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Teratogenic effect of phenolic metabolites on zebrafish (*Danio rerio*) embryos

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Abstract

The hydroxylated polybrominated diphenyl ethers (OH-PBDEs) 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 both general toxicity as well as teratogenecity of this metabolite, embryos of zebrafish were exposed to gradual concentrations of 6-OH-BD90. After 24, 48 and 72 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 ≥ 1 μM were lethal in the first hours of exposure while the doses 0.03 & 0.1 μM had no effects on the embryos. Doses in between caused developmental retardation and deformation in a dose-related fashion. In addition, these compounds induced several teratogenic effects. 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: Metabolites, Zebrafish, Toxicity, Developmental abnormalities

1. Introduction

Polybrominated diphenyl ethers (PBDEs) are widely used industrial chemicals that are produced in large quantities and are used extensively as flame-retardants in a wide range of products being ubiquitous in the environment (van Boxtel *et al*., 2008). Increasing concentrations of brominated flame retardants and their metabolites in the environment, human food chain and human tissues raise concern about possible neurotoxic effects and endocrine system malfunction (Legler and Brouwer, 2003). In fish, exposure to one of the most prevalent PBDEs in the environment, BDE47, resulted in effects on neural and cardiac development (Lema *et al*., 2007).

Although many PBDEs have not been well studied, new concern has been raised on their hydroxylated congeners. In organisms, PBDEs can be converted to mainly hydroxylated (-OH) forms after biotransformation, and their hydroxylated metabolites are found to be diverse among organisms (Hakk & Letcher, 2003). Hydroxylated polybrominated diphenyl ethers (OH-PBDEs) have been reported to be found in aquatic and terrestrial wild life and human plasma. Many of these compounds are naturally occurring while others are thought to be either metabolic or environmental transformation products of the commercially produced PBDEs. High concentrations of OH-BDEs have also been detected in algae and sponges as their natural anti-bacterial products to prevent infection (Sharma *et al*., 1970; Handayani *et al*., 1997; Malmvärn *et al*., 2005).

As a large number of PBDE congeners has been detected in a wide variety of wildlife species, including numbers of fish species (Marsh *et al*., 2004; Valters *et al*., 2005) and also in humans (Inoue *et al*. 2006), this study focused on the hydroxylated BDE 90 metabolite, an important metabolite within the pool of hydroxylated PBDEs retained in biological systems and have potential endocrine disrupting properties (Canton *et al*., 2005; Hamers *et al*., 2006). The main goal of this study was to determine the *in vivo* toxicity and teratogenic action of 6-OH-BD90 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).

2. Materials and Methods

2.1. Compounds

Five stock solutions (0.3, 1, 3, 10 and 25 mM) of 6-OH-BDE90 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).

Solvent (DMSO, 0.01%) and positive controls (6-OH-BDE47, its effect on Zebrafish embryos was studied in a preliminary experiment) 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).

2.1. Zebrafish toxicity experiments

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 these concentrations at the 4:8 – cell stage (1:1.25 hour post fertilization, hpf). Ten 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 edema, bent notochord, fin malformation, no pigmentation, incomplete head and eye development) were reported separately. 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.

2.2. 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 more understanding and characterizing the toxicity of these newly identified teratogens, embryotoxicity assay in the developing zebrafish was optimized. The dose-related effects of the teratogens were determined following 72 h exposure starting at 1 hour post fertilization (hpf). The results showed a very high teratogenic potential for this compound related to the levels (Figures 1-3).

For the groups treated with 0.03 and 0.1 μM 6-OH-BDE90, no effects were reported during all the experimental period, presenting embryos similar to those of the DMSO control group. Respecting to 0.3 μM, the 6-OH-BDE90 started its toxic non-lethal action at 48 hpf. Concentrations 1 and 2.5 μM were lethal within the first three hours of exposure, all embryos stopped their development in the epiboly stage. The developmental effects of 6-OH-BDE90 were dose dependent with an EC_{50} value of 0.19 μM for all endpoints and LC_{50} of 0.21 μM. Embryos exposed to 6-OH-BD47, showing 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.

