Toxicity of Brominated Flame Retardants in Fish, with Emphasis on Endocrine Effects and Reproduction

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Toxicity of Brominated Flame Retardants in Fish, with Emphasis on Endocrine Effects and Reproduction

Toxiciteit van gebromeerde vlamvertragers in vis, met bijzondere aandacht voor effecten op de hormoonhuishouding
(met een samenvatting in het Nederlands)

Proefschrift

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1

General introduction

Introduction

The increasing use of petroleum based polymeric materials in industry and every-day life necessitates addition of flame retardants to meet fire safety criteria. Around 25% of the flame retardant chemicals used are halogenated hydrocarbons, and the most important of these are the brominated flame retardants (BFRs). The total production of BFRs has been increasing since the early 1970s and exceeded 200,000 tons in 1999 (Bromine Science and Environmental forum, BSEF, 2000). Increasing production of BFRs has been followed closely by increasing contamination of both terrestrial and aquatic environments around the world, and BFRs and unintentional by-products (e.g. polybrominated dibenzo-*p*-dioxins and -furans) form a relatively large and recent antropogenic addition to the mixture of chemicals in our environment. Despite their clear benefit of reducing the risk of fires and associated personal accidents, concerns were raised on the potential disadvantageous effects associated with exposure, in humans and wildlife (Birnbaum and Staskal, 2004). Concurrent with mounting evidence for endocrine-related effects of a variety of antropogenic chemicals in wildlife including fish, increasing evidence was found for effects of BFRs on several endocrine systems (Vos et al., 2003). This thesis addresses the significance of BFRs as endocrine disrupters in the aquatic environment. The studies presented and discussed form part of the integrated risk assessment program "FIRE" (Flame Retardants Integrated Risk assessment for Endocrine effects), and focus in particular on possible toxic effects of BFRs in fish with emphasis on endocrine related effects.

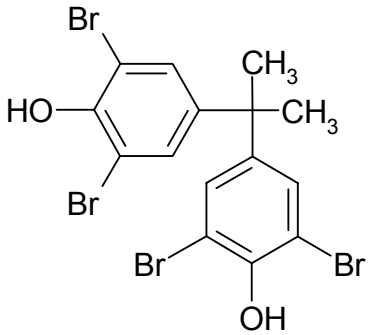
1 Brominated flame retardants

BFRs form a diverse group of organic chemicals, with over 100 different chemicals currently produced, which share a variable extent of bromine substitution as a functional feature. Heating of the relatively labile C-Br bond produces $Br^{\cdot}$ radicals that catalyze transfer of protons to scavenge highly reactive $H^{\cdot}$ and $HO^{\cdot}$ radicals that propagate the fire (Price, 1989). The most abundantly produced BFRs are members of three major chemical classes:

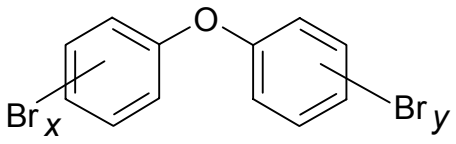
Tetrabromobisphenol A (TBBPA; Fig. 1). Currently the most abundantly produced BFR (59%, Bromine Science and Environmental Forum BSEF, 2000). Featuring two aromatic rings, the structure bears some resemblance to that of the polychlorinated biphenyls (PCBs), with the important distinctions that the rings are less closely positioned and the bromine substituents prevent the molecule from adopting a co-planar configuration necessary for arylhydrocarbon receptor (AhR) activation, one of the toxicological hallmarks of planar PCBs and dioxins. Because the molecule features two terminal reactive hydroxyl groups, the compound can be used reactively in a process where the molecule is covalently incorporated in the polymer matrix. However, 10% of the total production (a considerable amount still when compared to the total BFR use) is used additively, i.e. physically dispersed in the polymer, which increases the risk of leaching from the product (Birnbaum and Staskal, 2004). In the -aquatic- environment, the polar hydroxyl groups can increase water solubility and bioavailability of the chemical.

Polybrominated diphenylethers (PBDEs, Fig. 1). Prominently featuring two aromatic rings, the PBDEs also resemble PCBs; however, the opportunities for AhR activation are again reduced by the larger bromine substituents, especially when present adjacent to the ether linkage (ortho position), that in combination with the angle introduced by the ether linkage prevent a planar configuration by stereological interference. There are 10 positions for halogen substitution available, and congeners are identified by numbers analogous to the PCB numbering according to Ballschmiter and Zell (1980). PBDEs are subdivided in three major classes based on the average number of bromine substituents in the commercial product, which is typically a mixture of similarly sized congeners with on average 5, 8, or 10 bromine atoms: Penta-, Octa-, and DecaBDE (DecaBDE being currently the second most abundantly used BFR). Water solubility and bioavailability decrease with increasing bromine substitution. Lower brominated PBDEs (especially PentaBDE commercial product) are most frequently found in biota and generally regarded most potent and hazardous.

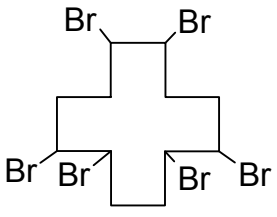
Hexabromocyclododecane (HBCD; Fig. 1). Today's third most abundantly produced BFR. Based on the respective orientation of the C-Br bonds, three diastereomers are recognized (α-, β-, and γ-HBCD), and the commercially sold technical product consists of a mixture with typically around 80% α-HBCD. There are no markedly polar sites in the molecule, and hence water solubility is low. However, HBCD is bioavailable and the compound's lipophilic nature and resistance to metabolism result in a relatively strong concentration in biota, as indicated by the relatively high bioconcentration factor (BCF) of around 20,000 (Veith et al., 1979; Drottar et al., 2001).



Tetrabromobisphenol A



Polybrominated diphenylethers



Hexabromocyclododecane

Figure 1. Chemical structures of common BFRs.

2 Exposure

BFRs may reach the aquatic environment via a number of ways, the relative importance of which depends on the physicochemical properties of the compound. Leaching from landfills and point sources like production sites may contaminate surface waters, and long range atmospheric transport may add to this in particular for the more stable compounds such as HBCD and PBDEs (Law et al., 2006). As a result of their limited solubilities in water, BFRs in the aquatic environment are predominantly found in sediment, and depending on their molecular size and half-life also in biota. Relatively high levels of lower brominated PBDEs and α-HBCD have recently been detected in fish (Law et al., 2006) and temporal trend studies have shown increasing levels in biota for the most bioaccumulative BFRs (e.g. 2,2',4,4'-tetraBDE (BDE-47), and α-HBCD; Sellström et al., 2003; Kierkegaard et al., 2004; Zhu and Hites, 2004). During the peak use of commercial pentamixtures in the 1980s, PBDE levels increased in fish liver while other common pollutants such as hexachlorobenzene (HCB) and dieldrin showed decreasing trends (de Boer et al., 1989). Following restrictions on the use of PBDEs in Scandinavia and probably as a result thereof, the increasing PBDE-levels in aquatic biota leveled off or even decreased starting from the mid-1980s in these areas (Sellström et al., 2003; Kierkegaard et al., 2004). The rapid response in environmental PBDE levels indicates that further reduction of the levels of these contaminants is to be expected following the voluntary cessation of production in the USA by the end of 2004. However, local sources, ongoing production elsewhere (Asia) and global redistribution may still result in continued exposure of relatively remote areas (de Boer, 1998; Birnbaum and Staskal, 2004; Kierkegaard et al., 2004; Vives et al., 2004).

Temporal data on HBCD levels in fish are more limited. HBCD levels have been rising in eggs of fish-eating birds (common guillemot, *Uria algae*) from the Baltic region and Norway already since the 1980s (Sellström et al., 2003; Føreid et al., 2006). Since HBCD was shown to biomagnify in the food chain (Sørmo et al., 2006), these results also indicate rising levels in fish. As more HBCD is still being released into the environment, future levels in fish may increase even further. In spite of higher production of TBBPA and DecaBDE, the levels of TBBPA and DecaBDE (BDE-209) in wild fish are generally low (Morris et al., 2004; Sørmo et al., 2006). However, the reasons for these lower levels can be quite different: TBBPA might be rapidly taken up but is equally rapidly excreted (half-life in fish <1 day, WHO, 1995) whereas DecaBDE has a longer half-life (26 days in lake trout, *Salvelinus namaycush*), but only limited amounts are taken up even after prolonged oral exposure due to its low solubility and large molecular size (Tomy et al., 2004). Levels of rapidly eliminated contaminants, such as TBBPA, are less indicative of cumulative exposure when compared to more bioaccumulating contaminants such as PCBs and lower brominated PBDEs. Hence the risks associated with exposure may be more easily underestimated, particularly when toxic metabolites are formed. In the case of DecaBDE, which has a longer half-life (Kierkegaard et al., 1999), general absence in wild fish strongly indicates limited bioavailability, and therefore toxicity of BDE-209 will not be addressed in this thesis.

3 Biological effects

It has become increasingly clear that BFRs can modulate different endocrine systems (Vos et al., 2003). *In vitro* studies with BFRs have shown that PBDEs, HBCD, and TBBPA may interfere in particular with the thyroid and sex steroid hormone systems. (Anti-)estrogenic properties of PBDEs or their hydroxyl metabolites were demonstrated in an estrogen responsive reporter gene assay (Meerts et al., 2001), and a number of (hydroxylated) PBDEs were shown to affect aromatase (estrogen synthetase) activity (Cantón et al., 2005). The structural resemblance of TBBPA and particularly hydroxylated PBDE metabolites with thyroid hormones may cause interference with binding of thyroid hormones to plasma transport proteins as was shown for human transthyretin (Meerts et al., 2000). TBBPA and HBCD augmented the response to the biologically active thyroid hormone triiodothyronin (T_3) *in vitro* (Kitamura et al., 2002; Schriks et al., 2006). In contrast, TBBPA antagonized T_3 responsive tail shortening in *Rana rugosa* tadpoles (Kitamura et al., 2005). As part of the FIRE program, extensive *in vitro* prescreening of individual BFRs was performed (Hamers et al., 2006; overview in Table 1). TBBPA, HBCD, as well as a number of environmentally prominent PBDEs (including the predominant constituents of commercial pentamix, BDEs -47, -99, and -100) were shown to interact with androgen, progesterone and estrogen receptor (AR-, PR-, and ER-) mediated effects, as well as the binding of thyroid hormone T_4 to plasma proteins (TBBPA and BDE-47). Potentiation of the active thyroid hormone T_3 was confirmed for TBBPA and HBCD, and a number of other BFRs including BDE-100. Consistent with earlier observations (Peters et al., 2004), none of these dominant BFRs showed notable activation of the arylhydrocarbon receptor (AhR). However, a number of environmentally important compounds including BDEs -47, -99, and -100 inhibited the AhR-mediated response induced by the model activating dioxin (2,3,7,8-tetrachloro-dibenzo-*p*-dioxin, TCDD). The biological impact of interactions of flame retardants and their possible metabolites with endocrine systems and their effects on related toxicological endpoints in entire organisms including fish are currently poorly understood.

A number of studies have addressed toxicity of BFRs in fish. TBBPA was acutely toxic in the mg/L range for a number of different species, including zebrafish (*Danio rerio*) (WHO, 1995). Intraperitoneal exposure to TBBPA increased hepatic gluthation reductase activity in rainbow trout, indicating oxidative stress, and this observation calls for evaluation of oxidative damage in TBBPA exposed fish (Ronisz et al., 2004). Induction of hepatic catalase activities, possibly indicating proliferation of peroxisomes, was inconclusive in rainbow trout (Ronisz et al., 2004). Possible oxidative damage or peroxisome proliferation by BFRs in fish have not been addressed in morphologic studies. Exposure to TBBPA or HBCD did not induce the estrogen dependent yolk precursor protein vitellogenin (VTG) in male rainbow trout (Christiansen et al., 2000; Ronisz et al., 2004), showing that these compounds do not exhibit direct ER-mediated effects. At present, thyroid-related effects and their possible impact on reproduction and development in fish have not been investigated.

Exposure to PBDEs reduced spawning and caused hepatic lipidosis in zebrafish (Holm et al., 1993; Norrgren et al., 1993). Activity of ethoxyresorufin-*O*-deethylase (EROD) was also increased, probably caused by contaminating AhR agonists (Norrgren et al., 1993). Indeed, polybrominated dibenzo-*p*-dioxins and -furans in commercial PBDE were responsible for AhR-mediated effects in rats (Sanders et al., 2005).

Table 1. *In vitro* effects of BFRs on selected endocrine endpoints. AR, PR, ER: androgen, progestagen, and estrogen responsive; E2SULT: estrogen sulfotransferase; TTR: transthyretin binding; T_4: thyroxin; T_3: triiodothyronin; AhR: aryl hydrocarbon receptor; DR: dioxin responsive; DHT: dihydrotestosteron; MPA: medroxy-progesteronacetate; E2: 17β-estradiol; PCP: pentachlorophenol (adopted from Hamers et al., 2006).

Technical product	Steroid hormones				Thyroid		AhR-mediated
assay (+,- reference)	AR-CALUX (DHT, flutamide)	PR-CALUX (MPA, RU-486)	ER-CALUX (E2, ICI 182,780)	E2SULT (PCP)	TTR binding (T_4)	T-screen (T_3)	DR-CALUX (TCDD, PCB-128)
Reference	↓ IC50=1.3μM	↓ IC50=0.2nM	↓ IC50=0.14nM ↑ EC50=4.0pM	↓ IC50=0.15μM	↓ IC50=55nM	↑ EC50=0.25nM	↓IC50=3.0μM
TBBPA	N.E.	N.E.	N.E.	↓ IC50=0.016μM	↓ IC50=0.031μM	↑ 23%[1]	N.E.
HBCD	↓ IC50>15μM	↓ IC50=1.6μM	↓ IC50>15μM	N.E.	N.E.	↑ 24%[1]	↓ IC50>15μM
PentaMix	↓ IC50=2.0μM	↓ IC50=10μM	N.E.	↓ IC50>15μM	N.E.	N.A.	↓ IC50=4.5μM
BDE-47	↓ IC50=1.0μM	↓ IC50>15μM	↑ EC50=7.0μM	↓ IC50=0.8μM	↓ IC50>25μM	N.E.	↓ IC50=2.7μM
BDE-99	↓ IC50=7.8μM	↓ IC50>15μM	N.E.	N.E.	N.E.	N.E.	↓ IC50=13μM
BDE-100	↓IC50=0.10μM	↓ IC50=3.4μM	↑ EC50=12μM	↓ IC50>15μM	N.E.	↑ 38%[1]	↓ IC50>15μM

[1]additional effect (%) of 1 μM of test chemical to 0.25 nM T_3 exposure in T_3 responsive rat pituitary adenoma cells.
N.E.: no effect.
N.A.: not analyzed.
IC50: concentration at which effect of positive control is reduced by 50%.
EC50: concentration at which 50% of maximum effect of positive control is reached.

In addition, PBDEs delayed hatching of killifish (*Fundulus heteroclitus*) embryos (Timme-Laragy et al., 2005). Although these results suggest effects of PBDEs on reproduction and development, straightforward evidence for disruption of underlying endocrine mechanisms is lacking. Furthermore, the suggested impact of contaminating AhR agonists has not been specifically investigated in fish *in vivo*.

4 Test species

As a result of the hydrophobicity of most BFRs, sediment-rich areas with relatively low flow rates such as river deltas and estuaries are the typical sites for exposure of aquatic species especially when close to industrial or heavily populated areas. It is for this reason that estuarine bottom-dwelling species such as the European flounder (*Platichthys flesus*) are suitable and popular for biomonitoring purposes and measuring of adverse effects. A number of (pathological) conditions in flatfish, like the flounder, in natural habitats as well as under laboratory conditions, were found to be associated with exposure to pollutants including polyhalogenated aromatic compounds (PHAHs). Pollutants such as PCBs, polyaromatic hydrocarbons (PAHs), DDT and its metabolites, and metals have been associated with e.g. a higher prevalence of skin lesions, and hepatic lesions including vacuolization of hepatocytes and bileduct epithelium, degeneration/necrosis, and (pre) neoplastic change in e.g. English sole (*Pleuronectes vetulus*), starry flounder (*Platichthys stellatus*) (Myers et al., 1994), and European flounder (Köhler et al., 1990, 2002; Vethaak et al., 1996; Vethaak and Jol, 1996; Vethaak and Wester, 1996; Stentiford, 2003). The prevalence of the common microsporidian parasite *Glugea stephani* may also be increased in relation to contaminant exposure, as was observed in winter flounder (*Pleuronectes americanus*) from localities where paper mill effluent was discharged. This effect has been proposed as an indicator for impaired immune function in affected flatfish (Kahn, 2004). More direct support for these observations and exposure to organohalogens was observed in large-scale mesocosm studies. European flounder that were exposed to contaminated harbor sludge showed accelerated oocyte ripening that could be attributed to a possible estrogenic effect of the contaminants present (Janssen et al., 1995, 1997). Hermaphroditism has also been reported in European flounder (Allen et al., 1999a; Minier et al., 2000) possibly associated with exposure to synthetic (xeno)estrogens. These parameters can be histologically classified according to severity in European flounder (Bateman et al, 2004). The latter is necessary for establishing dose-response relations that should be used to evaluate real world contamination levels in the aquatic environment. In addition, biochemical markers have been successfully applied in this species to provide insight in more specific toxic and biological mechanisms. Induction of the monooxygenase cytochrome P450 1A (CYP1A) enzyme and related EROD activity, particularly under controlled laboratory conditions, is a useful indicator of AhR activation by dioxin-like contaminants (Eggens et al., 1995; Besselink et al., 1997; Grinwis et al., 2000), and increased production of the yolk precursor VTG, particularly when observed in males, indicates exposure to estrogenic compounds. (Allen et al., 1999a,b; Lahr et al., in press; Vethaak et al., 2002).

Because of their ecological relevance with regard to the suspected endocrine effects of BFRs (Arcand-Hoy, 1998), reproductive parameters were also included in the experiments presented in this thesis. As was concluded earlier (Wester et al, 2002), the

larger size of the animals and relatively long reproduction cycle, as well as the obligate husbandry conditions (migration off-shore before spawning) makes the European flounder a less suitable sentinel species for investigating reproduction parameters. Histopathologic evaluation of a variety of small fish species including guppy (*Poecilia reticulata*), Japanese medaka (*Oryzias latipes*), and zebrafish (*Danio rerio*) was found to be a successful method to detect the toxicity of a variety of chemicals from different chemical classes, including the xenoestrogen β-hexaclorocyclohexane and the goitrogen sodium bromide (Wester and Vos, 1994; Wester et al., 2002.) In our studies, zebrafish was chosen as a smaller species in addition to European flounder, because it readily reproduces in the laboratory and allows for follow-up studies with offspring. An additional advantage of the small size is the use of whole body histology, which facilitates microscopic evaluation of a wide range of organs both in adults and juveniles. The histological alterations in zebrafish that have been exposed to (synthetic) estrogens are now well documented. Estrogen-induced hepatic VTG production is visible as increased basophilic staining of the liver (van der Ven et al., 2003a), and a relatively sensitive endpoint in zebrafish when compared to other popular test species including fathead minnow, medaka, and rainbow trout (Örn et al., 2006; Rose et al., 2002). Exposure to 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) also resulted in feminization of gonads in developing male zebrafish to a variable extent, ranging from a higher prevalence of a tentative female bilateral gonad attachment to skewed sex ratio indicative of total feminization of at least a number of males (Brion et al., 2004; Maack and Segner, 2004). Exposure of juveniles during development of gonadal sexual dimorphism to EE2 furthermore resulted in reduced fertilization of eggs produced as adults (Maack and Segner, 2004). Although this indicates a relatively delayed effect of exposure to estrogens, histological hallmarks of VTG induction respond much more acutely at similar exposure concentrations (3 ng/L; van der Ven et al., 2003a). Including histopathology in the study protocol can therefore reduce the need for more prolonged studies. In addition, exposure of zebrafish to the anti-estrogen tamoxifen resulted in other histopathological changes like reduced numbers of mature, and increased numbers of atretic oocytes in adults, predominance of male offspring and retarded gonad development in juveniles (van der Ven et al., 2003b; Wester et al., 2003). Sensitivity to thyroid-affecting compounds was indicated by the thyroid hypertrophy induced by the goitrogen propylthiouracil (van der Ven et al., 2006). Developing stages in particular also show well-documented sensitivity to planar PHAHs, with a mechanism of action mediated through the AhR. Although early life stage mortality resulting from exposure to AhR agonists is a less sensitive parameter in zebrafish than e.g. in fathead minnow, medaka, and rainbow trout (Elonen et al., 1998), exposure to tetrachlorodibenzo-*p*-dioxin (TCDD) causes impaired cardiovascular development, edema, hemorrhages and inadequate tissue perfusion (ischemia) in zebrafish early life stages (Carney et al., 2006). Furthermore, hepatic lipidosis and hypertrophy, hypertrophy of gill lamellae, and increased CYP1A enzyme has been observed in a variety of organs in adults (Buchmann, 1993; Zodrow et al., 2004). Exposure of adult zebrafish to a commercial mixture of polychlorinated bipenyls (PCBs) also resulted in increased CYP1A catalytic activity (as measured by EROD) and was accompanied by adversely affected reproductive performance, reduced bodyweight and hepato- and gonadosomatic indices, and reduced numbers of mature oocytes in ovaries (Örn et al., 1998). Since polybrominated dibenzo-*p*-dioxins and -furans (PBDDs and PBDFs) act similar to the polychlorinated dioxins and furans (PCDD/Fs) present in commercial PCBs mixtures, these compounds might have

been the cause for technical PBDE mixtures to also have AhR-agonistic activities (Birnbaum et al., 2003; Behnisch et al., 2003). In addition to inducing CYP1A, AhR agonists decreased the response to estrogenic compounds in isolated carp (*Cyprinus carpio*) hepatocytes (Smeets et al., 1999a,b). Adequate evaluation of toxicity of commercial PBDEs and the influence of contaminating AhR agonists on the endpoints mentioned above therefore necessitates chemical fractionation and clean-up when exposure studies are performed.

5. General experimental approach of the studies presented in this thesis

After a general screening for dioxin-like potency of purified BFRs in primary cultured fish hepatocytes, a comprehensive selection of technical BFR products was tested in semi-chronic exposure studies using both European flounder and zebrafish to investigate possible endocrine effects of BFRs. The exposure period aimed at in the flounder studies was 3 months. Partial life-cycle exposure in zebrafish was 4 weeks exposure of the parent generation, followed by exposure of eggs and developing juveniles until 6 weeks after hatching. The histological, biological and biochemical endpoints listed above (histology of endocrine and reproductive organs, monooxygenase type enzyme induction in zebrafish and flounder; VTG production in flounder; reproduction in zebrafish) were used as indicators for adverse health effects including endocrine disruption. The biochemical parameters and extensive use of histopathology in this approach can provide clues for underlying, possibly endocrine, mechanisms of action (Wester et al., 2002, 2003). In addition, chemical analyses were performed to evaluate the (internal) exposure concentrations, and relate this to possible observed effects, thus making the data available for risk assessment purposes. Dose-response modeling was performed according to the method published by Slob et al. (2002) using a bench-mark approach. This method enables the calculation of a critical dose level including 90% confidence limits at a predefined effect size. In this way an attempt was made to provide exposure limits for adverse effects, also in the case where no effects were observed for specific BFRs.

6. Thesis outline

This thesis is part of the extensive EU integrative risk assessment project FIRE (Flame retardants Integrated Risk assessment for Endocrine effects). The main purpose of the presented work was to specifically address the risks of exposure to BFRs in fish, in relation to environmental background levels. In addition, the relative importance of concurring AhR agonists was investigated, as well as interactions of these contaminants with PBDEs as a particular source. The potential of PBDEs to activate an AhR-mediated response and interact with the model AhR-agonist TCDD was investigated in primary carp hepatocytes (Chapter two). This sensitive *in vitro* method also provides the opportunity to test relatively small amounts of rare and expensive highly purified individual BFR congeners.

In Chapter three, the extent to which planar contaminants in a commercial pentabromodiphenylether mixture may influence *in vivo* AhR-mediated effects in fish was investigated. The outcome of the studies that are described in the chapters two and three underline the need for fractionation before bioassays with chemical mixtures such as commercial PBDEs are applied to identify possible hazardous chemicals (TIE: toxicity

identification and evaluation). The following three chapters (four to six) describe the actual testing of BFRs for biologically and ecologically relevant effects *in vivo*. TBBPA was tested first in zebrafish (Chapter four) using a partial life-cycle design. Long term studies on the effects of TBBPA and HBCD on the European flounder in a more environmentally relevant exposure situation were performed and are described in Chapter five. Finally, a chemically fractionated commercial mixture of pentabromodiphenylether (DE-71) was tested in the European flounder with exposure via feed and sediment. This purified PBDE mixture was also tested in zebrafish using the partial life-cycle design, which enabled monitoring of reproduction and juvenile development (Chapter six).

The results of the studies described in this thesis are discussed and summarized in Chapter seven.

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Some polybrominated diphenyl ether (PBDE) flame retardants with wide environmental distribution inhibit TCDD-induced EROD activity in primary cultured carp (*Cyprinus carpio*) hepatocytes

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Abstract

Ethoxyresorufin-*O*-deethylase (EROD) activity, a catalytic function of the cytochrome P450 1A (CYP1A) microsomal oxygenase subfamily, is a popular biomarker for exposure to xenobiotics, polyhalogenated aromatic hydrocarbons (PHAHs) in particular. It has found wide use in aquatic pollution assessment both *in vivo* and *in vitro*. In such studies, subjects are often exposed to complex mixtures where various constituents can interfere with EROD-activity, possibly resulting in inadequate estimation of toxic hazard or biological response. The present study investigates the effects of polybrominated diphenylethers (PBDEs), a relatively new and increasingly detected group of environmental contaminants, on the validity of EROD activity as exposure marker in carp (*Cyprinus carpio*) hepatocytes. Freshly isolated hepatocytes of a genetically uniform strain of male carp were co-exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) at concentrations of 0, 1, 3, 10, 30, and 100 pM, and one of the highly purified PBDE/PCB congeners (at concentrations of 0, 0.25, and 2.5 μM) or cleaned-up and untreated DE-71 samples (0, 0.1, and 1 μM). PBDEs were selected from the 209 possible congeners based on their relative abundance in environmental samples: BDE-47, BDE-99, BDE-100 and BDE-153. A tentative metabolite of BDE-47, 6OH-BDE-47, was also included. In addition, a commercial pentabrominated dipenylether mixture (DE-71) was tested for interference with EROD activity both with and without clean-up by carbon fractionating which removed possible planar contaminants. Polychlorinated biphenyl (PCB)-153, a reported inhibitor of EROD activity in flounder, was included for comparison. Cells were cultured for a total period of 8 days; exposure started at day 3 after cell isolation. After 5 days of exposure, cell pellets were frozen before EROD activity was determined. Upon exposure to TCDD, the cells responded with increased EROD activity as expected. Significant reduction of TCDD-induced EROD activity was found in the presence of BDE-47, BDE-99, and BDE-153, but not with BDE-100 and 6-hydroxylated BDE-47. Of these PBDE congeners, the most abundant congener in environmental samples, BDE-47, exhibited the strongest inhibition (down to 6% of the TCDD control value). The cleaned-up fraction of commercial penta-BDE (DE-71) mixture proved an even more potent inhibitor, resulting in reduction of EROD activity to 4% of the control values observed at 1.0 μM. BDEs -47 and -153 did not reduce TCDD-induced EROD activity when added shortly prior to measurement, suggesting possible interaction with TCDD at the level of CYP1A biosynthesis. PCB-153 did not show significant effects on EROD activity in carp in this study. The present results indicate that environmentally relevant PBDEs can interfere with determination of EROD activity *in vitro*, at levels reported earlier for PCBs. The observation that detected PBDE levels are rising, stresses the need for caution when interpreting EROD data on environmental samples.

Introduction

Induction of the CYP1A mediated EROD activity is a popular biomarker of exposure to aromatic hydrocarbon receptor (AhR) agonists in many vertebrate species. Typical AhR agonists are planar aromatic compounds with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) as the model activating substance. A wide variety of related polyhalogenated aromatic hydrocarbons (PHAHs), including polychlorinated and polybrominated biphenyls (PCBs and PBBs, respectively), have been shown to interact with the AhR (Safe, 1990; van den Berg et al., 1998). As PHAHs are commonly detected in the aquatic environment, measurement of EROD activity in fish and cultured fish cells has found wide use in ecotoxicologic exposure assessment studies (Whyte et al., 2000). Since these studies usually concern environmental samples that represent mixtures of different xenobiotic compounds, understanding of possible interactions of the various constituents is vital for adequate interpretation of EROD measurement. PCBs in particular have been under attention because of their typical presence in polluted areas. Indeed, apart from additive effects on CYP1A induction, partial AhR agonism and inhibition, mainly at high concentrations, have been observed for PCBs (Hahn et al., 1993; Besselink et al., 1998; Schlezinger and Stegeman, 2001). Recently, attention focussed on the chemically related polybrominated diphenylethers (PBDEs), which were found to inhibit AhR-mediated production of CYP1A protein in rat hepatocytes (Chen and Bunce, 2003). Information on the possible interference with EROD measurement in aquatic species is currently lacking.

PBDEs are high production volume chemicals, popularly used as additive flame retardants by the polymer industry (Bergman, 1989). During the past two decades, global production has been rising exponentially and levels detected in biota were found to double every 3-5 years (Meironyté, 1999; Ikonomou et al., 2002; Norstrom et al., 2002). While in the aquatic environment concentrations of important pollutants such as dieldrin and hexachlorobenzene (HCB) decreased during the last decades, increasing levels of PBDEs were detected in fish caught in the North Sea over the years 1977-1987 (de Boer, 1989). Detection in animals feeding in deep ocean waters indicates wide distribution of PBDEs in the aquatic environment (de Boer et al., 1998). The wide distribution and relative lack of toxicological information raises environmental concern (Vos et al., 2003). The PBDE congener found most predominantly, 2,2',4,4'-tetra BDE (BDE-47) was recently detected at levels comparable to those of PCB-153 in fish samples from the North Sea (Vos et al., 2003). Apart from BDE-47, in the majority of samples investigated, substantial amounts of BDEs -99, -100, and -153 were detected; occasionally, BDE-154 is found at levels comparable to those of BDE-153 (Sellström et al., 1993; Darnerud et al., 2001; Ikonomou et al., 2002; de Wit, 2002). Because of their structural resemblance to PCBs, PBDEs could act as inhibitors of EROD activity, thereby obscuring the possible presence of AhR agonist contaminants.

The present study addresses PBDEs as potential inhibitors of EROD activity using primary cultured carp hepatocytes (Carp HEP assay, Smeets et al, 1999). The hepatocytes were co-exposed to TCDD at concentrations ranging from 0 to 100 pM and several pure PBDE congeners at concentrations of 0, 0.25, and 2.5 μM. The following PBDEs were selected based on their abundance in environmental samples: BDE-47, BDE-99, BDE-100, BDE-153, and 6-hydroxylated BDE-47, a tentative BDE-47 metabolite. PCB-153 (a non-planar PCB) was reported to act as a selective CYP1A inhibitor (Petrulis et al., 2000) and

was added to the selection for comparison. In addition, a sample of DE-71, a commercially used pentabromodiphenylether mixture, was tested for its capability to affect dioxin-induced EROD response, before and after carbon filter clean-up to remove possible planar contaminants. The resulting AhR activation was evaluated by measuring *in vitro* induced EROD activity.

Materials and methods

Experimental animals
The genetically uniform strain of adult male carp *(Cyprinus carpio*; average body weight: 1 kg*)* used in these experiments was obtained from the hatchery of the Fish Culture and Fisheries research group at Wageningen Agricultural University. The fish were kept in Utrecht municipal tap water at a constant temperature of 24 °C.

Chemical pretreatment of DE-71 sample
A 45 mg subsample of DE-71 was dissolved in hexane and injected onto a porous graphitized carbon column. When the column was eluted with 50 ml cyclohexane/dichloro-methane (1:1 v/v), possibly contaminating brominated dibenzo-*p*-dioxins and dibenzofurans were retained. PBDEs were collected (cleaned-up DE-71) and after evaporation of the eluent, redissolved in dimethyl sulfoxide (DMSO). For comparison, 45 mg of untreated DE-71 was also dissolved in 50 ml cyclohexane/dichloromethane (1:1 v/v), and dissolved in DMSO after evaporation of the cyclohexane/dichloromethane. An average molecular mass of 565 was assumed for preparation of stock solutions.

Hepatocyte isolation and cell exposure
Carp hepatocytes were isolated (t=0 hrs) using a collagenase perfusion technique and cultured in phenol red-free DMEM/F12 medium (D2906, Sigma, St. Louis, MO), as described earlier (Smeets et al., 1999a). Isolated hepatocytes from one fish ($0.9x10^6$ cells/ml medium) were seeded in 96-well tissue culture plates (200 µl/well) (Greiner, Alphen a/d Rijn, The Netherlands). After 72 hours, 90% of the medium was changed. Subsequently, cells were exposed to a combination of one of the pure PBDE congeners BDE-47, BDE-99, BDE-100, BDE-153 and 6-OH-BDE-47, both an untreated and a cleaned-up sample of the commercial mixture DE-71; and TCDD by adding first the PBDE dissolved in 0.2 µl DMSO (Sigma-Aldrich, Germany), and subsequently TCDD in thousand-fold concentration in 0.2 µl DMSO. Final DMSO concentration was 0.2 %. TCDD concentrations were 0.0 (DMSO only), 1.0, 3.0, 10, 30, and 100 pM for each concentration of each BDE congener tested. Pure PBDEs were tested at concentrations of 0, 0.25, and 2.5 µM; cleaned-up and untreated DE-71 samples at concentrations of 0.10 and 1.0 µM. Plates were briefly shaken after dosing. Each combination of compounds was tested in 5 wells on at least one plate. The entire procedure was carried out in duplicate so as to monitor cell viability on duplicate plates via the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (see next section). TCDD was from Dow Chemical (Midland, USA); pure PBDE congeners were synthesized according to Marsh et al., 1999, 2003; and Örn et al., 1996, at the Department of Environmental Chemistry, Stockholm University. DE-71 (batch 25008) was a generous gift from Great Lakes Chemical Corp. (Herentals, Belgium). PCB-153 was synthesized at IRAS, Utrecht (99.5%

pure). Perfusion of a second fish was performed and cells were co-exposed in a similar manner to a range of TCDD concentrations and PCB-153 at concentrations of 0, 0.25, and 2.5 µM; co-exposure to TCDD and BDE-99 was repeated to compare responses between first and second carp. Hepatocytes of the second fish were additionally used to investigate the effect of PBDE congeners BDE-47 and BDE-153 on EROD catalytic activity when added after exposure to the TCDD range 80 minutes prior to EROD assessment. After 5 days of exposure, the medium was discarded from the plates for EROD assessment, and the cell monolayers were frozen at -70ºC prior to determination of EROD activity. The possibility of EROD inhibition at the catalytic level was evaluated for BDEs -47 and -153: plates were exposed to a range of TCDD concentrations, but at initial exposure, no PBDEs were added. After 120 hrs of exposure, cells were harvested and stored at -70ºC. After thawing the plates, BDE-99 and BDE-47 were added to the wells 80 minutes prior to EROD assessment in similar concentrations dissolved in a volume of 50 µl buffer. The present study was approved of by the Ethical Committee for Animal Welfare in Experiments of Utrecht University and complies with Dutch legislation.

