Chemosphere 73 (2008) 195–202

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Toxicity of analytically cleaned pentabromodiphenylether after prolonged exposure in estuarine European flounder (*Platichthys flesus*), and partial life-cycle exposure in fresh water zebrafish (*Danio rerio*)

Raoul V. Kuiper [a,*], A.D. Vethaak [b,1], Rocío F. Cantón [c], Henrique Anselmo [c,2], Marco Dubbeldam [d], Evert-Jan van den Brandhof [e], Pim E.G. Leonards [f,3], Piet W. Wester [e], Martin van den Berg [c]

[a] Department of Pathobiology, Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80158, 3508 TD Utrecht, The Netherlands
[b] National Institute for Coastal and Marine Management (RIKZ), P.O. Box 20907, 2500 EX The Hague, The Netherlands
[c] Institute for Risk Assessment Sciences (IRAS), Utrecht University, P.O. Box 80176, 3508 TD Utrecht, The Netherlands
[d] Grontmij-AquaSense Laboratory for Aquatic Ecotoxicology, Visserijweg 14a, 4486 CX Colijnsplaat, The Netherlands
[e] National Institute of Public Health and the Environment (RIVM), P.O. Box 1, 3720 BA Bilthoven, The Netherlands
[f] Wageningen IMARES, P.O. Box 68, 1970 AB IJmuiden, The Netherlands

ARTICLE INFO

Article history:
Received 14 November 2007
Received in revised form 28 April 2008
Accepted 28 April 2008
Available online 16 June 2008

Keywords:
Brominated flame retardants
BFR
PBDE
Fish
Risk assessment
Endocrine disruption

ABSTRACT

Residues of polybrominated diphenylethers (PBDEs), extensively applied as flame retardants, are widely spread in the aquatic environment and biota. The present study investigates effects of the environmentally relevant lower brominated diphenylethers in two fish species *in vivo* under controlled laboratory conditions. Euryhaline flounder (*Platichthys flesus*) and freshwater zebrafish (*Danio rerio*) were exposed to a range of concentrations of a commercial pentabromodiphenylether mixture, DE-71. Chemical analysis of exposed fish showed a pattern of PBDE congeners that was very similar to that in wild fish. The resulting range included environmentally relevant, as well as higher levels. Animals were investigated histopathologically with emphasis on endocrine and reproductive organs. In zebrafish, hatching of embryos and larval development were assessed. Biochemical parameters were investigated in flounder as markers for suggested dioxin-like activity (ethoxyresorufin-*O*-deethylase = EROD), and activation of endogenous estrogen synthesis (gonad aromatase activity). Thyroid hormones were analyzed in plasma in both species. Benchmark analysis using internal PBDE concentrations showed a mild dose-dependent decrease of hepatic EROD and ovarian aromatase activities, and plasma thyroxin levels in flounder, and an increase of plasma thyroid hormone levels in zebrafish. These trends did not result in statistically significant differences from control fish, and major histopathological changes were not observed. Reproduction in zebrafish appeared to be the most sensitive parameter with statistically significantly reduced larval survival and non-significant indications for decreased egg production at internal levels that were more than 55 times the highest environmental recordings. The present results indicate limited risk for endocrine or reproductive effects of current environmental PBDE contamination in fish.

1. Introduction

Polybrominated diphenyl ethers ((P)BDEs) are used as flame retardants in a wide number of synthetic applications such as building materials, furnishing textiles, and electronic equipment, to reduce the risk of fires. Losses at production sites and leaching from landfills has resulted in progressive contamination of the aquatic environment with predominant bioaccumulation of lower brominated congeners, predominating in commercial pentabrominated diphenylether mixtures (PeBDE), in aquatic biota (Birnbaum and Staskal, 2004).

Reported effects of PBDE-exposure include modulation of the thyroid and sex steroid endocrine systems. A number of wide spread tetra- and pentabrominated BDEs and commercial PeBDE showed competitive binding to both human and fish transthyretin, a major plasma thyroid hormone binding protein, *in vitro* (Hamers et al., 2006; Morgado et al., 2007). Interactions with sex steroid receptors were also observed *in vitro* (Hamers et al., 2006). In rats, oral exposure to commercial PeBDE (DE-71) decreased levels of plasma thyroid hormones (thyroxine: T_4, and triiodothyronine:

* Corresponding author. Tel.: +31302534362; fax: +31302534774.
E-mail address: R.V.Kuiper@uu.nl (R.V. Kuiper).
[1] Present address: Deltares, P.O. Box 177, 2600 MH Delft, The Netherlands.
[2] Present address: Institute for Marine resources and Ecosystem Studies (IMARES), P.O. Box 9101, 6700HB Wageningen, The Netherlands.
[3] Present address: Institute for Environmental Studies(IVM), VU University, De Boelelaan 1087, 1081 HV, Amsterdam, The Netherlands.

0045-6535/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.chemosphere.2008.04.079

T_3) and induced marked thyroid hyperplasia; anti-androgenic effects were noted in males (Stoker et al., 2004, 2005). Exposure to an uncleaned technical PeBDE mixture reduced egg production and caused hepatic lipidosis stickleback (Holm et al., 1993). Hepatic lipidosis was also observed in rainbow trout fry exposed to the same chemical mixture, although increased cytochrome P4501A (CYP1A) activity indicated possible contamination with planar polyhalogenated aromatic hydrocarbons (PHAHs) in that study (Norrgren et al., 1993). Neurodevelopmental defects and reduced juvenile survival were observed in killifish and zebrafish exposed to DE-71 (Timme-Laragy et al., 2006; Lema et al., 2007). The consequences of endocrine and neurobehavioral effects of (purified) PBDEs for the reproductive system and successful offspring production in exposed fish have not been investigated with regard to the tentative mechanisms involved at environmentally relevant exposure levels.

In the present study, possible target organs for PBDE toxicity with emphasis on endocrine effects, and reproduction-related parameters are investigated in two aquatic vertebrate species. The euryhaline European flounder (*Platichthys flesus*) is a common species on soft sediments in coastal and estuarine waters in Europe. Premature ovaries were observed in flounder with elevated 17β-estradiol levels after exposure to contaminated harbor sludge (Janssen et al., 1997). Zebrafish is a freshwater species sensitive to estrogenic and anti-thyroid effects (van der Ven et al., 2003a,b, 2006) and juvenile malformations caused by PBDEs (Lema et al., 2007). They were included in the study to evaluate reproductive performance and larval development at environmentally relevant exposure levels. Fish were exposed to DE-71, a classic PeBDE mixture. To avoid any influence of minor amounts of contaminating poly-brominated dibenzo-*p*-dioxins and -furans, DE-71 was purified before use. PBDE levels in exposed fish were analyzed to provide a dose background for observed effects, which then can be related to levels observed in biota in the environment.