24 hpf

No morphological alterations in the embryos were reported when concentrations of 0.03, 0.1 and 0.3 μM were applied, compared with those of the DMSO control group during the first 24 h of development, showing well developed healthy embryos with somites, yolk sac, tail, head, eyes, prominently sculptured brain and few pigment cells are present along the axis dorsal to the yolk extension and on the dorsal part of the yolk ball (Figure 1, A, B, C), similar to the control ones (Figure 1, E). While, embryos exposed to 1 and 2.5 μM showed coagulation resulted from development stop in epiboly stage (Figure 1, D). Embryos exposed to 6-OH-BD47, showing slow developed embryos with neither heart beat nor detached tail (Figure 1, F).

48 hpf

Embryos exposed to concentrations of 0.03 and 0.1 μM showing embryos with well-developed notochord, otolith, caudal fin, head, eyes and pigment extends the whole length of the body (Figure 2, A, B), similar to the control group embryos (Figure 2, D). The 0.3 μM treated-group showing slight delay in the growth with oedema and a slightly unstraight notochord with mal-formed tail (curved, short, no tail fin) in 90% of the embryos (Figure 2, C). However, blood circulates through a closed set of channels and clear heart beats were measured and ranged between 119-120 beats/ min., as all other groups. Embryos exposed to 6-OH-BD47, showing no more growth than recorded at 24 hpf (Figure 2, E).

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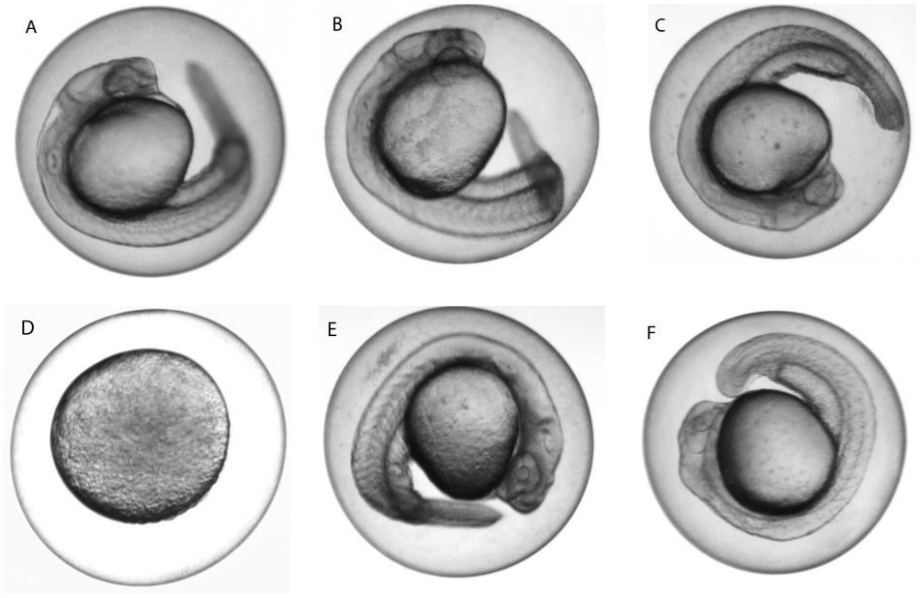

Figure 1: Morphological changes in zebrafish embryos exposed to different concentrations of 6-OH-BD90 and were photographed live in lateral orientation through a stereomicroscope at 24 h post fertilization (hpf). Embryos exposed to concentrations of 0.03 (A), 0.1 (B), 0.3 µM (C), showing well developed embryo with yolk sac, tail, head, eyes and pigmentation similar to the control group embryos (E). Embryos exposed to 1, 2.5 µM showing development stop in epiboly stage (coagulate, D). Embryos exposed to 6-OH-BD47, showing slow developed embryos with neither heart beat nor detached tail (F), (×4).

