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The cytotoxic effects of 5-methoxylated polybrominated diphenyl ether 47 (BDE47) on zebrafish (*Danio rerio*) embryos

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Abstract

The methoxylated polybrominated diphenyl ethers (MeO-BDEs) are important metabolites formed by *in vivo* metabolism of brominted diphenyl ethers and a natural product produced by marine organisms such as algae, so such ethers are ubiquitous in the environment. To monitor the toxic effect of this metabolite, embryos of zebrafish were exposed to gradual concentrations (0.03, 0.1, 0.3, 1 and 2.5 µM) of 5-MeO-BDE47. After 24, 48, 72 and 96 hours of exposure, general morphology and teratogenic effects were recorded. Both the general morphology score and the teratogenecity score were analyzed. Results showed that doses ≥ 5 µM had developmental effect at 72 hours of exposure, while the other doses had no effects on the embryos. At 96 hours of exposure, 100% mortality were recorded for embryos treated with 2.5 µM and 50% for those subjected to 1 µMIn addition, this compound seems to be less toxic compared with the hydroxylated metabolite 6-OH-BDE47, which presented toxic effect at the beginning of the test. This study shows that such metabolites should be included in the ecotoxiological studies for a proper risk assessment and more attention should be given to this class of chemicals in the aquatic environment.

Keywords: Phenolic metabolites, Zebrafish, Toxicity, Developmental abnormalities

1. Introduction

Polybrominated diphenyl ethers (BDEs) have been applied extensively as flame-retardants in a wide range of products as plastics, paints, foams and synthetic fibers (van Boxtel *et al*., 2008; An *et al*., 2010). Also, they are important metabolites formed by *in vivo* metabolism of brominated diphenyl ethers and a natural product produced by marine organisms such as algae, so such ethers are ubiquitous in the environment (Handayani *et al*., 1997; Malmvärn *et al*., 2005). Increasing concentrations of brominated flame retardants and their metabolites in the environment, human food chain and human tissues raise concern about the health impact and possible neurotoxic effects and endocrine system malfunction (Legler and Brouwer, 2003). The 2,2′,4,4′-tetrabromodiphenyl ether (BDE47), the most prevalent BDE congener found in the environment and human tissues, has been shown to be an endocrine disruptor (Canton *et al*., 2005; Hamers *et al*., 2006; Canton *et al*., 2008). It has also been reported to cause liver and neurodevelopmental toxicity (An *et al*., 2010). In fish, exposure to BDE47, had affected significantly on neural and cardiac development (Lema *et al*., 2007).

It is known that MeO-BDEs (methoxylated polybrominated diphenyl ether or brominated methoxy diphenyl ethers) exist together with their unmethylated OH-BDE homologues in sponges, algae and cyanobacteria. Also mollusks accumulate such compounds (Moore *et al*., 2004, Malmvarn *et al*., 2005).

Reports on residues of brominated flame retardants (BFRs) in the environment have increased of late, and it seems for lack of plausible other sources, that the unknown brominated compounds detected in marine organisms may be metabolited of BFRs (de Boer *et al*., 2000). So far little has been reported on the cytotoxicity of the metabolites. Very recently, El-Sayed Ali (2010) has studied the teratogenic effect of 6-OH-BDE47 and 6-OH-BDE90 on the zebrafish (*Danio rerio*) embryos.

It is necessary to study the toxic effect of MeO-BDEs on aquatic organisms thus provide useful toxicological data for the ecological risk assessment. Thus, the main objective of this study was to determine the *in vivo* toxicity of 5-MeO-BDE on developing zebrafish (*Danio rerio*) as a successful model used in toxicological studies of environmentally relevant substances (Scholz *et al*., 2008; Kammann *et al*., 2009), and to compare the results obtained for these treated embryos with those treated with 6-OH-BD47 to know a simple idea about the difference in the effect between the methyl and hydroxyl groups as a function group. Thus may help us for further investigations focusing on the route and mode of action on such metabolites in the fish body.