2. Materials and methods

2.1. Preparation of the DE-71 sample

Commercial pentabromodiphenylether (DE-71; lot 355OH29D) was obtained from Great Lakes Chemical Corporation (kindly provided by Dr. D. Sanders) and purified using activated charcoal according to Marsh et al. (1999) to remove all (brominated) dibenzodioxins and dibenzofurans, as well as any other coplanar molecules. Absence of dioxin-like activity (AhR-agonistic response) was confirmed by testing in a dioxin responsive reporter-gene cell line (DR-CALUX) as described by Hamers et al. (2006).

2.2. Exposure

2.2.1. Flounder

Juvenile flounder (159 days old) were obtained from a hatchery (Manx Mariculture Ltd., Isle of Man, UK) and grown under controlled conditions until the animals were 313 days of age at the start of the experiment, with an average weight of 48 ± 12 g (SD). Flounder were housed in aquariums (70 × 100 × 30 cm; width × length × height) containing 15 kg of (spiked) sediment and 160 l of water from the Eastern Scheldt (a tidal bay connected to the North Sea with a salinity of approximately 30‰; temperature was 15 °C; renewal rate: twice weekly via continuous flow through). A daily cycle of 16 h light, 8 h dark, was maintained. Exposure was for 101 days starting end of April 2005. A benchmark schedule was applied using single groups of ten animals per combined exposure dose of DE-71 in sediment (μg g^{-1} total organic carbon) and food (μg g^{-1} lipid): 0 + 0 (control); 0 + 0.014; 0.007 + 0.14; 0.07 + 1.4; 0.7 + 14; 7 + 140; 70 + 1400; and 700 + 14 000, respectively. For the spiking of food, cleaned DE-71 was dissolved in acetone (Promochem, Germany) and mixed with corn oil. Then the acetone was evaporated and the oil containing cleaned DE-71 was added to mixture of fish meal, mussels, and fish oil to prepare food pellets. The resulting lipid content of the food was 10%. Animals were fed three times a week at an estimated 1% of the total body weight until 5 days before the end of the study. For preparation of spiked sediment, sandy sediment was collected from a reference site in the Eastern Scheldt tidal zone (82% dry matter, 0.3% TOC). To 15 kg wet sediment, 10 ml acetone containing the desired amount of cleaned DE-71 (52 mg for the highest dose, 5.2 for the next etc.) and 1.5 l of salt water was added. This was stirred in 20 l glass bottles during 3 days before the spiked sediment was added to the aquariums. To allow for stabilization of the sediment concentration, fish were introduced two weeks after the sediment was added to the aquariums and flow through initiated.

2.2.2. Zebrafish

Eight months old reproducing zebrafish (*Danio rerio*), from a commercial importer (Ruinemans Aquarium BV, The Netherlands) were kept under quarantine conditions for a period of four weeks, and were acclimatized to the test system from 5 days prior to exposure. Twelve groups of four males and four females each were housed in all-glass aquariums measuring 25 × 18 × 22 cm; width × length × height, covered with a glass plate, containing 0.75 l test medium/fish. The water was aerated continuously via glass tubes and oxygen levels were >5 mg l^{-1} at all times. Temperature was maintained at 27 ± 2 °C, pH ranged from 7.2 to 8.4 and hardness was 214 mg $CaCO_3$ l^{-1}. A daily cycle of 14 h light, 10 h dark was maintained. Animals were fed *ad libitum* for 5 min twice a day with defrosted *Artemia* except on sundays. DE-71 stocks were prepared in dimethylsulfoxide (DMSO, Acros, 's Hertogenbosch, The Netherlands) and stored at room temperature in the dark. Experimental groups were exposed to waterborne DE-71 at nominal concentrations of 0 (control), 5, 16, 50, 160 and 500 μg l^{-1}. Exposure of adults was continued for 30 days by semistatic renewal twice a week. All concentrations were tested in duplicate except 50 μg l^{-1}, which was not replicated. Final DMSO concentration was 0.01% in all groups except in one of the controls. During exposure, animals were monitored daily for alertness, abnormal behavior or disease, and mortality.

Egg production and fertilization were evaluated directly after water renewal on Mondays and Thursdays, when both sexes were placed inside a nylon mesh (Ø: 3 mm) for 24 h with 6 l medium. Thereafter, the medium was split in equal volumes and sexes were separated until the next medium renewal. During the first three weeks eggs were collected in a calibrated glass tube for semi-quantitative monitoring of egg production. Egg fertilization ratios were estimated in four categories (resp. 25%, 50%,75%, 100%). During the last 10 days of adult exposure eggs were counted manually to determine the exact clutch size, fertilization ratio and hatchability.

During week 4 and 5 of exposure of adults, 4 replicate groups of approximately 50 viable eggs each were selected per adult group, and exposed to static waterborne DE-71 at the same nominal concentrations and ambient conditions as their parents, in 10 cm diameter glass petri dishes containing 60 ml exposure medium. From parents exposed to 50 and 500 μg DE-71 l^{-1}, insufficient eggs were obtained; therefore, eggs from parents exposed to 16 and 160 μg l^{-1}, were used for exposure of eggs and juveniles to 50 and 500 μg l^{-1}, respectively. In addition, two times 50 eggs from control parents were also exposed to the highest test concentration (500 μg l^{-1}) and two times 50 eggs from 500 μg l^{-1} parental exposure were also placed in control medium. Hatching was recorded and larvae were maintained in the petri dishes until one week post-hatching (PH) after which 2 replicates of 50 surviving larvae

per dose were randomly selected and transferred to all-glass aquariums containing 1.5 l of exposure medium under otherwise similar conditions as described for adults. From day 1 to 14 PH, larvae were fed 4 ml rotifer suspension (*Branchionus rubens*) daily per 50 larvae. Starting at day 7 PH, larvae were fed fresh *Artemia* nauplii 2 times a day (suspension adjusted to 17% dry matter, starting with 5 µl per larva, increasing to 120 µl per juvenile at the end of the exposure period). One day prior to euthanasia, feeding was discontinued. Exposure of juveniles was continued until 45 days PH. Juvenile fish were inspected daily for mortality, abnormal appearance and behavior. The studies were approved by the Ethical Committees for Animal Welfare in Experiments of RIVM and RIKZ and comply with Dutch legislation.