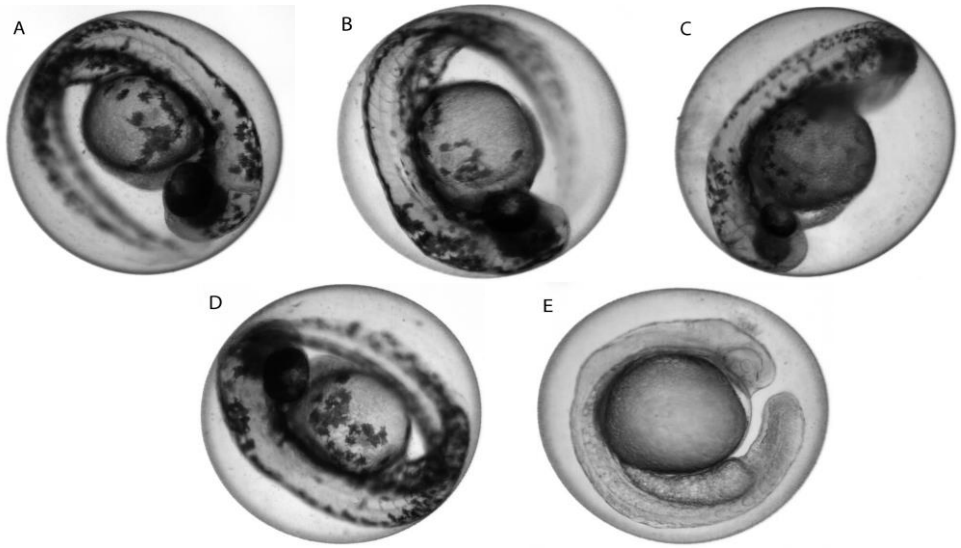

Figure 2: Morphological changes in zebrafish embryos exposed to different concentrations of 6-OH-BD90 and were photographed live through a stereomicroscope at 48 h post fertilization (hpf). Embryos exposed to concentrations of 0.03 (A), 0.1 (B) µM, showing embryos with well developed notochord with muscles, otolith, caudal fin, head, eyes and pigmentation similar to the control group embryos (D). 0.3 µM treated group, showing mal-formed embryos with oedema and mal-formed short tail with no tail fin (C). Embryos exposed to 6-OH-BD47, showing no more growth than recorded at 24 hpf (E), (×4).

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 and 0.1 μM of 6-OH-BDE90. 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 0.3 μM, slight growth retardation, delayed development of caudal fin and curved notochord were shown (Figure 3, B). Also reduction in heart beats number (80 beats/min.) was detected. Embryos exposed to 6-OH-BD47, showing no more growth than recorded at 24 hpf (Figure 3, C).

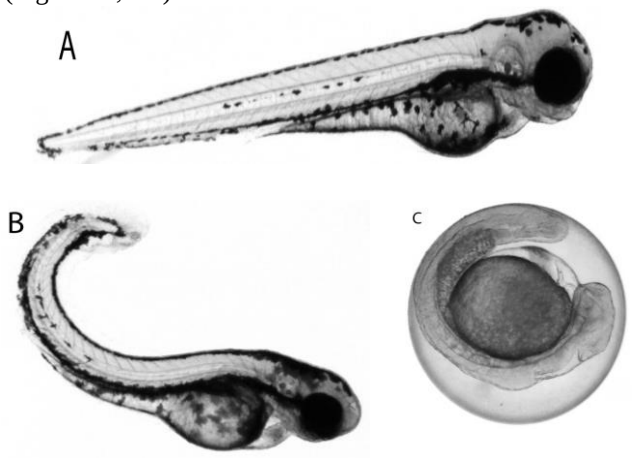

Figure 3: Morphological changes in zebrafish embryos exposed to different concentrations of 6-OH-BD90 and were photographed live in lateral orientation through a stereomicroscope at 72 h post fertilization (hpf). Embryos exposed to concentrations of 0.03, 0.1 μM showing well developed hatched larvae similar to the control group larvae (A, ×2). Embryos exposed to 0.3 μM showing hatched larvae with a curved notochord (B, ×2). Embryos exposed to 6-OH-BD47, showing no more growth than recorded at 24 hpf (C, ×4).

4. Discussion

Aquatic organisms can be exposed to the PBDE 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, it is necessary to study the toxic effect of OH-BDEs in aquatic organisms thus provide useful toxicological data for the ecological risk assessment. 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 embryonic fish and inducing several teratogenic effects such as malformations, scoliosis and edema to the embryos. Examination of the mechanism of action of 6-OH-BDE47 toxicity with microarray and subsequent biochemical analysis showed different genes regulated by 6-OH-BDE47 such as zebrafish dapper homolog 2 (Dact2) and retinoic acid receptor (Rara2) genes. In particular, one toxic mechanism which could explain the acute toxicity of the metabolite was revealed in their study, namely uncoupling of oxidative phosphorylation (OXPHOS). Such toxic effect of 6-OH-BDE47 was shown in the current study although this compound was not the main objective of this study.

