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Transcriptomic responses of European flounder (*Platichthys flesus*) liver to a brominated flame retardant mixture

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ABSTRACT

Male European flounder (*Platichthys flesus*) were exposed to a technical mixture of brominated diphenyl ethers (PDBEs, DE-71, Pentamix) that had been purified to remove contaminating dioxins. Controls were exposed to carrier solvent alone. Fish were exposed to decadally increasing concentrations of Pentamix via both sediment and spiked food. The GENIPOL *P. flesus* cDNA microarray, differentially expressed gene profiling (DEG) and quantitative PCR were employed to detect hepatic transcriptional differences between exposed fish and controls. Gene transcriptional changes were more sensitive to Pentamix exposure than biomarkers measured previously. Pentamix exposure induced transcripts coding for enzymes of xenobiotic metabolism (CYP1A, aldo-keto reductases) and elicited endocrine disruption (vitellogenin and thyroid hormone receptor alpha), with effects on CYP1A and VTG occurring at the highest exposure. Ontology analysis clearly showed dose-responsive changes indicative of oxidative stress, induction of mitochondrial dysfunction, and apoptosis. We conclude that exposure to PBDEs in both sediment and food has a significant adverse effect on a broad range of crucial biochemical processes in the livers of this widely distributed estuarine fish species, the flounder.

1. Introduction

The polybrominated diphenylethers (PBDEs) are brominated flame retardants (BFRs), formerly widely used to reduce the flammability of furniture, textiles and electronic equipment. Commercial PBDE mixtures are classified according to the degree of bromination; the 'Pentamix' (DE-71) contains mainly tetra- and penta-BDEs. Over the past 20 years there has been progressive contamination of the aquatic environment and bioaccumulation of lower brominated congeners in aquatic biota at various trophic levels (Boon et al., 2002a,b; Birnbaum and Staskal, 2004). The marketing and use of Pentamix has been banned in the European Union since 2004 (Kemmlein et al., 2009), but the lower brominated congeners are resistant to degradation, thus are likely to persist in the environment and pose an ongoing risk to aquatic organisms, particularly demersal fish.

Reported effects of acute PDBE exposure in mammalian systems include interactions with sex steroid and thyroid endocrine systems (Hamers et al., 2006; Pacyniak et al., 2007; Fery et al., 2009) and oxidative stress leading to liver damage (Albina et al., 2010; Bruchajzer et al., 2010), genotoxicity (Barber et al., 2006; Ji et al., 2011) and neurotoxicity (Branchi et al., 2003; Blanco et al., 2011). PBDE exposure has been associated with numerous transcriptional changes in mammals (Suvorov and Takser, 2010; Dunnick et al., 2012) and fish (Olsvik et al., 2009; Chen et al., 2010; Han et al., 2011; Softeland et al., 2011; Chan and Chan, 2012). Evidence for alteration of cytochrome P450 1A activity is mixed (Holm et al., 1994; Boon et al., 2002a,b; Chen and Bunce, 2003; Olsvik et al., 2009; Chen et al., 2010; Wahl et al., 2010; Softeland et al., 2011) and may be attributable to dioxin contamination of the commercial mixtures since purification of commercial Pentamix (DE-71) to remove planar components resulted in a lack of DR-CALUX response and only weak CYP1A immunoreactivity in exposed zebrafish (*Danio rerio*) gills (Kuiper et al., 2006), indicating that the majority of the AhR activation potential of this mixture is caused by non-BDE components. In an *in vitro* study

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Table 1
Experimental groups.

Pentamix sediment (μg/g TOC)	Pentamix feed (μg/g lipid)	Group ID	Muscle BDE47 (μg/g wet wt) (±SD)	Microarray replicates
0	0	SC	0.07 ± 0.07	5
0	0.014	UC	0.07 ± 0.06	–
0.007	0.14	0.007	0.09 ± 0.14	–
0.07	1.4	0.07	0.21 ± 0.21	5
0.7	14	0.7	0.22 ± 0.16[a]	4
7	140	7	0.34 ± 0.19[a]	5
70	1400	70	3.9 ± 2.8[a]	–
700	14,000	700	45 ± 56[a]	4

[a] Significantly different from solvent control ($P < 0.05$), TOC denotes total organic carbon. Adapted from our previous publication, Kuiper et al. (2008).

of a range of BFRs, 2,2′,4,4′-tetra-bromodiphenyl ether (BDE47) was found to be a potent inhibitor of estrogen sulfotransferase activity (Hamers et al., 2006). In reporter gene assays, other congeners found in Pentamix were found to be androgen receptor (AR) antagonists and variably, estrogen receptor (ER) and dioxin receptor (DR) agonists or antagonists (Hamers et al., 2006). After 3.5 months exposure to a PBDE mixture, spawning success in stickleback (*Gasterosteus aculeatus*) was reported to be dramatically reduced (Holm et al., 1993). Toxicity of BFRs was reviewed by Birnbaum and Cohen Hubal (2006) and their endocrine disrupting effects by Legler (2008).