Cell viability and determination of EROD activity

During the experiments, cell viability was assessed by microscopic examination of the cell morphology and the state of cell-to-cell contacts on days 0, 1, 3 and 7 of the assay. The viability of cells after exposure (on day 7) in the first experiment was also determined on duplicate plates by measuring the mitochondrial succinate-dehydrogenase activity using 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, Mo, USA) as substrate (Denizot and Lang, 1986). 170 µl medium was removed and 90 µl medium containing 0.65 mg MTT/ml, was added. After 1 hour incubation, the medium was removed completely and the blue reaction product was extracted by adding 150 µl isopropanol. The isopropanol was transferred to clean 96 wells plates and reaction product was measured spectrophotometrically at 595 nm. 20 unexposed wells were used to determine background MTT activity by removing the MTT medium immediately and subsequently adding the isopropanol in the otherwise unchanged procedure.

CYP1A activities, determined as the deethylation of ethoxyresorufin (EROD), were measured directly on the 96 well plates, using a method adapted from Smeets et al. (1999). Frozen plates were thawed and 100 µl of EROD reaction mixture was added, containing: 50 mM Tris buffer pH 7.8, 5 mM $MgCl_2$, 20 µM dicumarol (tris/dic), 2 µM 7-ethoxyresorufin and 1.5 mM NADPH. The increase in fluorescense (excitation: 530 nm, emission: 590 nm) was determined after 10 min incubation at 25ºC. Resorufin formation was calculated by comparing fluorescense to a resorufin standard dilution series. To investigate the effect of PBDEs on EROD catalytic activity after exposure, BDE-47 and BDE-153 were added in 50 µl tris/dic; PBDE concentrations were 0, 0.25, and 2.5 µM in 50 µl tris/dic. After briefly shaking the plates, they were left at room temperature for 80 minutes. Then 50µl tris/dic containing the remaining constituents of EROD reaction mixture was added and started the reaction. Average protein content per well was determined according to Lowry et al. (1951), using bovine serum albumin as the standard.

Statistical analysis

Statistical calculations were performed using the computer program SPSS (version 9.0). Statistical significance ($P \leq 0.01$) among the treatments was calculated for TCDD-responsive

values (i.e. TCDD concentrations 10 pM and higher) using one-way analysis of variance (ANOVA). EROD data were normalized by square root transformation. Post hoc testing for BDE effects was performed using Bonferroni correction for multiple comparisons when equal variance was assumed, based on Levene's statistic. MTT data were analyzed for significant decrease compared to controls by one-way ANOVA using Bonferroni correction for multiple comparisons. When equal variance could not be assumed, results were tested according to Games-Howell.

Results

Cell viability

Microscopic observation on days 0 and 1 revealed that on day 1 cells looked similar to freshly isolated hepatocytes. Cells were rounded and showed limited intercellular contact. Three days after seeding the hepatocytes had a slightly larger diameter suggesting attachment to the bottom of the wells. Cell borders showed frequent intercellular contacts. A similar picture was seen at the end of exposure. Cell layers remained attached to the bottom of the plates throughout the experiment.

Additionally, mitochondrial dehydrogenase activities of hepatocytes exposed to combinations of xenobiotic compounds were related to those of cells exposed to DMSO only (controls) using MTT. Compared to the controls, activities ranged from 56% to 201% Values below 100% were rare and did not consistently relate to high levels of BDE and/or TCDD exposure except in cells exposed to the highest TCDD concentration (100pM) in combination with BDEs -99 and -153 at concentrations of 0.25 and 2.5 µM where values down to 60% indicated reduced cell viability (not statistically significant). No negative effect on MTT values was observed in cells exposed to these BDEs combined with lower TCDD concentrations.

Effects of BDE co-exposure on TCDD induced EROD activity

Carp hepatocytes reacted to TCDD exposure alone with increased EROD activity in a dose dependent manner (EC_{50}: 19 pM). EROD activity was not observed in cells exposed to 0 pM of TCDD and any of the BDEs tested at concentrations of 0, 0.25, and 2.5 µM, indicating that none of the PBDEs tested in the present study induced EROD activity per se (Fig. 1). A dose dependent reduction in EROD response was observed after co-exposure to various PBDE congeners and TCDD at concentrations of 10, 30 and 100 pM (Fig. 1, Table 1). TCDD exposed cells, co-exposed to BDE-47 at concentrations of 0.25 µM and 2.5 µM, displayed EROD activities of 39-41%, and 6-9%, respectively, of the EROD activities when no PBDE was added at the corresponding TCDD levels (Table 1). BDE-99 reduced TCDD induced EROD activities to 29% and 13-19% at concentrations of 0.25 and 2.5 µM, respectively. Co-exposure to BDE-153 at concentrations of 0.25 and 2.5 µM resulted in EROD activities between 47% and 61%, and between 7% and 23% of the corresponding values when no BDE was added. Although used at lower concentrations, both cleaned-up and untreated samples of the technical penta-BDE mixture, DE-71, showed strong inhibiting effects on TCDD induced EROD activity in carp hepatocytes (Fig. 1, Table 1). In contrast, BDE-100 and 6-hydroxylated BDE-47 did not influence EROD activity consistently. EROD activity was reduced significantly in cells co-exposed to BDE-100 at

TCDD concentrations of 30 pM; this effect however was not dependent on BDE dose and was not present at TCDD levels of 10 pM and 100 pM.

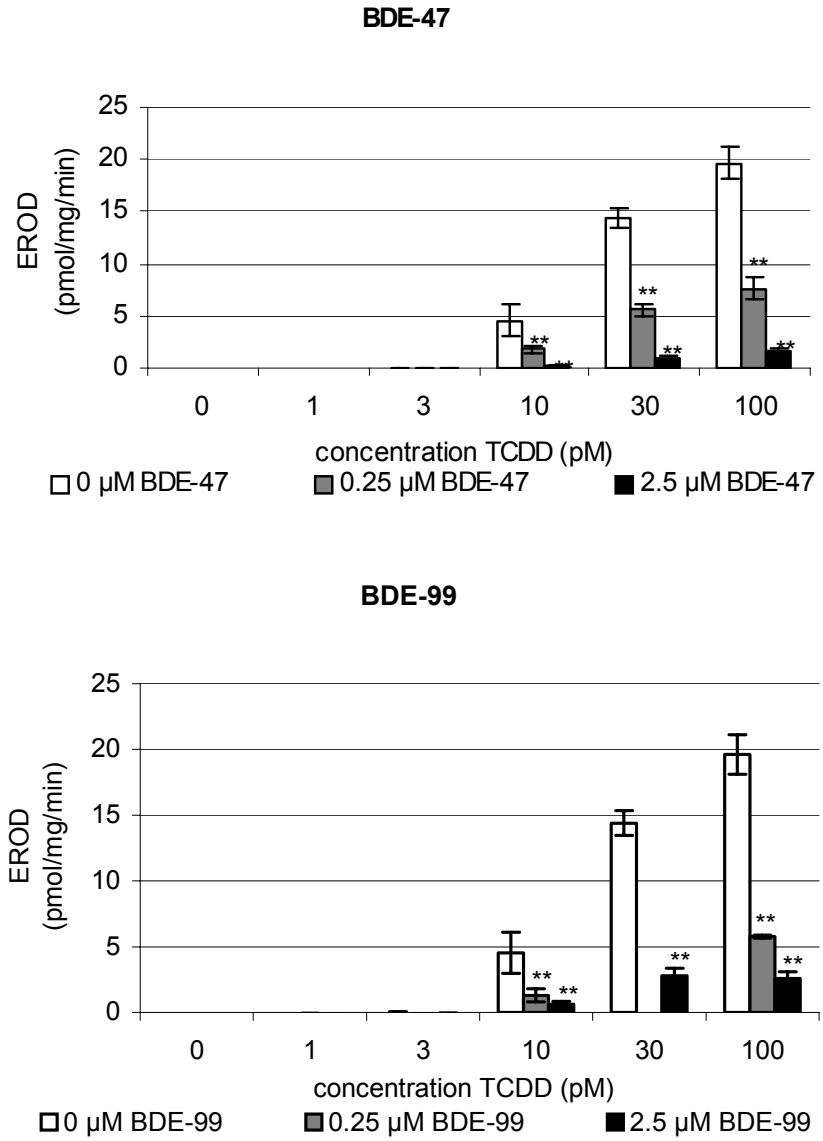
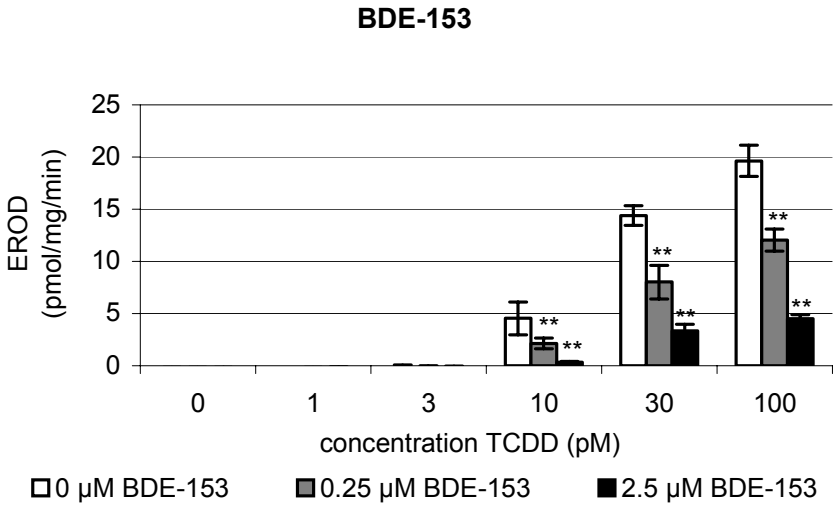
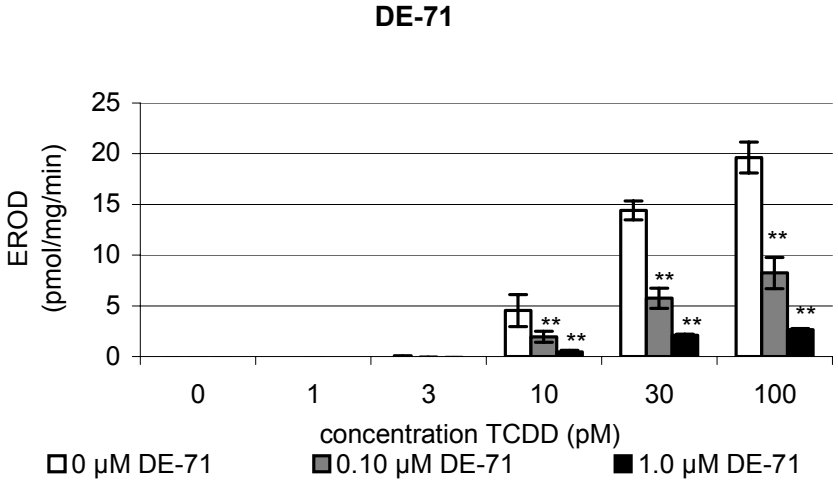


Fig. 1. Effects of various pure PBDE congeners, the commercial mixture DE-71 and cleaned-up DE-71 on EROD activity in carp hepatocytes co-exposed to PBDEs and a range of TCDD concentrations for 5 days. Error bars represent SD of 5 determinations except in controls (n=10). Controls were exposed to equivalent concentration of DMSO. Stars indicate statistically significant difference from corresponding TCDD concentration($p \leq 0.01$).





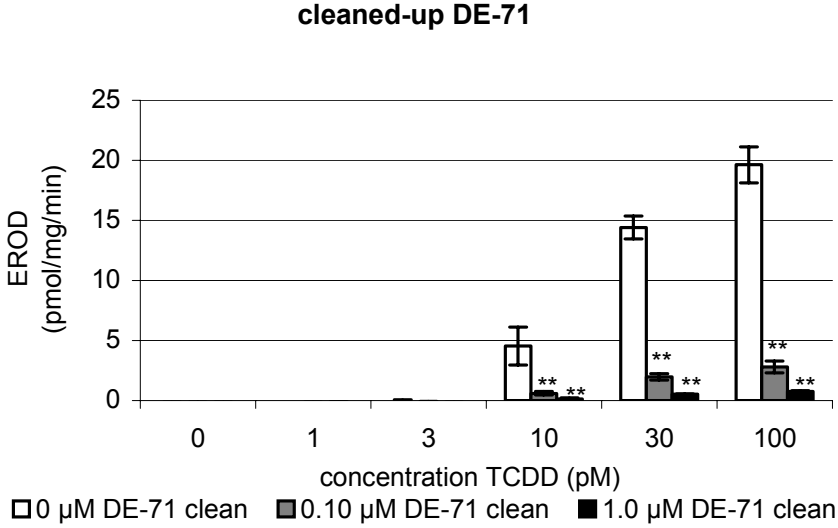


Fig. 1. (continued).

Except for BDE-100 and 6-hydroxylated BDE-47, the inhibitory effect became apparent at TCDD concentrations of 10 pM and higher and was observed from the lowest BDE concentration tested. For TCDD concentrations ranging up from 10 pM, the percentage by which EROD activity was reduced remained fairly constant (Table 1). While of the pure congeners, BDE-47 was the most potent inhibitor, co-exposure to cleaned-up DE-71 resulted in even stronger reduction of EROD activity (down to 14% and 4% at DE-71 concentrations of 0.10 and 1.0 µM, respectively).

Table 1. Inhibition of EROD in TCDD exposed carp hepatocytes by PBDE congeners relative to control (100%).

	Resulting EROD (%)		
	TCDD conc (pM)		
PBDE congener (µM)	10	30	100
control	100	100	100
BDE-47 (0.25)	41	39	39
BDE-47 (2.5)	6	7	9
BDE-99 (0.25)	29	-	29
BDE-99 (2.5)	14	19	13
BDE-100 (0.25)	84	59	86
BDE-100 (2.5)	77	72	83
BDE-153 (0.25)	47	56	61
BDE-153 (2.5)	7	23	23
DE-71 (0.10)	43	40	42
DE-71 (1.0)	10	14	13
DE-71 clean (0.10)	13	14	14
DE-71 clean (1.0)	3	4	4
6-HO-BDE-47 (0.25)	95	98	105
6-HO-BDE-47 (2.5)	99	88	86

Note: EROD activity is expressed as percentage of EROD induction at the corresponding TCDD concentration without PBDE exposure (control).

In an additional experiment, either one of the potent inhibitory PBDEs: BDE-47 and BDE-153, were added after TCDD exposure, shortly before EROD measurement. Also included in that experiment were co-exposure as in the first experiment to TCDD and BDE-99, and TCDD and PCB-153. Co-exposure to BDE-99 resulted in inhibition of TCDD-induced EROD response, reproducing the findings from the first experiment (not shown). EROD activity was not reduced in cells, co-exposed to TCDD and PCB-153 (Fig. 2). Addition of the inhibitory BDEs -47 and -153 shortly before measurement did not result in reduction of TCDD-induced EROD activity (Fig. 3).

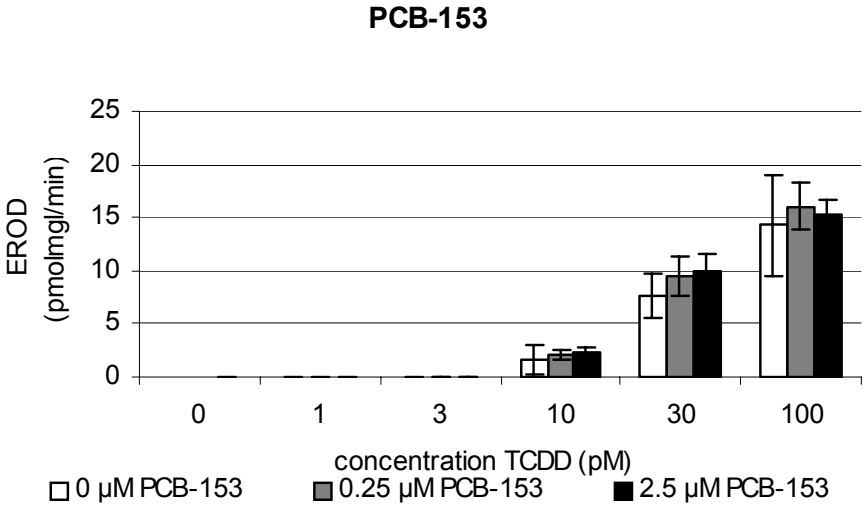


Fig. 2. EROD activity in carp hepatocytes co-exposed to PCB-153 and a range of TCDD concentrations did not show inhibition related to PCB-153 dose. Bars represent standard deviations of 5 determinations.

Discussion

Several PBDE congeners were observed to influence measurement of TCDD-induced EROD activity in carp hepatocytes when present in the exposure medium. EROD response of carp hepatocytes exposed to TCDD alone has not been reported but is consistent with what was described in primary isolated rat hepatocytes (Petrulis et al., 2001) and carp hepatocytes co-exposed to estradiol (Smeets, 1999). The MTT assay did generally not indicate negative exposure effects on cell viability at TCDD and BDE concentrations used in this study, except in the case of BDEs -99 and -153 when combined with 100 pM of TCDD. In an additional study, we exposed freshly isolated carp hepatocytes for 5 days to the individual PBDE congeners used in the present study at concentrations of 0, 2.5, and 7.5 µM. No cytotoxicity was observed for BDE-47 and BDE-100; BDE-99 and BDE-153 showed no cytotoxicty at the concentrations used in the present study but became cytotoxic at 7.5µM (data not shown). Reduced MTT values were not observed at any concentration of BDE-47 and DE-71. Microscopic examination of the cells did not reveal morphologic changes during exposure, and the cells remained attached to the bottoms of the wells throughout the entire procedure. We therefore conclude that EROD-activity in the hepatocytes was influenced in a congener- and concentration dependent manner, and not caused by cytotoxicity.

Inhibition by PHAHs could account for the lack of correlation found between measured EROD activity and total toxic equivalency quotient (TEQ) exposure determined by chemical analysis in a number of environmental studies (Whyte, 2000; Kennedy et al., 2003). Certain PCBs were shown to negatively influence CYP1A activity in *Poeciliopsis lucida* hepatoma cells co-exposed to TCDD (Hahn et al., 1993, 1995). Besselink et al. (1998) reported inhibition of TCDD-induced EROD activity by PCBs at IC_{50} concentrations ranging from 0.24 (PCB-126) to 31.88 (PCB-153) µM. In the present study, significant reduction of EROD activity by PBDEs was found from the lowest concentration

tested (0.25 µM), in most cases down to below 50% of the control value. Thus, our results indicate inhibitory potential similar to that of PCBs for some widely distributed PBDEs, including the predominantly detected congener, BDE-47. In fish from the North Sea, BDE-47 levels up to 194 ng/g lipid weight have been reported in pooled samples of 25 animals, similar to PCB-153 levels detected in the same study (Vos et al., 2003). Even higher concentrations (up to 9500 ng/g lipid weight for BDE-47, and up to 1200 ng/g lipid for DE-71 quantified as technical product) were reported in fish samples from fresh water (Allchin et al., 1999). These data indicate that PBDEs in environmental samples may at the least equally contribute to inhibition of EROD activity as was suggested for PCBs.

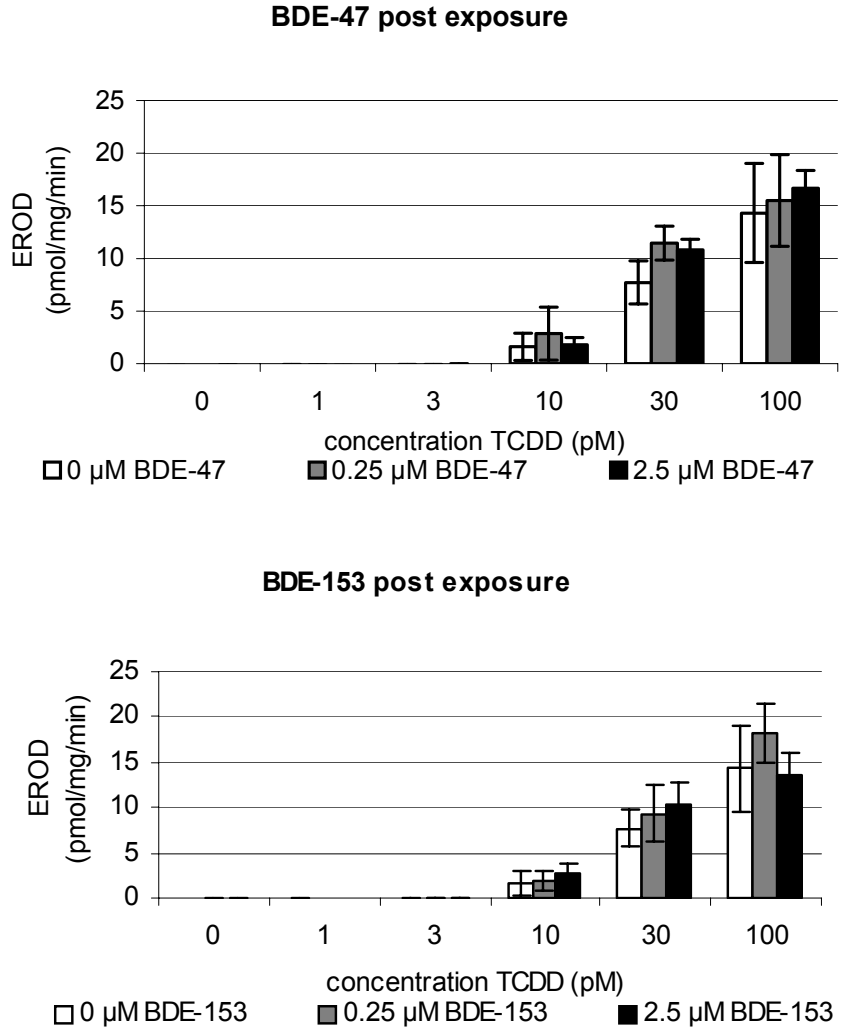


Fig. 3. EROD activity in carp hepatocytes exposed to a range of TCDD concentrations. BDE-47 and BDE-153, added 80 minutes prior to EROD measurement, did not show inhibition of TCDD induced EROD activity. Bars represent standard deviations of 5 determinations.

Cleaned up DE-71 showed by far the strongest inhibiting potential, even though tested at lower concentrations. A possible explanation could be the presence of other inhibitory PBDEs, possibly non-*ortho* substituted congeners. In the study by Besselink et

al. (1998), PCB-126 (non-*ortho* substituted) had a stronger potential to inhibit EROD response than PCB-153 (ortho substituted), which was a relatively weak inhibitor. No pure non-*ortho* substituted PBDEs were tested in the present study, and the composition of DE-71 has insufficiently been elucidated; however, non-*ortho* substituted PBDEs were no major constituents of Bromkal 70-5DE, another commercially produced PentaBDE mixture (de Wit, 2002).

Although weak induction of EROD activity has been reported for some PBDEs including BDEs -100 and -153 (Chen et al., 2001), the present lack of induction in cells exposed to low TCDD concentrations and individual congeners or the commercial penta mixture, DE-71, is consistent with literature (Chen and Bunce, 2003). Induction of EROD activity was reported in mouse liver after subchronic *in vivo* exposure to DE-71 (Fowles et al., 1994). This effect was not observed after acute exposure, indicating that repeated dosing was needed for sufficient (preferential) accumulation of CYP1A inducing potential (toxic equivalency, TEQ). In view of the present observations, one possible explanation is that an internal quenching effect by PBDEs obscures the presence of possibly planar (e.g. contaminating PBDDs and PBDFs) CYP1A inducers in commercial PBDE mixtures. Masking of TEQs by PBDEs in DE-71 is supported by our observation that cleaned-up DE-71 showed stronger inhibiting potential than untreated DE-71.

Several mechanisms have been proposed to explain less than additive observed EROD activity in combined PHAH exposure studies. Since the EROD assay measures CYP1A by its catalytic action, reduction of induced EROD-activity can be due to either a reduced amount of enzyme present, or a reduced catalytic activity. A number of studies address this issue. Schlezinger and Stegeman (2001) found that penta-chlorinated biphenyl (PCB-126) suppressed EROD activity in scup (*Stenotomus chrysops*) kidney, but not CYP1A protein, suggesting inhibition of CYP1A catalytic activity as at least one possible mechanism. These results are supported by the observation that PCBs may inhibit CYP1A activity in flounder (*Platichthys flesus*) hepatic microsomes (Besselink et al., 1998). In contrast, the expression of a luciferase reporter gene under transcriptional control of the AhR in mouse hepatoma cells by the di-*ortho* substituted PCBs-52 and -128 was reported by Aarts et al. (1995), evidencing pre-translational antagonism of AhR mediated gene expression. Accordingly, Yuan et al. (2001) report unexpectedly low CYP1A protein levels in Atlantic tomcod with high hepatic burdens of planar PHAHs. In the present study, TCDD-induced EROD activities were not influenced by pre-incubation with PBDEs in the EROD reaction mixture, shortly before measurement. Although nominal PBDE concentrations were equal, accumulation of PBDEs in the cells during exposure may result in higher final concentrations when EROD activity was determined. However, the absolute amount of PBDEs at the highest concentration added post exposure exceeded the total amount added during co-exposure at lower concentrations without reproducing the inhibitory effect observed at those concentrations. The present observation that inhibition of EROD activity by PBDEs requires presence of PBDEs during exposure to TCDD, supports a negative effect on CYP1A synthesis, possibly via the AhR. It appears that EROD activity as biomarker for exposure may be influenced via a number of ways. Although AhR-agonists structurally resemble CYP1A substrates, structure-activity relationships (SARs) may be different for the different mechanisms, resulting in inconclusive overall SARs for EROD inhibition. In future studies, analysis of mRNA should elucidate the mechanistic nature of the EROD inhibition by PBDEs in fish hepatocytes.

In conclusion, certain PBDEs can inhibit EROD activity at similar levels as reported for PCBs. Being relatively new xenobiotic compounds detected in the environment, global PBDE levels are rising and levels of individual congeners may equal or even exceed those of certain environmentally relevant PCB congeners. These observations again underline the need for caution when using EROD activity as biomarker in *in vitro* fish assays when monitoring exposure to environmental pollutants. If interaction at the level of the AhR is a possible cause for reduction of CYP1A activity, such interactions might also have consequences for AhR mediated toxicological end parameters. Further research is needed to elucidate such a possible mechanism of action.

Acknowledgement

Dr W. Traag at the Institute of Food Safety RIKILT, Wageningen, the Netherlands, is acknowledged for fractionating the DE-71 sample. The authors thank Ineke van Holsteijn at IRAS for vital assistance during the perfusion experiments. The present research was carried out in the EU-supported project "Toxicology of Brominated Flame Retardants as Suspected Endocrine Disruptors for Human and Wildlife Health" (acronym FIRE; EU contract number QLK4-CT-2002-00896).

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3

In vivo and *in vitro* Ah-receptor activation by commercial and fractionated pentabromodiphenylether using zebrafish (*Danio rerio*) and the DR-CALUX assay

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A**bstract**

The present study addresses the toxicity of a commercial pentabrominated diphenylether (PeBDE) flame retardant mixture, DE-71, in a model aquatic vertebrate. Four weeks' exposure of juvenile zebrafish (*Danio rerio*) to water-borne DE-71 resulted in dose-dependent induction of CYP1A immunoreactivity, predominantly in the endocardium and the endothelium of larger blood vessels, such as ventral aorta and branchial arteries, as well as the larger hepatic and pancreatic blood vessels.

To investigate the impact of possible contaminating PBDD/Fs in the DE-71 product, the study was repeated after DE-71 had been fractionated into a non-planar (cleaned PBDEs) and a planar fraction (PBDD/Fs). Zebrafish were exposed under similar conditions to the planar and cleaned DE-71 fractions, and to uncleaned DE-71. In addition, the above fractions were chemically analyzed and tested in a reporter gene assay (DR-CALUX) for their aromatic hydrocarbon-receptor (AhR) stimulating potencies. A relatively strong CALUX response was detected from the planar DE-71 fraction (19.7 ng TCDD equivalent (TEQ)/g DE-71), coinciding with a strong induction of CYP1A immuno-reactivity in zebrafish. CYP1A immunoreactivity in zebrafish exposed to uncleaned DE-71 was intense, although the CALUX response was tenfold less compared to the planar fraction. Only weak CYP1A immunoreactivity was found in fish exposed to cleaned DE-71, and none in control animals; no CALUX response was detected in cleaned DE-71.

The present findings indicate that chemical impurities of the commercial PeBDE product account for AhR-mediated effects. Analytical isolation of a planar fraction from the commercial product increased the *in vitro* (DR-CALUX) signal 10 times. Immunohisto-chemistry showed a strong tissue specific reaction to DE-71 *in vivo* at these relatively low TEQ levels regardless of chemical pretreatment of the mix, reflecting the sensitivity of CYP1A induction in juvenile zebrafish to AhR agonists.

Introduction

Polybrominated diphenylethers (PBDEs) are abundantly used as flame retardants in synthetic products such as electronic equipment, building materials, and textiles. Release of these additives into the environment has resulted in widespread aquatic contamination, as evidenced by their presence in remote areas (de Boer et al., 1998; Ikonomou et al., 2002; Vives et al., 2004). Particularly lower brominated PBDE congeners tend to bioaccumulate (de Wit, 2002; Burreau et al., 2004), and increasing levels were detected in aquatic biota in a number of temporal trend studies. Whereas environmental concentrations of major pollutants such as dieldrin and hexachlorobenzene (HCB) decreased, increasing levels of PBDEs were detected in livers from cod caught in the North Sea over the years 1977-1987 (De Boer, 1989). Similarly, PBDE concentrations in fish from the Great Lakes increased over the years 1980-2000 (Zhu and Hites, 2004). Increasing PBDE levels were also reported higher up the food chain (*eg.* in guillemot eggs from Sweden (1969–2000, Sellström et al., 2003), and in ringed seals from the Canadian Arctic (1981–2000, Ikonomou et al., 2002)). In fish, total PBDE levels as high as 4.6 μg/g lipid weight have been reported (Sellström et al., 1998). Despite the larger production volumes of higher brominated diphenylethers, tetra- and penta-brominated congeners predominate in biota, 2,2',4,4'-pentabromodiphenylther (BDE-47) being the most important (Andersson and Blomkvist, 1981; Watanabe et al., 1987; Sellström et al., 1993; Jansson et al., 1993; de Boer 1998). Since BDE-47 was not a breakdown product of higher brominated PBDEs by photolytic debromination (Söderström et al., 2004) or metabolism (Kierkegaard et al., 1999), commercial penta-brominated mixtures such as DE-71, which contain substantial amounts of BDE-47, are the most likely source. Although in Europe, reduced use of lower brominated PBDEs caused levelling off (Kierkegaard et al, 2004) or even reduction (Sellström et al., 2003) of PBDE levels in biota, high production volumes in other areas and global redistribution (Birnbaum and Staskal, 2004; Vives et al., 2004) present a continuous source for environmental exposure.

PBDEs may have adverse effects on biota in a number of ways. Several, particularly lower brominated, PBDE congeners, and in particular, their hydroxylated metabolites may modulate both estrogen receptor (Meerts et al., 2001; Nakari and Pessala, 2005) and thyroid receptor mediated effects (Schriks et al., 2006) *in vitro*. The detection of planar PHAHs (polybrominated dibenzo-p-dioxins, PBDDs, and dibenzofurans, PBDFs; Sanders et al., 2005; Sakai et al, 2001) in commercial diphenylether mixtures is of additional concern, since PBDD/Fs show comparable toxicity as their identically substituted chlorinated homologues (Hornung et al., 1996; Behnisch et al., 2003; Birnbaum et al., 2003). A number of reports on effects of PBDEs in fish are available. Prolonged oral exposure of sticklebacks (*Gasterosteus aculeatus*) to the commercial penta-BDE mixture, Bromkal 70-5DE, reduced spawning success and caused mild hepatic lipidosis and increased EROD activity (Holm et al., 1993); a mild induction of EROD activity was also reported in rainbow trout (*Oncorhynchus mykiss*) fry injected with Bromkal 70-5DE (Norrgren et al., 2003); these authors suggested that impurities in the commercial blends might be responsible. Conversely, EROD activity was decreased in rainbow trout orally exposed to tetra- and penta-BDE during 22 days; glutathion reductase activity was also decreased, whereas blood glucose and hematocrit showed a mild but statistically significant increase (Tjärnlund et al., 1998). Exposure of killifish (*Fundulus heteroclitus*) embryos to

DE-71 via the water up to 100 µg/l did not result in induction of EROD activity, but delayed hatching up to 4.5 days and decreased swimming in response to external stimuli in larvae (Timme-Laragy et al., 2005). The possible role of endocrine disruption on sex steroid and thyroid hormonal homeostasis *in vivo* remains to be identified and the impact of possible planar contaminants in commercial mixtures has not been properly evaluated in aquatic species.

The present study focuses on histopathological changes to determine target organs for subacute toxicity of commercial pentabromo diphenylether (DE-71) in a popular aquatic vertebrate model species, zebrafish (*Danio rerio*), between 4-8 weeks of age when gonad sexual dimorphism develops (Hsiao and Tsai, 2003). Whole body serial sections cut at 40 µm intervals were examined by light-microscopy with emphasis on thyroid, gonad and liver histology. Attention was also given to brain and pituitary gland, skin, gills, intestine, kidney, spleen and thymus. Immunohistochemistry using a monoclonal antibody directed against scup (*Stenotomus chrysops*) cytochrome P4501A (CYP1A) was performed to evaluate tissue patterns of CYP1A protein formation. The toxic potential of possible planar impurities (PBDD/Fs) was investigated in a separate *in vivo* experiment of similar design. The DE-71 sample was fractionated to separate PBDD/Fs from the (non-planar) PBDEs, based on the method developed for the separation of PCBs from PCDD/Fs by adsorption chromatography using Al_2O_3 (Ramos et al., 1997), and both fractions were chemically analyzed. Zebrafish were exposed to the non-planar fraction (further termed as cleaned DE-71), the planar fraction (PBDDs and PDDFs), and to uncleaned (untreated) DE-71, and PBDE levels were analyzed in whole fish to evaluate exposure. In addition, to assess dioxin-like potencies for each of the fractions *in vitro*, TCDD equivalents (TEQs) were determined in an AhR-activated reporter gene assay (DR-CALUX, Murk et al., 1996, 1998).

Materials and methods

Animals and husbandry

Zebrafish (*Danio rerio*) were bred at the Institute of Risk Assessment Sciences (IRAS), Utrecht. Breeding stock is maintained on live *Artemia salina* and dry flake fish food (King British, Bradford, England), supplemented with *Daphnia magna*. From 2 days after hatching, larvae are fed from a culture containing protozoa grown on hay and banana peel. From the second week they accept the same diet as the adults. At three weeks of age, the animals were transferred to the exposure vessels, where they were fed dry flake food only. Testing was started after one week of acclimatization.

Preparation of DE-71 stock solutions

The non-planar (PBDEs) and planar (PBDD/Fs) fractions of DE-71 were prepared using basic Al_2O_3 (Merck, Darmstadt, Germany, mesh size 63-200µm) activated at 150°C for 12 hrs. A stock solution of DE-71 (100 mg) in 50 ml hexane/dichloromethane (DCM) (48:2 v/v, Promochem, Wesel, Germany) was prepared. Five ml of this solution was added to a glass column filled with 25 g of activated Al_2O_3. PBDEs were eluted from the column using 150 ml hexane/DCM (48:2, v/v), followed by 200 ml of hexane/DCM (9:1, v/v) (non planar DE-71 fraction). PBDD/Fs were subsequently eluted with 200 ml of hexane/DCM (1:1, v/v) (planar fraction). This procedure was repeated ten times. All non-planar fractions

and all planar fractions were combined, and evaporated to 2 ml. An aliquot of the non-planar (1 ml) and an aliquot of the planar fraction (1 ml) was evaporated to dryness and again dissolved in 5 ml DMSO (HPLC grade, Baker, Germany) to prepare dosing solutions. For preparation of uncleaned DE-71, 50 mg DE-71 was dissolved in 50 ml hexane/DCM, evaporated to dryness, and again dissolved in 5 ml of DMSO.

