures was not investigated, exposure to the more common tetrabrominated PBDE congener (BDE 47) leads to a loss of flow of cerebrospinal fluid in the neural tube and brain ventricles, which may be a mechanism for the onset of dorsal curvatures in zebrafish (Lema *et al.*, 2007). Similar to zebrafish, young mice are vulnerable to the neurodevelopmental effects of BDE 47. Studies in mice indicate that developmental windows of PBDE susceptibility do occur in neonatal development that may be linked to tissue uptake and distribution in sensitive regions of the developing mouse brain (Eriksson *et al.*, 2001).

Cardiac toxicity of BDE 49

As reported by Lema *et al.* (2007), exposure to BDE 47 can perturb cardiac function by increasing atrium and ventricle heart rates (tachycardia), as well as causing arrhythmia and an atrial/ventricular A/V block. Accordingly, these endpoints were used to analyze the effects of BDE 49 on cardiac function. Adverse effects of BDE 49 on heart rates were observed at 6 dpf, but not at the earlier time point analyzed (3 dpf, Fig. 4). By 6 dpf exposure to 8 μ M BDE 49 caused a significant decrease in both atrial (72% decrease) and ventricle heart rates (78% decrease) in all surviving animals tested (Fig. 4). However, even at a low dose of 8 μ M, fish were beginning to show signs of severe physiological stress, and it was difficult to distinguish if the observed decrease in overall heart rates were associated with the onset of mortality. All embryos in the two highest BDE 49 exposure groups (16 and 32 μ M) ultimately did not survive to 6 dpf, and therefore no heart rate data was collected for these groups at this time point (Fig. 4B). We did not observe any embryos within BDE 49 treatment groups exhibiting significant tachycardia or arrhythmias at the two developmental time points analyzed, (Fig. 4).

Effect of BDE 49 on touch-escape responses

The developmental and neurobehavioral toxicity of PBDEs has been extensively studied in rodents, with several studies focusing on BDE 47. For example, exposure to BDE 47 in rats leads to alterations in motor and cognitive functions as manifested by spontaneous behavior, as well as learning and memory (Viberg *et al.*, 2002; Branchi *et al.*, 2003; Costa and Giordano, 2007). Neonatal exposure to BDE 47 as well as other common congeners can cause long-term behavioral abnormalities (McDonald, 2002, Eriksson, 2001 #2449). Although the mechanisms underlying the neurobehavioral injury of tetrabrominated PBDEs have not been well characterized, it appears that the disruption of thyroid hormone homeostasis associated with normal brain development, as well as disruption of neurotransmitter signaling are key contributors to the neurotoxicity of PBDEs (Costa and

Giordano, 2007; Dingemans *et al.*, 2011). In addition to studies in rodents, Atlantic killifish (*Fundulus heteroclitus*) larvae exposed to a commercial waterborne PBDE mixture (DE-71) exhibit lethargic behavior and impaired fright responses (Timme-Laragy *et al.*, 2006), indicating that the mechanisms underlying neurobehavioral injury from these compounds may share similarities across aquatic and mammalian species.

In the current study, we examined the impairment of touch-escape responses as an indicator of potential adverse effects of BDE 49 on zebrafish neurobehavior. The effects of BDE 49 exposures on zebrafish touch-escape responses were analyzed at 5 dpf, a time point that preceded the appearance of treatment related mortality. The touch-escape responses observed in the 8 μ M BDE 49 treatment groups were somewhat increased, but were not statistically different as compared to control animals. Exposure to the highest dose of BDE 49 (32 μ M) caused a significant decrease in touch-escape responses relative to those observed in control animals (Fig. 5). As well as having a decreased touch-escape response, larvae in the 32 μ M exposure group exhibited extensive tail curvatures, which caused the animals to swim in a circular motion. Furthermore, at 5 dpf, many of the animals exposed to the highest dose of BDE 49 (32 μ M) exhibited continuous muscle spasms, suggesting additional neurological injury. In addition, some of the larvae in the BDE 49 exposure groups showed lethargic movements prior to the onset of tail curvatures before 5 dpf, providing additional evidence of the capability of BDE 49 to cause neurodevelopmental injury. Similarly, both total swimming distance and percent active time were shown to be negatively correlated with tissue BDE-47 concentrations in zebrafish under chronic exposure conditions (Chou *et al.* 2011). From an ecological perspective, the impact of PBDEs on fish behaviors such as swimming speed, locomotion, ability to elicit a touch-escape, or other fright responses, can threaten survival under normal environmental conditions. These neurobehavioral abnormalities ultimately lead to an inability to avoid predators, as well as to locate prey (Holmes and McCormick, 2010; Floyd *et al.*, 2008).