In mammals, PBDEs can be metabolised to hydroxylated forms (Wang et al., 2012). Incubation of BDE47 with rat liver microsomes resulted in formation of a range of hydroxylated metabolites that exhibited increased transthyretin binding and estradiol-sulfotransferase inhibition; particularly 3-OH-BDE47 and 4′-OH-BDE49 (Hamers et al., 2008). Zhong et al. (2011) reported a study employing human L02 cells in which exposure to hydroxylated BDE47 metabolites elevated reactive oxygen species (ROS) levels and superoxide dismutase (SOD) activity, decreased glutathione (GSH) levels, induced apoptosis and inhibited cell proliferation. Additional metabolites of PBDEs include 2,4-dibromophenol, an uncoupler of oxidative phosphorylation (Hempfling, 1970). After acute exposure of zebrafish fibroblasts to 1 μM hydroxylated BDE47 (6-OH-BDE47), transcripts related to carbohydrate metabolism and proton transport were induced and uncoupling of oxidative phosphorylation elicited acute toxicity (van Boxtel et al., 2008), however, in medaka fish (*Oryzias latipes*), Wan et al. (2010) reported that BDE47 was not metabolised to 6-OH-BDE47.

Because of their high abundance, bottom-dwelling life style, and susceptibility to adverse environmental conditions, the European flounder (*Platichthys flesus*) is one of the species of choice for monitoring the effects of endocrine disruptors and other chemical contaminants in UK and European estuarine and coastal waters (Kirby et al., 2004; Vethaak et al., 2009). Flounders had been simultaneously exposed to "dioxin-free" Pentamix contaminated sediment and contaminated feed for three months, detailed in a previous publication (Kuiper et al., 2008). Briefly, this study showed that in flounder muscle tissue, BDE47 was the most abundant congener (63 ± 6%) with detectable levels of BDEs 49, 99, 100, 153 and 154. The profile of congeners detected in flounder muscle was different from that of the parent Pentamix (DE-71), with a notable decrease in the proportion of BDE99 and increase in proportion of BDE47, and the profile of congeners differed between flounder and zebrafish. Whilst there were no significant changes in length, weight, or relative liver and gonad weights of the fish there was significant accumulation of BDE47 in flounder muscle at higher exposure concentrations. However, the residue levels were extremely variable with differences of up to 20-fold between individuals within an exposure group. No significant changes in concentrations of plasma thyroid hormones T3 and T4, gonad aromatase activity or microsomal ethoxyresorufin O-dethylase (EROD), pentoxyresorufin-*O*-deethylase (PROD) or benzoxy-resorufin-*O*-deethylase (BROD) activities were found (Kuiper et al., 2008). Liver tissues from these animals were analysed in the present study to determine if gene transcriptional changes could be more sensitive indicators of Pentamix exposure after chronic sub-toxic exposure, to characterise any changes in terms of biological pathways potentially affected and thus to relate toxicogenomic data directly to both a pollutant and an organism of high environmental relevance.

2. Materials and methods

2.1. Fish exposures

Exposure conditions were described previously by Kuiper et al. (2008). Briefly, 159 days old artificially reared flounders obtained from Manx Mariculture Ltd. (Isle of Man, UK) (48 ± 12 g) were held in aquaria containing 15 kg of (Pentamix spiked) sediment and 160 L of water from the Eastern Scheldt (a tidal bay connected to the North Sea with a salinity of approximately 30%); (temperature was 15 °C; renewal rate was twice weekly via continuous flow-through) for 3 months. Fish were fed three times a week at an estimated 1% of body weight with Pentamix-spiked pellets prepared from fish meal, mussels and fish oil. Groups of ten animals were exposed to a range of sediment and food Pentamix concentrations as shown in Table 1. For convenience these are subsequently referred to as solvent control (SC), untreated sediment control (UC), and 0.007, 0.07, 0.7, 7, 70 and 700 μg/g groups. Fish metadata were presented previously (Kuiper et al., 2008). For transcriptional analysis, samples of liver tissue were frozen at −80 °C.

2.2. Differentially expressed gene profiling (DEG) and quantitative PCR (QPCR)

These procedures are more fully detailed in Supplementary File 1. Briefly, Total RNA was extracted (RNEasy Tissue Mini Kit; Qiagen, Crawley, UK) from liver tissue samples of all fish ($n = 10$ per group). Equal amounts of RNA from each fish were pooled by exposure group, reverse transcribed to cDNA and amplified with arbitrary primers in a two-step procedure using the Genefishing DEG Premix Kit (Biogene, Kimbolton, UK) according to the manufacturers' instructions. Products were visualised by agarose gel electrophoresis, apparently differentially expressed products were excised and purified (Geneclean III; Biogene or MinElute Gel Extraction; Qiagen), then cloned (TOPO TA cloning kit; Invitrogen, Paisley, UK), plasmids were purified from individual colonies (QIAprep Spin Miniprep kit; Qiagen) and 5–10 clones from each product were sequenced. Sequences were aligned (Sequencher; Ann Arbour, MI, USA) and putatively identified by BLAST (NCBI). Primers were designed from the sequences obtained, RNA from individual fish was reverse transcribed to cDNA and used for SYBR-green real-time QPCR (ABI7000, Applied Biosystems, Carlsbad, CA,