Chemical analysis of DE-71

Uncleaned DE-71 was analyzed using a gas chromatograph (GC, 6890, Agilent Technologies, Germany) coupled to a 5973 mass spectrometer (MS, Agilent Technologies) in the electron capture negative ionization mode (ECNI) mode (MSD transferline 290 ºC, source temperature 200 ºC, quadrupole temperature 106 ºC, electron energy 70 eV) with methane (3.25 ml/min) as a reagent gas. Quantification and identification of PBDEs was carried out with the Br isotope cluster (m/z 79 and 81). Helium gas was used as carrier gas at a constant flow of 1.2 ml/min. A 50 m x 0.25 mm x 0.25 µm CP-Sil 8 fused silica column was used. GC conditions were: oven temperature 90 ºC during 3 minutes, then increased with 30 ºC/min and kept at 210 ºC for 20 min; and finally increased to 290 ºC with 5 ºC/min. Injection was pulsed splitless (280ºC) and the carrier gas was helium (1.2 ml/min).

PBDD/Fs were determined in the planar fraction using GCxGC using a TRACE 2D GC (ThermoElectron, Milan, Italy) coupled to a time-off-flight mass spectrometer (ThermoElectron, Austin, TX, USA). The MS was operated in the electron impact (EI) and in the ECNI mode using methane gas as reagent gas with a flow rate of 3 ml/min. The data acquisition rate was 40 Hz, electron energy 65 eV, and a mass range of m/z 70-850 was used. The MS transfer line was set at 300 ºC and the ion source at 190 ºC. A 30 m x 0.25 mm x 0.25 µm DB-1 (J&W Scientific, Agilent) was used in the first dimension, and a 1 m x 0.1 mm x 0.1 µm 007-65HT column (Quadrex, New Haven, CT, USA) in the second dimension. Modulation was performed at the beginning of the second dimension column with a modulation period of 8 sec. One µl of the DE-71 fractions was splitless injected (injection temperature 280 ºC) into a PTV injector with the split opening after 2 minutes, using an autosampler. Helium was used as carrier gas at a flow rate of 1 ml/min. GC conditions were: oven temperature 90 ºC during 3 minutes, then increased with 30 ºC/min and kept at 210 ºC for 20 min; and finally increased to 290 ºC with 5 ºC/min. For more details on the GCxGC-ECNI-TOF-MS systems see Korytar et al. (2005). The following PBDD/Fs were present in the analytical standard which was used for identification and quantification: 2,3,7,8-TeBDD, 1,2,3,7,8-PeBDD, 1,2,3,4,7,8-HxBDD, 1,2,3,6,7,8-HxBDD, 1,2,3,7,8,9-HxBDD, OBDD, 2,3,7,8-TeBDF, 1,2,3,7,8-PeBDF, 2,3,4,7,8-PeBDF, 1,2,3,4,7,8-HxBDF, 1,2,3,4,6,7,8-HpBDF (all from Cambridge Isotope Laboratories, Inc.). Detection limits for tetra-, penta-, hexa-, and heptabrominated PBDD/Fs were 0.4, 0.02, 0.03, and 0.03 mg/kg DE-71, respectively.

Chemical analysis of zebrafish

Fish samples from experiment B (see below) were rinsed with demineralized water to remove any remaining exposure water. The pooled homogenate of 4 animals per group was weighed, dried with sodium sulphate (Merck), and stored for two hours. An internal standard (CB112, Promochem) was added to the sample followed by an extraction with 10 ml hexane/acetone (3:1, v/v) using a vortex mixer. Extraction was repeated after one hour

and on the next day. The extracts were combined and evaporated to 1 ml. An aliquot of the extract was dried under nitrogen flow for gravimetrical determination of the lipid content. The residual extract was fractionated by silica gel (deactivated with 1.8% H_2O, Merck) using 11 ml isooctane (Promochem) and 25 ml isooctane:diethylether (85:15, v/v; Promochem) as eluents. Finally, the silica eluate was evaporated to 1 ml and the final analysis was carried out by GC-MS in the ECNI mode using the same conditions as for the DE-71 analysis.

Experimental design
In vivo studies
Groups of 20 zebrafish each with a starting age of 3 weeks were kept in 3 l glass beakers containing 2.5 l copper-free tap water. They were placed in a conditioned room (ambient temperature 25°C, daily 12 hrs dark/ 12 hrs light regimen). Water was refreshed by semistatic renewal, i.e. 2 l of water were replaced by fresh, pre-heated copper-free tap water, three times weekly on Mondays, Wednesdays, and Fridays. Water temperature and oxygen content were checked immediately before each renewal. Acidity was monitored at regular intervals. The four-week test period (age: 4-8 weeks) was started after one week of acclimatization.

In the first study (experiment A), 6 groups of 20 zebrafish were exposed via the water to a commercial mixture of penta-BDE (DE-71, batch 25008, provided by Great Lakes Chemical Corp., Herentals, Belgium), at nominal water concentrations of 0.0, 0.010, 0.032, 0.10, 0.32, and 1.0 mg DE-71/l (Table 1a). Animals were randomly assigned to the different exposure groups and tanks were positioned in random order. Prior to initial addition to the water, dosing solutions were prepared by dissolving DE-71 in dimethylsulfoxide (DMSO, Baker, Germany), using glass vials. Vials were left in the dark at room temperature on a platform shaker for 24 hours. During the experiment, they were stored in the dark at room temperature. DMSO concentration in the exposure medium was 0.01% in all groups, including the control. Exposure was initiated by adding 250 µl of the dosing solution immediately after renewal. On subsequent renewals, 200 µl of dosing solution was added.

Table 1a. Nominal concentrations of DE-71 in water (0.01 % DMSO), mortality during, and size at the end of, experiment A. (* $p<0.05$).

Dose (mg DE-71/l)	Mortality	Length ± SD (mm)	Weight ± SD (mg)
0.0	0/20	15.4 ± 1.4	31.5 ± 9.1
0.010	0/20	16.3 ± 1.2	34.9 ± 8.9
0.032	0/20	15.3 ± 1.6	30.8 ± 9.9
0.10	0/20	14.7 ± 1.7	28.8 ± 11.4
0.32	1/20	14.9 ± 2.2	32.7 ± 16.4
1.0	3/20	12.9 ± 1.5*	19.8 ± 7.3*

The second study (experiment B) was carried out under identical conditions. Seven groups of 20 animals were exposed to either water-borne DE-71 in nominal concentrations 0.10 and 1.0 mg/l, the cleaned fraction of 0.10 and 1.0 mg DE-71/l, the

planar fraction of 0.10 and 1.0 mg DE-71/l, or DMSO only (DMSO at 0.01% in all groups, Table 1b).

This study was approved by the Ethical Committee for Animal Welfare in Experiments of Utrecht University and complies with Dutch legislation.

Table 1b. Nominal concentrations of DE-71 and equivalents of nominal concentrations of DE-71 fractions in water (0.01 % DMSO), mortality during, and size at the end of, experiment B.

Exposure		Mortality	Length ± SD (mm)	Weight ± SD (mg)
control		0/20	20.6 ± 1.7	66.1 ± 17.7
uncleaned DE-71	0.1 mg DE-71/l	0/20	21.2 ± 1.2	71.7 ± 11.6
	1 mg DE-71/l	0/20	19.6 ± 2.1	58.9 ± 17.4
cleaned DE-71	0.1 mg DE-71/l	0/20	20.3 ± 2.5	63.8 ± 22.2
	1 mg DE-71/l	0/20	19.4 ± 2.0	55.6 ± 19.1
planar fraction	0.1 mg DE-71/l	0/20	19.9 ± 2.0	62.1 ± 17.0
	1 mg DE-71/l	0/20	19.8 ± 2.2	59.6 ± 18.3

Sampling and histological technique

During the last day of the experiments, animals were not fed to minimize intestinal content. In this way, better fixation and less histological "dominance" of the intestines were achieved. At the end of the experimental periods, animals were euthanized in random order using a solution of 0.3 g TMS/l and 0.6 g $NaHCO_3$/l in preheated copper free tap water (TMS is 3-amino benzoic acid ethyl ester, methane sulfonate salt, Sigma-Aldrich, Steinheim, Germany). From all experimental groups, 16 animals were processed for histological examination. In experiment B, remaining animals were frozen at -20 °C for chemical analysis. For histology, entire animals were fixed in Bouin's fixative, immediately after euthanasia. Animals that died during the experiments were excluded from histological examination because rapid autolysis renders these animals unsuitable for histopathology. After twelve hours, fixed animals were transferred to 70% ethanol. Length and weight of the animals surviving the entire experimental period were then determined and the animals were routinely processed and embedded in paraffin (2 fish per paraffin block). Serial sections of approximately 4 µm thick were cut at 40 µm intervals to reveal small organs like thyroid follicles and pituitary gland. Hematoxylin-eosin (H&E) staining was used for routine microscopy. Sections were randomized and coded before histological examination. Histological alterations were marked as absent, present, or severe, by two independent histopathologists.

For immunohistochemistry, sections were mounted on slides coated with 0.01% poly-l-lysine (PLL; Brunswick Chemie, Amsterdam, The Netherlands), and deparaffinized. A mouse monoclonal anti-scup CYP1A antibody (kindly provided by J. J. Stegeman, Woods Hole Oceanographic Institution, Woods Hole, Mass., USA) was used as primary antibody. Immunoperoxidase staining was then performed according to Grinwis et al. (2000). A monoclonal mouse anti-proliferating cell nuclear antigen (PCNA) antibody (Dako, Glastrup, Denmark) with indirect immunoperoxidase staining (Grinwis et al., 2000) was used to examine possible effects on cell proliferation. To test for false positive results, negative controls were included in which the primary antibody was omitted or a primary monoclonal mouse anti-*Chlamydia* antibody (Clone C5, Argene Biosoft, Varilhes, France) was used in the procedure mentioned above. Proliferating testis was used as positive

internal control for PCNA staining. For the semi-quantitative assessment of CYP1A induction, a total of 3 unstained, PLL-mounted slides containing liver, heart and gills were selected from randomly chosen specimens of each dose-group in experiment A. Immunohistochemistry was performed on all 18 slides simultaneously to prevent technique induced staining differences. Staining intensities in various organs were qualitatively assigned as not detectable, low, intermediate or high by two independent histopathologists. As each slide contained two specimens, six animals were viewed for each exposure group. For statistical analysis these pairs were counted as single observations. During microscopic evaluation of all slides, observers were not aware of the exposure concentration from which the slides originated. A similar procedure was applied to 8 randomly selected PLL slides per group in experiment B.

In vitro study

Chemical-activated luciferase gene expression mediated by Ah-receptor activation (DR-CALUX) was used as described by Murk et al. (1998) to assess the AhR activating potential of DE-71, cleaned DE-71, and its planar fraction. In brief, H4IIE-cells transfected with the luciferase reporter gene plasmid: pGudLuc1.1 (H4IIE.luc cells), were exposed in triplicate to each of the mixtures in concentrations 0.008, 0.08, 1.6 and 2.4 mg DE-71/ml. A standard concentration series of TCDD ranging from 0.3 to 300 pM was included. After 24 hrs of exposure, the medium was discarded and cells were washed with low salt buffer and allowed to swell with 20 µl of demineralized water. Cell plates were frozen at -80 °C for at least 30 minutes to allow lysis of the cells. Luciferase activity was measured as light production after addition of luciferin assay mix (Promega) on an Amelite luminometer (Amersham). Responses of the compounds were compared to the responses of the TCDD standard curve.

Statistical analysis

Statistical analysis was performed using SPSS 12.0 software for windows (SPSS, Chicago, IL). Continuous data were analyzed by one-way analysis of variance (ANOVA). Semi-quantitative data were tested for significance using the Kruskal-Wallis (KW) test for all groups; for post hoc testing KW was used for 2 independent samples. A difference between groups was considered statistically significant when $p<0.05$.

Results

Chemical analysis of DE-71 and internal dose

Both fractions of DE-71 were analyzed to elucidate the composition of the DE-71 sample used in this study. Results are shown in Fig. 1, and Tables 2 and 3. From analysis of the planar fraction, it appears that most PBDEs were separated from the PBDD/Fs, with the exception of the lower brominated BDE-28, which was longer retained in the Al_2O_3 column and eluted together with PBDD/Fs. Five contaminating PBDDs (compounds 1-6; Table 2), and 2 PBDFs (compounds 7 and 8; Table 2) were detected which could not be further identified; the retention times of one PBDF showed overlap with the 1,2,3,7,8-PeBDF standard.

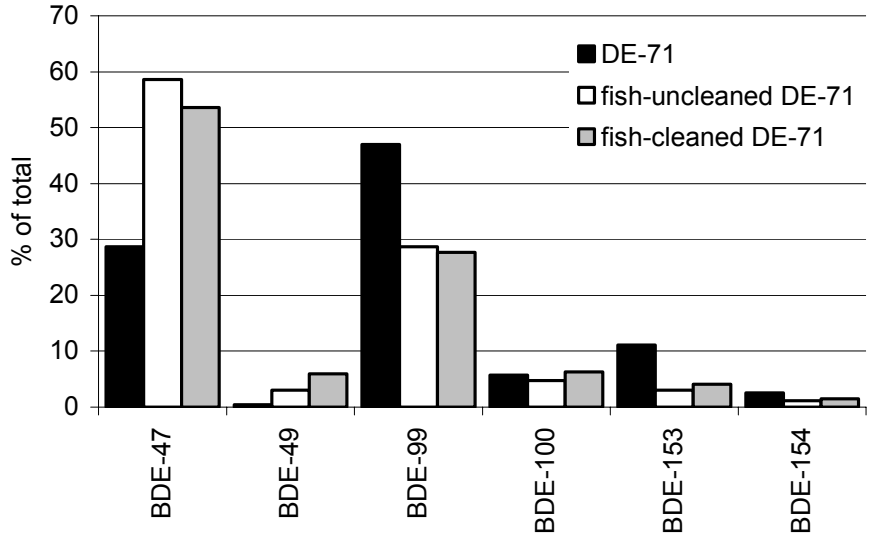

Fig. 1. Distribution of predominant BDE congeners in DE-71 and average distributions in zebrafish exposed to uncleaned and cleaned DE-71.

Table 2: Indicative concentrations of PBDD/F congeners in DE-71 based on quantification with a 2,3,7,8-congener standard.

Planar compound	Estimated concentration (mg/kg DE71)
1) tetrabromoDD	3
2) pentabromoDD	5
3) pentabromoDD	6
4) hexabromoDD	6
5) hexabromoDD	9
6) hexabromoDD	5
7) pentabromoDF*	3
8) pentabromoDF	5

* The retention time of this compound was very close to the retention time of 1,2,3,7,8-pentabromoDF.

Pooled samples of four fish per group from experiment B were analyzed for their PBDE content (Table 3). A weak background (predominantly BDEs -47 and -190) was detected in the control group. Animals exposed to uncleaned DE-71 and cleaned DE-71 showed similar patterns of the predominant congeners BDEs -47, -49, -99, -100, -153, and -154 (Fig. 1). BDE-28 levels in animals exposed to uncleaned DE-71 at 1 mg/l were low compared to the other groups. Animals exposed to only the planar fraction contained only very low amounts of PBDEs except for BDE-28, and less so for BDEs -47, -66, and -77.

Table 3. Analysis of individual BDE congeners from pooled sample of 4 fish per exposure group in experiment B. Amounts are in ng/g wet weight.

Exposure	Control	Uncleaned 0.1 mg/l	Uncleaned 1 mg/l	Non-planar 0.1 mg/l	Non-planar 1 mg/l	Planar 0.1 mg/l	Planar 1 mg/l
(% in DE-71)	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g
BDE-28 (0.1)	3.4	1300	250	120	270	1600	19800
BDE-47 (28.7)	36	297100	471100	270800	417400	160	670
BDE-49 (0.4)	1.5	2300	48100	34500	37400	3.0	12
BDE-66 (0.4)	<0.8	830	4300	310	1900	120	1300
BDE-71 (<0.03)	<0.8	62	470	92	410	<0.7	<0.8
BDE-75 (<0.03)	1.0	74	250	33	280	<0.7	<0.8
BDE-77 (<0.03)	<0.8	<0.9	<0.9	<0.9	<0.9	9.0	55
BDE-85 (2.9)	<0.7	180	1600	290	5400	<0.6	<0.7
BDE-99 (47.0)	5.6	138400	242400	124100	244400	39	<0.9
BDE-100 (5.7)	2.3	16700	51000	15900	77800	<0.7	27
BDE-119 (<0.03)	<0.8	26	73	31	71	<0.7	<0.8
BDE-138 (1.0)	<0.8	390	2200	390	4600	<0.7	<0.8
BDE-153 (11.1)	2.1	11800	30900	11300	49800	10	25
BDE-154 (2.6)	1.1	4400	10900	4200	17300	3.2	7.5
BDE-183 (<0.03)	<0.6	34	170	48	350	<0.5	<0.6
BDE-190 (<0.03)	47	<0.9	<0.9	<0.9	15	<0.7	<0.8

General toxicological parameters

During experiment A, mortality was low (Table 1a). In the first week, one animal in the highest and one in the second highest dose group died. In the highest dose group, three out of 20 animals developed erratic swimming behavior during the fourth (and last) week of the experiment. Affected animals were swimming on their sides or upside-down and showed prolonged violent swimming when roused. Two of these animals died shortly after these symptoms developed; a third animal died later during the same week. Deceased animals were not included in the analysis of size and weight at the end of the total exposure period. Slight but statistically significant growth retardation (body weight and length) was found in the highest dose group only (Table 1a). Animals in all groups were feeding normally, apart from the three that showed abnormal behavior, mentioned above. It should be noted that the highest concentration of DE-71 might have exceeded solubility in water containing 0.01% DMSO, judging from slight opacity of the water at the time of dosing. This opacity was of a transient nature and is not expected to have hindered the animals in feeding.

During experiment B, no mortality occurred. Transient water opacity was noted in the two groups of highest PBDE concentration (1 mg (un)cleaned DE-71/l, Table 1b). Differences in growth were not observed between groups.

Histopathology

Whole body histological examination of 16 animals per group was performed in both experiments A and B. Increased basophilia, indicative of highly active protein synthesis, of the liver was found in only 2 animals exposed to 0.010 mg DE-71/l in experiment A. Vacuolization of hepatocytes was abundant in all groups including controls in both experiments. Marked vacuolization of the exocrine pancreas was present in two animals (exposed to 0.010 and 0.10 mg DE-71/l) from experiment A. Thyroid epithelium retained its normal flat to cuboidal aspect in all groups and no apparent changes in follicular colloid were noticed. None of these changes were related to exposure.

Hermaphroditism (ovotestis) was found in 2 animals from the control group and 1 from each of the groups exposed to concentrations 0.010 mg/l, 0.032 mg/l, and 1.0 mg/l in experiment A. The condition was characterized by the presence of differentiated oocytes in a gonad that showed clear signs of male differentiation (spermatogenesis). None of these testis-ova contained yolk granules. In both experiments A and B, the undifferentiated nature of the gonad did not allow gender determination in part of the histologically examined animals. The prevalence of undifferentiated gonads or gender did not relate to dose group in either experiment.

In the gills, lifting of epithelium with subepithelial proteinaceous material (branchial edema) was occasionally present in all dose groups, although less evident in the controls. Severe edema of the majority of secondary lamellae was significantly more frequent in animals from the groups of highest DE-71 dosage in experiment A (KW: $p<0.01$). However, this finding was not reproduced in experiment B. Infiltration of the gill lamellae by lymphocytes was seen occasionally throughout all groups including controls in the present experiments. This condition was most pronounced at the bases of the primary lamellae, suggesting these lymphocytes are a functional part of the thymus, which particularly in juvenile zebrafish shows close anatomical association with the epithelial lining of the gill cavity. Since lymphocytes were rarely observed inside the epithelial layer, true inflammatory change (branchitis) is deemed absent in the present study.

A strong and clearly exposure-related statistically significant increase in immunoreactivity against CYP1A was observed in animals exposed to DE-71 in experiment A (Figs. 2 and 3). Increased CYP1A immunoreactivity was noted at the lowest DE-71 concentration (0.01 mg/l). This effect was most pronounced in endothelium of larger blood vessels throughout the body, particularly endocardium, ventral aorta, and branchial arteries. The well-vascularized gills were used for semi-quantitative analysis of CYP1A induction (Table 4a). In the higher dose groups, besides the endothelium lining blood vessels, the pillar cells in the secondary gill lamellae were positive (Fig. 2a). In the control groups, immunoreactivity was absent (Fig. 2b).

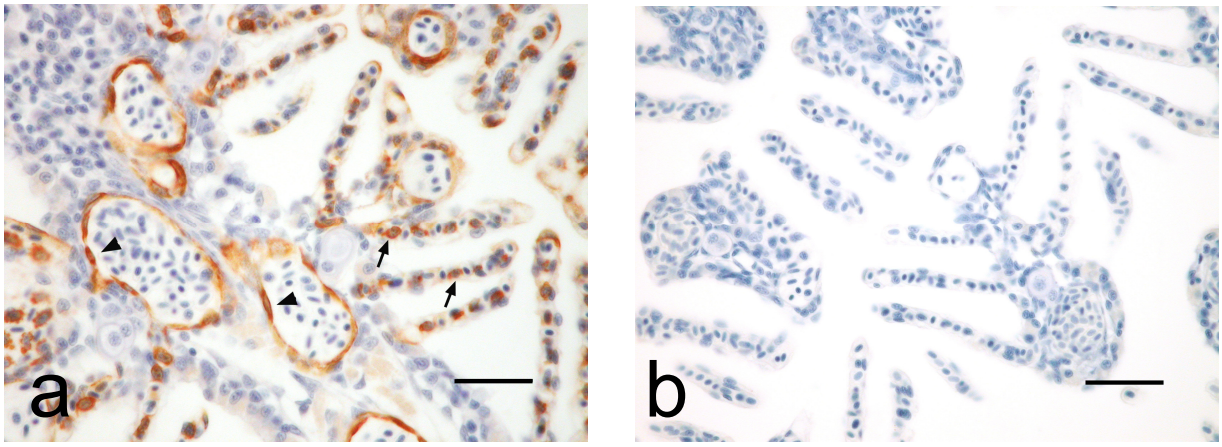

Fig. 2. (a) Gills from a zebrafish exposed to uncleaned DE-71 (1 mg/l). Immunoperoxidase staining using a primary antibody against CYP1A. Endothelium lining blood vessels (arrowheads) and pillar cells of secondary lamellae (arrows) show marked immunoreactivity. Hematoxylin counterstain, bar represents 25 µm. (b) Gills from a control fish, stained according to the same procedure as in Fig. 1a. Bar represents 25 µm.

A distinct positive reaction was also found in the endothelium of internal blood vessels. In the liver, only weak CYP1A immunoreactivity was detected in parenchyma of exposed animals (Fig. 3). As in other organs, endothelium lining hepatic vascular tissue showed dose dependent CYP1A immunoreactivity (Fig. 3). A less intense, but dose related induction of immunoreactive CYP1A was furthermore present in bile duct epithelium (Fig. 3), throughout the intestinal tract mucosa, in the epithelium of predominantly proximal kidney tubules, skeletal muscle and Leydig cells of the skin (schreck-substanz cells). CYP1A immunohistochemistry in animals exposed to uncleaned DE-71 (0.1 and 1 mg DE-71/l) and the planar fraction of DE-71 (1 mg DE-71/l) in experiment B confirmed the pattern described for experiment A. Exposure to cleaned DE-71 in experiment B resulted in only occasional weak CYP1A immunoreactivity (Table 4b).

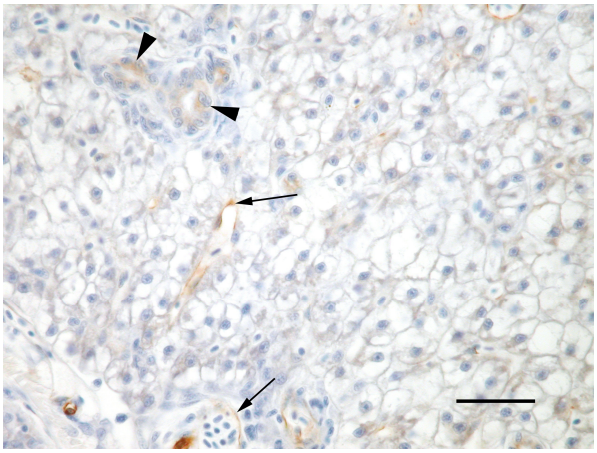

Fig. 3. Liver section from a zebrafish exposed to uncleaned DE-71 (1 mg/l). Indirect immunoperoxidase staining using anti-CYP1A as primary antibody. Diffuse grey-brown color of hepatocytes hints to some extent of immunoreactivity. Marked positivity in endothelium (arrow) and bile duct epithelium (arrowheads). Bar represents 25 µm.

Table 4a. Experiment A: endothelial CYP1A immunoreactivity (gill endothelium)

DE-71 dose (mg/l)	-	+/-	+	++
0.0	3			
0.010**		3		
0.032**			2	1
0.10**			1	2
0.32**			1	2
1.0**				3

Table 4b. Experiment B: endothelial CYP1A immunoreactivity (gill endothelium)

Exposure	-	+/-	+	++
control (DMSO only)	8			
uncleaned DE-71: 0.1 mg/l**			6	2
uncleaned DE-71: 1 mg/l**			2	6
non-planar fraction from 0.1 mg DE-71/l (cleaned DE-71)**$^{\#\#}$	1	7		
non-planar fraction from 1 mg DE-71/l (cleaned DE-71)**$^{\#\#}$	2	6		
planar fraction from 0.1 mg DE-71/l	7		1	
planar fraction from 1 mg DE-71/l**			4	4

numbers represent sets of 2 animals; **groups differ significantly from controls, $^{\#\#}$groups differ significantly from uncleaned DE-71 at 0.1 and 1 mg/l (K-W: $p<0.01$).

- no visible immunoreactivity
+/- sporadic immunoreactivity
+ general immunoreactivity
++ strong general immunoreactivity

Immunohistochemical staining for proliferating cell nuclear antigen (PCNA, exp A) revealed no dose-related alterations of cell proliferation in any of the organs examined. No indication was found for altered mitotic activity in the male gonads of exposed animals.

Determination of TEQs using the DR-CALUX assay

The TCDD toxic equivalency of uncleaned DE-71 was 2.0 ng TEQ/g DE-71, whereas the planar contaminants isolated from the same amount of DE-71 yielded a 10 times higher TEQ (19.7 ng/g; Table 5). Luciferase induction by cleaned non-planar DE-71 was below the limit of detection. There were no indications for cytotoxicity at the concentrations tested.

Table 5. Toxic equivalencies (TEQs) of the DE-71 stock solutions used in experiment B determined by the DR-CALUX assay (limit of detection: 0.8 ng/g).

Stock in DMSO	ng TEQ/g DE-71
untreated (uncleaned) DE-71	2.0
cleaned DE-71	<0.8
planar fraction of DE-71/ml	19.7

Discussion

BDEs -47 and -99 were the major components of the commercial PentaBDE mix, DE-71, which is in agreement with literature (Birnbaum and Staskal, 2004). The relative enrichment of BDE-47 in animals exposed to uncleaned DE-71 (Fig 1) has been noted before and may have resulted from preferential uptake (de Wit, 2002); this could also explain the relative increase of BDE-49 in exposed zebrafish. The total amount of contaminating PBDD/Fs in the uncleaned DE-71 sample was estimated at 42 µg/g (Table 2) and was responsible for all of the dioxin-like toxic potency detected *in vitro*. A peak representing 3 µg pentabrominated DF/g of DE-71 was detected eluting very close to 1,2,3,7,8-PeBDF. This could be consistent with the report of 1,2,3,7,8-PeBDF as predominantly detected dioxin-like contaminant in DE-71 (Sanders et al., 2005), although the level was lower in that study (19.8 ng/g). With a relative potency in the DR-CALUX assay of 0.14 compared to TCDD (at EC_{50} concentrations in pM; Behnish et al., 2003), the present level of 1,2,3,7,8-PeBDF could explain up to 225 ng TEQ/g DE-71.

The present lack of consistent negative effects of PBDEs on growth is in agreement with earlier results in rainbow trout after oral exposure to approximately 20 mg Tetra- or PentaBDE per kg for 22 days (Tjärnlund et al., 1998). Although in the present study, juvenile (growing) fish were used, and high internal PBDE concentrations were reached, mildly reduced length and weight (84% and 63% of the controls, respectively) were only observed in animals exposed to 1 mg DE-71/l in experiment A, indicating relatively limited toxic potential of lower brominated PBDEs in 4-8 weeks old zebrafish. Lack of toxic effects of planar contaminants in the present study may be consistent with the predominance of TCDD-induced developmental toxicity in even younger, embryonal stages of zebrafish (Carney et al., 2006), and the relative insensitivity of zebrafish early life stages to TCDD when compared to other fish species (Elonen et al., 1998). Absence of general toxicological signs during experiment B remains unexplained and a clinical lowest observed effect concentration (LOEC) was not established.

The frequent hepatocellular vacuolization observed in all groups including controls in the present study may have obscured a dose related hepatocellular lipidosis, as was reported in adult three-spined stickleback after 3.5 months of oral exposure to another commercial PentaBDE mixture, Bromkal 70-5DE (total dose: 6.29 and 10.39 mg /20 fish; Holm et al., 1993). Oral exposure in that study may also have resulted in a more direct route to the liver, and the time between exposure and examination of the fish was longer. However, since no mention of a cleanup procedure was made in that study, the lipidosis could have resulted from exposure to planar contaminants as was observed in rainbow trout exposed to TCDD (Walter et al., 2000; van der Weiden et al., 1992). Again, zebrafish may be less sensitive to dioxin-like chemicals compared to sticklebacks, or the contaminant levels in DE-71 may have been lower than in Bromkal 70-5DE.

The absence of morphologic thyroid changes indicates that exposure to commercial PentaBDE did not affect thyroid function to a major extent in developing zebrafish. Although thyroxin (T_4) levels were decreased in juvenile lake trout (*Salvelinus namaycush*) after single oral exposure to a mix of 13 BDE congeners including DecaBDE (BDE-209), triiodothyronin (T_3) levels were unaffected (Tomy et al., 2004). Since T_3 is the more active form of thyroid hormone (Brown et al., 2004), morphologic changes in the thyroid indicative of functional disorder tentatively relate to altered levels of T_3 and TSH,

rather than T_4. In pubertal rats however, exposure to DE-71 decreased plasma levels of both thyroid hormones (T_3 and T_4), and increased plasma thyroid stimulating hormone (TSH) levels, resulting in thyroid morphology changes consistent with goiter after 3-4 weeks of exposure to a daily oral dose of 60 mg/kg (Stoker et al., 2004). Although zebrafish is capable of developing goiter when exposed to the anti-thyroid drug propylthiouracil (van der Ven et al., 2006) the lack of histological changes in the thyroid gland may be consistent with a relatively low sensitivity of fish to goitrogenic environmental contaminants (Leatherland, 1993).

In spite of (anti)-estrogenic properties of PBDEs *in vitro* (Meerts et al., 2001; Nakari and Pessala, 2005), *in vivo* exposure to DE-71 did not produce morphologic evidence for endocrine disruption on the reproductive level in the present study. Mitotic activity in male gonads, evaluated by PCNA immunostaining, was similar in all groups. Liver H&E staining, a sensitive indicator for production of vitellogenin in zebrafish exposed to known estrogens (van der Ven et al, 2003) was not dose-dependently affected. Lack of effects in our experiments may reflect the rather low relative (anti-)estrogenic potencies of PBDEs, and the low uptake of weak ER agonist BDEs compared to inactive ones (e.g. BDE-47; Meerts et al., 2001).

The dose-dependent induction of immunodetectable CYP1A in endothelium in the gills, heart and remaining internal organs, hepatocytes, bile duct epithelium, and epithelium of the gastro-intestinal tract and kidney of animals exposed to uncleaned DE-71 resembles CYP1A induction patterns found in TCDD exposed fish remarkably well (Buchmann et al., 1993; Zodrow, 2004, *Danio rerio*; Stegeman et al., 1991, *Stenotomus chrysops*; Grinwis et al., 2000, 2001, *Platichthys flesus*). The markedly reduced CYP1A induction in animals exposed to cleaned DE-71 indicates that the planar contaminants in DE-71 were largely responsible. Although analysis of fish indicated a relatively large amount of BDE-28 in the planar fraction, BDE-28 was not a potent AhR agonist in primary cultured rat hepatocytes (Chen and Bunce, 2003), and recently presented as an AhR antagonist (Hamers et al., 2006). Presently, CYP1A induction after exposure to water-borne DE-71 or the planar fraction of DE-71 was most conspicuous in gill endothelium, whereas previously, in adult zebrafish exposed to TCDD via intraperitoneal injection, immunodetection of CYP1A was more intense in internal organs (kidney, liver, gastrointestinal tract; Zodrow et al., 2004). Water-borne exposure may have resulted in a relatively higher exposure of the gills in the present study. In addition, the gills are highly vascularized, and in lake trout early life stages, endothelium was shown to be sensitive to induction of CYP1A by TCDD (Guiney et al., 1997). In that study, vascular immunodetection of CYP1A coincided with pericardial, yolk sac and meningeal edema, indicating a relation with vascular functional disorder. In zebrafish, pericardial and cranial edema was reported in newly hatched larvae exposed to TCDD (Wannemacher et al., 1992) and in larvae exposed during the first 10 days after fertilization (Henry et al, 1997), suggesting a similar target for AhR-mediated toxicity in this species. Since hypoxia was shown to reduce AhR-mediated effects and edema in zebrafish early life stages exposed to TCDD (Prasch et al., 2004), locally higher oxygen levels may result in a relatively high sensitivity of the gills compared to internal organs in post-hatch fish. Although dose dependent branchial edema was only found in experiment A, it is tempting to assume a relation with AhR-mediated toxicity.

The importance of planar contaminants for *in vivo* induction of CYP1A by uncleaned DE-71 was confirmed *in vitro* by the lack of an AhR-mediated DR-CALUX

response to cleaned DE-71, in spite of the presence of some AhR agonist PBDE congeners (namely BDEs -66, -85, -100 and -153; Chen and Bunce, 2003). The planar fraction isolated from the DE-71 stock however induced a 10 times stronger AhR-agonist response compared to the original uncleaned stock. The relatively low induction by uncleaned DE-71 possibly resulted from inhibition of AhR-mediated gene expression by some of the PBDEs present (Chen and Bunce, 2003; Kuiper et al., 2004; Peters et al, 2004). In spite of the lower DR-CALUX response, zebrafish exposed to uncleaned DE-71 showed an equally strong immunostaining of CYP1A protein as fish exposed to the planar components alone. This is consistent with the failure of DE-71 to reduce β-naphtoflavone-induced EROD activity *in ovo* in killifish (Fundulus heteroclitus) within one day of exposure (Timme-Laragy et al., 2006). The apparent lack of attenuation of AhR mediated CYP1A induction in both the present and the killifish studies is possibly caused by preferential uptake of planar components; in the present study, the longer exposure period of the fish when compared to the H4IIE-luc cells could have enhanced this effect, resulting in stronger AhR activation *in vivo*. Because of the low absolute doses of PBDD/Fs and the small volume of the samples, PBDD/Fs were expected to be below the limits of detection and therefore not analyzed in the fish in the present study, and a ratio between planar and non-planar components was not established.