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2. Materials and methods

2.1. Chemicals

Five stock solutions (0.3, 1, 3, 10 and 25 mM) of 5-MeO-BDE47 were dissolved in dimethyl sulfoxide (DMSO, 0.01%) immediately prior to use and then directly diluted 10000 times in Dutch standard water (nominal concentrations: 0.03, 0.1, 0.3, 1 and 2.5 µM). The effect of solvent (DMSO, 0.01%) and positive (6-OH-BDE47) controls on Zebrafish embryos was studied in a preliminary experiment and were incorporated in the experiment.

2.2. Fish maintenance

Zebrafish (*Danio rerio*) were raised and kept under standard laboratory conditions at about 26 °C ± 1 °C and a photoperiod of 14:10 h. light: dark (Brand *et al*., 2002) in the Institute of Environmental Studies, Vrije University, Amsterdam, The Netherlands. Fish were fed with dry fish feed, Tetra-Pro Flakes (Tetra GmbH, Germany) in the morning and hatched brine shrimp (Artemia cysts from INVE, Grantsvillle, UT, USA) in the afternoon. The fish were acclimated in glass aquaria containing copper free water. Typically, the eggs were spawned synchronously at dawn of the next morning. One hour later, eggs quality has been checked under the microscope (Leica MZ 75), being sure to select the healthy, fertilized eggs-can be easily identified by their transparency- for the experiment. Fish breeding and embryo manipulation were conducted according to Westerfield *et al*. (1997).

2.3. Embryos toxicity test

Selected eggs (1 hour post fertilization, hpf) were placed in 24-well cell culture sterilized plates (one embryo/well) with self-adhesive foil. Embryos were exposed to the previous concentrations at the 4:8 – cell stage (1:1.25 hour post fertilization, hpf). Twenty embryos/concentration were used and incubated at 26 °C ± 1 °C for 72 hrs. Control of the light cycle to 14 h light and 10 h dark is achieved by keeping the eggs in a separate room equipped with an automatic light control. Embryos/larvae were screened daily and scored for survival, alterations in morphology, developmental abnormalities and endpoints of toxicity (Nagel, 2002). Toxic/lethal end points (coagulation, missing heart beat, missing somites, missing tail detachment, missing spontaneous movement) and non-lethal malformations (pericardial or yolk sac oedema, notochord, fin malformation, loss of pigmentation, incomplete head and eye development) were separately reported. The experiment was repeated twice.

Developed embryos/larvae were examined and photographed daily by stereo microscope. Paintshop Pro. 8 image analysis software was utilized to control a Roper digital camera on the microscope. Images were depicted at all treatment levels to complete the picture of the morphological abnormalities in different organs.

2.4. Calculation of LC_{50} and EC_{50}

The LC_{50} and EC_{50} were calculated at 3-days post fertilization from concentration-% lethality and concentration-% effect curves, respectively for all end points separately as well as for the sum of lethal affected embryos.

3. Results

For further understanding and characterizing the toxicity of these newly identified metabolite, embryotoxicity assay in the developing zebrafish was optimized. The dose-related effects of 5-MeO-BDE47 were determined following 96 hour of exposure starting at 1 hour post fertilization (hpf). The results showed no differences at the first 48 h. of exposure and no lethality was recorded till the 96 h. of exposure (Figures 1-3).

For all groups treated with 6-MeO-BDE47, no effects were reported the first 48 h. of exposure, presenting embryos similar to those of the DMSO control group. At 72 h. of exposure, abnormalities were recorded only for embryos treated with 2.5 µM 6-MeO-BDE47. Concentrations 0.3, 1 and 2.5 µM were lethal only at 96 h., presenting 50% mortality for the first 2 concentrations and 100% for the later one. The LC_{50} of 0.3 µM was calculated at 96 h. after exposure. Embryos exposed to 6-OH-BD47, showing 50% death of the embryos, the rest showed slow developed embryos with neither heart beat nor detached tail from the beginning and being in this form during all the test period without further growth, it's used only for checking the validity of the test condition during the study period and to compare between the hydroxylated polybrominated diphenyl ethers and polybrominated methoxy diphenyl ethers, as both of them are the metabolites of BDE47.