2.3. Sampling procedure

2.3.1. Flounder

Directly following euthanasia in random order using MS222 (Sigma–Aldrich, Germany), animals were weighed and length was determined. Blood was sampled from the caudal spinal venous plexus and plasma samples with 30 µl aprotinin (Sigma; 0.1 mg ml^{-1} in 0.9% NaCl) added per ml plasma were flash frozen and stored at −70 °C for analysis of thyroid hormones. Gonads and liver were excised and weighed; the topmost gonad and cranial one third of the liver was frozen in liquid nitrogen and stored at −70 °C for preparation of microsomes. Remaining abdominal organs including kidneys, and branchial arches including the thyroid region were fixed in formalin and routinely processed and paraffin embedded, and histological sections were routinely stained with hematoxylin and eosin (H& E). Flank muscles (“fillet”) were stored at −20 °C for chemical analysis.

2.3.2. Zebrafish

Immediately after euthanasia with MS222, length and weight were determined, and blood was sampled from the clipped tail peduncle of adults using heparinized glass capillaries. An equal volume of aprotinin (6 µg ml^{-1} in 0.01 M PBS, pH 7.2) was added and the cellular component was separated using a haematocrit centrifuge (12 000 rpm for 5 min.). Plasma was stored at −70 °C for determination of thyroid hormones. Immediately after blood sampling, the fish were fixed in Bouin’s fixative for 24 h after which they were transferred to 70% ethanol until routine histological processing. Whole body sections were stained with H&E. Immunohistochemical staining was performed using polyclonal rabbit anti-zebrafish VTG, in a 1:50 dilution (as otherwise described previously by van der Ven et al. (2003a)) and monoclonal mouse anti-scup CYP1A (Kuiper et al., 2006). Sections from adult females, and zebrafish exposed to uncleaned DE-71 (containing 1,2,3,7,8-pentabromodibenzo-furan) were included as positive controls for VTG and CYP1A staining, respectively. Fish for chemical analysis were rinsed in aquadest twice and dipped dry before storing at −20 °C until analysis.

2.4. Chemical analyses

Determination of internal PBDE levels was previously described (Kuiper et al., 2006). Briefly, approximately 7 g of flounder muscle from each animal was dried with sodium sulphate (Merck), and stored for 2 h prior to extraction and clean-up. Analysis was performed with GC–MS (ECNI mode, *m*/*z* 79) using an internal standard (BDE-58) and external calibration points. From zebrafish, one male and one female per exposure concentration were weighed and whole bodies were prepared as described for flounder tissue; from juvenile zebrafish, a pooled sample of four fish was prepared per exposed group of 50 juveniles. For quality control and quality assurance, blanks, duplicate analysis of a sample,

Table 1
Internal PBDE concentrations and biochemical parameters in flounder exposed to DE-71 during 101 days

Dose sediment (µg/g TOC)	Dose food (µg/g lipid)	µg/g wet weight internal ∑8BDE (mean ± SD)	µg/g wet weight internal BDE 47 (mean ± SD)	µg/g lipid internal BDE 47 (mean ± SD)	Plasma T_3 (ng/ml ± SD)	Plasma T_4 (ng/ml ± SD)	Aromatase gonad (pmol/h/mg ± SD)		EROD(pmol/min/mg ± SD)
							Male	Female	
Background	0	0.10 ± 0.11 (0.02–0.32; *n* = 10)	0.07 ± 0.07 (0.02–0.21)	4.9 ± 3.7 (2.2–14)	5.5 ± 1.6 (*n* = 10)	18 ± 9 (*n* = 10)	2.1 ± 0.8 (*n* = 3)	1.1 ± 0.5 (*n* = 5)	11 ± 13 (*n* = 9)
Background	14×10^{-3}	0.10 ± 0.09 (0.01–0.3; *n* = 10)	0.07 ± 0.06 (0.01–0.20)	7.1 ± 7.2 (0.64–21)	6.8 ± 1.5 (*n* = 10)	15 ± 5 (*n* = 10)	1.04 (*n* = 2)	2.4 ± 1.3 (*n* = 6)	13 ± 11 (*n* = 10)
7×10^{-3}	14×10^{-2}	0.13 ± 0.20 (0.01–0.64; *n* = 9)	0.09 ± 0.14 (0.01–0.45)	8.2 ± 11 (0.71–38)	6.2 ± 2.2 (*n* = 9)	20 ± 10 (*n* = 9)	0.65 ± 0 (*n* = 4)	1.6 ± 1.5 (*n* = 6)	15 ± 7 (*n* = 7)
7×10^{-2}	14×10^{-1}	0.30 ± 0.30 (0.01–0.84; *n* = 10)	0.21 ± 0.21 (0.00–0.60)	20 ± 22 (0.43–68)	5.4 ± 2.5 (*n* = 10)	9.2 ± 6.1 (*n* = 10)	0.06 ± 0.55 (*n* = 3)	1.5 ± 0.5 (*n* = 5)	10 ± 9 (*n* = 10)
7×10^{-1}	14	0.34 ± 0.25* (0.04–0.74; *n* = 8)	0.22 ± 0.16* (0.02–0.48)	19 ± 14* (2.9–40)	4.9 ± 1.9 (*n* = 10)	16 ± 12 (*n* = 10)	1.6 ± 3.5 (*n* = 4)	1.3 ± 0.7 (*n* = 5)	8.0 ± 6.9 (*n* = 9)
7	140	0.53 ± 0.28* (0.28–1.11; *n* = 8)	0.34 ± 0.19* (0.18–0.74)	24 ± 15* (11–57)	4.8 ± 1.7 (*n* = 10)	11 ± 6 (*n* = 10)	ND	1.4 ± 1.3 (*n* = 3)	4.1 ± 3.9 (*n* = 10)
70	1400	5.9 ± 4.3* (0.85–14; *n* = 10)	3.9 ± 2.8* (0.50–9.20)	331 ± 207* (56–657)	5.6 ± 1.4 (*n* = 9)	7.8 ± 3.9 (*n* = 9)	1.31 (*n* = 2)	0.91 ± 0.65 (*n* = 7)	2.9 ± 1.2 (*n* = 8)
700	14000	71 ± 86* (19–308; *n* = 10)	45 ± 56* (11–200)	2691 ± 2714* (833–10000)	5.6 ± 1.2 (*n* = 10)	13 ± 5 (*n* = 10)	0.07 ± 0.55 (*n* = 3)	0.77 ± 0.24 (*n* = 6)	2.5 ± 2.6 (*n* = 10)

ND not detected.
* Significantly different from background PBDE levels, $p < 0.05$.

recovery measurements, and an internal reference material (eel) were analysed each batch of 12–15 samples. Recoveries of 15 PBDEs were >80%, variation on duplicate analysis was less than 20%, and no or very low traces of BDE-47 and BDE-99 were found in procedural blanks (concentrations were corrected for blanks).