Conclusions

Commercial pentabromodiphenylether induces CYP1A in various tissues in juvenile zebrafish (*Danio rerio*) in a similar pattern as observed after exposure to dioxin-like compounds, with endothelium as the predominantly reacting cell-type. Although weak AhR activation has been reported for several PBDE congeners, in the present study cleaned DE-71 did not induce a significant DR-CALUX response, and only limited CYP1A immunoreactivity was observed in juvenile zebrafish. Based on the DR-CALUX response to the planar fraction alone, planar contaminants accounted for 19.7 ng TEQ/g DE-71. The present findings confirm 1,2,3,7,8-PeBDF as an important dioxin-like contaminant in DE-71. Although the levels of planar contaminants in commercial pentabromo-diphenylether mixtures may vary, they are the likely cause for AhR mediated effects in this and probably other studies with uncleaned PBDE mixtures. This should be taken into account when interpreting TCDD equivalencies reported for commercial PBDE mixtures. Immunohistochemical analysis of CYP1A proved a sensitive method for detection of AhR agonists in fish exposed to complex mixtures where the overall composition may modulate dioxin-like activity.

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Toxicity of tetrabromobisphenol A (TBBPA) in zebrafish (*Danio rerio*) in a partial life cycle test

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Abstract

Toxicological effects of the widely used flame retardant tetrabromobisphenol A (TBBPA) were assessed in a partial life-cycle test with zebrafish (*Danio rerio*). Exposure of adult fish during 30 days to water-borne TBBPA in nominal concentrations ranging from 0 (control) to 1.5 µM was followed by exposure of offspring early life stages up to 47 days post hatching to the same concentrations. Adults exposed to 3 and 6 µM showed severe disorientation and lethargy shortly after beginning of exposure and were euthanized. Because semistatic exposure resulted in fluctuating water concentrations, pooled fish samples were chemically analyzed for internal dose assessment. Egg production was decreased in fish exposed to TBBPA concentrations of 0.047 µM and higher, and a critical effect level of 7.2 µg/g lipid with a lower 5% confidence limit of 3.9 µg/g lipid for 50% decreased egg production was calculated. Histology of adult ovaries indicated a relative increase of premature oocytes in 2 surviving females exposed to 1.5 µM. Hatching of TBBPA exposed larvae was decreased except in animals exposed to 0.375 µM. Early post hatching mortality was high (81%) in larvae in from the highest exposure concentration and surviving juveniles showed a significant predominance of the female phenotype. Exposure of eggs from control parents to up to 6 µM TBBPA resulted in increasing malformation and pericardial fluid accumulation from 1.5 µM; at higher concentrations, all embryos failed to hatch. The presented results indicate decreased reproductive success in zebrafish at environmentally relevant TBBPA concentrations.

Introduction

Tetrabromobisphenol A (2,2-bis-(3,5-dibromo-4-hydroxyphenyl)-propane; TBBPA) is today's most abundantly used brominated flame retardant (BFR). Annual consumption of TBBPA in Europe alone was 7.800 tons in 2002, representing 6% of the total global use (BSEF 2004). TBBPA is applied in a wide variety of consumer products. Its predominant use (e.g. in circuitry boards) is as reactive flame retardant, i.e. the flame retardant is covalently bound to the polymer. However, approximately 10% is used as additive flame retardant in a wide variety of consumer products including housings of domestic electric/electronic appliances (BSEF 2004), facilitating the release of TBBPA into the environment (Birnbaum and Staskal, 2004). TBBPA may leach from landfills (Osako et al., 2004), and consequently end up in surface waters. As a result, TBBPA has been found in aquatic sediment samples from all over the world (Sellström et al., 1995; Watanabe et al., 2003; Morris et al., 2004) and although usually below the limit of detection in the water phase, TBBPA has repeatedly been detected in fish (Asplund et al., 1999; Ohta et al., 2004; Morris et al., 2004) in levels up to 63 ng/g lipid weight in eel from fresh water and up to 245 ng/g lipid in whiting (muscle) from the North Sea (Morris et al, 2004); recently, 583 ng TBBPA/g lipid was detected in a pooled sample of 13 sand eel from the Waddensea (RIVO 2003; unpublished data).

In fish, TBBPA is acutely toxic at high concentrations as shown in a number of studies. LC_{50} concentrations reported include 0.51 mg/L (0.9 µM) in bluegill sunfish, 0.40 mg/L (0.7 µM) in rainbow trout (WHO 1995), and a relatively high 3 mg/l (5.5 µM) in zebrafish (Lee et al., 1993). Evidence for TBBPA toxicity at lower doses is limited and points to modulation of the thyroid and reproductive hormonal systems: TBBPA competes with the *in vitro* binding of thyroxin to its human plasma transporter protein, transthyretin (Hamers et al., 2004; Meerts et al., 2000), and the rat thyroid receptor (Kitamura et al., 2002), and increases the response to T_3 in cultured GH3 rat pituitary adenoma cells (Kitamura et al., 2005a). However, 10^{-8} M (54 ng/L) TBBPA and higher may suppress T_3-dependent tail shortening in *Rana rugosa* tadpoles, indicating anti-thyroid activity (Kitamura et al., 2005b).

Cultured MCF-7 breast cancer cells show a weak estrogenic response to TBBPA (Körner et al., 1998; Olsen et al., 2003), and TBBPA inhibits *in vitro* estradiol sulfonation (Hamers et al., 2004), indicating potential for both direct and indirect estrogenic activity *in vivo*. Increased uterus weights in ovariectomized mice exposed to TBBPA for 3 days (20 mg/kg/day, i.p.) confirm estrogenicity *in vivo* (Kitamura et al., 2005a).

Since sex steroids and thyroid hormones influence reproduction in aquatic vertebrates and may interact during larval development (Cyr and Eales, 1996; Arcand-Hoy and Benson, 1998), the presence of TBBPA in aquatic environments raises concern as to their possible impact on reproductive health in fish. Regarding endocrine effects of TBBPA in fish, the focus has been on the estrogen dependent production of the yolk precursor vitellogenin (VTG) which was not affected by intraperitoneal exposure to TBBPA in rainbow trout and eelpout (Christiansen et al., 2000; Ronisz et al., 2004); however the combined effects of possible disruption of estrogen or thyroid hormonal systems on ecologically relevant parameters like reproduction and early-life stage development in fish exposed to TBBPA have not been taken into account.

In the present study, zebrafish (*Danio rerio*) were exposed to TBBPA in a partial life-cycle test using a benchmark approach to further investigate possible ecotoxicological consequences. Egg production and fertilization ratio were used to monitor reproduction, and development and mortality were investigated in offspring until juvenile stage (47 days post hatch). Whole body sections of both adults and offspring were examined histologically with special attention for reproductive and endocrine target organs. TBBPA concentrations were determined in water and fish (adult and juvenile) for accurate dose-response assessment.

Materials and methods

TBBPA (99.17% pure, containing tribromobisphenol-A and o,p'-TBBPA as impurities; HPLC-analyzed) was obtained as a composite mix of technical products from various producers through BSEF (kindly provided by Dr Klaus Rothenbacher).

Exposure of adults

Adult reproductive zebrafish (*Danio rerio*) were supplied by a commercial importer (Ruinemans Aquarium BV, Montfoort, The Netherlands) and kept under quarantine conditions for a minimum period of 4 weeks. Seven days prior to testing the animals were transferred to the test system containing standard formulated water (van der Ven et al., 2006). Each group was kept in 6 L water in an all-glass aquarium, covered by a glass plate. Water was continuously aerated via glass tubes and water temperature was maintained at 27±2°C, pH ranged from 7.2 to 8.4 and oxygen levels were >5 mg/L at all times. Nitrate levels were checked before each renewal and were usually below 0.5 mg/L, never exceeding 0.8 mg/L. A daily cycle of 14 hrs light, 10 hrs dark was maintained.

Adults were exposed to water-borne TBBPA during 30 days using dimethyl sulfoxide (DMSO, Acros, 's Hertogenbosch, The Netherlands) as solvent (final DMSO concentration: 0.01% in all groups including controls). A benchmark dosing regimen was applied using nominal concentrations of 0 (control), 0.023; 0.047; 0.094; 0.188; 0.375; 0.750; 1.50; 3.00; and 6.00 µM (Table 1) in a single experimental unit per test concentration consisting of 3 males and 3 females, except for the control group which was in duplicate. Animals were fed defrosted *Artemia* (Landman BV, Hoevelaken, The Netherlands) twice a day in an amount that was consumed completely within 5 minutes. Males were kept separate from females inside a nylon mesh net (mesh size: 3mm). Exposure was semi static by medium renewal every 3 to 4 days. At the end of week 4, water samples were collected in clean glassware immediately after renewal (0 hrs), and immediately before the end of exposure in week 5 (96 hrs later). Water samples were also collected from 2 aquariums under identical conditions, but without fish, at 0, 72, and 96 hrs after renewal. Water samples were stored at -20°C until chemical analysis.

Reproduction was monitored one day after each water renewal, when both sexes were placed inside the nylon mesh for 24 hours, after which clutch size, fertilization ratio, and hatching were determined. During exposure, animals were monitored daily for (abnormal) behavior or disease, and mortality. After 30 days, adults were euthanized in 100 mg tricaine methanesulphonate/L (MS222, Sigma-Aldrich, Zwijndrecht, the Netherlands) buffered with 500 mg $NaHCO_3$/L, and length and weight were determined, from which a condition factor (CF; weight/length^3) was derived. From each group, one male and female

were rinsed with water and dipped dry before freezing and storage at -20°C for chemical analysis; remaining animals were fixed in Bouin's fixative, and after 24 hrs transferred to 70% ethanol until histological processing.

Table 1. Internal tetrabromobisphenol A (TBBPA) concentrations in male and female adult zebrafish

Nominal water concentration (µM TBBPA)	TBBPA (ng/g ww)		TBBPA (ng/g lw)	
	male	female	male	female
Control-A	<11	<6.3	<262	<166
Control-B	<11	<7.7	<262	<175
0.023 µM	120	190	3871	6129
0.047 µM	590	1500	16857	30612
0.094 µM	2300	NA	71875	NA
0.188 µM	1800	9800	36735	376923
0.375 µM	4000	3900	114286	59091
0.75 µM	9100	6200	178431	193750
1.5 µM	12000	23000	285714	489362

ww=wet weight; lw=lipid weight; NA not analyzed; <(number): below limit of detection. Measurements were in single fish.

Exposure of juveniles

During weeks 4 and 5 of adult exposure, replicate groups of 50 fertilized eggs each (or less when not available) were randomly selected per dose and placed in 10 cm diameter glass petri dishes containing 60 mL DSW with the same nominal TBBPA concentrations as their parents. Hatching was recorded and a target number of 2x 50 larvae was sampled for continued exposure by semi static renewal (twice weekly). From 1 to 14 days post hatching (dph), larvae were fed fresh rotifer suspension (*Branchionus rubens*) daily. Starting at 7 dph, larvae were fed newly hatched *Artemia* nauplii two times a day (suspension adjusted to 17% dry matter, starting with 5 µl per larva, increasing to 120 µL per juvenile at 46 dph). At 15 dph the animals were transferred to all-glass aquariums under similar conditions as described for adults, containing 1.5 L water per 50 animals; this volume was increased to 3 L at 21 dph. Water samples were collected during week 5 of juvenile exposure from one control group and dose groups 0.094; 0.375 and 1.5 µM TBBPA directly after renewal (t=0) and 72 hrs later (t=72), and stored at -20°C for chemical analysis. On 46 dph, feeding was discontinued and animals were euthanized on day 47 post hatching. Larvae were monitored for abnormal behavior and growth, malformations, and survival. Juveniles were euthanized in buffered MS222, and length and weight were determined. Four animals per replicate were rinsed in clean water, dipped dry, frozen and stored at -20°C for chemical analysis (TBBPA body burden). Remaining animals were fixed in Bouin's for histological processing.

In addition, eggs from control parents were exposed for 3 days using 96 well plates (Greiner, Alphen a/d Rijn, the Netherlands). Fertilized eggs were individually placed in round bottom wells containing 200 µL of DSW with 0, 0.094, 0.188, 0.375, 0.75, 1.5, 3.0, or 6.0 µM TBBPA. Twelve eggs per concentration were randomly assigned to the plate; concentrations were also randomized. Development and survival *in ovo* was monitored three times a day using an inverted microscope. The procedure was carried out in duplicate. The present study was approved of by the Ethical Committee for Animal Welfare in Experiments of RIVM and complies with Dutch legislation.

Histological processing and evaluation
After routine tissue processing and paraffin embedding, whole body serial sections (4 µM) were cut at approximately 40 µM intervals from all adults except those sampled for chemical analysis, and a randomly chosen sub sample of on average 30 juveniles per concentration group, including cross sections through gonads and thyroid. Sections were stained with hematoxilin/eosin (H&E) and evaluated in random order by two independent observers.

Chemical analysis of fish and water samples
Total body analysis was performed on all frozen samples. Per adult concentration group, one male and one female were analyzed individually. Because of small size, four juvenile fish from each concentration group were pooled. Fish were thawed and dried with $NaSO_4$, and stored for two hours. The dried sample was extracted with a Soxhlet apparatus using hexane:acetone (3:1, v/v, Promochem, Wesel, Germany). An internal standard (1 mL of 200 ng/mL 13C-TBBPA, CIL, Andover, MA) was added to the extract, which was subsequently evaporated to 10 mL. An aliquot of the extract was dried with nitrogen and the lipid content was gravimetrically determined. The residual extract was acidified (pH=2), and sulphuric acid was added to remove the lipids. The cleaned extract was further purified with silica gel using isooctane and isooctane:diethyl ether (85:15, v/v, Promochem) as eluents. Finally, the silica eluate was evaporated to dryness with nitrogen, and 1 mL of methanol was added. TBBPA was determined with LC-MS/MS (LCQ Advantage, Thermo Finnigan) using electrospray ionization. Identification of TBBPA was based on the bromine cluster at m/z 444-452, and for quantification the daughter ion m/z 447 of the parent molecular ion (m/z 543) was used. A Zorbax, Eclipse XDB-C18 column was used with a gradient of ammonium chloride (0.1 mM) and acetonitrile (ACN) starting from 0 to 6 min. at ammonium chloride:ACN (3:1, v/v); followed by ammonium chloride:ACN (1:9, v/v) from 6 to 10 min.

Water samples were analyzed in duplicate. An aliquot of the samples was taken, and 1 mL of 200 ng/mL 13C-TBBPA was added as an internal standard. After the water was acidified (pH=2) and hexane was added, it was shaken for 2 minutes. The organic layer was separated. This procedure was repeated twice to ensure complete extraction of the water sample. The combined hexane extract was evaporated to dryness and methanol was added. TBBPA was determined with LC-MS/MS.

Statistics
Continuous data were explored by Analysis of Variance (ANOVA) and linear regression, using SPSS 12.0.1 software (SPSS Inc., Chicago, USA). Kruskall-Wallis and Fisher's exact tests (SPSS 12.0.1) were used for analysis of non-parametric parameters. Geometrical means of the successive clutch sizes (eggs produced per group when reproduction was successful) were used to compare the egg production between groups since within groups these observations were not independent. A fitted dose-response model (PROAST software, RIVM, Bilthoven, The Netherlands, Slob, 2002) was used to calculate the concentration leading to a 50% reduction in clutch size, together with a lower 5% confidence limit for that concentration.

Results

Exposure of adults

Chemical analysis
Results from chemical analysis of adult fish are shown in Table 1. TBBPA levels in animals, determined on both wet and lipid weight basis, showed a strong linear relation with the nominal water concentration (linear regression: R^2=0.80 and 0.65, respectively); correction for lipid content did not change regression statistics significantly; TBBPA content was not related to gender. Less then 1 % of the total amount of TBBPA added was found in the animals after 30 days of exposure. Water concentrations immediately after renewal were 96±11% (SD) of the nominal concentrations. During the exposure cycles, water TBBPA concentrations dropped and reached a minimum after 96 hrs at 5.2±2.7% (SD) of the nominal values; a gradual decrease was measured at 24, 72, and 96 hrs after renewal in a pilot study (data not shown). TBBPA levels in aquariums without fish decreased to 71% and 61 % of the initial levels after 72 hrs and 96 hrs, respectively.

General parameters
During adult exposure, abnormal behavior was noted within 1 hour from the start of the experiment, in animals exposed to 3.0 and 6.0 μM TBBPA, starting with slow-down of swimming and reduced respiration, followed by progressive loss of equilibrium. After a few hours, these animals were recumbent on the bottom of the tank and were euthanized for ethical reasons, and fixated for histological processing only; water samples were not collected. Apart from these euthanized animals, there was no exposure related morbidity or mortality. No dose dependent differences in weight, length or condition factor (CF) were noted in either sex.

Reproduction and hatching
Although the number of eggs produced per group on days when reproduction was monitored was variable throughout the experiment and between concentration groups, the total number of eggs produced by all groups exposed to TBBPA was lower than the number produced by each control group (Fig 1). There was substantial variation in the number of clutches produced with a relatively high number in adults at 1.5 μM TBBPA (Fig 1).

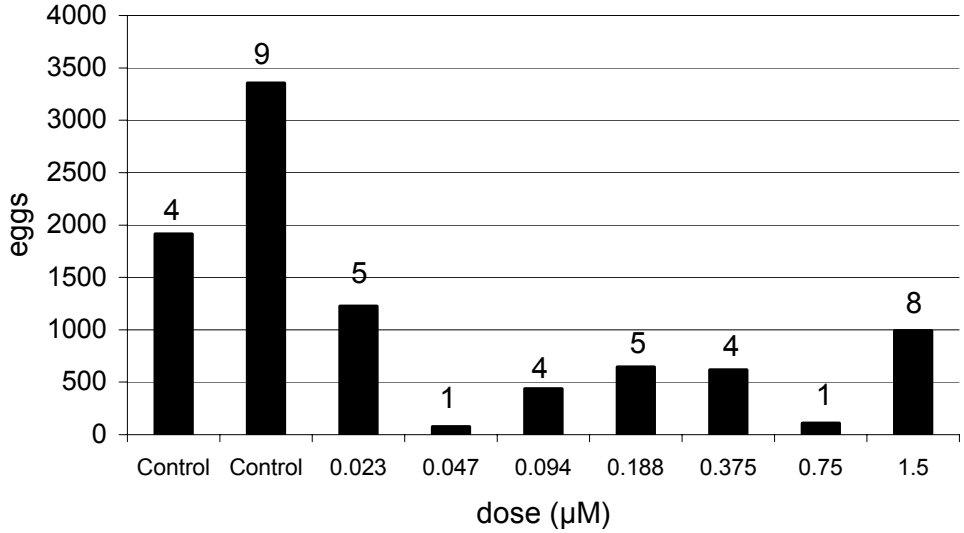

Fig. 1. Total egg production in zebrafish resulting from during 30 days exposure of adults. Numbers above bars represent number of clutches (3 females combined).

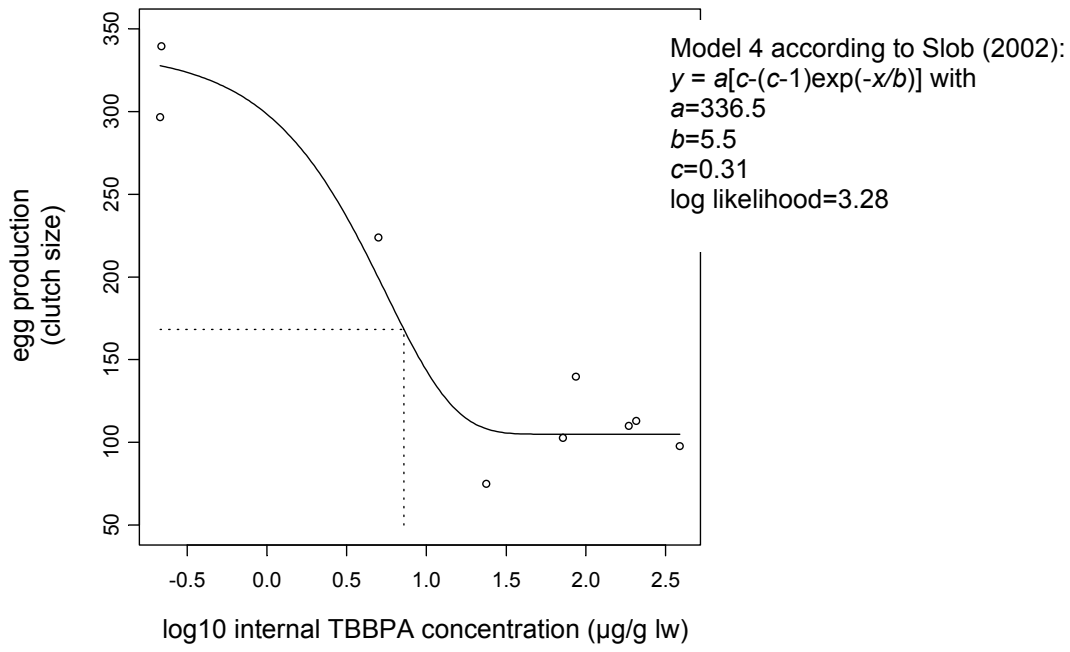

Fig. 2. Geometric mean of number of eggs per clutch (3 females combined) as a function of tetrabromobisphenol A (TBBPA) concentration, with fitted dose-response model. A 50% reduction was estimated at a critical internal level of 7.2 µg/g lipid weight (stippled lines).

Average clutch size showed a more consistent dose response and was well below 200 eggs/clutch (3 females) from 0.047 µM TBBPA onwards. A fitted dose-response model using internal concentrations calculated 50% reduction of clutch size (geometric mean) at an internal concentration of 7.2 µg TBBPA/g lipid with a lower 95% confidence limit of 3.9 µg/g lipid (Fig. 2). Egg fertilization ratio was not affected by TBBPA (Fig 3). Hatching of embryos from exposed eggs was decreased, except in embryos exposed to 0.375 µM TBBPA (Table 2), which hampers interpretation of dose-response characteristics.

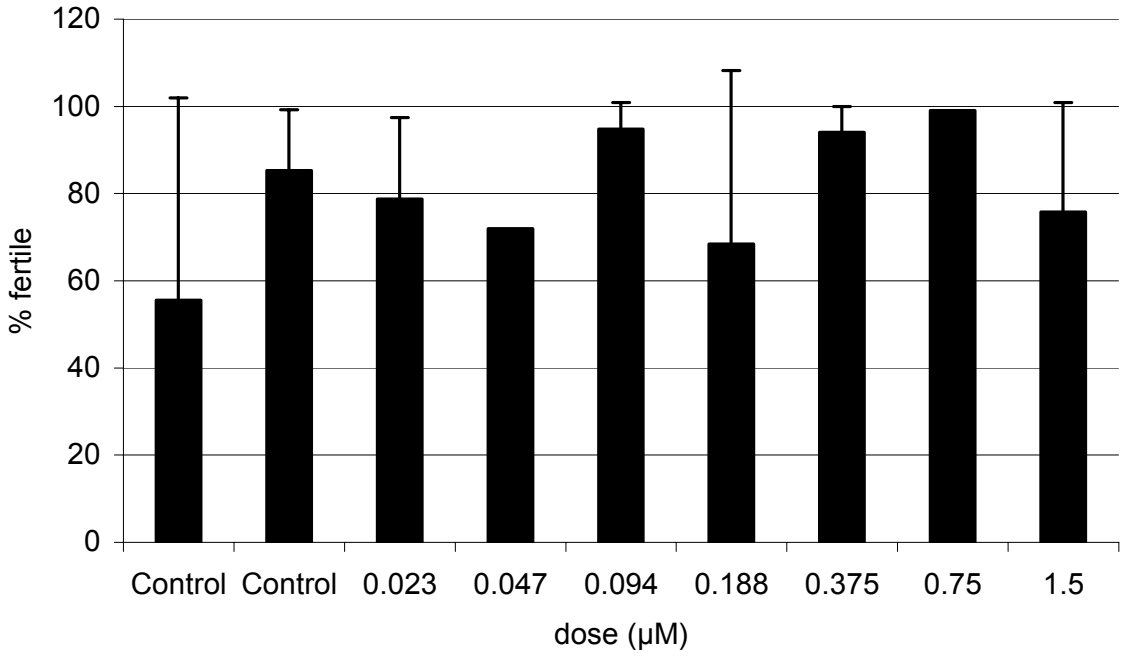

Fig. 3. Fertilization ratios in eggs from zebrafish exposed to TBBPA; egg deposition was in exposure medium. Error bars represent standard deviations of fertilization ratios from successive clutches.

Adult histology

Whole body histological sections revealed an increased estimated area occupied by previtellogenic oocytes in both microsopically analyzed females that had been exposed to 1.5 µM TBBPA during 30 days; this condition was seen in one out of four control females and did not occur in intermediate dose groups (Fig. 4).

Early atresia of oocytes characterized by hypertrophy and vacuolization of surrounding granulosa cells, hyalinization and fragmentation of the zona radiata, and disruption of yolk vesicles, was observed in females from all groups (35% overall prevalence), and was slightly less frequent (one out of four; not statistically significant) in the control groups. No dose-related changes were found in male gonads. Thyroid tissue appeared similar throughout all groups, showing variably sized colloid containing follicles lined by cubic epithelial cells, interpreted as moderately active. Without exception, female livers stained more strongly basophilic than male livers; differences in staining intensity were not dose related. Fragmentation of chromatin (karyorrhexis) in renal tubular epithelium was observed in 76% of the females but not in males, and was not related to TBBPA dose. The acute behavioral effects in animals exposed to 3.0 µM TBBPA and higher were not accompanied by histopathological changes.

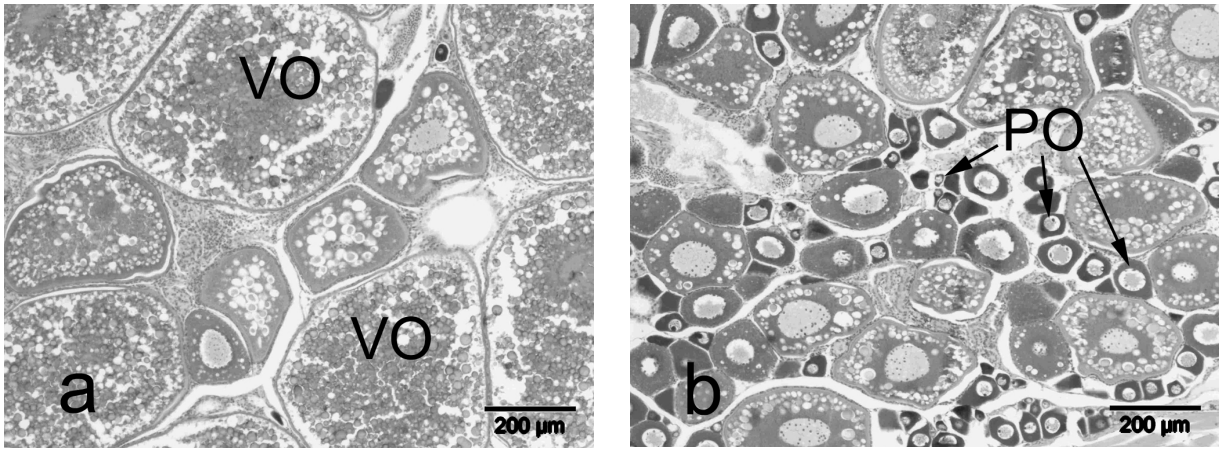

Fig. 4. Ovary of control (a) and exposed (1.5 µM tetrabromobisphenol A, b) zebrafish. Note the relative increase of the smaller, non-granulated previtellogenic oocytes in the exposed ovary. VO=vitellogenic oocytes; PO=previtelogenic oocytes.

Exposure of juveniles

Chemical analysis

In juvenile zebrafish, TBBPA levels ranged from below the detection limit to 5.6 µg/g lipid in animals exposed to a nominal dose of 1.5 µM.

TBBPA levels in exposure water directly after renewal were 80±16% (SD) of the nominal dose. After 72 hrs, levels had dropped to 22±6% (SD) of the nominal dose. Water was not analyzed at 96 hrs after renewal.

Juvenile growth, development, survival

Juveniles from dose groups 0, 0.023, 0.094, 0.375, and 1.5 µM were exposed to similar nominal concentrations as their parents until 47 dph; in adult dose groups 0.047, 0.188 and 0.75 µM, egg production during the last week of adult exposure was insufficient for subsequent exposure of offspring. Hatching ratios were significantly reduced in all exposed groups except when exposed to 0.375 µM (Table 2); a dose-response relation was not observed. Whereas at lower doses post hatching mortality was low, at 1.5 µM 81% of all larvae died during the first week of exposure (Table 2). Mortality was preceded by retardation of development (smaller animals, no swim-up even after 72 hrs post hatching) and malformations (abnormally curved spines, accumulation of clear fluid in the pericardial region and body cavity). Larvae at lower exposure levels showed no malformations and no effects on length, weight or CF at the end of the exposure period were noted.

Table 2. Hatching of embryos from adults during week 4 of exposure, and survival of hatched juveniles after the first week of exposure to tetrabromo-bisphenol A (TBBPA)

Nominal water concentration (µM TBBPA)	Eggs sampled during week 4	Hatching (%)	Post hatch survival (%)
0	600	79.7	97
0.023	100	43.0**	98
0.047	54	33.3**	NA
0.094	164	20.1**	100
0.188	300	13.0**	NA
0.375	369	80.0	96
1.5	315	58.1**	19**

** significantly decreased compared to controls (Fisher's exact test: $p<0.01$); NA not applicable.

Exposure of eggs from untreated parents resulted in severe retardation of development (delayed closure of hindgut), caudal and cranial malformation as well as edema in the pericardial and cranial yolk sac region (Fig. 5), and failure to hatch at concentrations of 3.0 and 6.0 µM. The number of developmentally retarded embryos was significantly increased in eggs exposed to 3.0 µM TBBPA and higher from 23 hrs post fertilization (Fisher's exact test: $p=0.01$); after 47 hrs, all embryos exposed to 6.0 µM had died. Surviving embryos including controls hatched at 55 hrs post fertilization; hatching of normally developing embryos was not delayed.

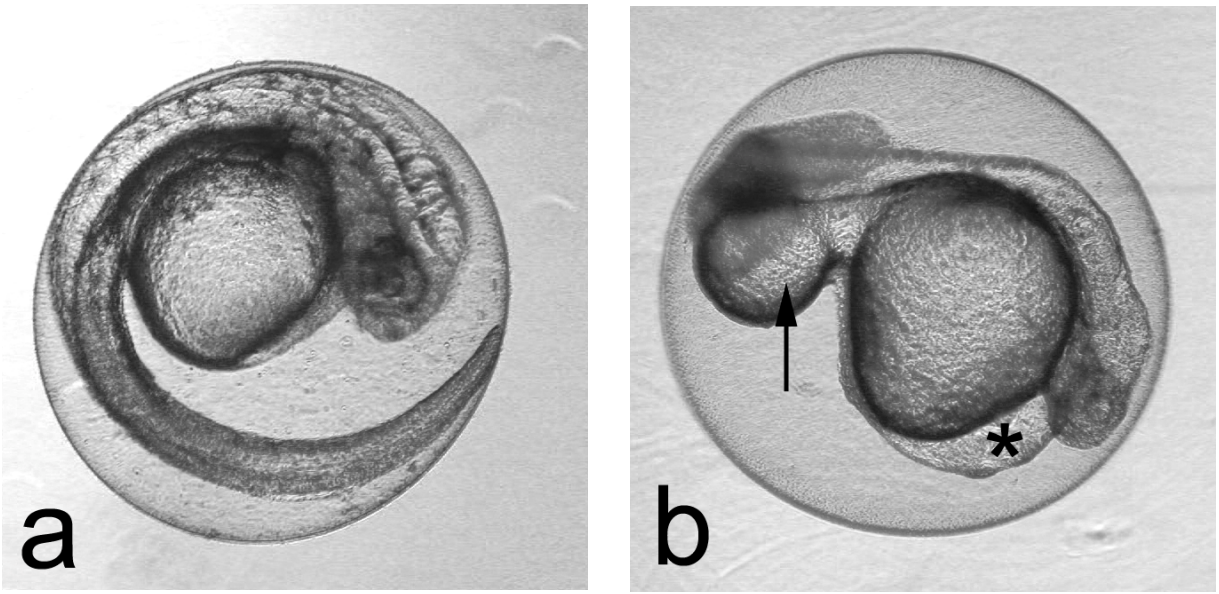

Fig. 5. Embryo of control (a) and exposed (6.0 µM tetrabromobisphenol A; b) zebrafish at 28 hrs post fertilization. Note caudal and cranial malformation, delayed closure of hindgut (arrow) and distension of the cranial yolk sac wall (edema; asterisk) in TBBPA-exposed specimen.

Juvenile histology

No dose related changes were observed in surviving juveniles after 49 days of exposure. Gonad development was sufficient to allow for sex differentiation; only premature gonad tissue was observed. Whereas in all other groups male differentiation was observed in 50-55% of the animals, in the 1.5 µM group (internal concentration: 5.6 µg/g lw), a statistically significant lower number was found (Fig. 6) and more female gonads were observed (79%; abundant oocytes of perinucleolar stage).

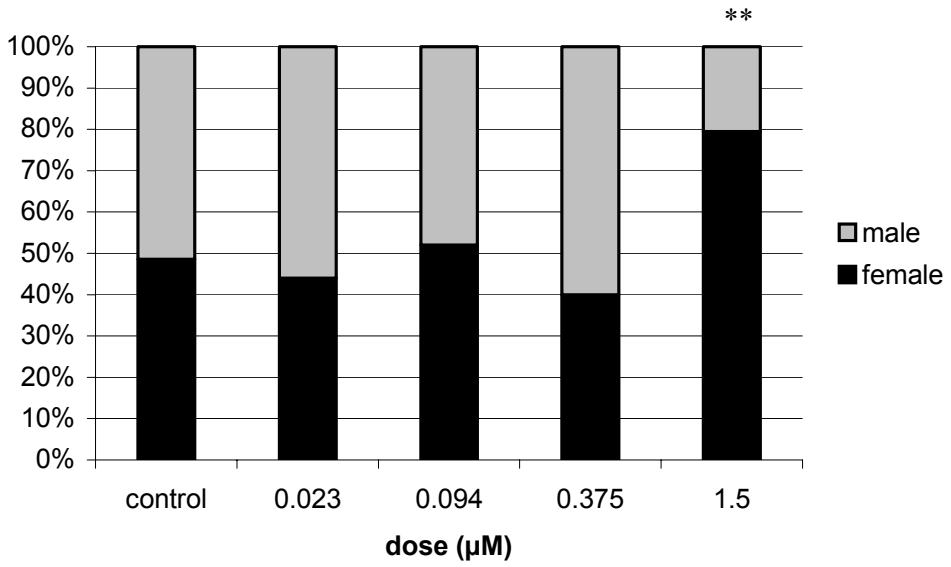

Fig. 6. Development of gender characteristics in gonads from TBBPA-exposed juvenile zebrafish at 47 dph. **Significantly different from control ($P<0.01$, Fisher's exact test).

As in adults, thyroid tissue presented moderate activity in all groups; there was no difference in the estimated number of follicles throughout the groups. A striking variation of staining intensity and intracellular vacuolization of liver was noted throughout all groups, which was not dose or gender related.