Despite the discovery that 6-OH-BDE47 causes acute toxicity through the uncoupling of OXPHOS, little is known about the toxicity of other OH-BDEs. Based on the findings of 6-OH-BDE47 by van Boxtel *et al*. (2008), it is plausible that other OH-BDEs 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).

According to Terada's theory (1990), a high active uncoupler should have an acidic group for the proton delivery, bulky liphophlic groups for a better binding to membrane and a strong electron withdrawing group for the delocalization of the charge of the anion. Although the structures of OH-BDEs meet these requirements, the activities of the functional groups in their molecules may not be the same. In a study of the effects of hydroxylated organohalogens on endocrine disrupting effects, Kester *et al*. (2000) proposed that the cooperative effect of –OH and –Br groups could lead to different potencies of endocrine disruptors. This may give some hints that the position of hydroxyl group is not the only determinant for the uncoupling activity of OH-BDEs, the relative position of substituted bromine atom is also an important factor. Future chemical studies on OH-BDEs may help to understand these structure-specific activities.

The present study confirms that 6-OH-BDEs -90 can cause lethal sudden effect at the beginning of exposure

to doses equal or more than 1μM. Meanwhile, developmental abnormalities in zebrafish embryos were shown at doses lower than 1μM. Such lower doses was far less toxic than their equivalents of 6-OH-BDE47 which had an action in earlier stages than shown in the current study. The abnormalities were related to the concentration and exposure period to such compound. 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 but still at doses lower than that used in the present study. Under the pressure of edema, heart can be malformed during development. The increasing pressure can also reduce heartbeat and blood circulation, which finally leads to death.

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 three congeners have previously been observed for cytotoxicity in human adrenocortical carcinoma cells (Canton *et al*., 2005).

From the present study reveals, for the first time, the teratogenic effect of 6-OH-BDEs -90 and focusing on the toxicity of such metabolites in living organism. Generally, there is scare information about the toxic effect of phenolic PBDE in living organism. Further studies are required to follow up the mode of action of such compound although the findings of the present study put a possibility of similarities of action between 6-OH-BDE90 and6-OH-BDE47.

Also, this study raises the issue of the environmental risks of OHBDEs. In addition, more research into the environmental levels and toxicity of phenolic PBDEs is necessary to confirm the risks of different OH-BDEs on different organisms.

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التاثيرات التشويهيه لنواتج الايض الفينوليه علي أجنة أسماك الزيبرا

تعتبرمركبات الايثر ثنائيه الفينيل البروموهيدروكسيلية مركبات أيضيه هامة والتي تنتج عن التحولات الأيضيه لمركبات الايثر ثنائيه الفينيل البرومونيه كما انها تنتج طبيعيا بواسطه الطحالب البحريه لذا فهي موجوده في البيئات المائيه . لدراسه التأثير السام لهذه المركبات و تأثيرها التشويهي على أجنة أسماك الزيبرا, تم تعريض أجنة أسماك الزيبرا لتركيزات مختلفة من 6-OH-BD90 أعتبارا من ساعة و حتي 72 ساعة بعد تلقيح بيض هذا النوع من الأسماك وتم الفحص بعد 24 ساعة -48 ساعة -72 ساعة. ولقد أوضحت الدراسة أن إستجابة هذة الأجنة تعتمد علي درجة تركيزات الجرعة المستخدمة. كما أوضحت هذة الدراسة أيضا أن التعرض المبكر لهذا النوع من المركبات لة تاثير مباشر علي موت هذة الأجنة عند تعرضها لجرعات اكبر او تساوي 1µ مول. بينما الجرعات التي تسبب تشوهات للأجنة تتراوح بين 0.1 : 1µ مول بعد ثلاثه ايام من التعرض. و خلاصة هذة الدراسة تشير أنة يجب أعطاء المزيد من الانتباة لهذا النوع من المركبات لتقليل التاثيرات الضارة لها في البيئات المائية.