2.5. Thyroid hormone analyses

The levels of plasma T_3 and T_4 were analyzed by radioimmuno assay according to Friedrichsen et al. (2003), and as described by van der Ven et al. (2006) for flounder and zebrafish, respectively.

2.6. Determination of hepatic (EROD, PROD, BROD) and gonadal (aromatase) enzyme activities in flounder

Microsomes were prepared from gonads and rostral parts of the livers, and ethoxy-, pentoxy-, and benzoxy-*O*-deethylase (EROD, PROD and BROD) activities were determined in hepatic microsomes, and aromatase activity was determined in gonad microsomes as previously described (Kuiper et al., 2007).

2.7. Statistical analysis

Internal PBDE concentrations, thyroid hormone levels and enzyme activities were log-transformed prior to one-way analysis of variance (ANOVA; SPSS 12.0 for windows, SPSS, Chicago, IL) to compare exposure groups to controls (Dunnet's post hoc analysis); differences were considered statistically significant when $p < 0.05$. Zebrafish juvenile survival was evaluated using Kaplan-Meier analysis (SPSS 12.0); groups were considered statistically different when 95% confidence intervals showed no overlap. Dose–response modeling was performed on continuous endpoints according to Slob (2002) using PROAST software (RIVM, The Netherlands). Likelihood ratios were calculated to determine if an extension of the model resulted in a statistically significant improvement of the fit ($\alpha = 0.05$).

3. Results

3.1. Chemical analysis of exposed fish tissues

Both in flounder and zebrafish, internal PBDE levels increased with dose (Tables 1 and 2). BDEs-47, -49, -99, -100, -153, and -154 were the most abundant congeners in exposed fish, accounting for over 95% of the total PBDEs detected (Fig. 1). Gender-related differences in PBDE levels were not observed in exposed flounder or zebrafish. The proportion of BDE-47 was relatively constant in both species (87 ± 3% in zebrafish and 63 ± 6% in flounder (average ± SD)) but showed a mild (not statistically significant) decline in flounder exposed to 0.07 μg g^{-1} TOC in sediment and 1.4 μg g^{-1} lipid weight (lw) in food, and higher.

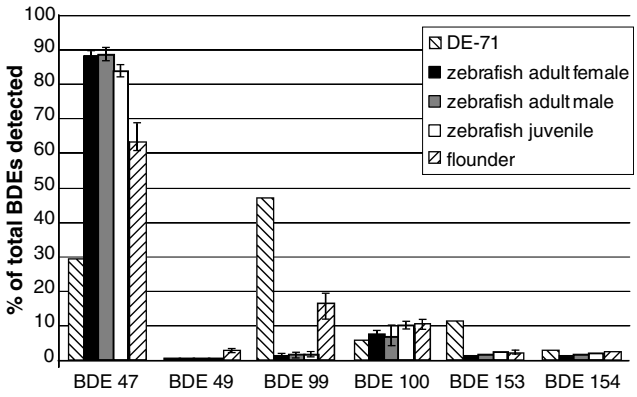


Fig. 1. Distribution of predominant PBDE congeners in DE-71-exposed flounder and zebrafish. Error bars represent standard deviations of percentages between the various dose groups.

Levels of BDE-49 were higher in flounder compared to zebrafish, and in the latter species BDE-49 did not substantially contribute to the total PBDE body burden except in adult controls where low levels of BDEs-28 and -49 were detected (<LOD-49 and 100 ng g^{-1} wet weight (ww), respectively). Although present at relatively low amounts, a clear dose-related increase was observed for BDE-183 in zebrafish (linear regression: $p < 0.01$, $R^2 = 0.88$), whereas BDE-183 was not detected in flounder. The sum of the eight most important PBDEs found in feral fish (∑8BDE: BDEs-28, -47, -49, -99, -100, -153, -154, and -183) is used to indicate the total PBDE levels in experimentally exposed fish. ∑8BDE levels in flounder muscle ranged between 0.10 and 308 μg g^{-1} ww and were significantly increased compared to background levels in animals exposed to the combination of 14 μg g^{-1} lw in food and 0.7 μg g^{-1} TOC in sediment, and higher (Table 1). The average internal level in the highest exposed flounders was almost three orders of magnitude greater than the background level; the total range was four orders of magnitude wide. In zebrafish, ∑8BDE levels were below the detection limit in adult controls and ranged up to 560 μg g^{-1} ww. Analysis of juvenile zebrafish indicated similar levels as were found in adults; levels in juveniles from exposed parents that were raised in control medium were within the range of the other

Table 2
internal PBDE concentrations and plasma thyroid hormone levels in adult zebrafish exposed to DE-71 for 30 days

Dose (μg DE-71/L)	μg/g wet weight internal ∑8BDE (mean ± SD)	μg/g wet weight internal BDE 47 (mean ± SD)	μg/g lipid internal BDE 47 (mean ± SD)	T_3 ng/ml plasma (mean ± SD)	T_4 ng/ml plasma (mean ± SD)
0	<LOD (<LOD-3.2; $n = 4$)	<1.3 (<1.3–2.5)	<27 (<27–57)	<1 ($n = 8$)	<5 ($n = 8$)
5	8.8 ± 4.4 (3.7–12; $n = 3$)	7.7 ± 3.7 (3.4–10)	246 ± 138 (97–370)	7.0 ± 6.0 ($n = 8$)	<5 ($n = 8$)
16	15 ± 3.8 (12–21; $n = 4$)	13.3 ± 3.4 (10–18)	404 ± 191 (196–619)	9.9 ± 2.1 ($n = 7$)	23 ± 20 ($n = 6$)
50	125.9 (78–174; $n = 2$)	114 (68–160)	8273 (2000–14545)	11 ± 5 ($n = 6$)	<5 ($n = 5$)
160	236 ± 46 (203.6–303.9; $n = 4$)	210 ± 41 (180.0–270.0)	6978 ± 2009 (4878–9310)	13 ± 5 ($n = 8$)	30 ± 19 ($n = 7$)
500	460 ± 82 (376–560; $n = 4$)	408 ± 79 (320–500)	9653 ± 3444 (5926–12941)	19 ± 9 ($n = 8$)	35 ± 18 ($n = 8$)

∑8BDE: the sum of BDEs-28, -47, -49, -99, -100, -153, -154, and -183.

controls and these groups were treated as similar for statistical analysis. All detectable levels of BDEs in zebrafish were highly linear with the nominal DE-71 exposure concentrations (BDE-47: $R^2 = 0.89$). Correction for lipid content of the fish improved regression statistics slightly in adult zebrafish ($R^2 = 0.93$, $p = 0.007$) but not in juveniles.