Discussion

TBBPA levels in fish were linear to the nominal concentrations (Table 1); the amount of TBBPA recovered from fish represented <1% of the total amount offered per fish, indicating minimal bioaccumulative potential of TBBPA in zebrafish. This observation is consistent with the short half-life of TBBPA reported in other fish species (WHO 1995; Morris et al., 2004). The absence of a significant difference in TBBPA levels between males and females may indicate that oviposition is not a major route of excretion in zebrafish. The relatively low recovery of TBBPA from fish together with of the dramatic drop of TBBPA water levels during the exposure cycles points to substantial loss of added TBBPA during exposure. Together with the limited decrease of TBBPA levels in aquariums without fish, these data suggest metabolism of the compound by the fish. Major TBBPA metabolites have not been characterized in fish and were beyond the scope of the present study. Methylation of the compound has been described in experimental aquatic exposure; methylated TBBPA is more hydrophobic and as a consequence, bioaccumulation

may exceed TBBPA bioaccumulation (Hakk and Letcher, 2003). If the animals in the present study accumulated methylated forms as a function of TBBPA exposure, part of the effects discussed below should perhaps be attributed to these metabolites.

The acute effects observed following exposure of adult animals to 3.0 µM TBBPA and higher are in agreement with previous observations (Lee et al., 1993). According to Veith and Broderius, (1990) baseline toxicity should be considered at these TBBPA concentrations. The high mortality in larvae during the first week of exposure to 1.5 µM TBBPA indicates a higher sensitivity to toxic effects of TBBPA compared to older juveniles and adults; accumulation of clear fluid (edema) in the coelomic cavity and pericardial area in developing embryos and larvae exposed to high TBBPA concentrations indicate vascular or osmoregulatory dysfunction.

Although both reduced egg production and hatching indicated a negative effect of TBBPA on reproductive success, a consistent dose-response was only observed for the decreased egg production. Because of the normally abundant and variable egg production in zebrafish, a relatively large critical effect size of 50% clutch size reduction was favored for dose response modeling. In a study where zebrafish were orally exposed to PCBs (Örn et al., 1998), 30-60% reduced egg production was accompanied by a reduction of mature oocytes in the ovaries. The relative overrepresentation of immature oocytes and incidental severe oocyte atresia in zebrafish exposed to 1.5 µM TBBPA may be consistent with the decreased egg production; there were no indications for a unifying mechanism, particularly since the histological observations were not obviously concentration dependent. Although a reduction in mature oocytes was noted in ovaries of zebrafish exposed to estrogens (van den Belt et al., 2002; van der Ven et al., 2003), in the present study there were no indications for an increase of estrogen dependent production of the yolk precursor vitellogenin (liver cell morphology and staining properties). Chromatin fragmentation (karyorrhexis) in kidney tubules has been reported as related to estrogen exposure in zebrafish (Olsson et al., 1999). The karyorrhexis observed in this study more likely reflects endogenous estrogen levels than estrogenic activity of TBBPA, since it was restricted to adult females, and there was no relation to TBBPA exposure. Juvenile gonad development may be a more sensitive parameter for estrogenic effects (Brion et al., 2004; Maack and Segner, 2004). Presently, female developmental stages predominated at an internal concentration of 5.6 µg TBBPA/g lipid, a similar level at which clutch size in adults was reduced. It remains unclear whether this observation was a result of preferential development of a female phenotype, or of a higher mortality rate in males. Although the above explanation is favored by the present observation of progressed oocyte development, alternatively, when zebrafish are protogynous (Maack and Segner, 2003), the condition may also represent delayed development, consistent with the observed abnormal development during the first week in larvae exposed to 1.5 µM and close observation of eggs individually exposed to higher doses. Both delayed development and preferential development of female phenotype have been observed in zebrafish exposed to E2 (Brion et al., 2004). Particularly in juvenile fish where plasma estradiol levels can be very low (0.3 pmol/g tissue in juvenile steelhead trout; Yeoh et al., 1996), an internal concentration of 5.6 µg TBBPA/g lipid (approximately 0.4 nmol/g wet weight) could have some estrogenic influence. The fact that thyroid hormones are also crucial for development in fish (Power et al., 2001), and may act synergistically with gonadotropic hormones (Cyr and Eales, 1996), hampers interpretation of the present results in mechanistic terms; however, in spite of

tentative anti-thyroid effects of TBBPA in tadpoles (Kitamura et al., 2005b), none of the findings in this study were consistent with the effects in zebrafish exposed to the anti-thyroid drug propylthiouracil in a partial life-cycle assay (van der Ven et al., 2006).

Conclusion

The present study indicates that exposure of zebrafish to TBBPA can result in effects on population relevant parameters as egg production, juvenile survival and gender development of offspring at TBBPA body burdens around 5-7 µg/g lipid. In feral fish, levels one order of magnitude lower have been reported. Although the results could be consistent with estrogenic activity of TBBPA, the extent to which such a mechanism plays a role remains unclear without further supportive evidence. There were no indications for anti-thyroid activity.

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5

Long-term exposure of European flounder (*Platichthys flesus*) to the flame retardants tetrabromobisphenol A (TBBPA) and hexabromocyclododecane (HBCD)

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Abstract

Tetrabromobisphenol A (TBBPA) and hexabromocyclododecane (HBCD) are widely used flame retardants that have increasingly been found as contaminants in the aquatic environment. In the present study, European flounder (*Platichthys flesus*) were chronically exposed to TBBPA (105 days), or HBCD (78 days), in a wide range including environmentally relevant concentrations. TBBPA was administered via the water, whereas HBCD was administered in food and sediment, or in sediment alone. Chemical analysis of muscle showed an average increase in internal concentrations of approximately two orders of magnitude for both compounds tested. Animals exposed to HBCD via sediment alone (8000 µg/g total organic carbon, TOC) showed a proportional increase of α-HBCD in muscle compared to animals exposed via food and sediment. In both studies, exposure to the test compounds did not affect general health and toxicity parameters (behavior, survival, growth rate, relative liver and gonad weight). Hepatic microsomal enzyme activities (TBBPA: EROD; HBCD: EROD, PROD, and BROD) were not induced by any of the tested chemicals. Aromatase activity in male gonads showed a mild increase with rising TBBPA levels. There were no morphological and immunohistochemical indications for increased production of the yolk precursor protein vitellogenin (VTG) in animals exposed to TBBPA and HBCD; immunochemical analysis of plasma VTG levels showed no dose response in animals exposed to TBBPA. In animals exposed to TBBPA, levels of the thyroid hormone thyroxin (T_4) increased with internal concentrations of the test compound, possibly indicating competition of TBBPA for plasma protein binding. Triiodothyronin (T_3) levels were not affected and histology showed no signs of altered thyroid gland activity. Other organs investigated (liver, gills, kidney, skin, and gonads) revealed no histological changes related to TBBPA or HBCD exposure. Overall, the present results indicate limited endocrine effects of these widely used flame retardants in a test species representative of European estuaries at environmentally relevant exposure levels and at internal levels up to 4300 ng TBBPA/g wet weight, and 446 µg HBCD/g lipid weight in flounder muscle.

Introduction

Brominated flame retardants (BFRs) are widely used in polymer based consumer products to reduce the risk of fires. Tetrabromobisphenol-A (TBBPA) and hexabromocyclododecane (HBCD) are currently among the most widely used BFRs, with an estimated annual global use of 130,000 and 16,700 tons, respectively (Bromine Science and Environmental Forum BSEF; 2003, 2004). TBBPA can be used reactively, *i.e.* it may be covalently bound to the polymer in which it is applied, or additively. Approximately 10% of TBBPA is used as an additive BFR (BSEF, 2004). HBCD is typically used as an additive BFR.

Environmental contamination may result when flame retardants, particularly when not covalently bound, are released from the product both during and after use (Birnbaum and Staskal, 2004). Both TBBPA and HBCD have been found in the aquatic environment (De Wit, 2002; Morris et al., 2004; Janák et al., 2005). Reported levels of TBBPA and HBCD in river and estuarine sediments are in the µg/kg (dry weight) range, with exceptionally high levels (9750 µg/kg and 1680 µg/kg dry weight, respectively) recorded in the river Skerne in England, close to a BFR production site (Morris et al., 2004). Levels are generally lower in marine sediments, where TBBPA has not been detected and HBCD concentrations up to 6 µg/ kg dry weight have been reported, with the highest levels occurring near river mouths (Klamer et al., 2005). The strong association of TBBPA and HBCD with estuarine sediments and lipophilicity (log K_{ow}s (octanol-water partiotoning coefficients) are 4.5, and 5.8, respectively; De Wit, 2002) indicate a potentially high exposure risk for bottom-feeding fish such as the European flounder (*Platichthys flesus*). In aquatic biota, TBBPA is present in relatively low levels, mainly as a result of the short biological half-life (WHO, 1995). As a result of its slightly higher K_{ow} and possibly of more limited metabolism, HBCD has more frequently been found in aquatic biota and biomagnifies in the aquatic food chain (Morris et al., 2004, Sørmo et al., 2006). HBCD levels as high as 1110 ng/g lipid have been reported in muscle of the estuarine flatfish sole (*Solea solea*) from the Western Scheldt (Janák et al., 2005).

In vitro studies indicate that the endocrine system, particularly with respect to reproductive and thyroid hormonal function, are a target of sub-lethal exposure to these chemicals. TBBPA and HBCD inhibit estradiol sulfotransferase activity (Hamers et al., 2006). Weak estrogenic effects of TBPA were also observed in cultured MCF-7 human breast cancer cells (Körner et al., 1998; Olsen et al., 2003). TBBPA and HBCD can compete with the binding of T_4 to plasma transport proteins such as transthyretin (Hamers et al., 2006; Meerts et al., 2000). At high doses, both TBBPA and HBCD enhance thyroid hormone dependent growth of GH3 rat pituitary tumor cells (Kitamura et al., 2005a; Hamers et al., 2006; Schriks et al., 2006).

Few studies have addressed sub-lethal effects of TBBPA and HBCD in aquatic vertebrates *in vivo*. TBBPA reduces T_3-induced tail-shortening, a hallmark of metamorphosis, in *Rana rugosa* tadpoles (Kitamura, 2005b). Juvenile rainbow trout showed increased glutathione reductase activities after 4, 14 and 28 days following intra-peritoneal exposure to 100 mg TBBPA/kg, which could indicate oxidative stress (Ronisz et al., 2004). In the same study, HBCD was found to increase the hepatosomatic index (HSI) in rainbow trout 28 days after intra-peritoneal exposure to 50 and 500 mg HBCD/kg. Furthermore, hepatic catalase activity was increased, but not in a consistent manner. Both TBBPA and HBCD appeared to reduce β-naphtopflavone-induced CYP1A catalytic activity, but the mechanism

and consequences of these observations are not clear. The apparent lack of induction of estrogen dependent synthesis of the yolk precursor protein vitellogenin (VTG) in that study indicates limited estrogenic potency of TBBPA and HBCD in rainbow trout. No effects were found on hatching, swim up, larval and fry survival, and growth in rainbow trout early life stages eposed to HBCD (Drottar et al., 2001). However, exposure to TBBPA reduces egg production and juvenile survival in zebrafish (Kuiper et al., in press). No data are available on biological consequences of potential endocrine effects in marine aquatic vertebrates exposed to environmentally realistic concentrations of TBBPA and HBCD.

The present paper investigates endocrine effects of prolonged exposure in European flounder to TBBPA and HBCD. The flounder is a bottom-dwelling flatfish that commonly occurs in coastal waters, estuaries and large fresh water bodies in western Europe (Vethaak and Jol, 1996) where sediment can be an important source of exposure to BFRs, particularly HBCD (Morris et al., 2004). Flounder were exposed to TBBPA (for 105 days) via the water phase in a continuous flow-through system, and to HBCD for 78 days via spiked food and sediment to mimic a more particle dependent exposure route. In both studies, a benchmark exposure protocol was used to enable accurate dose-response modeling. Toxicological endpoints included general biometric variables, histology of target organs, production of the yolk precursor vitellogenin (VTG), microsomal enzyme activities, and plasma thyroid hormone levels. Muscle samples were chemically analyzed for TBBPA and HBCD to provide a dose background for any observed effects, and to make data available for risk assessment.

Materials and methods

Test animals

Juvenile flounder (314 days old) were obtained from a hatchery (Manx Mariculture Ltd, Isle of Man, UK) and held at the RIKZ field station (Jacobahaven, the Netherlands) until the start of the experiments (husbandry conditions: 15°C, 16/8 hrs light/dark regimen). Flounders were 683 and 940 days of age at the start of the TBBPA and HBCD experiments, respectively. The animals were weighed per groups of 10 before the experiments started; average body masses were 92±26 and 86±26 g (values ± standard deviations (SD) of 7 respectively 8 groups of 10 animals).

Exposure

TBBPA

Tetrabromobisphenol A (TBBPA; technical mixture, homogeneous with a purity of 99.17% and containing tribromoBPA and o,p'-TBBPA as impurities, verified by HPLC analysis) was obtained as a composite mix through BSEF. Groups consisting of 10 animals were exposed to water borne TBBPA at nominal concentrations of 0; 0.54; 5.4; 54.4; 109; 218 and 435 ng/mL for a period of 105 days (Table 1). The exposure systems consisted of glass aquariums measuring 100*70*30 cm (l*w*h), containing 10 kg of sediment and 160 L water, both from the Eastern Scheldt (a relatively pristine tidal bay connected to the North Sea with a salinity of ca. 3.2%). The water was renewed by continuous flow-through at a rate of 160 L per day. The water temperature was 21±1°C (n=39 measurements), pH ranged from 7.19 to 7.65 and O_2 saturation was 73±13.3% (n=44). The relatively high temperature was chosen because of poor solubility of TBBPA at lower temperatures. TBBPA was

dissolved in dimethylsulfoxide (DMSO, Acros, Geel, Belgium) and continuously added to the inflow water. The final DMSO concentration was 0.1‰ in all tanks including the control aquarium. Water samples were collected at 5 equally spaced intervals during exposure and stored at -20°C for analysis of TBBPA concentration. To saturate all active sites in the test system to which TBBPA could be lost by absorption, flow through with TBBPA dosed water was started two weeks prior to the introduction of the fish. Fish were acclimated by raising the temperature of the stock aquariums to 18°C two weeks prior to exposure. During exposure, the animals were fed pellet feed (Trouvit; Seafarm, Kamperland, the Netherlands) three times a week at an estimated 1% of the total body mass at the start of the experiment, and inspected daily for behavioral changes and signs of diseases. A 16/8 hrs light/dark regimen was maintained throughout the experiment. When necessary, accumulating organic debris was removed from the tanks. Feeding was discontinued five days prior to the end of exposure. Effluent was cleaned over charcoal filters and absence of TBBPA was confirmed by chemical analysis.

HBCD

Hexabromocyclododecane (HBCD; SAYTEX; technical mixture) was obtained as a composite mix through BSEF. The average composition was 10.28, 8.72, and 81.01% for the α-, β-, and γ-HBCD diastereomers, respectively. The animals were kept in 160 L of Eastern Scheldt water at a temperature of 15°C (renewal twice weekly via continuous flow-through) under otherwise similar ambient conditions as described for TBBPA. The aquariums contained 15 kg of HBCD-spiked sediment. Groups consisting of 10 animals were exposed for 78 days to the combined exposure doses of HBCD in food (µg/g lipid) and sediment (µg/g total organic carbon (TOC)): 0+0 (control); 0.3+0.08; 3+0.8; 30+8; 300+80; 3,000+800; and 0+8,000 (Table 2). The highest sediment dose was not accompanied by a feeding dose because of solubility problems. Once, during the second week of the experiment, flounder from one of the control groups were accidentally fed food spiked with 30 µg HBCD/g lipid. For the spiking of food, HBCD was dissolved in acetone (Promochem, Wesel, Germany) and mixed with corn oil. Then the acetone was evaporated and the oil containing HBCD was added to food pellets. The resulting lipid content of the food was 10% (determined according to Bligh and Dyer, 1959). Animals were fed 3 times a week at an estimated 1% of the total body mass at the start of the experiment, increased to 2% after 21 days until 5 days before the end of the study, when feeding was discontinued. For preparation of spiked sediment, sandy sediment was collected from a relatively clean tidal zone in the Eastern Scheldt (78.3% dry matter, 0.3% TOC). TOC in sediment was estimated as half the loss on ignition after heating of dried sediment at 900°C for two hrs. To 15 kg wet sediment, 10 mL acetone containing the desired amount of HBCD (282 mg for the highest dose, and serial 10x dilutions for the lower doses) and 1.5 L of salt water were added. This was stirred in 20 L glass bottles for 3 days before the spiked sediment was added to the aquariums. To allow for stabilization of the sediment concentration, fish were not introduced until 2 weeks later. The effluent was cleaned using charcoal filters.

Sampling procedure

Sampling was similar in both experiments. Directly following euthanasia in random order using MS222 (Sigma-Aldrich, Steinheim, Germany), blood was sampled from the caudal vein using a 2 mL syringe with a 0.6 mmØ needle, and transferred to heparinized vials.

After centrifuging, plasma samples were stored at -70°C for analysis of thyroid hormones, and a subsample was stored at -70°C for analysis of VTG, after addition of 30 µL aprotinin (Sigma-Aldrich, 0.1 mg/mL in 0.9% NaCl) per mL plasma. The coelomic cavity was opened and after the bile was removed with a syringe, the liver was weighed and divided in a rostral, middle and caudal part. The rostral part was frozen in liquid nitrogen and stored at -70°C for preparation of microsomes during the subsequent first month. The caudal part was frozen at -20°C for future chemical analysis, and the middle part was fixed in 4% neutral buffered formaldehyde (10% formalin) for histological processing. Gonads were excised and weighed. The upper most gonad was frozen in nitrogen and stored at -70°C for preparation of microsomes, whereas the lower (heterolateral) gonad was fixed in formalin. The remaining internal organs including kidneys were also fixed in formalin. Branchial arches and the thyroid region were fixed in formalin. From animals exposed to TBBPA, the brains were also sampled and frozen in nitrogen for microsome preparation. Finally, flank muscles ("fillet") were excised and frozen at -20°C for chemical analysis.

Chemical analysis

TBBPA

TBBPA was analyzed in muscle, and collected water samples as previously described (Kuiper et al., 2006). Approximately 7 g of thawed muscle from each individual fish was homogenized and extracted with a Soxhlet apparatus using hexane:acetone (3:1, v/v, Promochem, Wesel, Germany) for 12 hours. The lipid content was determined gravimetrically. Water samples were extracted in duplicate by twice repeated shaking (for each replicate separately) with hexane. TBBPA was analyzed using LC-MS/MS.

HBCD

HBCD was analyzed in thawed muscle as described for TBBPA (Kuiper et al., 2006). One mL containing 100 ng/mL of each of the three 13C-HBCD diastereomers (CIL, Andover, MA) was added as internal standard to each of the samples. Purification over silica gel (5% deactivated with water) included two eluate fractions: the first fraction was eluted with 11 mL isooctane and 29 mL isooctane:diethyl ether (85:15, v/v, Promochem), followed by 5 mL diethyl ether which was collected as the second fraction. Finally, the combined silica eluates were evaporated to almost dryness with nitrogen, and 1 mL of methanol was added. The α,β,γ-HBCD isomers were determined with LC-MS/MS (LCQ Advantage, Thermo Finnigan) using electron spray ionization, using the same mobile phase solvents and gradient, and HPLC column as described for TBBPA. Identification of HBCD was based on the retention times of the ^{13}C-labelled compounds and the bromine clusters of the adduct ion m/z 677 and fragment ion m/z 644. The m/z 644 ion was used for quantification.

Microsome preparation

Microsomes were prepared from gonads and liver samples, and from brains (TBBPA study only). Tissues were thawed in random order, homogenized in buffer (50 mM Tris-HCl, pH 7.4, 1.15% KCl) and centrifuged at 10,000 g for 25 min at 4°C. The supernatant was then centrifuged at 30,000 g for 1 hour and 15 min at 4°C. The microsomal pellet was harvested, resuspended in 0.25 M sucrose and frozen at -70°C until further analysis. Protein concentrations were determined according to Lowry et al. (1951) using bovine serum albumin (Sigma A7030) as a standard.

EROD, PROD and BROD activities
Determination of ethoxyresorufin-*O*-deethylase (EROD) activity was based on Fent et al. (1998). Microsomes were thawed on ice and 10 µL of each sample (10-40 µg microsomal protein) was added to a well of a 96 well plate (Greiner, Alphen a/d/Rijn, The Netherlands), together with 40 µL of 20 µM dicumarol (Sigma) in Tris buffer (Tris/dic: 100 mM Tris, 0.1 M NaCl, pH 7.8, 5 mM MgCl). 4 µM 7-ethoxyresorufin (Sigma) was prepared in Tris/dic, and 2.5 mg NADPH (Sigma)/mL was added shortly before use. Addition of 50 µL of this solution to each well started the reaction. The resorufin produced was measured real-time on a fluorescence plate reader at 530 nm excitation and 590 nm emission wavelengths and calculated using a resorufin standard curve (0; 1.56; 3.13; 6.26; 12,5; 25; 50 and 100 pmol resorufin in 90 µL Tris/dic with 10 µL microsomes added). For determination of pentoxy- and benzoxyresorufin-*O*-deethylase (PROD and BROD) activities, 7-pentoxyresorufin and 7-benzoxyresorufin (Sigma) respectively replaced 7-ethoxyresorufin in the otherwise unchanged procedure.

Determination of aromatase (CYP19)-activity
The catalytic activity of aromatase was determined based on the tritiated water-release method of Lephart and Simpson (1991). In brief, 15 µL of microsomes (5-10 mg protein/mL) were added to 175 µL Hepes/$MgCl_2$ buffer (50mM Hepes, 5 mM $MgCl_2$, pH=7.8 in 100mL milliQ water) containing 6.1µL ^3H labeled androstenedione ([1ß-^3H(N)]-Androst-4-ene-3,17-dione; 28.8 Ci/mmol, New England Nuclear Research Products, Boston, MA, USA) per mL buffer, and the total volume was adjusted to 225 µL by addition of Hepes/$MgCl_2$ buffer. After addition of 25 µL NADPH (5 mM in Hepes/$MgCl_2$ buffer), the samples were incubated for 1 hr (25°C, 5% CO_2) during which radiolabeled androstenedion is aromatized to estradiol, and tritiated water is released. Following incubation, 200 µL of the reaction mixture was used for measuring the level of radioactivity. Corrections were made for background radioactivity, dilution factor, and specific activity of the substrate. 4-hydroxy-androstenedione (4-HA, 1 µM) was used as a positive control for aromatase catalytic inhibition (Heneweer et al., 2004).

Determination of VTG (TBBPA experiment)
Vitellogenin (VTG) was determined by competitive enzyme-linked immunosorbent assay (ELISA) as described by Lahr et al (in press) using 45,000x diluted polyclonal rabbit anti-turbot VTG (CS-2, Biosense, Norway) as a primary antibody. VTG was quantified using a standard dilution series of flounder VTG (CEFAS, Lowestoft, UK).

Determination of thyroid hormones T_4 and T_3
Quantitative measurement of thyroxin (T_4) and triiodothyronin (T_3) in plasma from animals exposed to TBBPA and HBCD was performed using solid-phase ^{125}I radioimmunoassay (RIA; Coat-a-count, Diagnostic Product Corporation, DPC, Los Angeles, USA), specific for T_4 and T_3. To test if TBBPA binds to the T_4 antibodies in the kit, a plasma sample from one of the control animals from the HBCD study was spiked with TBBPA. TBBPA (0.4 µg/mL) and T_4 standard (10 ng/ml) in distilled water were also tested to test for binding to T_4 antibodies. All samples were analyzed in duplicate. The amount of bound radioactive antigen was quantified for 1 min each, using a gamma counter (Cobra Auto Gamma,

Packard Instrument Company, Dowers Grove, IL, USA). The detection limits for T_4 and T_3 were 0.18 and 0.017 ng/mL, respectively.

Histology and immunohistochemistry

Tissues sampled for histological evaluation were routinely processed and paraffin embedded. Branchial arches and thyroid region were decalcified in 10% neutral buffered ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, St. Louis, USA) for 72 hrs before tissue processing. Tissue sections were cut at 3 µm thickness and stained with hematoxylin and eosin (H&E). Microscopic evaluation was performed in a blinded and randomized fashion. For VTG immunohistochemistry, paraffin sections were mounted on 0.01% poly-l-lysine covered glass slides. After deparaffinization and rehydration, slides were heated for 25 min in distilled water using a microwave oven (700-1000W). After 30 min incubation with 1% H_2O_2 in methanol, the slides were incubated overnight at 4°C with a polyclonal rabbit anti-turbot VTG (CS-2, Biosense, Norway; dilution: 1:500). After rinsing with PBS:Tween, slides were incubated for 30 min with horseradish peroxidase-labeled goat anti-rabbit (Dako, Glastrup, Denmark). The slides were rinsed in PBS:Tween and incubated with freshly prepared avidin biotin complex for 30 min. After rinsing with PBS, immunoreactive VTG was visualized by incubating the slides for 10 min in 0.3% H_2O_2 and 0.5% 3,3-diaminobenzidine tetrahydrochloride (Sigma, Zwijndrecht, the Netherlands) in 0.5 M Tris:HCl buffer (pH: 7.6). A liver section from a male flounder, exposed to 1 mg/kg 17β-estradiol (Sigma, Zwijndrecht, the Netherlands) in corn oil via two intraperitoneal injections (one week interval) and sacrificed two weeks after initial injection, was used as a positive control.

Statistics

Between group differences in internal (bio)chemical parameters were compared using one-way analysis of variance (ANOVA) after log transformation using Levene's test for equality of variance and normal probability plots to evaluate a normal distribution of transformed data. Post hoc testing was performed using Dunnet's test for comparison with a single control group. Relations between continuous effect parameters and internal concentrations were explored by linear regression; Pearson correlation was used for relations between different continuous effect parameters. When appropriate, Fisher's exact test was applied to non-continuous end points (histopathology). PROAST software, (RIVM, Bilthoven, The Netherlands) was used according to Slob et al. (2002) to fit Hills' and exponential dose-response models to T_4 levels and aromatase activities respectively, in fish exposed to TBBPA. For all calculations, values below the limit of detection (LOD) were set to half the LOD.

Results

Exposure levels

Actual TBBPA levels in exposure water were within a factor 0.25 of the nominal concentrations (Table 1). Levels in fish were linearly related to nominal and actual water levels (R^2=0.67 and 0.63, respectively; $p<0.01$), but substantial variation was observed within the groups (Table 1). TBBPA levels in muscle were significantly higher in fish from all of the exposed groups as compared to the control group (ANOVA: $p\leq0.02$) except for the group exposed to a nominal concentration of 0.01 µM (5.4 µg/L). The lipid content in muscle was 0.7 ± 0.3 % (SD). TBBPA levels in muscle were not significantly influenced by lipid content. Average TBBPA levels in muscle samples from fish exposed to the highest concentration were 200 times higher than the value in unexposed animals after 105 days of exposure.

Internal HBCD levels are presented in relation to lipid content for comparison to exposure levels (Table 2). Average lipid content in muscle was 0.9 ± 0.4 % (SD) and did not significantly influence internal HBCD concentrations. γ-HBCD levels in all exposed groups, including the control group where accidental exposure to contaminated food had occurred, were significantly higher than in the remaining control group. An unexpected background of γ-HBCD was present in these control animals (from <LOD to 1.8 µg/g lipid). Total HBCD levels (ΣHBCD, estimated using half the detection limits for α-, and β-HBCD when values were below LOD) were significantly increased in animals exposed to 8.0 µg/g TOC in sediment combined with 30 µg/g lipid in food and higher (ANOVA: $p<0.01$). A maximum level of 446 µg ΣHBCD/g lipid was reached in muscle from an animal exposed to the combination 3000 µg/g lipid in feed and 800 µg/g TOC in sediment. The average internal γ-HBCD level in that group was 316 times greater than the average background level from the remaining, unexposed control group. The proportions of the diastereomers α-, β-, and γ-HBCD in animals with detectable ΣHBCD levels were similar to those present in the original technical mixture (mainly γ-HBCD), indicating a similar dose-dependent increase of all diastereomers, with a notable exception in animals exposed via spiked sediment only. In these animals, the levels of α-HBCD were similar to the levels of γ-HBCD (Fig. 1).

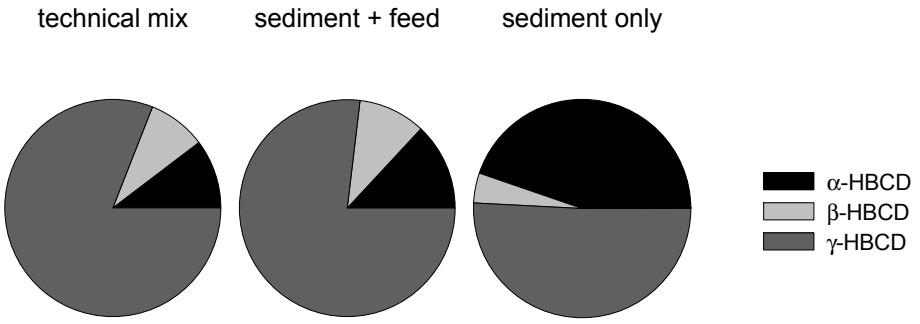

Figure 1: Proportions of the diastereomers α-, β-, and γ-hexabromocyclododecane (HBCD) in the technical mixture, in animals exposed via sediment and food, and in animals exposed via sediment alone after 78 days.

Table 1: Chemical analysis and biochemical effect parameters in European flounder exposed to water-borne tetrabromobisphenol A (TBBPA) for 105 days.

Nominal TBBPA conc (ng/mL)	Actual TBBPA conc[1] (ng/mL)	TBBPA muscle median; range (ng/g ww)	Plasma VTG ± SD (ng/mL)		Plasma T_3 ± SD (ng/mL)	Plasma T_4 ± SD (ng/mL)	Aromatase activity ± SD (pmol/hr/mg)		EROD activity ± SD (pmol/min/mg)
			Male	Female			Male	Female	
0	<0.01	7.7 (<1-42) (n=10)	807 ± 778 (n=5)	2322 ± 1863 (n=5)	4.6 ± 1.1 (n=9)	n.d (n=8)	0.27 (n=2)	0.48 ± 0.37 (n=5)	21 ± 14 (n=10)
0.54	0.13 ± 0.07	21.5* (7-98) (n=10)	1245 (n=2)	1281 ± 481 (n=8)	4.1 ± 1.2 (n=9)	0.3 ± 0.5 (n=10)	0.10 (n=2)	0.50 ± 0.53 (n=8)	28 ± 41 (n=10)
5.4	1.36 ± 0.83	11 (<6-16) (n=10)	1072 ± 240 (n=5)	5270 ± 5684 (n=5)	5.1 ± 1.6 (n=10)	0.2 ± 0.3 (n=10)	0.39 ± 0.31 (n=3)	0.83 ± 0.52 (n=5)	29 ± 23 (n=7)
54.4	11.02 ± 7.88	91** (58-300) (n=8)	1550 ± 1261 (n=5)	7617 ± 627 (n=3)	5.0 ± 1.2 (n=8)	2.5 ± 2.1** (n=8)	0.16 (n=2)	0.60 ± 0.18 (n=3)	29 ± 28 (n=8)
109	27.46 ± 18.62	195** (84-530) (n=10)	610 ± 368 (n=3)	11234 ± 25487 (n=7)	4.9 ± 1.4 (n=9)	2.3 ± 1.6** (n=8)	0.54 (n=1)	0.38 ± 0.17 (n=7)	16 ± 12 (n=10)
218	114.74 ± 50.54	465** (110-1400) (n=10)	664 ± 458 (n=4)	6350 ± 12116 (n=6)	4.6 ± 1.2 (n=8)	7.9 ± 3.6** (n=9)	0.40 ± 0.31 (n=4)	0.69 ± 0.37 (n=4)	23 ± 8 (n=10)
435	193.47 ± 66.6	1950** (690-4300) (n=10)	1259 ± 738 (n=6)	2090 ± 828 (n=4)	5.1 ± 1.9 (n=9)	16.6 ± 3.7** (n=8)	1.04 ± 0.96 (n=5)	0.30 ± 0.29 (n=4)	25 ± 22 (n=9)

[1]average ± SD of 5 consecutive samplings. */** significantly increased compared to control at $p<0.05/0.01$, respectively

Table 2: Chemical analysis and biochemical effect parameters in European flounder exposed to hexabromocyclododecane (HBCD) technical mix during 78 days via food and sediment.

Nominal HBCD exposure		HBCD muscle median; range (µg/g lipid)[2]			T_3 ± SD (ng/mL)	T_4 ± SD (ng/mL)	Aromatase ± SD (pmol/hr/mg)		EROD ± SD (pmol/ min/mg)	BROD ± SD (pmol /min/mg)	PROD ± SD (pmol/ min/mg)
Sediment (µg/g TOC)	Food (µg/g lipid)	α	β	γ			Male	Female			
Reference A	0	<2.2	<2.2	<0.9 <0.9-1.8	6.7 ± 2.7 (n=10)	6.4 ± 8.3 (n=9)	0.04 (n=2)	1.06 ± 0.79 (n=4)	16 ± 7 (n=10)	0.24 ± 0.06 (n=10)	0.16 ± 0.09 (n=10)
Reference B[1]	0	<2.1	<2.1	2.1** <0.4-8	5.7 ± 2.3 (n=10)	3.3 ± 2.5 (n=10)	0.03 ± 0.06 (n=4)	0.25 ± 0.09 (n=4)	16 ± 10 (n=10)	0.25 ± 0.15 (n=10)	0.17 ± 0.11 (n=10)
0.08	0.3	<4.3	<4.3	1.4** <1.1-8.8	6.9 ± 1.4 (n=10)	3.2 ± 2.6 (n=8)	0.02 ± 0.02 (n=4)	0.20 (n=2)	14 ± 8 (n=10)	0.21 ± 0.08 (n=10)	0.10 ± 0.06 (n=10)
0.8	3.0	<0.7 <0.7-1.4	<0.7	2.0** 1.1-18.9	7.8 ± 2.1 (n=10)	4.6 ± 3.0 (n=8)	0.14 ± 0.29 (n=6)	n.a.	15 ± 8 (n=10)	0.21 ± 0.05 (n=10)	0.09 ± 0.03 (n=10)
8	30	0.9 <0.3-1.9	<0.4 <0.4-0.5	12.5** 6.1-20.7	6.1 ± 1.5 (n=10)	2.4 ± 2.0 (n=9)	n.d. (n=4)	0.68 ± 0.47 (n=3)	12 ± 5 (n=10)	0.17 ± 0.05 (n=10)	0.09 ± 0.07 (n=10)
80	300	18.3* 8.0-28.6	4.5* 2.3-6.4	106.4** 70.9-183.3	5.8 ± 2.2 (n=10)	2.6 ± 2.7 (n=8)	n.d. (n=3)	0.19 (n=2)	12 ± 10 (n=10)	0.15 ± 0.04 (n=10)	0.12 ± 0.06 (n=10)
800	3000	44.2* 29.3-98.6	57.3* 34.3-91.4	172.7** 93.3-300	7.1 ± 1.9 (n=8)	8.3 ± 9.8 (n=5)	0.02 ± 0.02 (n=4)	0.93 ± 0.46 (n=4)	14 ± 10 (n=8)	0.20 ± 0.07 (n=8)	0.19 ± 0.08 (n=8)
8000	0	33.0* 17.3-41.7	2.8* 1.6-6.1	36.7** 23.6-46.3	6.9 ± 2.1 (n=9)	5.2 ± 2.9 (n=8)	0.05 (n=2)	0.66 ± 0.36 (n=5)	11 ± 8 (n=10)	0.22 ± 0.12 (n=10)	0.25 ± 0.17 (n=10)

[1]once exposed to food with 30µg HBCD/g lipid during the second week of the experiment.
[2]10 animals used for chemcal analysis in all groups except in animals exposed to 800/3000 and 8000/0 µg/g TOC/lipid, respectively (9 animals in both groups); n.d. not detected; n.a. not analyzed due to insufficient samples. */** significantly increased compared to reference A at $p<0.05/0.01$, respectively.

