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

R.V. Kuiper[a], R.F. Cantón[b], P.E.G. Leonards[c], B. M. Jenssen[d], M. Dubbeldam[e], P.W. Wester[f], M. van den Berg[b], J.G. Vos[a,f], A.D. Vethaak[g]

[a] *Department of Pathobiology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands*
[b] *Institute for Risk Assessment Sciences (IRAS), Utrecht University, Utrecht NL, The Netherlands*
[c] *Institute of Environmental Studies (IVM), Amsterdam, The Netherlands*
[d] *Department of Biology, Norwegian University of Science and Technology, Trondheim, Norway*
[e] *Grontmij-AquaSense Laboratory for Aquatic Ecotoxicology, Colijnsplaat, The Netherlands*
[f] *National Institute of Public Health and the Environment (RIVM), Bilthoven, The Netherlands*
[g] *National Institute for Coastal and Marine Management (RWS-RIKZ), The Hague, The Netherlands*

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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 metamorph-osis, 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).

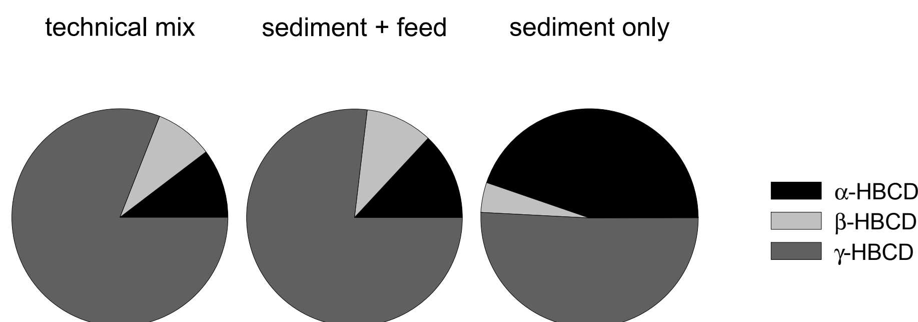

Figure 1: Proportions of the diastereomers α-, β-, and γ-hexabromocyclododecane (HBCD) in the technical mixture, in animals exposed via sediment and food, and in animals exposed via sediment alone after 78 days.

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]			$T_3 \pm$ SD (ng/mL)	$T_4 \pm$ 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.

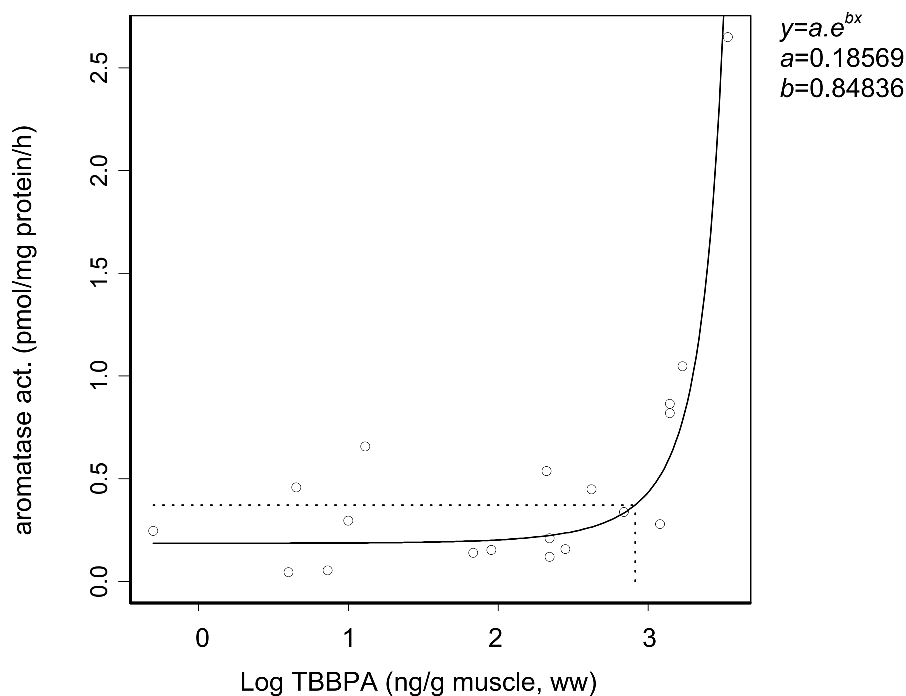

Figure 2: Aromatase activity in gonads of male flounder in relation to internal TBBPA concentration. Fitted dose-response model: $y=a.e^{bx}$; dotted lines represent two times the basal level and corresponding internal dose (820 ng/g muscle, wet weight).

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.

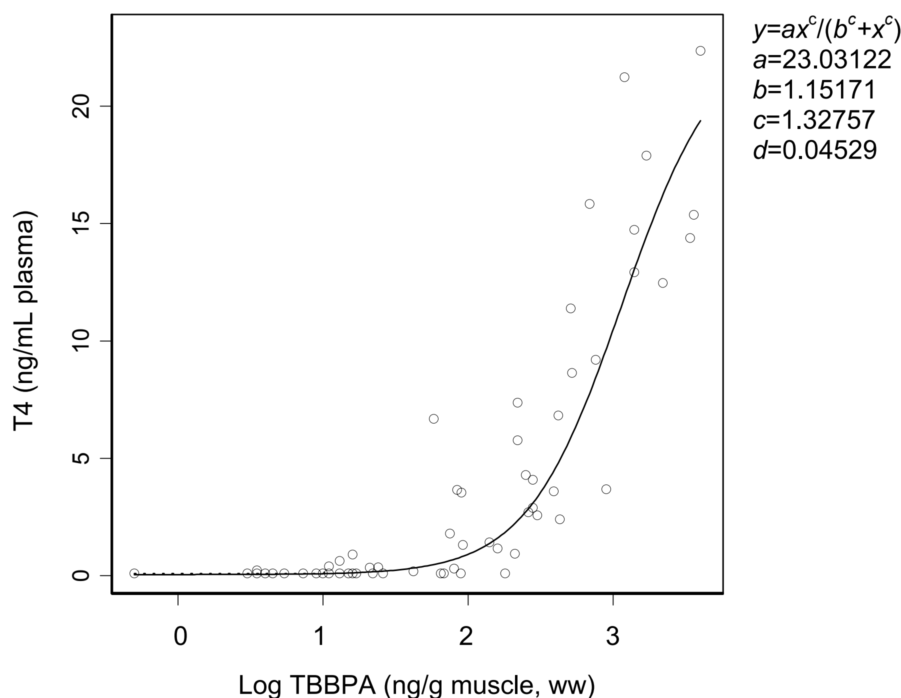

Figure 3: Relation between plasma T_4 levels and internal TBBPA concentrations in flounder. Fitted dose-response model: $y=ax^c/(b^c+x^c)$

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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