3.2. Gross observations, reproduction and development (zebrafish), histopathology

Flounder exposed to 0.007 μg g^{-1} TOC + 0.14 μg g^{-1} lipid (resp. sediment and food) and higher were slightly less active than control animals and animals exposed to 0.014 μg g^{-1} lipid (food only) at the start of exposure; after 2.5 weeks, this unexplained difference in behavior was no longer observed. Length, weight, and relative weights of livers and gonads were not related to exposure.

During exposure of adult zebrafish, no gross abnormalities were noted, and no exposure related differences in length and weight were observed. The total cumulative egg production showed a decrease in adults exposed to concentrations of 50 μg DE-71 l^{-1} and higher (Fig. 2). However, due to the large variation between groups this decrease was not statistically significant and a dose–response model could not be fitted consistently. Fertilization and hatching were not affected (Table 3). Exposure of juvenile zebrafish resulted in retardation of development followed by increasing mortality during the first week after hatching in animals exposed to 160 and 500 μg l^{-1}. The median post-hatching survival times in larvae exposed to 160 and 500 μg DE-71 l^{-1} were significantly and dose-dependently decreased (Table 3), and mortality was 100% after 10 days in affected groups. Juveniles from parents that had been exposed to 500 μg DE-71 l^{-1} placed in control medium developed normally and hatching and survival were not different from the controls. The lengths and weights of surviving juveniles after 45 days of exposure (averages: 16.8 ± 2.0 (SD) mm and 72.2 ± 25.0 (SD) mg, respectively) were similar in all groups and were not affected by exposure.

Apart from a mild diffuse hepatocellular hydropic swelling in surviving exposed zebrafish juveniles, dose-related histological changes were not detected in either species. Immunohistochemistry showed no evidence for increased levels of VTG or CYP1A in exposed zebrafish. Incidental pathological findings were limited and similar to previous reports (Kuiper et al., 2006, 2007).

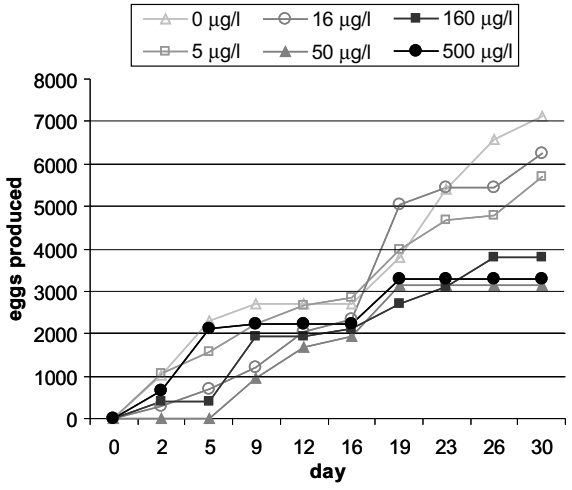
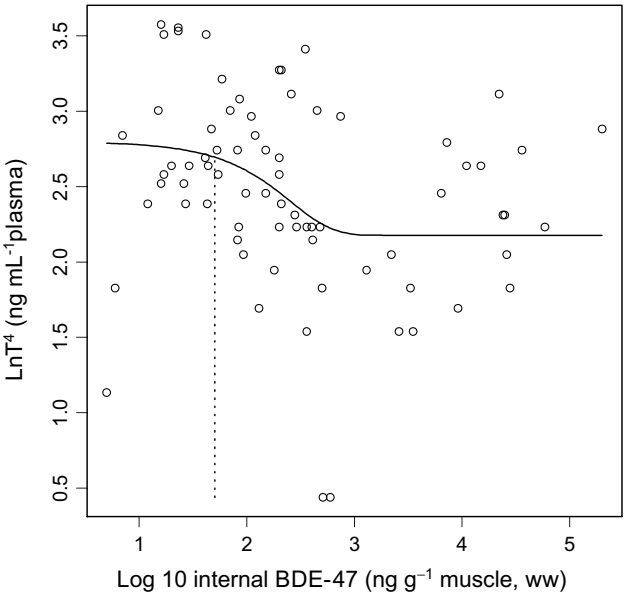


Fig. 2. Total cumulative egg production in zebrafish exposed to DE-71 for 30 days.



Model 4 according to Slob (2002):
$y = a[c\text{-}(c\text{-}1)\exp(\text{-}bx)]$ with
$a = 16.44$
$b = 4.8$
$c = 0.54$
log likelihood = –65.85

Fig. 3. Relation between T_4 and internal BDE-47 concentrations in flounder (muscle). The dotted line indicates the BDE-47 concentration at which the T_4 concentration is reduced by 10%.

Table 3
fertilization, hatching, median survival, and internal BDE 47 concentrations and size of surviving juvenile zebrafish after 45 days of exposure to DE-71

Dose (μg DE-71/l)	Fertilization (% ± SD)	Hatching (% ± SD)	Median survival (days)[a]	μg/g wet weight internal BDE-47 (mean ± SD)
0	96 ± 13	92 ± 5	>14	0.1 ± 0.2 (<0.02–0.5; $n = 6$)
5	91 ± 24	89 ± 9	>14	13 ± 4 (11–19; $n = 4$)
16	95 ± 15	92 ± 4	>14	42 (33–50; $n = 2$)
50[b]	100	86 ± 6	>14	73 ($n = 1$)
160	97 ± 9	93 ± 6	8 (7.96–8.04)[d]	NA
500[a]	95 ± 11	91 ± 4	6 (5.38–6.62)[d]	NA
500[c]	96 ± 13	92 ± 5	7[e]	NA

[a] Daily survival was counted during the first 2 weeks post hatching. Groups with median survival times >14 all showed >97% survival at the end of the 45 day exposure period.
[b] Due to insufficient egg production in these parent groups, eggs from the lower parent concentration group were used (16 and 160 μg l^{-1}, respectively).
[c] Eggs from control parents exposed to 500 μg l^{-1}.
[d] 95% confidence interval.
[e] All larvae died between day 6 and 7 post hatching; a 95% c.i. was not calculated.

3.3. Thyroid hormone concentrations in flounder and zebrafish

Levels of plasma thyroid hormones (T_3 and T_4) are shown in Table 1 (flounder) and 2 (zebrafish). Plasma T_3 levels in the flounder from this study ranged from 2.8 to 17.2 (average: 8.6) nM and T_4 levels ranged from 2.0 to 46.0 (average: 17.6) nM. A significant negative dose–response relation was detected only for T_4 (Fig. 3), indicating a 10% decrease at an internal concentration of 51 ng BDE-47 g^{-1} muscle (wet weight), with a lower 95% confidence limit (c.l.) of 19 ng g^{-1}. However, the estimated maximal decrease of 46% did not result in significant differences between group averages.