3.1. At age 24 hpf

All concentrations applied ranged from 0.03 to 2.5 µM of 5-MeO-BDE47, showing no morphological alterations in the embryos were reported. Well developed embryos compared with those of the DMSO control group. At 24 h of development, embryos are well developed with somites, head, eyes, trunk region, yolk sac, tail, prominently sculptured brain and few pigment cells are present along the axis dorsal to the yolk extension and on the dorsal region of the yolk sac (Figure 1, A). While, embryos exposed to 6-OH-BD47, showing slow developed embryos with neither heart beat nor detached tail (Figure 1, B&C).

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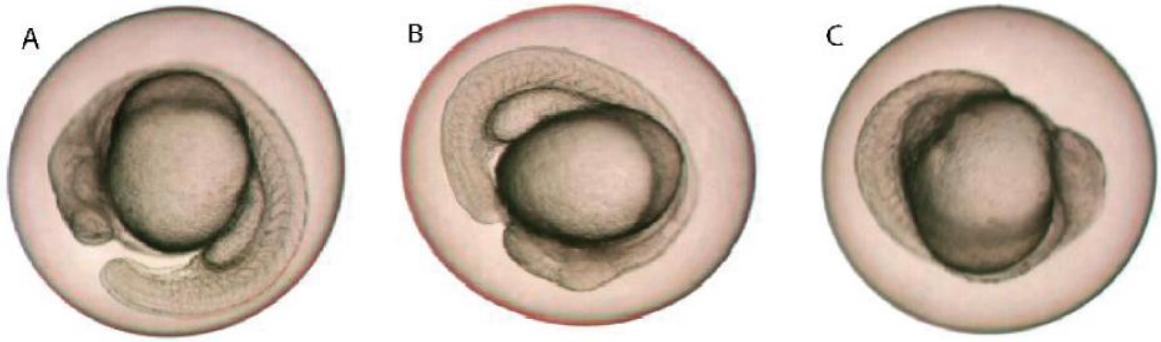

Figure 1. Morphological changes in zebrafish embryos exposed to different concentrations of 5-MeO-BDE47 and were photographed live in lateral orientation through a stereomicroscope at 24 h post fertilization (hpf). Embryos exposed to all concentrations (A) showing well developed embryo with yolk sac, tail, head, eyes and pigmentation similar to the control group embryos. Embryos exposed to 6-OH-BD47, showing slow developed embryos with neither heart beat nor detached tail (B) or showing development stop in early stage (death), (×4).

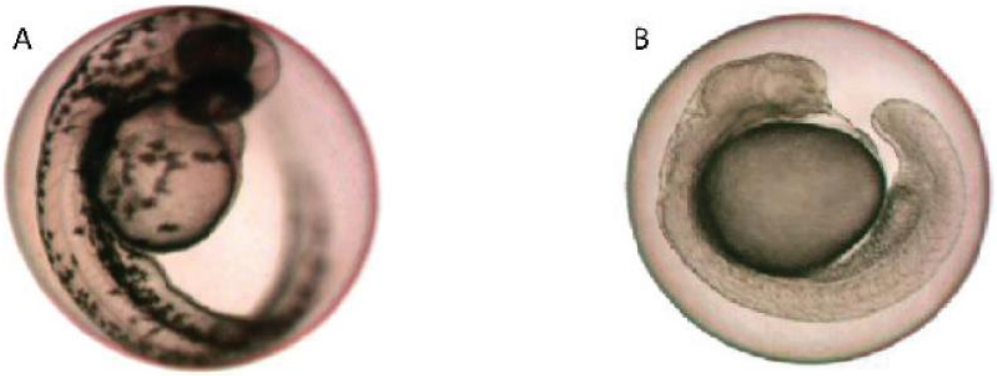

Figure 2. Morphological changes in zebrafish embryos exposed to different concentrations of 5-MeO-BDE47 and were photographed live through a stereomicroscope at 48 h post fertilization (hpf). Embryos exposed to the different concentrations showing embryos with well developed notochord with muscles, otolith, caudal fin, head, eyes and pigmentation similar to the control group embryos (A). Embryos exposed to 6-OH-BD47, showing no more growth than recorded at 24 hpf (B), (×4).