Conclusions

The results of our studies indicate that exposure to BDE 49, a relatively understudied tetrabromodiphenyl ether, can cause developmental abnormalities in zebrafish. The spectrum of developmental defects observed consisted of a loss of cardiac function associated with severe physiological stress, altered neurosensory ability, and morphological impairments as reflected by abnormal dorsal curvatures of the tail. The observed developmental toxicities were followed by a dose-dependent mortality, indicating that BDE 49 is a developmental toxicant with the potential to cause subacute toxicity and ultimately acute toxicity. Although our focus was to determine the potential for developmental toxicity of this compound, we did not investigate if BDE 49 exposures could elicit developmental and toxicological effects at low, more environmentally relevant concentrations below our lowest dose tested (<4 μ M, 2000 μ g/L), or under chronic exposures. Accordingly, additional research is needed in these areas. However, it should be noted that PBDEs do not reach high concentrations in surface waters due to poor water solubility, and it is assumed that dietary exposures are more relevant for fish. Also, exposure to 4 μ M BDE 49, the lowest dose tested in the present study, caused 100% mortality at 7 dpf and >90% of the animals showed tail curvatures at 6 dpf, suggesting that the observed effects may have environmental relevance. From an ecological risk assessment perspective, the present report and those generated from other laboratories indicates that tetra brominated diphenyl ether fire retardants (BDE 47, 49), as well as other less brominated congeners, are developmental toxicants in zebrafish. The fact that some more fully brominated PBDEs (i.e. BDE 153 and BDE 183 (Usenko *et al.*, 2011) do not exhibit the potent neurodevelopmental effects in zebrafish, assessing ecological risk of exposure to PBDEs should be approached with caution and with consideration to other structure-activity relationships. Because these brominated compounds

occur as mixtures similar to other halogenated compounds such as furans and dioxins, the Toxicant Equivalency Factor (TEF) methodology may be of use for evaluating the toxicity of environmental PBDE exposures.

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Fig. 1.
Structure of the pentaPBDE congeners BDE 47, BDE 49 and BDE 99.

Fig. 2. Acute toxicity of BDE 49 in zebrafish embryos. All treatments caused decreased survivorship at 6 dpf. The data represent pooled trials from two replicate experiments containing n=18–19 embryos in each experimental group and controls.

Fig. 3. BDE 49 induction of tale curvatures. Embryos exposed to BDE 49 showed an increased incidence of dorsal curvatures of the tail with increasing dose. The data represent pooled trials from two replicate experiments containing n=18–19 embryos in each experimental group and controls. Insert shows representative tail curvatures observed in larval zebrafish during BDE 49 exposures. As described in methods, animals were scored as exhibiting either clear presence/absence of tail curvature.

Fig. 4.

Cardiac defects in zebrafish exposed to BDE 49. No changes in overall heart rates were observed in the PBDE 49 exposed groups relative to control animals at 3 dpf (A). By contrast, a significant decrease in heart rate was observed at the 8 μM treatment at 6 dpf (B) in both atrial (black columns) and ventricular (gray columns) heart rates. In panel B, zebrafish embryos exposed to 16 μM or 32 μM PBDE 49 concentrations did not survive (dns) to 6 dpf, therefore, no data are shown. Values presented reflects the mean $\pm$ SEM of $n=4$ individuals. Asterisks denote $p \leq 0.05$.

Fig. 5. Effect of exposure to BDE 49 on larval touch escape responses. The highest dose of PBDE 49 tested caused significant impairment of larval zebrafish escape response. All exposures for analysis of touch escape responses were initiated at 24 hpf with analysis of experimental data collected at 5 dpf. Values presented are the mean $\pm$ SEM of $n=18$ individuals in each experimental group and controls. Asterisks denote $p \leq 0.05$.