In contrast, in adult zebrafish, a mild but statistically significant positive dose–response was noted for plasma $T_{3/4}$ with increasing external and internal BDE-47 levels in adults (Table 2). T_4 concentrations doubled at a calculated internal dose of 116 (lower 95% c.l.: 79) μg BDE-47 g^{-1} tissue (wet weight). Due to substantial variation in T_3 concentration in control animals, a similar value for T_3 could not be confidently calculated. In both species, thyroid hormone levels did not depend on gender. Thyroid hormones were not analyzed in juvenile zebrafish.

3.4. Microsomal enzyme activities (flounder)

Hepatic EROD activities were generally low, ranging from undetectable to 39 pmol mg^{-1} min^{-1} with an overall average of 8.1 pmol mg^{-1} min. EROD activity showed a decreasing trend with increasing BDE-47 concentrations in muscle (Table 1). PROD and BROD activities were very low and did not relate to nominal or internal PBDE concentrations, but were weakly correlated to EROD activity ($R = 0.6$ and 0.3, respectively, $p < 0.01$; data not shown). Aromatase activities in female gonads generally exceeded those in male gonads. No relation between gonadal aromatase activity and BDE-47 concentrations in muscle was observed in male flounder, but a significant ($p < 0.05$) dose–response model indicated a reduction of 10% aromatase activity in female flounder already at a BDE-47 concentration of 40 ng g^{-1} muscle (wet weight; Fig. 4).

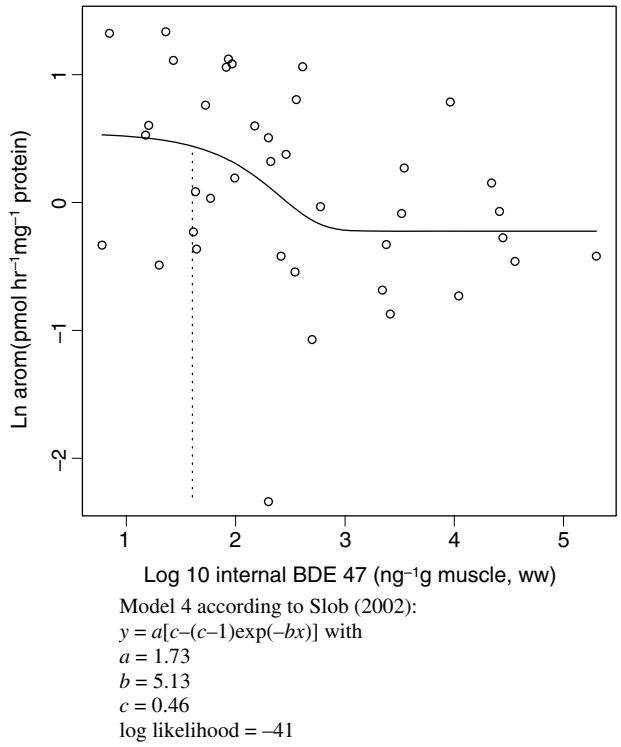


Fig. 4. Relation between aromatase activity in ovaries and internal BDE-47 concentrations in muscle of female flounder. The vertical dotted line indicates the internal BDE-47 concentration at which a 10% decrease of aromatase activity was calculated.

4. Discussion

The present results confirm uptake of PBDEs by fish not only via exposure of contaminated food and sediment (flounder), but also via the water phase (zebrafish). The observed congener pattern with predominance of BDE-47, followed by BDEs-99, -100, -153, and -154 in exposed flounder and zebrafish is in general agreement with reported patterns in both salt- and freshwater wild fish (Law et al., 2006), supporting a predominant role for commercial penta-BDE mixtures as a major PBDE source in aquatic biota in the environment. Relative loss of BDE-99 compared to the ∑8PBDEs in zebrafish is consistent with a higher debromination rate in other freshwater cyprinids as was shown in the intestine of common carp (*Cyprinus carpio*; Stapleton et al., 2004). The dose-related increase in the heptabrominated congener BDE-183 in whole body homogenates of zebrafish exposed to (another sample of) DE-71 was noted before (Kuiper et al., 2006), and indicates that BDE-183 is present in, and bioavailable from, this commercial pentabromodiphenylether mixture. Hence, in wild aquatic biota, BDE-183 may not exclusively originate from higher brominated products (octaBDE mixtures). Presently, low levels of lipophilic BDE-183 may have remained undetected in flounder muscle. The ratios between PBDE congeners were very constant, regardless of the total amount of PBDEs present, except for the lower brominated BDEs-49 and -47, and in zebrafish, BDE-28, which were relatively high when detected in control or lower dosed groups. This trend indicates that the background PBDE levels did not originate from the exposure mixture and were likely present before the start of the experiments, reflecting the ubiquitous nature of these lower brominated PBDEs in biota. Dose–response modeling of continuous parameters was based on internal wet weight concentrations of BDE-47, the predominant BDE congener found in biota in the marine environment (Birnbaum and Staskal, 2004).

The lack of grossly observable effects in both adult flounder and zebrafish exposed to high doses of DE-71 is consistent with existing literature (Birnbaum and Staskal, 2004; Kuiper et al., 2006). However, the high mortality in juvenile zebrafish exposed to 160 and 500 μg pentaBDE l^{-1} points to higher sensitivity of juvenile fish, and levels at which toxicity became apparent are consistent with the observations of Lema et al. (2007). Hepatocellular swelling was difficult to interpret due to the low numbers of surviving juveniles at higher exposure doses; however, the histological appearance was not consistent with fatty vacuolation as reported in earlier studies (Holm et al., 1993; Norrgren et al., 1993) and could reflect increased glycogen reserves in survivors. Juvenile development in our study was not influenced by the exposure status of the parents, indicating that transfer of PBDEs via the egg does not contribute to exposure-associated risk. Although juvenile mortality at 50 μg l^{-1} was not different from the controls, a not statistically significant reduction of cumulative egg production at that concentration indicates that the dose at which reproductive success would be affected could be slightly lower. The average internal ∑PBDE level in parents (126 μg g^{-1} ww) exposed to that concentration exceeded environmental top levels (2270 ng g^{-1} ww in burbot, *Lota lota*, from lake Mjøsa, Norway; Mariussen et al., 2003) 55-fold. Internal PBDE levels in marine fish vary normally in the low ng g^{-1} ww range (e.g., de Boer, 1989; Boon et al., 2002; Voorspoels et al., 2004), indicating a lower risk for the marine environment. Recent discontinued production of PeBDE mixtures has halted further rise of lower brominated PBDEs in

environmental samples (Gauthier et al., 2008). Taken together these data indicate a limited risk for reduced production of offspring even under current heavily contaminated field conditions.