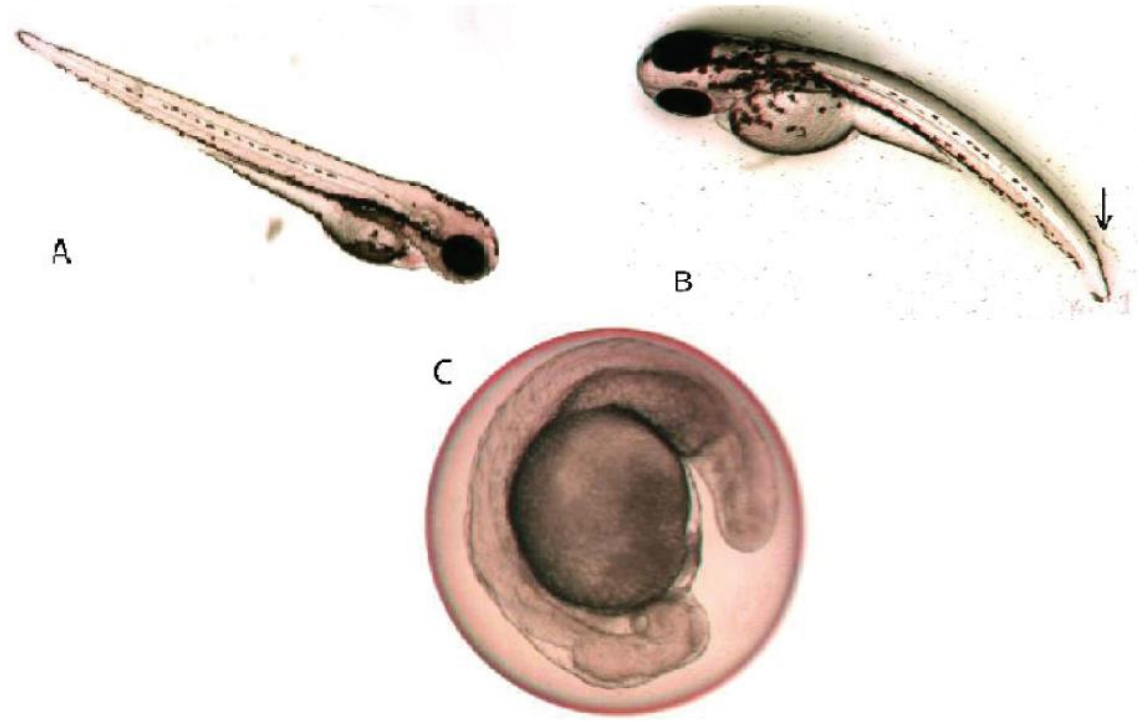

Figure 3. Morphological changes in zebrafish embryos exposed to different concentrations of 5-MeO-BDE47 and were photographed live in lateral orientation through a stereomicroscope at 72 h post fertilization (hpf). Embryos exposed to concentrations of 0.03, 0.1, 0.3, 1 µM showing well developed hatched larvae similar to the control group larvae (A, ×2). Embryos exposed to 2.5 µM showing hatched larvae with a curved notochord, deformed caudal fin (arrow) (B, ×2). Embryos exposed to 6-OH-BD47, showing no more growth than recorded at 24 hpf (C, ×4).

3.2. At age 48 hpf

Embryos exposed to the different concentrations of 5-MeO-BDE47, showing well developed embryos with normal notochord, auditory capsules with otolith, head, eyes, caudal fin and pigment extends the whole length of the body (Figure 2, A), similar to the control group embryos. Blood circulates through a closed set of channels and clear heart beats were measured and ranged between 119-120 beats/ min., in all groups. Embryos exposed to 6-OH-BD47, showing no more growth than those recorded at 24 hpf (Figure 2, B).

3.3. At age 72 hpf

Hatched larvae with quite elongated pectoral fin buds and vigorous heart beats were observed in the DMSO control group and those treated with 0.03, 0.1, 0.3, 1 µM of 5-MeO-BDE47. Also, it was shown that the yolk sac started to be shrunk making the pericardial cavity more conspicuous (Figure 3, A). For the embryos treated with 2.5 µM, slight growth retardation, delayed development of caudal fin and curved notochord were shown in 100% of the embryos (Figure 3, B). Embryos exposed to 6-OH-BD47, showing no more growth than recorded at 24 hpf (Figure 3, C).