Apart from a slight, but statistically significant relative increase in levels of β-HBCD in animals with highest ΣHBCD in muscle, (exposed to the combination 800 µg TOC (sediment) and 3000 µg/g lipid (food)), there was no relation between ΣHBCD in muscle and the proportions of different diastereomers.

Gross observations
During exposure, no abnormal behavior was noted in animals exposed to TBBPA and HBCD. Two animals exposed to an intermediate dose of TBBPA (0.1 µM) died during exposure, without previous signs of stress or disease and without signs in the remaining animals of that group. No gross lesions were observed upon exterior inspection and during dissection in TBBPA exposed animals. One animal from an intermediate HBCD exposure group (80 µg/g lipid and 300 µg/g TOC) showed multiple pale foci of approximately 1 mm in diameter in the liver. No important differences in somatic weight, total fish length, liver weight, gonad weight or somatic indices (GSI, HSI) and condition factor (CF), were observed in relation to exposure to TBBPA or HBCD. Average total body weights ± standard deviations at the end of the TBBPA and HBCD experiments were 95 ± 26 (n=68) and 111 ± 28 (n=78), respectively; condition factors were 1.0 ± 0.2 and 1.1 ± 0.1.

EROD (TBBPA and HBCD studies), and PROD and BROD activities (HBCD study)
EROD-activities in analyzed liver microsomes from all TBBPA and HBCD exposed animals were consistently low, with averages of 24.1 ± 22.8, and 13.8 ± 8.3 pmol/mg protein/min, respectively. The large variation was equally distributed throughout the groups and EROD activities did not relate to external dose or internal TBBPA or HBCD concentrations in either gender (Tables 1, 2). Furthermore, EROD activities in fish exposed to HBCD via sediment alone were not significantly different from the other groups (Table 2). BROD and PROD activities in animals exposed to HBCD did not show exposure-related changes (Table 2), and showed a strong (BROD: $R=0.9$, $p<0.01$) or mild (PROD: $R=0.5$, $p<0.01$) correlation with EROD activity. However, the proportion of HBCD-γ in muscle of animals where all HBCD diastereomers were above the LOD (n=30) showed a weak negative correlation ($R=0.5$, $p<0.01$) only with hepatic PROD activity.

CYP19 (aromatase) activity
Catalytic P450arom activity was detected in most gonads assayed (Tables 1 and 2), and was decreased in samples to which a known catalytic aromatase (CYP19) inhibitor (4-HA) was added (data not shown). Activities were generally lower in testes than in ovaries, except in animals exposed to TBBPA where aromatase activities in testes increased with increasing TBBPA nominal and internal concentrations and a statistically significant dose-response was detected (Fig. 2). Based on the fitted dose-response model, a doubling of basal aromatase activity in testis occurs at 0.82 µg TBBPA/g muscle (ww; lower 95% confidence limit (c.l.) =0.60 µg/g). Exposure to HBCD did not affect aromatase activities in testes or ovaries. Microsomes prepared from brains from animals exposed to TBBPA did not exhibit detectable aromatase activity. No brain microsomes were tested in the HBCD study.

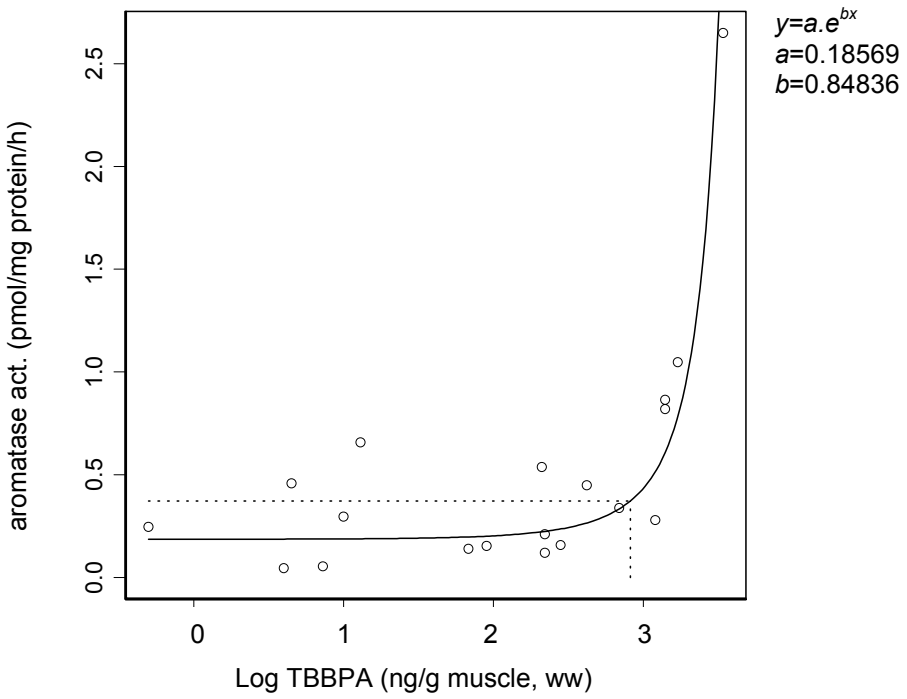

Figure 2: Aromatase activity in gonads of male flounder in relation to internal TBBPA concentration. Fitted dose-response model: $y=a.e^{bx}$; dotted lines represent two times the basal level and corresponding internal dose (820 ng/g muscle, wet weight).

Plasma VTG levels (TBBPA study only)

Levels of VTG in plasma from flounder from the TBBPA study are presented in Table 1. The overall range in individual fish was from <LOD to 3690 ng/mL in males, and from <LOD to the exceptionally high 68969 ng/mL in females. Relatively high concentrations of VTG (>2x SD) were found in 2 females exposed to a nominal concentrations of 0.2 and 0.4 µM TBBPA. These females did not have particularly well-developed ovaries. VTG levels were not related to exposure concentrations or to TBBPA levels in muscle in either gender. There was a positive relation between plasma levels of VTG and T_3 (see below) in females (Pearson correlation: $R=0.58$; $p<0.01$; $n=60$), but not in males. No relations were observed between VTG levels in plasma and CYP19 activity in ovaries or testes.

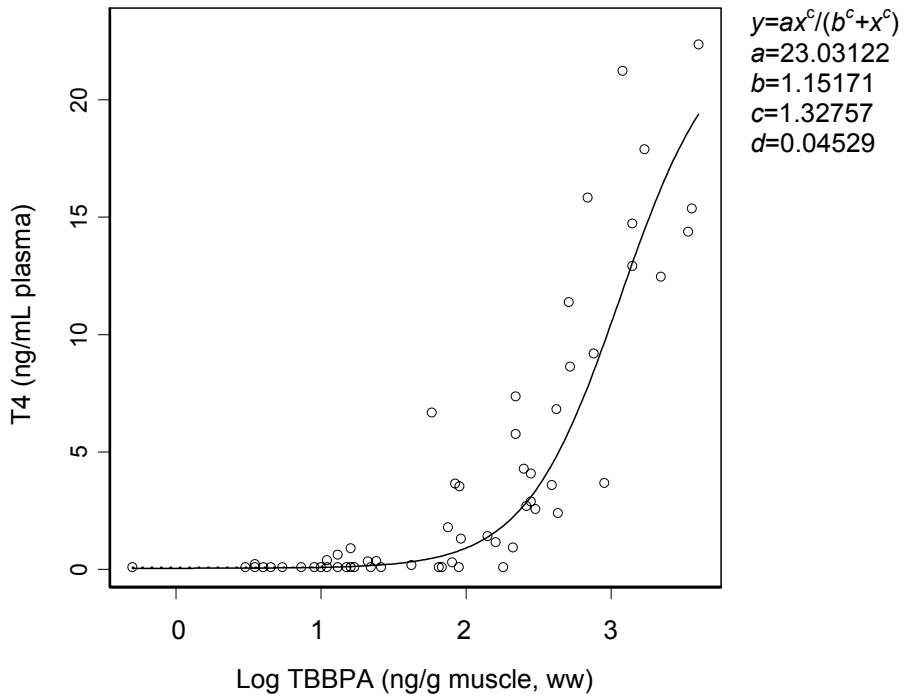

Figure 3: Relation between plasma T_4 levels and internal TBBPA concentrations in flounder. Fitted dose-response model: $y=ax^c/(b^c+x^c)$

Plasma T_3/T_4 levels

Mean plasma T_3 and T_4 levels in animals exposed to TBBPA and HBCD are presented in Tables 1 and 2. Gender related differences in plasma thyroid hormone levels were not observed. Plasma T_3 levels remained fairly constant in all animals and ranged from 1.6 to 11.8 ng/mL in fish from the TBBPA study, (mean ± SD = 7.4 ± 1.6; n=62), and from 3.2 to 18.2 ng/mL (mean ± SD = 10.1 ± 3.3; n=77) in fish from the HBCD study. In animals from the TBBPA study, plasma T_4 levels in control animals were lower than in control animals from the HBCD study. Plasma T_4 levels in animals exposed to TBBPA ranged from <LOD to 22.4 ng/mL, and were significantly increased in groups exposed to nominal concentrations of 54.4 ng/mL and higher. A statistically significant dose-response was detected using TBBPA concentrations in muscle (Fig. 3). Because basal levels of T_4 were mostly below the detection limit, the internal concentration at which a doubling of T_4 occurred could not be accurately estimated. A surplus TBBPA added to plasma from an unexposed animal in duplicate increased the measured T_4 concentration from <LOD to 8.2 and 19.2 ng/mL. TBBPA in water did not result in a measurable response. T_4 levels in plasma from fish from the HBCD study ranged between <LOD and 8.5 ng/mL and were not related to exposure or HBCD concentrations in muscle.

Histology

There were no dose-related histopathological changes in internal organs including liver, spleen, kidney, gonad and thyroid gland in fish exposed to TBBPA or HBCD. The numbers of macrophage centers in liver, spleen and kidney varied greatly. Swelling of the epithelium of intrahepatic bileducts accompanied by accumulation of large, clear intracytoplasmic vacuoles ("ballooning") was frequently observed in both studies (present in 23 of the 68 animals examined from the TBBPA study, and 24 out of 78 in the HBCD study) throughout all dose groups. Fibrillar cytoplasmic inclusions (previously described by Vethaak and Wester, 1996) were observed in hepatocytes of the majority (TBBPA: 59%; HBCD: 83%) of the animals. In 15% and 36% of the livers from the TBBPA and HBCD studies, respectively, accumulation of bright yellow pigment was observed inside macrophages. The pigment was fluorescent in UV light and stained bright red with a prolonged Ziehl-Neelsen procedure, consistent with ceroid/lipofuscin. Six animals from both studies showed intracellular accumulation of protozoan parasites (1-2 μM diameter round structures with a clear center consistent with *Glugea stephani*) in hepatic macrophage aggregates as described among others by Khan (2004).

Most animals, both males and females, had immature gonads. In the TBBPA study, 26 of 34 evaluated ovaries showed previtellogenic ova and only 2 showed signs of deposition of yolk (early cortical alveolus stage), and among 25 evaluated testes, 2 showed overt signs of advanced spermatogenesis. Advanced oocyte development was present in only 5 out of 33 ovaries, and advanced spermatogenesis in 21 of the 35 evaluated testes from the HBCD study. Developmental stages of gonads did not correlate to TBBPA or HBCD exposure levels.

Thyroid histological appearance was variable in both the TBBPA and HBCD studies, showing marked ballooning of follicular epithelium in 36% and 38% of all cases. This observation was not related to gender, dose group or exposure levels.

VTG immunohistochemistry

Whereas in liver sections from the male flounder exposed to 17β-estradiol imunoreactivity was apparent in plasma within the hepatic sinusoids, there was no immunohistochemical detection of VTG in animals from either gender exposed to TBBPA or HBCD.

Discussion

The present study confirms that TBBPA and HBCD are bioavailable to a lower aquatic vertebrate species, European flounder, when exposed in an environmentally relevant test setup. The levels reached in the animals represent a wide range including concentrations that were reported in the environment. In the Dutch Wadden Sea, TBBPA was detected up to 14 ng/g wet weight in wild fish (pooled sample of 13 sandeel (*Ammodytes tobianus*) (pooled sample; unpublished data, RIVO, 2003). The highest HBCD levels in fish were reported in sole (*Solea solea*) from the Dutch Western Scheldt estuary. In pooled muscle samples from two locations, 139 and 1110 ng ΣHBCD/g lipid were detected (Janák et al., 2005). The average levels in the control groups in the present study represent background Eastern Scheldt levels. Background TBBPA levels were low relative to the levels reported in wild fish. Levels of HBCD in the control groups from our study were in the range of reported environmental levels (Morris et al., 2004; Janák et al., 2005), possibly as a result

of the bioaccumulative potential of this compound (Sørmo et al, 2006). The average levels in the highest exposure groups were approximately two orders of magnitude higher than the levels of TBBPA and HBCD found in the control fish. Judging from the relatively low ΣHBCD levels in fish exposed via sediment alone, spiked food was the more important exposure source for HBCD in our study. However, the total cumulative oral HBCD dose exceeded the sediment dose by two orders of magnitude, and the similar recoveries from fish exposed to the highest combined exposure doses and from fish exposed via sediment alone (0.2% of the cumulative oral dose plus total amount added to sediment, or total amount added to sediment only, respectively) indicate that oral and sediment exposure contributed equally to the internal ΣHBCD levels. It should be noted, however, that exposure via spiked sediment alone resulted in a significant relative increase of α-HBCD. This strongly indicates that the route of exposure influences the diastereomeric composition of HBCD residues in biota. Since α-HBCD is the predominantly detected congener in biota in the environment (Birnbaum and Staskal, 2004; Morris et al, 2004; Janák et al., 2005), we conclude that in field situations, sediment exposure is likely to be important as an HBCD source in bottom-dwelling fish such as the flounder.

Exposure doses up to 435 ng TBBPA/L and 800 µg HBCD/g TOC in sediment combined with 3000 µg HBCD/g lipid in food did not result in overt toxicological signs in the flounder. Hepatic microsomal enzyme activities were generally low, possibly due to low background levels of typical inducers such as coplanar PCBs and dioxins in these laboratory-reared animals. Even at relatively high exposure levels in the present study, TBBPA, or HBCD did not induce EROD activity. A decrease in hepatic EROD activity as was shown in rainbow trout exposed to TBBPA and HBCD (Ronisz et al., 2004) was not observed. However, the low background activities may have reduced the sensitivity for decreased EROD activity in the present study. The lack of EROD induction by TBBPA and HBCD is consistent with *in vitro* data (Hamers et al., 2006), and suggests that the test compounds were no major source for planar contaminants (*e.g.* brominated dibenzo-*p*-dioxins or -furans.

Careful use of PROD and BROD activities have been suggested to indicate induction of CYP2B like enzymes in fish (Addison, 1993). In mammals, CYP2B like enzyme activity may be involved in the relative abundance of α-HBCD in biota, as was suggested by the preferential metabolism of γ- and β-HBCD by phenobarbital-induced rat microsomes (Zegers et al., 2005). In our study with HBCD, we found a strong correlation between BROD and EROD activities, and a less strong correlation between the activities of PROD and EROD. Similarly, Addison et al. (1991) observed that PROD activity could be induced in European flounder by the CYP1A inducer β-naphtoflavone, indicating that PROD activity may at least in part be a function of CYP1A enzyme. Like EROD activity, PROD and BROD activities were not related to HBCD absolute exposure levels in the present study. However, a weak but statistically significant proportional decrease of γ-HBCD was found only with increasing hepatic PROD activity. Thus, although PROD activity is not induced by HBCD, other chemicals may affect HBCD metabolism via modulation of CYP2B activity.

Exposure to TBBPA or HBCD did not affect the reproductive system of flounder in our study to a major extent. Although the interpretation of aromatase activity in gonads of male flounders exposed to TBBPA is hampered by the lack of data on when these levels become adverse, the observed increase was relatively mild. Using the highest TBBPA

internal levels reported in the environment (14 ng/g wet weight), the dose-response model estimates a very limited 1.2% increase in aromatase activity (higher 95% c.l.=1.6%), which is much smaller than the variation observed between activities in individual animals in the lower dose groups. The observation that even much higher aromatase activities were not accompanied by a dose dependent increase of plasma VTG levels indicates limited functional consequences (production of estrogens) at these levels of TBBPA exposure. Alternatively, increased aromatase activities did not result in an increase of endogenous estrogen production sufficient to drive increased VTG expression, due to low substrate levels (androgen levels were not analyzed, as judging from the immature male gonads the levels were expected to be below the detection limit).

The VTG levels reported here in both sexes are consistent with levels reported in immature male and female flounder from various, including contaminated, estuarine sites around the North Sea (Allen et al., 1999a,b; Lahr et al., in press). The large majority of gonads from the present study should indeed be regarded as immature, as indicated by our histological findings. Still, VTG levels in females were consistently higher than in males in our study, indicating responsiveness to (sexually dimorphic) estrogen levels. Although in cultured male flounder from our study, VTG levels were generally higher than those reported in (immature) male flounder from a reference site in the UK, they were usually below the threshold value for estrogenic exposure of 1000 ng/mL suggested by Lahr et al. (in press). In contrast, immature male flounder from contaminated sites were capable of increasing VTG levels 10^6-fold resulting in much higher concentrations (mg/mL range; Allen et al., 1999a). These results indicate that the flounder in our study were of a sensitive developmental stage regarding VTG production. Lack of *in vivo* VTG induction by high levels of TBBPA and HBCD in flounder in our study is in agreement with the findings in rainbow trout and eelpout (*Zoarces viviparus*; Ronisz et al., 2004) and indicates that TBBPA and HBCD have no or little estrogenic action in these fish *in vivo*. As testis ova in wild flounder were only detected at sites where also much higher VTG levels (exceeding 10 mg/mL on average) occurred, and are likely related to exposure at an earlier age (Allen et al., 1999a,b) absence of this gonadal abnormality in our study is not surprising. However, the absence of more acute morphologic changes associated with (pseudo)estrogen exposure such as premature oocyte ripening (Janssen et al., 1995), and the limited biochemical effects even at high internal concentrations indicate that exposure to TBBPA or HBCD were not major causes for previously reported pollution-related effects on the reproductive system of flounder.

Possible interaction of TBBPA and HBCD with the thyroid hormone system *in vivo* was evaluated by analysis of thyroid hormones and thyroid histology. Plasma thyroid hormone levels were in the ng/mL range, which is consistent with existing literature on thyroid hormones in a number of marine teleosts (Bjørnsson et al., 1998; Cyr et al., 1998; Pavlidis et al., 2000). The levels of T_4 in the control and two groups of lowest exposure in the TBBPA study were low compared to the values in the other experiments. Although the TBBPA and HBCD studies were conducted in spring and winter, respectively, seasonal influences are thought not to have played a role because of the similar controlled husbandry conditions. However, the animals exposed to TBBPA were kept at a higher temperature (21°C in TBBPA, compared to 15°C in the HBCD study). Although literature suggests an influence of temperature on teleost thyroid status, plasma levels of T_4 itself were largely unaffected by temperature after prolonged acclimation in marine cod (*Gadus morhua*), and

acute change in rainbow trout (*Salmo gairdneri*) (Eales et al., 1982; Cyr et al., 1998), and the influence of temperature on the present T_4 levels remains speculative.

Given the lower background in the TBBPA study, the very high T_4 levels in animals exposed to the highest TBBPA dose are remarkable, and may be explained in a number of ways. Since the assay was designed to measure total T_4 levels (plasma protein bound and free T_4) in the presence of blocking agents that compete for plasma binding sites, apart from indicating an absolute increase of total T_4 levels, additional competition of TBBPA with plasma binding sites could enhance the sensitivity of the assay. This explanation is supported by the increased detection of T_4 in flounder plasma after direct addition of TBBPA (which was itself not detected), and is consistent with displacement of T_4 from T_4 binding proteins *in vitro* (Hamers et al., 2006). The biological consequences of this observation *in vivo* are not clear. The fitted dose-response model indicates a T_4 level of 1.2 ng/mL, at an environmentally relevant internal concentration of 14 ng TBBPA/g wet weight, which would represent a relatively strong increase when compared to the (undetectable) levels in the control group.

Thyroid hormones have been associated with vitellogenesis and oocyte development in a number of studies, although mostly at higher levels. Accelerated oocyte development as was reported in guppy (*Poecilia reticulata*) exposed to T_4 (Lam and Loy, 1985) was not observed in the present study. Moreover, in the present study, T_3, but not T_4, concentrations showed a mild but statistically significant correlation with VTG concentrations. T_3 levels correlated with rising VTG levels during annual reproductive cycling in the marine fish common dentex (*Dentex dentex;* Pavlidis et al., 2000). High T_3 (32 ng/mL) and low T_4 (<10 ng/mL) levels were measured in Atlantic halibut (*Hippoglossus hippoglossus*) in the months prior to spawning (Bjørnsson et al., 1998). Studies in *Xenopus* showed that VTG gene activation by 17β-estradiol was potentiated by T_3 (Rabelo et al., 1993), indicating a possible unifying mechanism. It is not inconceivable that a similar mechanism operates in the flounder and thyroid hormones (notably T_3) may enhance vitellogenesis and oocyte ripening. Moreover, association of T_3 and T_4 with VTG plays an important role in transfer of maternal thyroid hormones to ripening oocytes (Monteverdi and Di Gulio, 2000), and possible dissociation of T_4 could affect offspring. However, a partial life-cycle study using zebrafish (*Danio rerio*) showed only limited effects of parental TBBPA exposure in juvenile development at very high internal TBBPA concentrations (no consistent dose-related effect on hatching, 80% mortality and skewed sex ratio at an internal dose of 5.6 µg TBBPA/g lipid in juveniles but not at lower doses) (Kuiper et al., 2006). Plasma concentrations of the more biologically active hormone T_3 (Brown et al., 2004) were not affected by the two tested compounds, and based on lack of general effects (e.g. behavioral activity) and histological indications for altered thyroid activity in flounders exposed to TBBPA, the observed effects on plasma T_4 concentrations did not appear to have functional consequences in the animals.

Histological examination did also not reveal major long-term adverse effects in any of the other organ systems evaluated. The frequency of observed *Glugea stephani* xenomas in livers and kidneys, which may have derived from the exposure water, was not related to controlled exposure levels indicating that exposure to TBBPA or HBCD do not play a major role in pollution-associated *Glugea* infestation observed in the wild (Khan, 2004). Accumulation of ceroid pigment and the numbers of melanomacrophage clusters, which have been associated with oxidative damage of lipid cellular components (Wolke et

al., 1985), were not significantly different between groups or related to internal concentrations of TBBPA or HBCD, and there were no accompanying hallmarks of hepatocellular degeneration. Thus, we found no histopathologic support for oxidative damage, despite suggested oxidative stress in rainbow trout exposed to TBBPA and HBCD (Ronisz et al., 2004).

Conclusion

Environmentally relevant internal levels of TBBPA were associated with minor changes in aromatase activities and mildly elevated thyroxin levels without apparent biological consequences; exposure to HBCD did not affect any of the investigated parameters. Interactions of TBBPA with binding of thyroid hormones to plasma proteins, the yolk precursor vitellogenin in particular, should be subject to further investigation. Variation in histological appearance of investigated tissues and prevalence of macroscopic and microscopic lesions were not associated with exposure to TBBPA or HBCD, indicating no major functional implications of the limited biochemical changes observed. The present findings indicate limited potential for *in vivo* endocrine disruption of the reproductive and thyroid hormonal systems by the flame retardants TBBPA and HBCD in immature European flounder over a wide range of internal concentrations including reported maximum levels in feral fish: from not detectable to 4300 ng TBBPA/g wet weight, and from not detectable to 446 µg ΣHBCD/g lipid weight.

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6

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*)

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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. European flounder (*Platichthys flesus*) were exposed via spiked food and sediment for 101 days, and adult zebrafish (*Danio rerio*) were exposed via the water for 30 days followed by exposure of juveniles until 45 days post hatching (partial life-cycle approach) to a range of concentrations of a commercial pentabromodiphenylether mixture, DE-71. Chemical analysis of exposed animals shows that internal concentrations increased with dose and included environmentally relevant levels as well as higher levels. The congener profile in exposed fish was also very similar to that in wild fish. Animals were investigated histopathologically with emphasis on endocrine organs. Additional biochemical parameters were investigated in flounder as markers for suggested dioxin-like activity (EROD), activation of endogenous estrogen synthesis (gonad aromatase activity), and thyroid hormonal status. In zebrafish, hatching of embryos and larval development were assessed. In flounder, plasma thyroid hormone (T_4) concentrations, and hepatic EROD and ovarian aromatase activities showed a mild decrease as internal PBDE concentrations increased, but without accompanying general or histopathological changes. Adult zebrafish showed a mild increase of plasma thyroid hormone levels increasing internal PBDE concentrations, and mild indications for decreased egg production. Reduced larval survival was observed at internal levels that were more than 55 times the highest environmental recordings.

Introduction

Polybrominated diphenyl ethers (PBDEs) 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. Because of their application as additive flame retardants, PBDEs are not structurally bound to the polymer matrix, and tend to escape from the target products during and after use. Landfills and production sites provide major sources for contamination of rivers and surface waters (Renner, 2000; Birnbaum and Staskal, 2004), and rising levels of PBDEs in the aquatic environment have been reported since their introduction in the early 1970s (De Boer, 1989; Zhu and Hites, 2004). Depending on the substitution pattern, 209 possible PBDE congeners have been defined and numbered analogously to polychlorinated biphenyls (PCBs) according to Ballschmiter and Zell (1980). The bioavailability of PBDEs decreases with increasing molecular size, and particularly lower brominated congeners are detected in biota (de Wit, 2002; Burreau et al., 2004). Although debromination of higher brominated PBDEs may occur, this is thought not to result in formation of the major contaminating PBDEs in biota, such as BDEs -47 and -99 (Söderström et al., 2004). BDEs -47 and -99 predominate in commercial penta-brominated diphenylether (PentaBDE) and these technical mixtures are regarded as the major source of PBDEs in biota (Birnbaum and Staskal, 2004). Until recently, continued production and global redistribution of bioaccumulating and relatively stable lower brominated diphenylethers has resulted in progressive contamination of aquatic biota (Ikonomou et al., 2002; Vives et al., 2004).

Effects of exposure to particularly lower brominated BDEs point to modulation of the thyroid and sex steroid endocrine systems, as well as retardation of neurobehavioral development. *In vitro* studies have shown interactions with binding to the transport protein transthyretin (TTR) and (anti)estrogenic effects for a number of PBDE congeners and (hydroxylated) metabolites (Meerts et al., 2000, 2001; Hamers et al., 2006). Decreased levels of plasma thyroid hormone (thyroxine, T_4) were reported in mice and rats after exposure to lower brominated diphenyl ethers (Zhou et al., 2002; Stoker et al., 2004; Skarman et al., 2005). Reduced T_4 and triiodothyronine (T_3) levels coinciding with increased circulating thyroid stimulating hormone (TSH) levels and marked thyroid hyperplasia were observed in pubertal rats of both sexes orally exposed to the commercial PentaBDE mixture DE-71 (Stoker et al., 2004). Furthermore, anti-androgenic effects were observed in male rats (Stoker, 2005). Exposure to BDE-99 reduced sperm production and decreased circulating sex steroid levels in male offspring, and ovarian development in female offspring from dams exposed during gestation (Kuriyama et al., 2005; Lilienthal et al., 2006), indicating effects on sexual development. These authors also report behavioural alterations (hyperactivity in offspring from exposed dams, and increased, normally sexually dimorphic, sweet preference in males). Neurobehavioral effects of exposure to PBDEs were furthermore observed in mice (Eriksson et al., 2002; Viberg et al., 2002), and fish (Timme-Laragy et al., 2006).

With regard to fish, reduced spawning success and hepatic lipidosis were observed in stickleback exposed to an uncleaned technical PBDE mixture (Holm et al., 1993). Hepatic lipidosis, together with increased cytochrome P4501A (CYP1A) activity indicating possible contamination with planar PHAHs, was also observed in rainbow trout fry exposed to the same chemical mixture (Norrgren et al., 1993). 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 common in European estuaries and is susceptible to a number of conditions including (pre)neoplastic lesions, in association with exposure to environmental pollutants such as polyhalogenated aromatic hydrocarbons (PHAHs) and organotins (Vethaak et al., 1996; Grinwis et al., 2000; Stentiford et al. 2003), and histologically premature ovaries associated with elevated 17β-estradiol levels were observed in flounder exposed to contaminated harbor sludge (Janssen et al., 1997). Zebrafish were included as a freshwater species sensitive to estrogenic and anti-thyroid effects (van den Belt et al., 2002; van der Ven et al., 2003b, 2006), and to evaluate reproductive performance and larval development as ecologically relevant parameters. For analogy to environmental exposure, a classic pentabrominated diphenylether mixture, DE-71, was used as contaminant source. Because some of the biological effects previously reported in animals exposed to commercial PentaBDE mixtures may have been caused by minor amounts of contaminating poly-brominated dibenzo-*p*-dioxins and -furans (Sanders et al., 2005; Kuiper et al., 2006), the commercial product was purified to remove possible planar contaminants. 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.

Materials and methods

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 dibenzodioxins and -furans, 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).

Exposure of flounder

Juvenile flounder (159 days old) were obtained from a hatchery (Manx Mariculture Ltd, Isle of Man, UK) and held at the RIKZ field station (Jacobahaven, the Netherlands) until the start of the experiment. The animals were 313 days of age at the start of the experiment, with an average weight of 48 ± 12g (SD). Flounder were housed in a conditioned room with a 16 hrs light, 8 hrs dark daily cycle. Aquariums (70*100*30 cm w*l*h) contained 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 3.2% (temperature 15°C; renewal twice weekly via continuous flow through). Exposure was for 101 days starting end of April 2005. A benchmark schedule was applied using 10 animals per combined exposure dose of DE-71 in sediment (µg/g total organic carbon) and food (µg/g lipid): 0+0 (control); 0+0.014; 0.007+0.14; 0.07+1.4; 0.7+14; 7+140; 70+1400; and 700+14000, respectively.

For the spiking of food, cleaned DE-71 was dissolved in acetone (Promochem, Wesel, Germany) and mixed with corn oil. Then the acetone was evaporated and the oil containing HBCD was added to mixture of fish meal, mussels, and other substances to prepare food pellets. The resulting lipid content of the food was 10%. Animals were fed 3 times a week at an estimated 1% of the total bodyweight 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.

Exposure of zebrafish

Eight months old reproducing zebrafish (*Danio rerio*), from a commercial importer (Ruinemans Aquarium BV, Montfoort, The Netherlands) were kept under quarantine conditions for a period of 4 weeks, and were acclimatized to the test system from 5 days prior to exposure. 11 groups of 4 males and 4 females each were housed in all glass aquariums measuring 25x18x22 cm, covered with a glass plate, containing 3 L of test medium (0.75 L/fish). The water was aerated continuously via glass tubes and oxygen levels were >5 mg/l 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. A daily cycle of 14 hrs light, 10 hrs dark was maintained. Animals were fed ad libitum for 5 minutes 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. Groups consisting of 4 males and 4 females were exposed to waterborne PentaBDE at nominal concentrations of 0 (control), 5, 16, 50, 160 and 500 µg/L. Exposure of adults was continued for 30 days by semistatic renewal twice a week. All concentrations were tested in duplicate except 50 µg/l, which was not replicated. Final DMSO concentration was 0.01% in all groups except in 1 of the controls. During exposure, animals were monitored daily for alertness, abnormal behavior or disease, and mortality.

Reproduction was monitored directly after water renewal on Mondays and Thursdays, when both sexes were placed inside a nylon mesh (3 mmØ) for 24 hours 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.

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 Σ8BDE (mean±SD)	µg/g wet weight internal BDE-47 (mean±SD)	µg/g lipid internal BDE-47 (mean±SD)	Plasma T3 (nM±SD)	Plasma T4 (nM±SD)	Aromatase gonad ± SD (pmol/hr/mg)		EROD ± SD (pmol/min/mg)
							Male	Female	
Background	0	0.10 ± 0.11 (0.02-0.32; n=10)	0.07 ± 0.07 (0.02-0.21)	4.92 ± 3.66 (2.19-14.43)	8.4 ± 2.4 (n=10)	23.1 ± 11.0 (n=10)	2.13 ± 0.84 (n=3)	1.06 ± 0.52 (n=5)	10.9 ± 12.5 (n=9)
Background	$14*10^{-3}$	0.10 ± 0.09 (0.01-0.3; n=10)	0.07 ± 0.06 (0.01-0.20)	7.11 ± 7.20 (0.64-20.80)	10.5 ± 2.3 (n=10	19.4 ± 6.2 (n=10)	1.04 (n=2)	2.41 ± 1.31 (n=6)	12.6 ± 10.8 (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)	8.20 ± 11.40 (0.71-37.49)	9.5 ± 3.4 (n=9)	25.7 ± 12.7 (n=9)	0.65 ± 0.48 (n=4)	1.62 ± 1.48 (n=6)	14.5 ± 6.7 (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)	19.58 ± 21.56 (0.43-67.45)	8.3 ± 3.8 (n=10)	11.8 ± 7.8 (n=10)	0.06 ± 0.55 (n=3	1.49 ± 0.49 (n=5)	10.1 ± 8.7 (n=10)
$7*10^{-1}$	14	0.34 ± 0.25* (0.04-0.74; n=8)	0.22 ± 0.16* (0.02-0.48)	18.83 ± 13.64* (2.88-4.00)	7.6 ± 2.9 (n=10)	20.6 ± 15.1 (n=10)	1.57 ± 3.51 (n=4)	1.29 ± 0.74 (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)	24.09 ± 14.48* (11.25-56.92)	7.4 ± 2.6 (n=10)	13.9 ± 7.6 (n=10)	ND	1.43 ± 1.26 (n=3)	4.1 ± 3.9 (n=10)
70	1400	5.91 ± 4.27* (0.85-13.8; n=10)	3.86 ± 2.81* (0.50-9.20)	330.70 ± 207.14* (55.56-657.14)	8.7 ± 2.1 (n=9)	10.0 ± 4.9 (n=9)	1.31 (n=2)	0.91 ± 0.65 (n=7)	2.9 ± 1.2 (n=8)
700	14000	70.6 ± 85.9* (18.7-308; n=10)	44.6 ± 56.2* (11.0-200)	2690.8 ± 2714.4* (833.33-10000)	8.6 ± 1.9 (n=10)	16.4 ± 6.4 (n=10)	0.07 ± 0.55 (n=3)	0.77 ± 0.24 (n=6)	2.5 ± 2.6 (n=10)

*significantly different from background PBDE levels, $p<0.05$.
Σ8BDE: the sum of BDEs -28, -47, -49, -99, -100, -153, -154, and -183.

During weeks 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 of exposure medium. From parents exposed to 50 and 500 µg DE-71/L, insufficient eggs were obtained; therefore, eggs from parents exposed to 16 and 160 µg/L, were used for exposure of eggs and juveniles to 50 and 500 µg/L, respectively. In addition, 2 times 50 eggs from control parents were also exposed to the highest test concentration (500 µg/L) and 2 times 50 eggs from 500 µg/L 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.