The changes in thyroid hormone levels are contradictory between the two species under investigation. Although the mild decrease in T_4 observed in flounder did not result in statistically significant differences between exposure groups, a decrease in T_4 would be consistent with previous observations in rats and fish exposed to PBDEs (Stoker et al., 2004; Tomy et al., 2004). Moreover, the dose–response model indicates that a decrease may occur at concentrations that are occasionally observed in the environment. In contrast, dose–response modeling significantly indicated a dose-related increase of T_3 and T_4 in zebrafish in the present study. The internal PBDE concentrations reached in zebrafish were higher than those in flounder and the limited number of observations in the environmentally relevant range in zebrafish renders comparison of these species somewhat difficult. Interpretation of the zebrafish data is furthermore hampered by the large variation especially in T_3 values at the lower, environmentally relevant internal BDE-47 concentrations. This variation may have resulted from sampling errors, given the very small plasma volumes obtained from the zebrafish. Reduced growth in zebrafish larvae has been observed after induced hypothyroidism, but without significant mortality as observed in the present study (van der Ven et al., 2006). The impact of increased thyroid hormone levels on growth and reproduction has not been investigated in fish. Although the lack of coincident histological changes in the thyroid glandular tissue in both species indicates a minor impact on functional thyroid status, a role for altered activities of thyroid hormone metabolizing enzymes at the level of target tissues like the teleost ovary (Brown et al., 2004) cannot be ruled out as a possible mechanism behind the observed mild (not statistically significant) reduction in egg production. It is noteworthy that T_3 inhibited aromatase activity in cultured rat sertoli cells (Ulisse et al., 1994) possibly resulting in reduced production of estrogens. However, apart from the indicated mild reduction of egg production, no anti-estrogenic effects were noted in exposed fish. Aromatase activities were not related to levels of $T_{3/4}$ in flounder in the present study, and there were no histological indications for estrogen dependent production of the yolk precursor VTG. Rather, in flounder ovaries, aromatase activity decreased with exposure pointing to possible indirect anti-estrogenicity already at relatively low exposure concentrations. Due to the low oogenic activity observed in the flounders, we can at present not exclude anti-estrogenic effects in that species on the basis of morphology. Further study of androgen/estrogen ratios in reproducing females is needed to evaluate the physiological relevance of this observation.

The lack of induction of EROD activity in flounder or production of immunodetectable CYP1A protein in zebrafish exposed to cleaned DE-71 is consistent with earlier results both *in vitro* and *in vivo*. DE-71 or the environmentally relevant highly purified PBDE congeners did not activate the AhR pathway in different cell lines including carp hepatocytes (Kuiper et al., 2004; Hamers et al., 2006). Cleaned DE-71 did furthermore not induce CYP1A protein formation in zebrafish (Kuiper et al., 2006). The present results confirm that the cleaning of our DE-71 sample was adequate and excludes AhR activation as a possible mediator of the observed effects on thyroid hormone levels and aromatase activities. The low PROD and BROD activities and their correlation with EROD indicates that these marker reactions are partly related to CYP1A activity and have no additional value in the evaluation of exposure to PBDEs.

In summary, our results confirm that exposure to commercial pentaBDE results in the congener pattern observed in wild fish. The dose-dependent increase of BDE-183 in zebrafish indicates that the presence of this congener in wild fish may also originate from the late use of PeBDE. Although histopathological changes, or statistically significant changes to thyroid hormone levels or microsomal enzyme activities were not observed in exposed fish, dose–response modeling indicated that a mild decrease of T_4 concentrations in plasma, and aromatase activity in ovaries might occur at environmentally relevant levels in flounder. Survival of juvenile zebrafish was the most sensitive parameter for statistically significant adverse effects on reproductive success, but reduced egg production is indicated at slightly lower internal PBDE concentrations; internal concentrations at which these effects occurred were at least 55 times higher than the highest levels reported in wild fish. No indications were found for dioxin-like effects of cleaned PBDEs in fish.

Acknowledgement

To the authors thank Åke Bergman and co-workers (Stockholm University, Sweden) for the purification of the DE-71 sample. Timo Hamers (VU University, Amsterdam, The Netherlands) confirmed absence of AhR-agonists. Theo Visser (Erasmus Medical Center, Rotterdam, The Netherlands), and Deborah Power (University of Algarve, Faro, Portugal) performed the thyroid hormone analyses in flounder and zebrafish. This study was part of the EU-sponsored project "Flame-retardants Integrated Risk Assessment" (FIRE), Contract No. QLK4-CT-2002-00596.

References

Birnbaum, L.S., Staskal, D.F., 2004. Brominated flame retardants: cause for concern? Environ. Health Perspect. 112, 9–17.

Boon, J.P., Lewis, W.E., Tjoen-A-Choy, M.R., Allchin, C.R., Law, R.J., de Boer, J., ten Hallers-Tjabbes, C.C., Zegers, B.N., 2002. Levels of polybrominated diphenyl ether (PBDE) flame retardants in animals representing different trophic levels of the North sea food web. Environ. Sci. Technol. 36, 4025–4032.

Brown, S.B., Adams, B.A., Cyr, D.G., Eales, J.G., 2004. Contaminant effects on the teleost fish thyroid. Environ. Toxicol. Chem. 23, 1680–1701.

de Boer, J., 1989. Organochlorine compounds and bromodiphenylethers in livers of Atlantic cod (*Gadus morhua*) from the North sea, 1977–1987. Chemosphere 18, 2131–2140.

Friedrichsen, S., Christ, S., Heuer, H., Schäfer, M.K.H., Mansouri, A., Bauer, K., Visser, T.J., 2003. Regulation of iodothyronine deiodinases in the Pax-/- mouse model of congenital hypothyroidism. Endocrinology 144, 777–784.

Gauthier, L.T., Hebert, C.E., Chip Weseloh, C.V., Letcher, R.J., 2008. Dramatic changes in the temporal trends of polybrominated diphenylethers (PBDEs) in herring gull eggs from the Laurentian Great Lakes: 1982–2006. Environ. Sci. Technol. 42, 1524–1530.