3.4. At age 96 hpf

Further regular development was observed in 0.03, 0.1, 0.3 and 1µM of 5-MeO-BDE47 treated groups, although 50% mortality of 1 µM treated embryos was shown. 100% mortality was recorded in the embryos treated with 2.5 µM. Embryos exposed to 6-OH-BD47, showing no more growth than recorded at 24 hpf.

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4. Discussion

Aquatic organisms can be exposed to the BDE congeners from both metabolic and natural sources, thus facing even more risks than terrestrial organisms. Since many different hydroxylated BDE metabolites have been identified in both human and wild life (Bergman *et al*., 2006; Malmberg *et al*., 2005; Marsh *et al*., 2004; Valters *et al*., 2005) and given the obvious structural similarities. van Boxtel *et al*. (2008) have revealed that one metabolite of BDE47, 6-OH-BDE47 was highly toxic to zebrafish, *Danio rerio* with multiple manifestations of toxicity, such as lethal to the adult and embryos and inducing several teratogenic effects such as malformations, scoliosis and oedema to the embryos. For more accurate assessment of the harm effect of BDE47, it must be mentioned that BDE47 can be metabolized to 6-OH-BDE47 and 6-MeO-BDE47.

Despite the discovery that 6-OH-BDE47 causes acute toxicity through the uncoupling of OXPHOS, little is known about the toxicity of other Me-BDEs. Based on the findings of 6-OH-BDE47 by van Boxtel *et al*. (2008), it is plausible that other OH-BDEs or MeO-BDE47 may also have a common mode of action, due to the similarities in their molecular structures. Furthermore, little information is known about the potential of such metabolites to accumulate in organisms, and it has been suggested that continuous exposure may result in tissue retention of phase I hydroxylated metabolites (Hakk and Letcher, 2003).

Moreover, An *et al*. (2010) studied the cytotoxicity of different doses of 6-OH-BDE47 and 6-MeO-BDE47 on human hepatoma cell line. They demonstrated that exposing to these compounds was able to induce inhibition of cell viability, increase of apoptosis rate, cell cycle block, and DNA damages.

The present study shows that 5-MeO-BDEs-47 cause developmental abnormalities in zebrafish embryos only at dose 2.5 µM after 72 h. of exposure. Thus, the abnormalities were related to the concentration and longer exposure period for such compound compared with the hydroxyl compounds (El-Sayed Ali, 2010). Growth retardation, malformations of yolk, heart, tail and head were also recorded by van Boxtel *et al*. (2008) when applied 6-OH-BDE47 on zebrafish embryos at doses lower than that used in the present study. Under the pressure of oedema, heart can be malformed during development. The increasing pressure can also reduce heartbeat and blood circulation, which finally leads to death, the issue which explains the sudden 100% death after 96 h. of exposure.

According to Canton *et al*. (2005), both BDE47 and 6-MeO-BDE47 were not toxic to either embryos or adult zebrafish at concentrations up to 10 µM, indicating that the hydroxyl group is critical for eliciting toxic effects. These structural requirements for the biological activity of the congeners have previously been observed for cytotoxicity in human adrenocortical carcinoma cells. These findings partially agree with the results obtained in the present study. As this experiment reveals that no toxic effect at all concentrations applied till the first 72 h. of exposure. Only abnormalities were shown at the highest concentration at 72h. of exposure. At 96 h., a sudden mortality for all individuals at concentrations of 2.5 µM and 50% mortality at the concentrations of 0.3 and 1 µM. Confirming the previously mentioned, the hydroxylated compound (6-OH-BDE47) caused a stop of development at the beginning of the experiment.Thus confirming the severe action of OH group compared with Me group especially at the beginning of exposure (72 h.).

Generally, there is a scarce of information about the toxic effect of phenolic BDE in living organism. Further studies are required to follow up the mode of action of such compounds although the findings of the present study put a possibility of differences of action between hydroxylated and methoxylated metabolites, even at the exposure duration level.

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