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 Σ8BDE (mean±SD)	µg/g wet weight internal BDE-47 (mean±SD)	µg/g lipid internal BDE-47 (mean±SD)	Plasma T_3 ng/ml (mean±SD)	Plasma T_4 ng/ml (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 (3.7-11.5; n=3)	7.7 ± 3.7 (3.4-10.0)	246 ± 138 (97-370)	7.0 ± 6.0 (n=8)	<5 (n=8)
16	15.2 ± 3.8 (11.5-20.5; n=4)	13.3 ± 3.4 (10.0-18.0)	404 ± 191 (196-619)	9.9 ± 2.1 (n=7)	22.8 ± 20.3 (n=6)
50	125.9 (78.1-173.8; n=2)	114.0 (68.0-160.0)	8273 (2000-14545)	10.5 ± 5.2 (n=6)	<5 (n=5)
160	236.3 ± 45.7 (203.6-303.9; n=4)	210.0 ± 40.8 (180.0-270.0)	6978 ± 2009 (4878-9310)	13.0 ± 4.8 (n=8)	30.2 ± 19.3 (n=7)
500	459.9 ± 81.6 (376.2-560.3; n=4)	407.5 ± 78.9 (320.0-500.0)	9653 ± 3444 (5926-12941)	19.0 ± 8.5 (n=8)	34.6 ± 18.4 (n=8)

Σ8BDE: the sum of BDEs -28, -47, -49, -99, -100, -153, -154, and -183.

Sampling procedure

Flounder

Directly following euthanasia in random order using MS222 (Sigma-Aldrich, Steinheim, Germany), animals were weighed and length was determined. Blood was sampled from the caudal spinal vein using a 2 mL syringe with 0.6 mmØ needle, and transferred to a heparinized container. After centrifuging, plasma was collected and 30 µL aprotinine (Sigma; 0.1 mg/mL in 0.9% NaCl) per mL plasma was added. Plasma samples were then

flash frozen and stored at -70°C for analysis of thyroid hormones. The fish was placed with its eyes facing up, and the coelomic cavity was opened and after the bile was removed with a syringe, the livers were weighed and divided in a rostral, middle and caudal part. The rostral parts were frozen in liquid nitrogen and stored at -70°C for preparation of microsomes; the caudal parts were collected in RNeasy buffer (Quiagen) stored at -20°C for future analysis of gene expression, and the middle parts were fixed in 4% neutral buffered formaldehyde (formalin) for histological processing. Fish were again weighed after removal of the intestinal tract. Gonads were excised and weighed; the topmost gonad was frozen in liquid nitrogen and stored at -70°C for preparation of microsomes, and the lower gonad was fixed in formalin together with the remaining internal organs including kidneys. The heads were removed and branchial arches including the thyroid region were fixed in formalin. Finally, flank muscles ("fillet") were excised and stored at -20°C for chemical analysis.

Zebrafish
Immediately after euthanasia with MS222, length, weight and condition factor (CF; weight/length^3) were determined, and blood was sampled from the clipped tail peduncle of adults using heparinized glass capillaries. An equal volume of aprotinin (Sigma; 6 µg/mL in 0.01 M PBS, pH7.2) 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 for 24 hrs after which they were transferred to 70% ethanol until routine histological processing. Fish for chemical analysis were rinsed in aquadest twice and dipped dry, and stored at -20°C until further processing.

Histology and immunohistochemistry
Sections were cut from paraffin embedded tissues from flounder, and whole zebrafish, at 3-4 µm thickness, and were routinely stained with hematoxylin and eosin (H&E). Additional immunohistochemical staining was performed on liver sections of male flounder from the highest exposure and control groups, using polyclonal rabbit anti-turbot vitellogenin (VTG; CS-2, Biosense laboratories, Bergen, Norway) as a primary antibody as described previously (chapter 5), and on selected whole zebrafish sections 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 as described previously (Kuiper et al., 2006). For VTG staining, (tissue of) flounder exposed to E2, and adult reproducing female zebrafish were included as positive controls.

Chemical analysis
Determination of internal BDE levels was previously described (Kuiper et al., 2006). Briefly, approximately 7 grams of flounder muscle from each animal was weighed, dried with sodium sulphate (Merck), and stored for two hours prior to extraction and clean-up. Analysis was performed with GC-MS. From zebrafish, 2 adults (1 male, 1 female) per exposure concentration were weighed and whole bodies were prepared as described for flounder tissue; from juvenile zebrafish, a pooled sample of 4 fish was prepared per exposed group of 50 juveniles.

Analysis of thyroid hormones
The levels of plasma thyroid hormones triiodothyronin (T_3) and thyroxin (T_4) were analyzed by radioimmuno assay (RIA). Flounder plasma was analyzed according to Friedrichsen et al. (2003), and zebrafish plasma was analyzed as described by van der Ven et al. (2006).

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 EROD, PROD and BROD activities were determined in hepatic microsomes and aromatase activity was determined in gonad microsomes as previously described (Kuiper et al., Chapter 5).

Statistical analysis
Internal PBDE concentrations were log-transformed prior to one-way analysis of variance (ANOVA) to compare exposure groups to controls (Dunnet's post hoc analysis). Zebrafish juvenile survival was evaluated using Kaplan-Meier analysis. Dose response modeling was performed on continuous endpoints according to Slob et al. (2002), using PROAST software (RIVM, 2002) to relate biochemical parameters to internal BDE-47 concentrations.

Results

Chemical analysis of exposed fish

Both in flounder and zebrafish, internal PBDE levels increased with dose (Tables 1 and 2, Figs. 1, 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. 3). 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 (82 ± 13% 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 TOC in sediment and 1.4 µg/g lipid weight (lw) in food, and higher.

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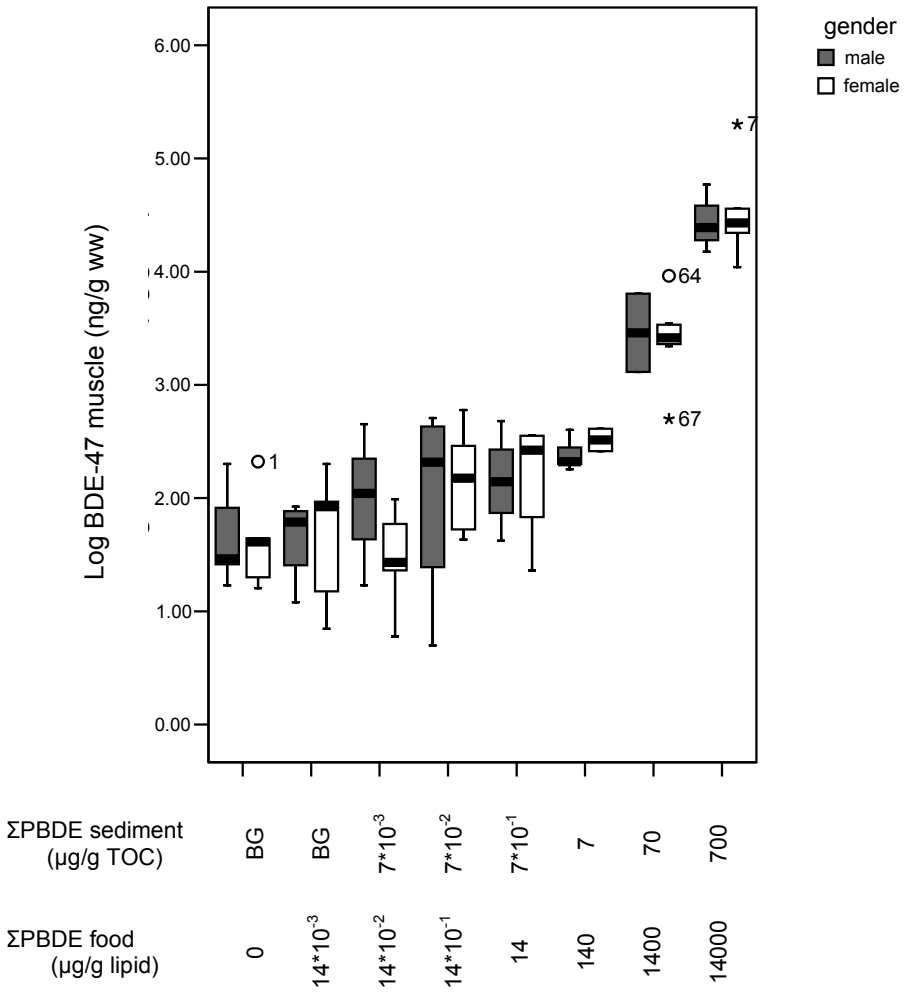

Figure 1. Internal BDE-47 concentrations in flounder exposed to commercial PentaBDE for 101 days.

Levels of BDE-49 were somewhat 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 were low levels of BDEs -28 and -49 were detected (<LOD-49 and

100 ng/g wet weight (ww), respectively). Although present at relatively low amounts, a clear dose-related increase was observed for BDE-183 in zebrafish, whereas BDE-183 was not detected in flounder. The sum of the eight most important BDEs found in feral fish (Σ8BDE: BDEs -28, -47, -49, -99, -100, -153, -154, and -183) is presently used to indicate the total PBDE levels in experimentally exposed fish. Σ8BDE levels in flounder muscle ranged between 0.10 and 308 µg/g ww and were significantly above the background levels in animals exposed to the combination of 14 µg/g lw in food and 0.7 µg/g 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, Σ8BDE levels were below the detection limit in adult controls and ranged up to 560 µg/g 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 controls and these groups were treated as similar for statistical analysis. All detectable levels of BDEs in zebrafish were highly linear with the nominal PentaBDE exposure concentrations (adults shown in Fig. 2; 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. In exposed zebrafish, an unidentified brominated compound, probably a PBDE metabolite, was detected which was particularly prominent in juveniles exposed to nominal concentrations of 16 and 50 mg DE-71/L (overall proportion 5%; 13% in top dose).

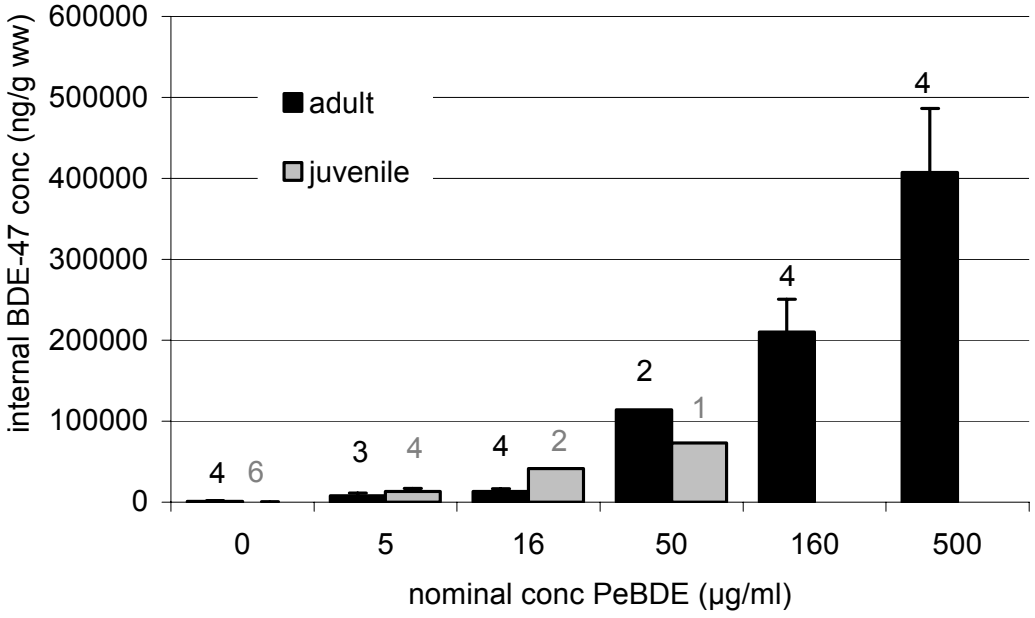

Figure 2. Internal BDE-47 concentrations in zebrafish exposed to commercial PentaBDE for 30 days (adults: R^2=0.89) and 45 days (juveniles; R^2=0.89). Error bars represent standard deviations; numbers above bars are number of replicates.

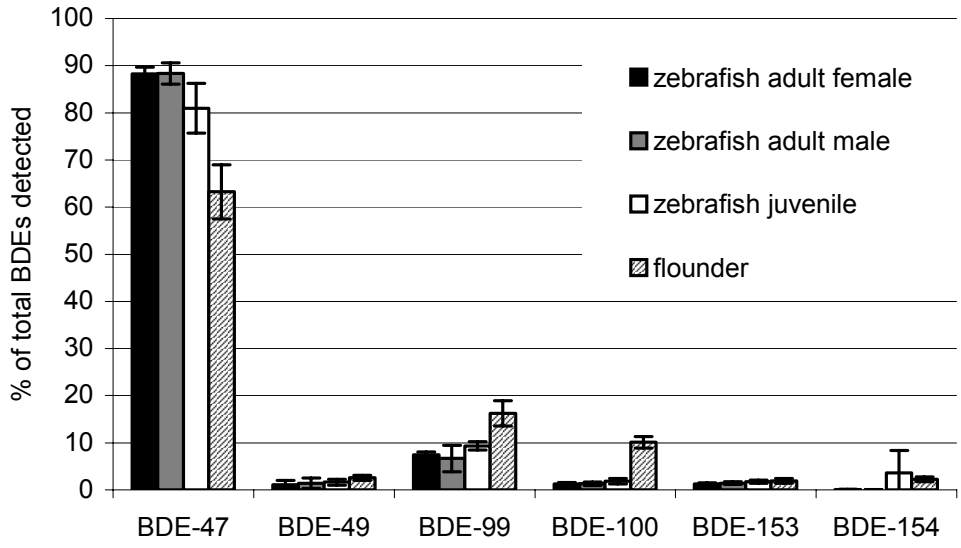

Figure 3. Distribution of predominant BDEs in DE-71, exposed flounder, and zebrafish. Error bars represent standard deviations of percentages between the various dose groups.

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)[1]	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	13.0 ± 4.0 (11.0-19.0; n=4)
16	95 ± 15	92 ± 4	>14	41.5 (33.0-50.0; n=2)
50[2]	100	86 ± 6	>14	73.0 (n=1)
160	97 ± 9	93 ± 6	8 (7.96-8.04)[4]	NA
500[2]	95 ± 11	91 ± 4	6 (5.38-6.62)[4]	NA
500[3]	96 ± 13	92 ± 5	7[5]	NA

[1]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.
[2]due to insufficient egg production in these parent groups, eggs from the first lower parent concentration group were used (16 and 160 µg/L, respectively)
[3]eggs from control parents exposed to 500 µg/L
[4]95% confidence interval
[5]all larvae died between day 6 and 7 post hatching; a 95% c.i. was not calculated.

Gross observations, zebrafish reproduction and development, histopathology
Flounder exposed to 0.007 µg/g TOC + 0.14 µg/g lw (sediment and food, respectively) and higher were slightly less active than control animals and animals exposed to 0.014 µg/g lw (food only) at the start of exposure; after 2.5 weeks, this difference in behavior was no longer observed. Length, weight, condition factor, 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 mild decrease in adults exposed to concentrations of 50 µg DE-71/L and higher (Fig. 4). A dose response model could not be fitted consistently and a critical effect dose was not reliably determined. Fertilization and hatching were not affected (Table 3).

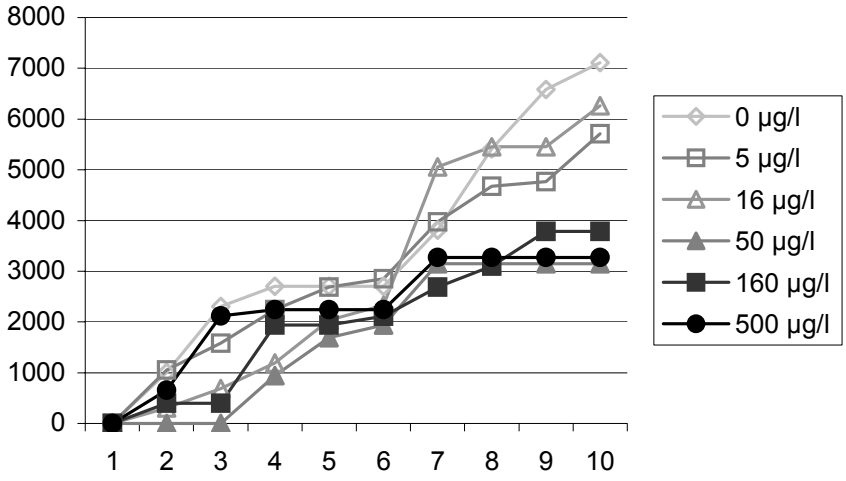

Figure 4. Total cumulative egg production in zebrafish exposed to DE-71 for 30 days.

Exposure of juvenile zebrafish resulted in retardation of development followed by increasing mortality during the first week PH in animals exposed to 160 and 500 µg/L. The median post-hatching survival times in larvae exposed to 160 and 500 µg DE-71/L were significantly and dose dependently decreased (Table 2), and mortality was 100% after 10 days in affected groups. Juveniles from parents that had been exposed to 500 µg DE-71/L 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 did not relate to exposure levels.

Dose-related histopathological changes were not detected in either species, including zebrafish juveniles. Immunohistochemistry showed no evidence for increased levels of VTG in exposed flounder or zebrafish, or CYP1A in zebrafish. Background pathology was low and similar to previous reports (Kuiper et al, 2006; Chapter 5).

Thyroid hormone concentrations
Levels of plasma thyroid hormones (T_3 and T_4) are shown in Tables 1 (flounder) and 2 (zebrafish). Plasma T_3 levels in the flounder from this study ranged from 2.8-17.2 (average: 8.6) nM and T_4 levels ranged from 2.0-46.0 (average: 17.6) nM. A significant negative dose response relation was detected only for T_4 (Fig. 5), indicating a 10% decrease at an internal concentration of 51 ng BDE-47/g muscle (wet weight), with a lower 95% confidence limit (c.l.) of 19 ng/g. However, the estimated maximal decrease of 46% did not result in significant differences between group averages.

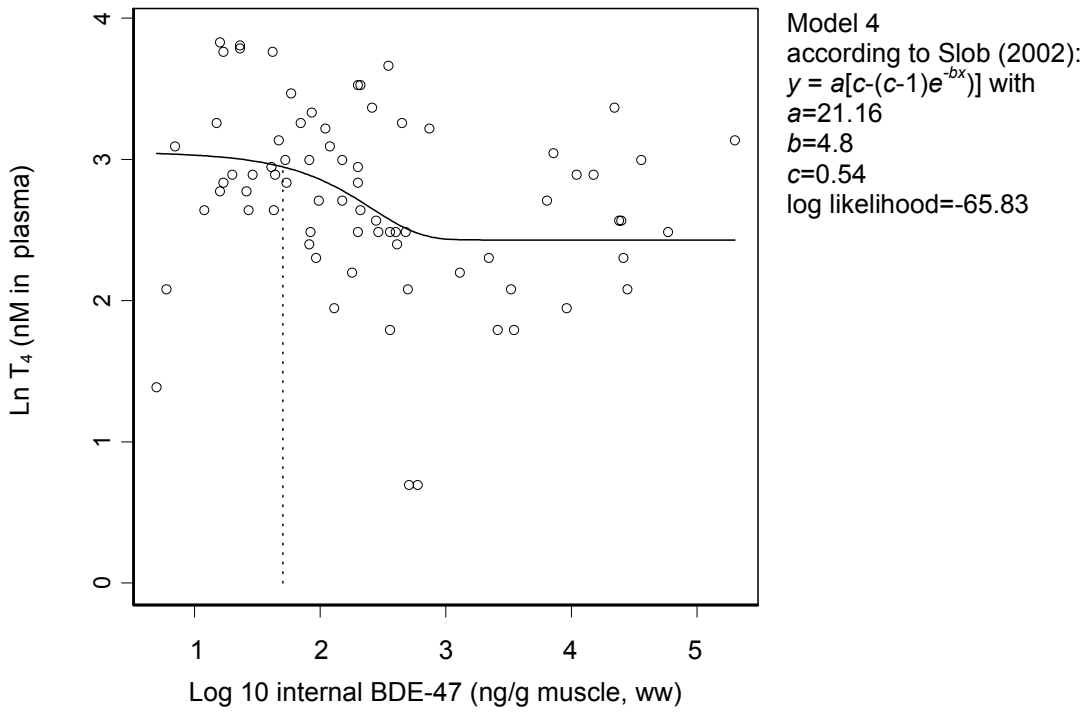

Figure 5. Relation between T_4 and internal BDE-47 concentrations in flounder (muscle). The stippled line indicates the BDE-47 concentration at which the T_4 concentration is reduced by 10%.

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 (Fig. 6a,b). T_4 concentrations doubled at a calculated internal dose of 116 (lower 95% c.l.: 79) µg BDE-47/g 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 hormone levels were not analyzed in juvenile zebrafish.

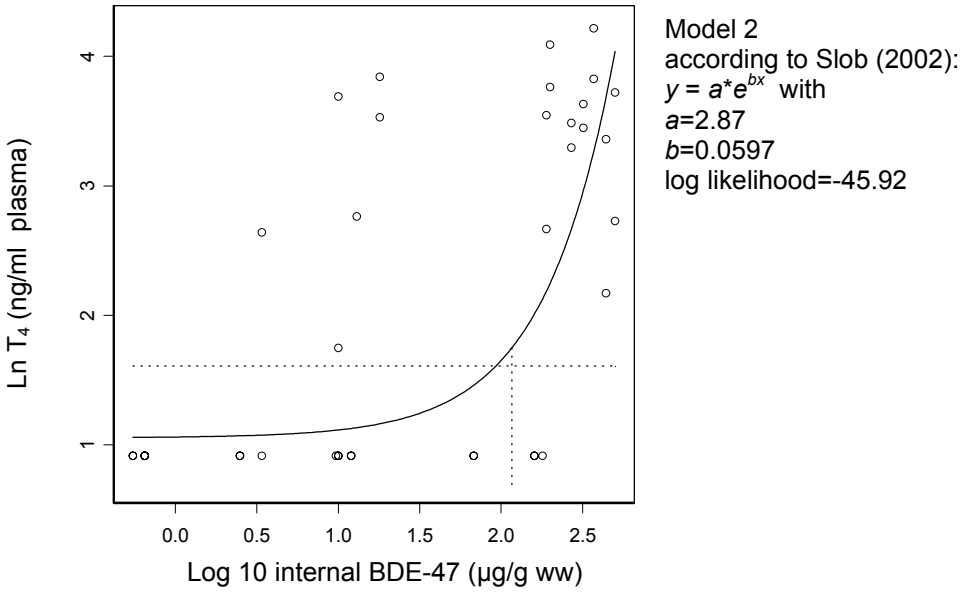

Figure 6a. Relation between T_4 and internal BDE-47 concentrations in zebrafish (whole body homogenate). The vertical stippled line indicates the BDE-47 concentration at which the T_4 concentration is doubled; the horizontal stippled line indicates the T_4 detection limit.

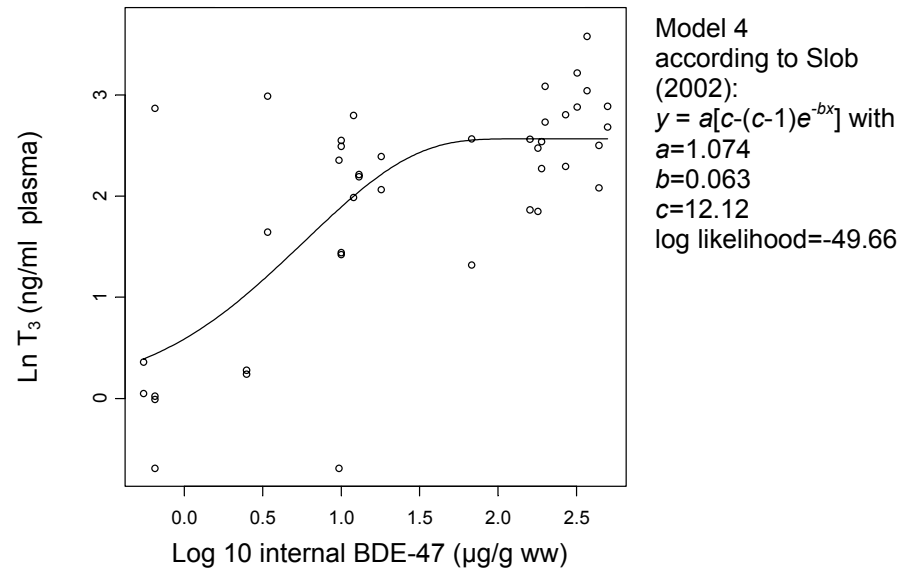

Figure 6b: Relation between T_3 and internal BDE-47 concentrations in zebrafish.

microsomal enzyme activities (flounder)
Hepatic EROD activities were generally low, ranging from undetectable to 39 pmol/mg/min with an overall average of 8.1 pmol/mg/min. EROD activity decreased with increasing BDE-47 concentrations in muscle (Fig. 7). PROD and BROD activities were very low and did not relate to nominal or internal PBDE concentrations, but were significantly correlated to EROD activity (R=0.6 and 0.3 respectively, $p<0.01$; data not shown).

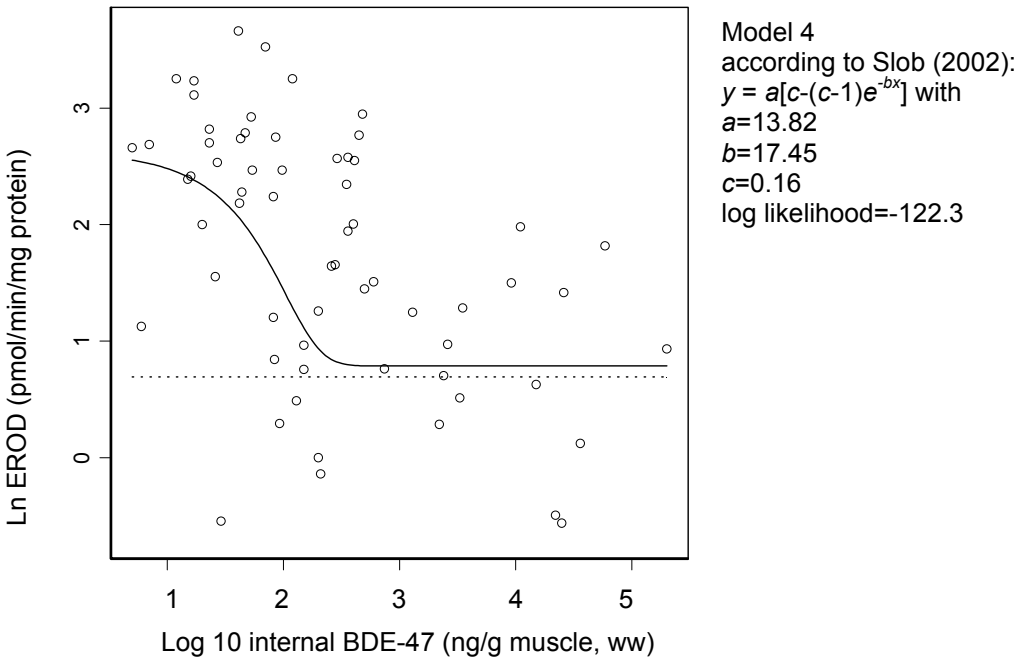

Figure 7. Relation between hepatic EROD activity and internal BDE 47 concentrations in flounder muscle.

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 aromatase activity decreased with increasing muscle BDE-47 concentrations in female flounder (Fig. 8). A 10% reduced aromatase activity was calculated already at a BDE-47 concentration of 40 ng/g muscle (wet weight), levels that were currently observed as background values.

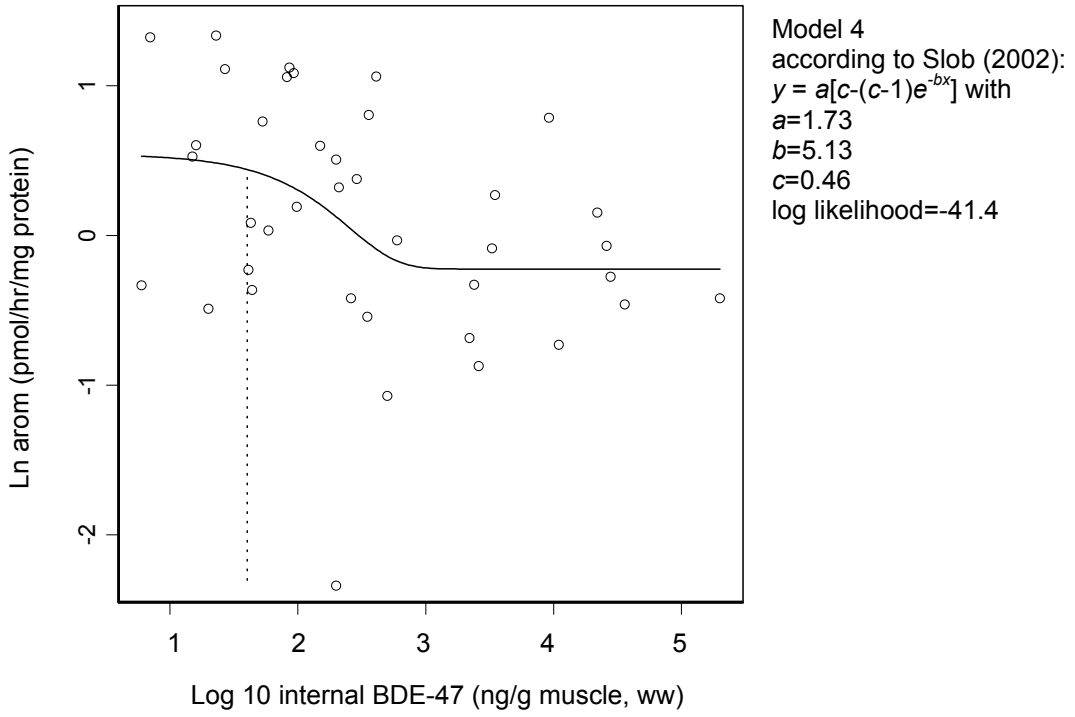

Figure 8. Relation between aromatase activity in ovaries and internal BDE-47 concentrations in muscle of female flounder. The vertical stippled line indicates the internal BDE-47 concentration at which a 10% decrease of aromatase activity was calculated.

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 -100, -153, -154, and -99 in exposed flounder and zebrafish is in general agreement with reported patterns in both salt- and freshwater wild fish (Boon et al., 2002; Law et al., 2006), supporting a predominant role for commercial PentaBDE mixtures as a major PBDE source in aquatic biota in the environment. Relative loss of BDE-99 compared to the Σ8PBDEs 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, BDE-183 may have remained undetected in flounder because of its relatively low affinity

for muscle (Voorspoels et al., 2003). 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 (de Wit et al., 2002; Law et al., 2006).

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 points to higher sensitivity of juvenile fish, which was apparently not influenced by the exposure status of the parents. Although juvenile mortality at 50 µg/L was not different from the controls, a mild reduction of cumulative egg production at that concentration indicates that the dose at which reproductive success would be affected could be slightly lower. However, the average internal ΣBDE level in parents (126 µg/g ww) exposed to that concentration exceeded environmental top levels (2270 ng/g ww in burbot, *Lota lota*, from lake Mjøsa, Norway; Mariussen et al., 2003) 55 fold, the highest average levels found in brown trout (*Salmo trutta*) from this lake (353 ng/g ww) are 357 fold lower. The risk for the marine environment is even lower as internal BDE levels in fish usually vary in the low ng/g ww range (e.g. de Boer 1989; Boon et al., 2002; Voorspoels et al., 2004; Leonards et al., 2004). This combined information indicates a limited risk for reduced production of offspring even under heavily contaminated field conditions.

The changes in thyroid hormone levels are somewhat contradictory between the two species used in this study. 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 (up to around 2 µg ΣBDE on wet weight basis in fresh water fish). In contrast, a dose-related increase in T_3 and T_4 was found 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 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. The lack of coincident histological changes in the thyroid glandular tissue in both species indicates a minor impact on functional thyroid status. It has been suggested that teleosts are relatively insensitive to the effects of goitrogenic environmental pollutants (Leatherland et al., 1993), and experimental parental hypothyroidism did not affect reproduction and offspring in PTU-exposed zebrafish (van der Ven et al., 2006). The impact of increased thyroid hormone levels on reproduction has not been investigated in fish, but research in other vertebrates indicates an association with disturbed estrous cycling in females and reduced fertility in males, and

increased estrogen/androgen ratios (Choksi et al., 2003). In this respect, it is noteworthy that T_3 inhibited aromatase activity in cultured rat Sertoli cells (Ulisse et al., 1994). Aromatase activities were not related to levels of $T_{3/4}$ in either gender in the present study, and treatment related changes in Sertoli cell morphology or quantity of ripe sperm were not observed.

Histopathological examination of the gonads in both species otherwise presented no indications for altered activities in any of the exposed animals compared to the controls, and absence of immunohistochemical indications for production of the yolk precursor VTG point to limited or no direct estrogenic action of PBDEs or possibly formed metabolites in the two species investigated *in vivo*. Although a mild (not statistically significant) reduction of egg production was indicated, there were no frank histological indications for anti-estrogenicity in exposed zebrafish (e.g. degeneration of oocytes, granulosa cell hypertrophy or Leydig cell hyperplasia, asynchronous sperm development, or relative increase of male offspring; Wester et al., 2003). However, the observed decrease in the activity of aromatase, which catalyzes androgen to estrogen transformation, in flounder ovaries, may point to an indirect anti-estrogenic effect 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, and 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 immuno-detectable CYP1A protein in zebrafish exposed to cleaned DE-71 is consistent with earlier *in vitro* and *in vivo* results. Both the technical mixture DE-71 and the dominating highly purified PBDE congeners did not activate the AhR pathway in a human hepatocyte-based DR-CALUX reporter gene assay, or induce detectable EROD activities in primary isolated carp hepatocytes (Hamers et al., 2006; Kuiper et al., 2004, 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 fish.

The mild decrease in EROD activities in flounder from higher PBDE exposure levels may be consistent with earlier observations that DE-71 and its major constituents may inhibit activation of the AhR pathway (Hamers et al., 2006; Kuiper et al., 2004; Peters et al., 2004). EROD inhibition was previously reported in fish *in vivo* and *in vitro* after exposure to environmental contaminants such as PCBs (Besselink et al., 1998; Eggens et al., 1996; Hahn, 1993). Although inhibition of the AhR pathway by PBDEs was less apparent *in vivo* by immunohistochemical detection of CYP1A in zebrafish (Kuiper et al., 2006), quantitative measurement of EROD activity may be more sensitive than semi-quantitative immunohistochemisty for detection of AhR antagonism *in vivo*, and EROD activity in flounder may be more sensitive to inhibiting contaminants compared to cyprinids (Eggens et al., 1996).

Conclusions

Exposure to a commercial pentabromodiphenylether mixture resulted in a dose-dependent increase in internal levels of PBDEs with a similar congener profile as is found in wild fish. BDE-183 was dose dependently increased in zebrafish, indicating that presence of this congener in wild fish may also find its origin in the late use of PentaBDE products. Our present results indicate an increase of thyroid hormone levels in plasma from zebrafish with increasing internal PBDE concentrations, without associated changes in thyroid morphology. At slightly lower, environmentally relevant exposure levels, a mild decrease of T_4 levels and ovarian aromatase activities were observed in flounder. Although the present morphologic results do not indicate (anti-)estrogenic effects of PBDEs in the two species investigated, the impact of the biochemical changes observed at environmentally relevant PBDE concentrations on reproductive health remains to be investigated. Survival of 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, but similar to PCB mixtures, PBDEs may reduce *ex vivo* EROD activity in flounder.

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7

General discussion

1. Introduction

During the past decades, increasing use of flammable polymers in construction materials and every-day life products such as furniture, clothing and personal electr(on)ic equipment has necessitated increasing use of flame retardant chemicals. Brominated flame retardants (BFRs) constitute the major part of these. It has now become clear that a number of these BFRs, most pronounced the group of polybrominated diphenylethers (PBDEs), show persistence and bioaccumulative behavior in the environment (de Wit, 2002; Birnbaum and Staskal, 2004). These characteristics allow classification of at least some BFRs as persistent organic pollutants (POPs). Past experience with adverse effects of important POPs such as the chemically related polychlorinated biphenyls (PCBs) fuels concern as to the safety of the use of large amounts of BFRs. Although bioaccumulative behavior of other popular BFRs such as tetrabromobisphenol A (TBBPA) and hexabromocyclododecane (HBCD) is less well documented, their large production volumes (130,000 and 16,700 tons per year, BSEF 2003, 2004) and release into the environment warrant careful evaluation of (environmental) safety.