Hamers, T., Kamstra, J.H., Sonneveld, E., Murk, A.J., Kester, M.H.A., Andersson, P.L., Legler, J., Brouwer, A., 2006. In vitro profiling of the endocrine disrupting potency of brominated flame retardants. Toxicol. Sci. 92, 157–173.

Holm, G., Norrgren, L., Andersson, T., Thurén, A., 1993. Effects of exposure to food contaminated with PBDE, PCN or PCB on reproduction, liver morphology and cytohrome P450 activity in the three-spined stickleback, *Gasterosteus aculeatus*. Aquat. Toxicol. 27, 33–50.

Janssen, P.A.H., Lambert, J.G.D., Vethaak, A., Goos, H.J.T., 1997. Environmental pollution caused elevated concentrations of oestradiol and vitellogenin in the female flounder, *Platichthys flesus* (L). Aquat. Toxicol. 39, 195–214.

Kuiper, R.V., Bergman, Å., Vos, J.G., van den Berg, M., 2004. Some polybrominated diphenyl ether (PBDE) flame retardants with wide environmental distribution inhibit TCDD-induced EROD activity in primary cultured carp (*Cyprinus carpio*) hepatocytes. Aquat. Toxicol. 68, 129–139.

Kuiper, R.V., Murk, A.J., Leonards, P.E.G., Grinwis, G.C.M., van den Berg, M., Vos, J.G., 2006. In vivo and in vitro Ah-receptor activation by commercial and fractionated pentabromodiphenylether using zebrafish (*Danio rerio*) and the DR-CALUX assay. Aquat. Toxicol. 79, 366–375.

Kuiper, R.V., Cantón, R.F., Leonards, P.E.G., Jenssen, B.M., Dubbeldam, M., Wester, P.W., van den Berg, M., Vos, J.G., Vethaak, A.D., 2007. Long-term exposure of European flounder (*Platichthys flesus*) to the flame retardants tetrabromobisphenol A (TBBPA) and hexabromocyclododecane (HBCD). Ecotoxicol. Environ. Safety 67, 349–360.

Law, R.J., Allchin, C.R., de Boer, J., Covaci, A., Herzke, D., Lepom, P., Morris, S., Tronczynski, J., de Wit, C.A., 2006. Levels and trends of brominated flame retardants in the European environment. Chemosphere 64, 187–208.

Lema, S.C., Schultz, I.R., Scholz, N.L., Incardona, J.P., Swanson, P., 2007. Neural defects and cardiac arrhythmia in fish larvae following embryonic exposure to 2,2′,4,4′-tetrabromodiphenyl ether (PBDE-47). Aquat. Toxicol. 82, 296–307.

Mariussen, E., Fjeld, E., Strand-Andersen, M., Hjerpset, M., Schlabach, M., 2003. Spatial distribution of polybrominated diphenyl ethers in trout from Norwegian lakes. Organohalogen Compd. 61, 69–72.

Marsh, G., Hu, J.W., Jakobsson, E., Rahm, S., Bergman, A., 1999. Synthesis and characterization of 32 polybrominated diphenyl ethers. Environ. Sci. Technol. 33, 3033–3037.

Morgado, I., Hamers, T., van der Ven, L., Power, D.M., 2007. Disruption of thyroid hormone binding to seabream recombinant transthyretin by ioxinyl and polybrominated diphenylethers. Chemosphere 69, 155–163.

Norrgren, L., Andersson, T., Björk, M., 1993. Liver morphology and cytochrome-P450 activity in fry of rainbow-trout after microinjection of lipid-soluble xenobiotics in the yolk-sac embryos. Aquat. Toxicol. 26, 307–316.

Slob, W., 2002. Dose–response modeling of continuous endpoints. Toxicol. Sci. 66, 298–312.

Stapleton, H.M., Letcher, R.J., Baker, J.E., 2004. Debromination of polybrominated diphenyl ether congeners BDE 99 and BDE 183 in the intestinal tract of the common carp (Cyprinus carpio). Environ. Sci. Technol. 38, 1054–1061.

Stoker, T.E., Cooper, R.L., Lambright, C.S., Wilson, V.S., Furr, J., Gray, L.E., 2005. In vivo and in vitro anti-androgenic effects of DE-71, a commercial polybrominated diphenyl ether (PBDE) mixture. Toxicol. Appl. Pharm. 207, 78–88.

Stoker, T.E., Laws, S.C., Crofton, K.M., Hedge, J.M., Ferell, J.M., Cooper, R.L., 2004. Assessment of DE-71, a commercial polybrominated diphenyl ether (PBDE) mixture, in the EDSP male and female pubertal protocols. Toxicol. Sci. 78, 144–155.

Timme-Laragy, A.R., Levin, E.D., Di Giulio, R.T., 2006. Developmental and behavioral effects of embryonic exposure to the polybrominated diphenylether mixture DE-71 in the killifish (*Fundulus heteroclitus*). Chemosphere 62, 1097–1104.

Tomy, G.T., Palace, V.P., Halldorson, T., Braekevelt, E., Danell, R., Wautier, K., Evans, B., Brinkworth, L., Fisk, A.T., 2004. Bioaccumulation, biotransformation, and biochemical effects of brominated diphenyl ethers in juvenile lake trout (*Salvelinus namaycush*). Environ. Sci. Technol. 38, 1496–1504.

Ulisse, S., Jannini, E.A., Carosa, E., Piersanti, D., Graziano, F.M., Darmiento, M., 1994. Inhibition of aromatase-activity in rat sertoli cells by thyroid-hormone. J. Endocrinol. 140, 431–436.

van der Ven, L.T.M., Holbech, H., Fenske, M., van den Brandhof, E.J., Gielis-Proper, F.K., Wester, P.W., 2003a. Vitellogenin expression in zebrafish *Danio rerio*: evaluation by histochemistry, immunohistochemistry, and in situ mRNA hybridisation. Aquat. Toxicol. 65, 1–11.

van der Ven, L.T.M., Wester, P.W., Vos, J.G., 2003b. Histopathology as a tool for the evaluation of endocrine disruption in zebrafish (*Danio rerio*). Environ. Toxicol. Chem. 22, 908–913.

van der Ven, L.T.M., van den Brandhof, E.J., Vos, J.H., Power, D.M., Wester, P.W., 2006. Effects of the antithyroid agent propylthiouracil in a partial life cycle assay with zebrafish. Environ. Sci. Technol. 40, 74–81.

Voorspoels, S., Covaci, A., Schepens P., 2004. PBDEs in sediments from a polluted area in Europe: The Belgian North Sea, the Western Scheldt estuary and tributaries. In: Proceedings of the Third International Workshop on Brominated Flame Retardants BFR 2004, Toronto, Canada, June 6–9, pp. 133–136.