This thesis is part of an EU-sponsored integrative BFR risk assessment program with particular focus on endocrine effects (FIRE: www.rivm.nl/FIRE). Results from previous *in vitro* studies, and in *vivo* experiments with rodents and amphibians, pointed to interactions with key endocrine systems like thyroid and sex steroid hormones (e.g. Meerts et al., 2000, 2001; Kitamura et al., 2002, 2005; Hamers et al, 2006; Schriks et al., 2006a,b). The strong functional similarities between the thyroid and reproductive hormone systems of fish and other vertebrates (Arcand-Hoy and Benson, 1998; Power et al., 2001), together with the steady increase of BFR levels in fish and aquatic food chains, raised concern also for the safety of BFRs from an ecotoxicological viewpoint. The studies presented in this thesis examine endocrine effects of exposure to three classes of BFRs with major environmental importance (PBDEs, TBBPA and HBCD), and associated risks for fish health and reproduction, in model freshwater and estuarine fish. In addition, activation of the aryl hydrocarbon receptor (AhR) pathway, typically involved in responses to dioxin(-like) chemicals and a number of polycyclic aromatic hydrocarbons (PAHs), by these BFRs was evaluated.

2. Involvement of the AhR in response to BFR exposure in fish

Activation of the AhR pathway is a classical hallmark of exposure to a widespread group of persistent pollutants that include dibenzodioxins and -furans and a number of PCBs (dioxin-like compounds; van den Berg et al., 1998, 2006). AhR activation has been associated with a wide range of effects including disruption of thyroid and estrogen endocrine systems (Safe, 1998; Smeets, 1999; Hagmar, 2003), reproductive and developmental defects (Prasch et al., 2003; Carney et al., 2006), and induction of cytochrome P450 (CYP) isoenzymes, specifically CYP1A in a variety of vertebrates including European flounder and zebrafish (Besselink et al, 1997; Grinwis et al., 1998; Whyte et al., 2000; Hill et al., 2005). Since these biological endpoints are included in our studies, the role of AhR activation in fish exposed to BFRs was evaluated using CYP1A induction as a biomarker.

Specific attention was given to CYP1A induction in the case of exposure to PBDEs in Chapters two and three. PBDEs share some structural similarity with PCBs and some of the more uncommon PBDE congeners were observed to activate the AhR pathway (Behnish et al., 2003; Chen and Bunce, 2003). Based on these observations, it has been proposed that these compounds should be included in the toxic equivalency factor (TEF) concept for dioxin-like compounds (TEF; see also van den Berg et al., 2006). More importantly, like technical PCB mixtures, PBDEs may be contaminated with minor amounts of very potent coplanar halogenated aromatic hydrocarbons such as brominated dibenzo-*p*-dioxins and -furans (Sakai et al., 2001; Sanders et al., 2005). Recent studies have shown that when PBDEs were extensively charcoal purified, the AhR-mediated activities in several mammalian *in vitro* systems were absent or only very minor (Peters et al., 2004). As a result the World Health Organization has recently suggested that PBDEs should not be included in the toxic equivalency concept for dioxin-like compounds (van den Berg et al 2006).

Ethoxyresorufin-*O*-deethylation (EROD), a CYP1A catalytic activity, in primary cultured carp (*Cyprinus carpio*) hepatocytes was selected as *in vitro* endpoint to study the effects of PBDEs on the AhR pathway in fish (Kuiper et al., 2004; Chapter two). Exposure of these cells showed that the individual environmentally most important PBDE congeners BDEs -47, -99, -100, and -153 as well as the original commercial product PentaBDE (DE-71) did not induce CYP1A catalytic activity in carp hepatocytes. These findings are in general agreement with reports in cells from rat, cynomolgus monkey, and human (Chen and Bunce, 2003; Peters et al., 2004, 2006). Moreover, co-exposure of fish hepatocytes to PBDEs and tetrachlorodibenzo-*p*-dioxin (TCDD) as model CYP1A inducer revealed inhibition of EROD activity by these environmentally relevant PBDEs, including the commercial mixture DE-71, with exception of BDE-100. These results with carp hepatocytes are also in agreement with those observed in mammalian cells (Chen and Bunce, 2003; Peters et al., 2004, 2006). Experimental work showed that this inhibition by individual PBDEs can not be attributed to cytotoxicity or direct catalytic inhibition of CYP1A activity. This combined information points to a mechanism where the PBDEs appear to interact with AhR activation by dioxin-like compounds, although other mechanisms like deranged synthesis of heme (the prosthetic group of the CYP enzymes) have also been suggested (Eggens et al., 1996; Hahn et al., 1993).

Interestingly, the potent dioxin-like contaminants present in DE-71 (see Chapter three) were insufficient to induce CYP1A activity in carp hepatocytes when exposed to the untreated commercial product. Consistent with inhibition of the AhR activation, it is conceivable that the large amount of PBDEs that form the bulk of the mix may have masked the dioxin-like activity of present contaminants. Obviously, this mechanism of CYP1A inhibition is still not entirely clear and even a mixed mode of action may underlie the inhibitory effects of PBDEs on AhR-mediated effects. Our results from the carp hepatocyte assay are therefore insufficient to draw firm conclusions on weak AhR interaction by minor amounts of individual PBDEs without further analysis of AhR-pathway components.

The *in vivo* potency of the commercial pentabrominated diphenylether mixture DE-71 for AhR-mediated CYP1A induction was investigated in juvenile zebrafish, before and after chemical fractionation and separation from dioxin-like compounds (Kuiper et al., 2006a; Chapter three). Concurrent *in vitro* testing was performed using an AhR-activated

luciferase reporter gene assay (DR-CALUX). Induction of CYP1A was not detected in CALUX cells or fish exposed to purified DE-71. These results indicate that planar contaminants may be solely responsible for AhR activation observed before in fish exposed to commercial PBDE mixtures (Holm et al., 1993; Norrgren et al., 1993), as has been shown in mammals (Sanders et al., 2005). Chemical analysis of the isolated planar fraction indicated that 1,2,3,7,8-pentabromodibenzofuran is the most likely AhR agonist present in DE-71. These observations underline the need for thorough chemical clean up of PBDEs, when studies with these compounds are designed for testing of exclusively PBDE-related effects.

Reduced luciferase activity in CALUX cells exposed to untreated DE-71 when compared to the isolated planar fraction is in agreement with inhibition of AhR-regulated gene expression by the PBDEs present in the mixture. A difference in immunohistochemical staining of CYP1A was less apparent between zebrafish exposed to the same fractions. Since the proportional composition of DE-71 (PBDEs vs. dioxin-like contaminants) can be expected to be similar in both approaches, these results suggest that the use of zebrafish *in vivo* is less sensitive to inhibition of CYP1A induction by PBDEs, whatever mechanisms may be involved. Therefore, the lack of immuno-detectable CYP1A induction in zebrafish (Chapter three) exposed to cleaned-up DE-71 strongly supports the absence of AhR-agonist potency from environmentally relevant PBDEs and indicates that not attributing a TCDD equivalent factor (TEF) to these PBDEs (van den Berg et al., 2006) is appropriate for fish as well.

Consistent with these results in zebrafish, there was no positive correlation between hepatic EROD activity and internal doses of cleaned PBDEs in European flounder (Chapter six). However, a mild but significant negative correlation of EROD activity with increasing PBDE levels was found, possibly indicating AhR antagonism as was earlier observed for environmentally relevant multiple ortho substituted PCBs (Eggens et al., 1996; Besselink et al., 1998). Assuming that the response of the flounder to PBDEs is more comparable with that of multiple ortho substituted PCBs than the dioxin-like non ortho PCBs, like PCB-126, CYP1A induction is clearly not a suitable biomarker for these BFRs in fish. Consistent with *in vitro* studies (Hamers et al., 2006), exposure of European flounder to TBBPA and HBCD did also not result in induction of hepatic EROD activity (Chapter five). In conclusion, our results do not support direct involvement of the AhR in responses to any of the investigated BFRs in fish.

3. Are other hepatic cytochrome P450 isoenzymes useful biomarkers for evaluation of BFR exposure in fish?

Since most of the environmentally relevant PBDEs are multiple ortho substituted and evidently do not induce AhR-mediated enzyme induction, a profile more typical of exposure to multiple ortho rather than non, or mono ortho substituted PCBs may be expected (Sanders et al., 2005). This would include induction of (hepatic) CYP450 isoenzymes such as CYP2B and CYP3A, rather than CYP1A. Activity of pentoxyresoryfin-*O*-deethylase (PROD) has been successfully used to indicate induction of CYP2B in rats and mice exposed to PBDEs (Zhou et al., 2001; Stoker et al., 2004; Staskal et al., 2005).

Inducible forms of CYP2B- and CYP3A-like proteins have been shown in several fish species (Fent et al., 1998; Bainy et al., 1999), and PROD activity has been used in a

variety of species including European flounder (Addison et al., 1991). In our studies with flounder, PROD and BROD (benzoxyresorufin-*O*-deethylase) activities were low and significantly correlated with EROD activity. These results are in agreement with earlier findings and indicate that pentoxy- and benzoxyresorufin are poor and rather unspecific substrates for CYP2B-like enzymes in fish (Addison et al., 1991; Fent et al., 1998). Determination of PROD and BROD activities in livers of flounder exposed to PentaBDE (Chapter six) could not measure inducible activity of CYP450 isoenzymes which would in mammalian systems be more typical for exposure to multiple ortho substituted PCB-like chemicals.

4. Effects of BFRs in fish *in vivo*

The *in vivo* toxicity of TBBPA, HBCD, and PentaBDE, was also investigated with an emphasis on endocrine and reproductive effects (Chapters four, five and six). Exposure to TBBPA had a negative effect on egg production and juvenile survival in zebrafish exposed to relatively high concentrations (Kuiper et al., 2006b; Chapter four). The reduced juvenile survival was accompanied by a significant change in gender distribution. However, this observation should be interpreted with care as an indicator for endocrine effects since the gender distribution in zebrafish is notoriously variable (Örn et al., 2003, 2006). Histological examination of thyroid, liver and gonads did not reveal changes associated with disrupted thyroid or reproductive hormone function, apart from a relative decrease of mature oocytes in top dose females. This finding however was not statistically analyzed due to insufficient observations. A significant dose response model indicated that egg production was the most sensitive parameter, and calculated 50% reduced egg production at an internal TBBPA concentration of 7.2 µg TBBPA/g lipid (approximately 150 ng/g wet weight, ww). There were no histological indications for increased production of the estrogen dependent yolk precursor vitellogenin (VTG) in zebrafish exposed to TBBPA.

To include a more quantitative measure of this biomarker for estrogenicity in our studies with TBBPA, VTG concentrations were determined in plasma samples from exposed flounder (Chapter five). No relation was observed between plasma VTG levels and TBBPA exposure or internal concentrations. This result was in agreement with the zebrafish studies and indicates that TBBPA has no estrogenic activity in fish *in vivo*. Aromatase (CYP19, estrogen synthase) activity was measured in flounder gonads to evaluate a possible interaction with steroidogenesis. A significant dose response model indicated a mild increase of aromatase activity in testes from flounders exposed to TBBPA, however without apparent (indirect) estrogenic effects. Although as in zebrafish, no morphological alterations of thyroid tissue were associated with increasing internal TBBPA concentrations in flounder (Chapter five), an increase in total plasma thyroxin (T_4) level was evident (group average significantly increased at an actual water concentration of 11 ng TBBPA/mL, with an average internal concentration of 153 ng TBBPA/g ww). TBBPA also increased T_4 levels in flounder plasma when added after sampling, which could be the result of competition between TBBPA and T_4 for binding to plasma proteins. In agreement with this observation, TBBPA competed with binding of T_4 to the major T_4 binding protein in human plasma, transthyretin (TTR) *in vitro* (Hamers et al., 2006). As insufficient flounder plasma was available to evaluate T_4 protein binding, the influence of TBBPA on

the binding of T_4 to plasma proteins in this fish species remains an interesting issue for further investigation.

Exposure to HBCD did not appear to affect any of the investigated parameters in flounder (Chapter five). The relative distribution of diastereomers (α-, β- and γ-HBCD) was dependent on the route of exposure in our study: exposure via sediment resulted in a relative predominance of α-HBCD. Predominance of α-HBCD has consistently been found in several wild fish species (Morris et al., 2004; Janák et al., 2005). This observation in our study with European flounder underlines the suitability of the use of sediment exposure in a test protocol with bottom dwelling fish, as an experimental design with high environmental relevance.

Finally, additional endocrine and reproductive parameters in relation to PBDE exposure were investigated in a zebrafish partial life-cycle and a flounder exposure study (Chapter six). A technical pentabromodiphenylether mixture (DE-71) was selected as test substance for exposure to environmentally relevant PBDEs, since the most bioaccumulative congeners are represented in this technical product. The mixture was thoroughly cleaned from possible dioxin-like contaminants as discussed above, in order to interpret any observed effects as exclusively PBDE-related. Congener specific chemical analysis of both zebrafish and flounder showed that the relative distribution of individual PBDEs in exposed fish was very similar to patterns observed in the field. Absence of CYP1A induction in both species confirmed successful removal of dioxin-like contaminants and was consistent with the lack of CYP1A induction by cleaned PBDEs in zebrafish (Kuiper et al., 2006a; Chapter three).

Several adverse effects were observed in zebrafish at relatively high exposure levels. Survival of larvae exposed to a nominal water concentration of 160 µg DE-71/L and higher was reduced. Survival of unexposed larvae from exposed parents (500 µg/L) was unaffected, indicating that larval mortality is largely independent of the exposure status of the parents, possibly as a result of only limited transfer of PBDEs via the eggs. Cumulative egg production during the study showed a mild, dose-related decrease; although this effect was not statistically significant, the data indicate that this may occur at somewhat lower concentrations than larval mortality (nominal parent exposure of 50 µg DE-71/L).

There were no indications for (anti-)estrogenic effects of PBDEs in either species, but a mild decrease of aromatase activity in the ovaries of exposed flounder was observed with increasing exposure levels. Using a significant dose response model, a 10% decrease of aromatase activity was calculated at an internal total BDE concentration (ΣPBDE) of 63 ng/g ww. Although this could indicate an indirect anti-estrogenic effect of PBDEs, zebrafish exposed to high PBDE concentrations did not show any signs of anti-estrogenicity (predominance of male offspring, increased atresia and degeneration of oocytes, and granulosa cell hypertrophy in adult females, Leydig cell hyperplasia and asynchronous sperm production in mature males or reduced reproductive success; Wester et al., 2003).

In adult zebrafish, increasing PBDE levels were associated with an increase in the levels of thyroid hormones T_3 and T_4 (Chapter six). Due to the limited sample size and the large variation, mechanistic interpretation of these data is difficult. Increased levels of thyroid hormones in fish exposed to environmental contaminants have not been reported before, and possible effects on reproduction should be further investigated before firm conclusions on the relation with a possible reduced egg production are drawn. In contrast,

and at lower (internal) PBDE concentrations, T_4 levels in flounder showed a significant negative dose response with increasing PBDE concentrations. This observation is more consistent with the effects previously observed in mice and rats (Zhou et al., 2002; Stoker et al., 2004; Skarman, 2005), and fish (Tomy et al., 2004). The decrease appeared to level off at 54% of the calculated original T_4 levels. A 10% decrease was calculated at a corresponding internal concentration of 81 ng ΣPBDE/g ww. In contrast to observations in rats (Stoker et al., 2004), the decrease in T_4 was not accompanied by thyroid hyperplasia even at he highest PBDE concentrations.

Although the studied BFRs clearly do not show dioxin-like activity and appear to have limited potential for endocrine effects *in vivo*, other types of effects have been described for these compounds. Recent behavioral studies indicate adverse effects of PBDEs in rats (Kuriyama et al., 2005; Lilienthal et al., 2006), mice (Eriksson et al., 2002; Viberg et al., 2002) and zebrafish (Timme-Laragy et al, 2006). The combined results indicate that neurobehavioral development may be a sensitive endpoint for non AhR-mediated toxicity of particularly PBDEs in mammals as well as fish. Although we observed no gross behavioral changes in our exposed fish, automated procedures allowing more dedicated monitoring of fish behavior are becoming available, and monitoring fish behavior could provide an important tool for hazard identification.

5. Risk of endocrine and reproductive toxicity by BFR exposure in fish

To conclude, risks for endocrine effects and reproductive disturbance in fish exposed to the three major groups of BFRs investigated in this thesis, are described and their relevance is discussed. A benchmark study design was used which in combination with chemical analysis of BFR concentrations in fish allowed calculation of critical (internal) effect dose (CED) for a defined critical effect size. An important advantage of the benchmark approach in our studies is that in contrast to a NOEL, the CED does not change with increasing variation (*e.g.* Zhou et al., 2001). This allowed calculation of CEDs in a number of instances in our studies where large variation was observed. Exposure of zebrafish to high levels of TBBPA and PentaBDE resulted in reduced juvenile survival. The internal concentrations at which zebrafish larval mortality occurred were approximately 120 ng TBBPA/g and >91,000 ng ΣPBDE/g ww. Keeping in mind that these values were based on a limited number of replicates, and in the case of PBDEs was estimated based on the highest level in surviving, healthy juveniles, they exceed current highest observed levels in the West European aquatic environment (14 ng TBBPA/g ww in sandeel, unpublished data from RIVO, 2003; 2270 ng PBDE/g ww in burbot, Law et al., 2006) by approximately one order of magnitude. A significant dose-response model showed adverse effects on egg production in zebrafish exposed to TBBPA at internal concentrations that are in the same range with a lower 95% confidence limit of 82 ng TBBPA/g ww. Although a statistically significant dose-response model could not be fitted, egg production also showed a decreasing trend in zebrafish exposed to PentaBDE, first occurring at internal ΣPBDE concentrations between 15 and 126 µg/g ww. Currently reported concentrations of TBBPA and PBDEs in freshwater fish are usually respectively 2-3, and 1-2 orders of magnitude lower than the exceptional concentrations mentioned above (Morris et al., 2004; Law et al., 2006). Therefore, concentrations at which effects on population level should be evident are unlikely to occur.

As discussed above, morphological indications for endocrine effects in zebrafish exposed to the studied BFRs were limited. The role for an endocrine mechanism behind the observed effects on zebrafish reproduction and juvenile survival at very high levels of exposure to TBBPA and PBDEs remains therefore unclear.

Dose-response modeling in the flounder studies indicated that thyroid hormone (T_4) concentrations in plasma and aromatase activity in gonads can be affected by TBBPA and PentaBDE at internal concentrations that are inside the range reported in wild fish (Chapters 5, 6). The predicted increase of aromatase activity at 14 ng/g TBBPA ww internal concentration was a limited 1.2%; both aromatase activities and T_4 concentrations remained within the range of control values at environmentally relevant TBBPA exposure levels. Aromatase activities and T_4 concentration in flounder already decreased at internal PBDE concentrations that were 1-2 orders of magnitude below the highest levels in fresh water fish. However, since current PBDE levels in marine fish are lower than in freshwater fish (in the lower ng/g ww range; Boon et al., 2002; Leonards et al., 2004; Voorspoels et al., 2004), these results indicate a limited risk for endocrine effects in estuarine species. In agreement with the results in zebrafish, even at the highest TBBPA and PentaBDE exposure levels, these biochemical changes were not accompanied by morphologic indications for endocrine effects.

There were no indications for any adverse effects on endocrine or reproductive systems in flounders exposed to HBCD up to an average maximum level of 3,0 µg ΣHBCD/g ww, which is similar to the highest (average) environmental level now reported in fresh water fish (2,3 µg ΣHBCD/g ww.; Law et al, 2006). Highest reported concentrations in marine fish were one order of magnitude lower, and it is therefore unlikely that current HBCD exposure levels present a risk for fish endocrine and reproductive health. However, as production this BFR with clearly bioaccumulating properties (Sørmo et al., 2006) continues or even increases, future environmental levels may rise. Continuous monitoring of HBCD levels in biota is therefore recommended and additional studies including perhaps even higher exposure levels may be needed in the future.

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Nederlandse samenvatting
Toxiciteit van gebromeerde vlamvertragers in vis, met bijzondere aandacht voor effecten op de hormoonhuishouding

Inleiding

Het sterk toegenomen gebruik van brandbare polymeren heeft toevoeging van grote hoeveelheden beschermende vlamvertragers aan gebruiksartikelen noodzakelijk gemaakt. Gebromeerde vlamvertragers (brominated flame retardants, BFRs) vertegenwoordigen daarvan een grote groep. Een aantal belangrijke BFRs blijkt te accumuleren in het milieu en wordt in toenemende mate teruggevonden in bewoners van het aquatische milieu, waaronder vissen. Uit eerdere studies met geïsoleerde cellijnen en knaagdieren waren aanwijzingen naar voren gekomen voor hormoonverstorende werking van sommige vlamvertragers, met name betreffende geslachts- en schilklierhormonen. Omdat tevens aanwijzingen gevonden werden voor toenemende hormoonverstoring bij vissen in het wild werd besloten te onderzoeken in hoeverre blootstelling aan, wat betreft het voorkomen in het milieu, belangrijke BFRs hierbij een rol kan spelen. De resultaten van dit onderzoek zijn gepresenteerd in dit proefschrift.

Twee vissoorten werden gekozen waarvan de normale fysiologie en (microscopische) anatomie relatief goed bekend zijn: de bot (*Platichthys flesus*), die van nature voorkomt in het vaak relatief vervuilde brakke water van rivierdelta's, en de zebravis (*Danio rerio*), als model voor reproductie in een zoetwatervis. Deze dieren werden onder goed gecontroleerde laboratorium omstandigheden blootgesteld aan de meest vervuilende broomhoudende vlamvertragers van dit moment, tetrabroombisphenol A (TBBPA), hexabroomcyclododecaan (HBCD) en een mix van veel voorkomende gebromeerde diphenylethers. Ter controle van de blootstellingsniveau's en om eventuele effecten te kunnen relateren aan veldgegevens zijn na afloop van de blootstellingsstudies teststofgehaltes (interne doses) bepaald in volledige, gehomogeniseerde zebravissen, en in spier (filet) van bot.

Effecten van gebromeerde vlamvertragers op vissen

Blootstelling aan TBBPA via het water veroorzaakte een dosis-afhankelijke eilegdaling bij de zebravis, en larvale sterfte en relatieve overmaat aan ontwikkelende vrouwtjes (hoofdstuk 4). De interne gehaltes waarbij 50% eileg reductie, en effecten op de larvale sterfte en ontwikkeling werden waargenomen, waren tenminste 10 keer hoger dan de gehalten in vissen gemeten in de Nederlandse kustwateren en elders. Histologisch onderzoek van schildklier, lever en gonaden gaf, afgezien van de scheve geslachtsverhouding van juvenielen bij hoge TBBPA concentratie, geen aanwijzingen voor een schildklier- of geslachtshormoon-gerelateerd achterliggend mechanisme.

Als een meer kwantitatieve maat voor mogelijke oestrogene effecten is het vitellogenine (VTG) gehalte van bloedplasma in blootgestelde botten gemeten (hoofdstuk 5). VTG is een voorloper van dooier eiwit, en wordt in de vissenlever geproduceerd na stimulatie door oestrogenen. Het VTG gehalte vertoonde geen relatie met blootstelling aan TBBPA of interne concentraties in de spier, en gaf zodoende evenmin aanwijzingen voor

een direct oestrogeen effect in deze diersoort. De activiteit van het cytochroom P450 enzym CYP19 (aromatase, verantwoordelijk voor de transformatie van androgenen in oestrogenen) vertoonde een zeer geringe toename in de testes van botten met stijgende TBBPA concentraties. Deze toename resulteerde echter niet in voldoende endogene productie van oestrogenen voor een toegenomen VTG productie. De concentraties van schildklierhormoon T_4 namen sterk toe met toenemende TBBPA blootstelling en interne concentraties. Dit is mogelijk veroorzaakt door competitie van TBBPA met plasma eiwitten waardoor meer ongebonden T_4 werd gemeten. De fysiologische betekenis van een dergelijk effect lijkt op dit moment gering gezien de afwezigheid van histologisch waarneembare schildklier-gerelateerde effecten, maar verdere opheldering van het achterliggende mechanisme, bijvoorbeeld door bepaling van plasma T_4 bindingscapaciteit, verdient aanbeveling.

Blootstelling aan HBCD via voedsel en/of sediment (in het kader van dit proefschrift alleen gerapporteerd in de bot) resulteerde niet in enige afwijking van de onderzochte parameters (hoofdstuk 5). Opmerkelijk was dat experimenteel een relatieve toename van α-HBCD werd gevonden ten opzichte van de andere twee diastereomeren, β- en γ-HBCD, in bot waarvoor met HBCD bewerkt sediment de belangrijkste blootstellingsroute was. Dit gegeven sluit aan bij veldobservaties waar HBCD vaak vooral in sediment wordt aangetroffen en in de praktijk voornamelijk α-HBCD wordt gevonden in aquatische dieren.

Tenslotte zijn aanvullende endocriene en reproductie parameters onderzocht in bot en zebravis na blootstelling aan PBDE's (hoofdstuk 6). Een technisch mengsel met de PBDE congeneren die ook in het milieu het meest gevonden worden als belangrijkste bestanddelen (DE-71, Great Lakes Chemical Corporation), werd vooraf gezuiverd van mogelijke dioxine-achtige contaminanten (zie hoofdstuk 3). Dit PBDE mengsel werd toegediend aan de zebravis via het water, en aan de bot via sediment en voer. Relatief hoge concentraties PBDE in het water (160 µg DE-71/L water) leidden bij zebravis tot larvale sterfte gedurende de eerste week na verlaten van het ei. Hoewel door het beperkte aantal waarnemingen niet statistisch significant, leek er daarnaast een dosis gerelateerde daling van de eileg op te treden vanaf 50 µg PBDE/L door een kleiner aantal legsels bij ouderdieren. In de bot was er een lichte daling van de aromatase activiteit in ovaria met toenemende interne PBDE concentraties. Zowel bij de zebravis als de bot werden zowel macro- als microscopisch geen morfologische aanwijzingen gevonden voor een (anti-) oestrogeen effect.

Bij beide diersoorten was er een effect van PBDE's op de concentraties van schildklierhormonen in bloedplasma, hoewel tegengesteld. Bij de zebravis werd na blootstelling aan hoge PBDE concentraties een toename van de concentraties van zowel T_3 als T_4 gevonden. De grote variatie in de data en het feit dat een dergelijk effect in geen enkele diersoort eerder is beschreven als gevolg van PBDE blootstelling bemoeilijkt de interpretatie. Verder onderzoek moet het belang van deze waarneming voor bijvoorbeeld de mogelijk verminderde reproductie ophelderen.

In de bot werd een milde daling van het shildklierhormoon T_4 gemeten in bloedplasma, wat overeenkomst vertoont met waarnemingen bij rat, muis en Amerikaanse meerforel (*Salvelinus namaycush*) na blootstelling aan PBDE's. Het effect was beperkt tot maximaal een halvering van de hormoonconcentraties en leidde ook bij de laagste T_4

waarden in de vis niet tot reflectoire hyperplasie van de schildklier zoals wel bij ratten werd vastgesteld na blootstelling aan PBDE's.

AhR-gemedieerde effecten

Vanwege hun structurele overeenkomsten met dioxines is behalve direct hormoon-gerelateerde effecten onderzocht of gebromeerde diphenylethers de aryl hydrocarbon receptor (AhR, ook dioxine receptor genoemd) kunnen activeren. Activatie van deze receptor is herhaaldelijk in verband gebracht met indirecte verstoringen van met name de steroïdhuishouding. Niet alleen dibenzodioxines maar ook de verwante dibenzofuranen en bijvoorbeeld een aantal polychloorbiphenylen (PCB's) zijn in staat deze receptor te activeren. In onze studies is de productie van cytochroom P4501A (CYP1A) gebruikt als maat voor AhR activatie. CYP1A is een enzym dat voorkomt in lever van zowel vissen als zoogdieren (in vissen ook in de kiewen, bloedvaten en andere organen), en geïnduceerd wordt via activatie van de AhR. Door gebruik te maken van geïsoleerde levercellen van karper was het mogelijk om dit aspect te onderzoeken met gebruik van minimale hoeveelheden van moeilijk verkrijgbare sterk opgezuiverde individuele gebromeerde diphenylethers.

De blootstelling van karper hepatocyten aan (gezuiverde) PBDE's (tot 2.5 μM) leidde niet tot een significant verhoogde activiteit van CYP1A (gemeten als de-ethylering van het substraat 7-ethoxyresorufine, EROD), terwijl bij veel lagere concentraties van de model stof tetrachloordibenzo-*p*-dioxine (TCDD) wel een duidelijke respons werd gemeten (vanaf 10 pM; hoofdstuk 2). Echter, bij blootstelling van karper cellen aan combinaties van TCDD en PBDE's bleken veel voorkomende PBDE's de inductie van CYP1A door TCDD te remmen. Dit is in overeenstemming met resultaten van soortgelijke studies in celcultures van knaagdieren en primaten inclusief de mens.

Bij *in vivo* blootstelling van zebravissen aan een commercieel mengsel van PBDE's werd in eerste instantie wel een toename van CYP1A gevonden (door middel van immunohistochemische kleuring, hoofdstuk 3). Echter, bij herhaling van dat experiment na zuivering van dit mengsel van mogelijke contaminanten als gebromeerde dioxines of furanen, werd geen CYP1A inductie meer gezien. Tests van ongezuiverde en gezuiverde PBDE's op een getransfecteerde reporter-gen cellijn met de AhR (DR-CALUX) gaven hetzelfde resultaat. Na chemische analyse van het oorspronkelijke mengsel bleek een dioxine-achtige stof, 1,2,3,7,8-dibenzofuraan de waarschijnlijk belangrijkste veroorzaker van de eerder waargenomen CYP1A inductie. Bij botten die blootgesteld werden aan een gezuiverd PBDE mengsel was er zelfs eerder een afname te zien in lever CYP1A activiteit, waarvoor op dit moment geen afdoende wetenschappelijke verklaring kan worden gegeven (hoofdstuk 6). Deze resultaten bevestigen het geringe vermogen van PBDE's om de AhR te activeren en manen tot voorzichtigheid bij de interpretatie van andere onderzoeken met ongezuiverde mengsels van deze PBDE's.

Bovengenoemde resultaten impliceren tevens dat het meten van CYP1A activiteit geen geschikte indicator is voor blootstelling aan PBDE's. Ook blootstelling aan TBBPA en HBCD resulteerde bij bot niet tot een meetbare verandering van lever CYP1A activiteit (hoofdstuk 5). In ratten en muizen werden echter wel andere cytochroom P450 isoenzymen, met name CYP2B en CYP3A, succesvol geïnduceerd door PBDE's. De conventionele

methode voor de bepaling van CYP2B activiteit met pentoxy- of benzoxyresorufine als substraat bleek echter onvoldoende specifiek te zijn in de bot (hoofdstuk 5, 6).

Conclusies

De belangrijke gebromeerde vlamvertragers PBDE's, HBCD en TBBPA zijn geen AhR agonisten en daarmee geassocieerde dioxine-achtige toxiciteit is dan ook niet waarschijnlijk. Het gebruik van EROD, PROD en BROD activiteiten is niet geschikt voor vaststelling van blootstelling aan BFRs bij vissen, en kan in geval van CYP1A remming zelfs misleidend zijn of aanleiding tot antagonisme geven.

De risico's voor negatieve effecten op de reproductie en overleven van larven bij blootstelling aan TBBPA, HBCD en PBDE's in concentraties zoals deze momenteel in het veld worden aangetroffen lijken beperkt. Larvale sterfte bij de zebravis trad op bij interne concentraties van 120 ng TBBPA/g en >91000 ng PBDE/g (nat gewicht). De daling in eileg in zebravis was tamelijk steil met een onderste 95% betrouwbaarheidslimiet van 82 ng TBBPA/g. Maximaal gemeten gehalten in het veld bedragen 14 ng TBBPA/g in spiering uit de Waddenzee, en 2270 ng/g PBDE in kwabaal uit het Noorse meer Mjøsa. Aangezien deze veldwaarden uitzondelijk zijn (in zoetwatervis zijn gehaltes van TBBPA en PBDE's meestal een tot drie ordes lager) is het minder waarschijnlijk dat deze effecten als gevolg van blootstelling aan TBBPA of PBDE's in het milieu zullen optreden.

De aanwijzingen voor hormoonverstorende effecten zijn eveneens beperkt: hoewel een sterke T_4 stijging op lijkt te treden in de bot na TBBPA blootstelling, is de voorspelde toename bij de hoogst gemeten veldwaarde slechts 1,2%, terwijl ook bij veel hogere T_4 waarden geen verdere aanwijzingen voor een gestoorde schildklierfunctie gevonden werden. Een toename van aromatase activiteit in testis was niet vast te stellen bij blootstellingswaarden die in het veld worden gevonden. Ook hoge aromatase activiteiten gemeten bij hogere TBBPA concentraties gingen niet gepaard met toegenomen VTG productie of andere afwijkingen. De daling van T_4 concentraties bij botten na blootstelling aan het commerciële PBDE mengsel (10% afname bij 81 ng/g nat gewicht), evenals afgenomen aromatase activiteit in ovaria (10% afname bij 63 ng/g nat gewicht) lijkt reeds bij lagere interne concentraties op te treden. Echter, aangezien de PBDE gehaltes in zoutwater vis over het algemeen lager liggen dan in zoetwater (ng/g range), is ook de indicatie voor risico voor het mariene milieu gering.

Er waren geen aanwijzingen voor hormoonverstorende effecten in botten blootgesteld aan HBCD. De maximale HBCD concentraties in de blootgestelde dieren (3 µg/g nat gewicht) waren vergelijkbaar met de hoogste waarden in vis uit zoet water, en 10 keer hoger dan in vis uit zout water milieu. Ook voor HBCD geldt dus bij huidige blootstellingsniveau's een beperkt risico voor hormoonverstoring bij vis. Met name voor HBCD is echter waakzaamheid geboden omdat bij voortschrijdende productie van deze bioaccumulerende stof in de toekomst mogelijk ook in vissen hogere concentraties gevonden zullen worden.

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Curriculum Vitae

Raoul Valentin Kuiper was born in Venray, the Netherlands, on the 6th of June, 1974. In 1992 he graduated from gymnasium "Het Boschveld College" in Venray, and in the same year started his education in veterinary medicine at the Veterinary Faculty of Utrecht University, the Netherlands. He graduated in 1998 with a degree of MSc. in Veterinary Science, resulting from an excellent tracé in environmental aquatic toxicology at the Department of Pathobiology. During the subsequent two years he took part in an analytical study of malachite green residues in farmed eel at the department of Public Health and Food, and completed the education program for veterinary practice. From 2000 he has been employed as researcher and trainee in veterinary pathology by the Department of Pathobiology. During this period he performed and directed research addressing possible adverse effects of brominated flame retardants on aquatic wildlife reproductive health. These studies (a dedicated work package of the project "FIRE", a program supported by the European Community) were a collaborative effort by the Institute of Risk Assessment Sciences of Utrecht University (IRAS), National Institute for Public Health and the Environment (RIVM), and the National Institute for Coastal and Marine Management (RWS-RIKZ). The presented thesis is a compilation of results from the FIRE program studies.

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