

Accumulation and Debromination of Decabromodiphenyl Ether (BDE-209) in Juvenile Fathead Minnows (*Pimephales promelas*) Induces Thyroid Disruption and Liver Alterations

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Polybrominated diphenyl ether (PBDE) flame retardants are known to affect thyroid hormone (TH) regulation. The TH-regulating deiodinases have been implicated in these impacts; however, PBDE effects on the fish thyroid system are largely unknown. Moreover, the liver as a potential target of PBDE toxicity has not been explored in young fish. This study measured decabromodiphenyl ether (BDE-209) effects on TH regulation by measuring deiodinase activity in juvenile fathead minnows (*Pimephales promelas*). Dietary accumulations and debromination of BDE-209 were also measured, and the morphology of thyroid and liver tissues was examined. Juvenile fathead minnows (28 days old) received a 28-day dietary treatment of BDE-209 at $9.8 \pm 0.16 \mu\text{g/g}$ of food at 5% of their body weight per day followed by a 14-day depuration period in which they were fed clean food. Chemical analysis revealed that BDE-209 accumulated in tissues and was metabolized to reductive products ranging from penta- to octaBDEs with 2,2',4,4',5,6'-hexabromodiphenyl ether (BDE-154) being the most accumulative metabolite. By day 28 of the exposure, rates of outer and inner ring deiodination (ORD and IRD, respectively) of thyroxine (T4) were each reduced by ~74% among treatments. Effects on T4-ORD and T4-IRD remained significant even after the 14-day depuration period. Histological examination of treated fish showed significantly increased thyroid follicular epithelial cell heights and vacuolated hepatocyte nuclei. Enlarged biliary passageways may be the cause of the distinctive liver phenotype observed, although further testing is needed. Altogether, these results suggest that juvenile fish may be uniquely susceptible to thyroid disruptors like PBDEs.

Key Words: deiodinase; endocrine disruption; metabolism; polybrominated diphenyl ether; thyroid hormone.

Decabromodiphenyl ether (BDE-209) is the fully brominated polybrominated diphenyl ether (PBDE) congener that constitutes > 97% of the PBDE mixture, decaBDE. It is the only PBDE commercial mixture still used today, primarily as an additive in high impact polystyrene in electronic casings and textile backcoatings. Increasing levels of BDE-209 continue to be measured in soils and sediments (de Wit *et al.*, 2006; Hale *et al.*,

2006), and studies indicate that BDE-209 is bioavailable to humans (Bi *et al.*, 2007; Lunder *et al.*, 2010) and wildlife (Gauthier *et al.*, 2008; Law *et al.*, 2008). Due to concerns about environmental persistence, bioaccumulation, and toxicity, decaBDE is now scheduled for phaseout in the United States by the end of 2013. However, environmental contamination is expected to continue as products containing decaBDE continue to be used, recycled, and discarded. In addition, decaBDE remains in use in other countries, presenting ongoing global contamination issues. BDE-209 may also break down photolytically (Stapleton and Dodder, 2008) and microbially (Gerecke *et al.*, 2005) to more persistent lower-molecular weight PBDEs that have a greater potential for bioaccumulation and toxicity.

The liver is the major site of xenobiotic metabolism, and PBDEs have been shown to biotransform in animals to persistent and bioactive metabolites. Studies in fish have shown that metabolism occurs primarily via reductive debromination, with little to no formation of hydroxylated PBDEs (Kierkegaard *et al.*, 1999; Roberts *et al.*, 2011; Stapleton *et al.*, 2004). This piscine metabolic pathway is distinguished from that of rodent and human metabolism whereby oxidative cytochrome P450-mediated pathways dominate to produce hydroxylated PBDEs (Germer *et al.*, 2006; Richardson *et al.*, 2008; Stapleton *et al.*, 2009).

Although reductive debromination appears to be the major metabolic pathway in fish, there continues to be a poor understanding of the role of specific enzymes in PBDE metabolism. Studies conducted in our laboratory and by others suggest a possible role for iodothyronine deiodinase (DI) enzymes in catalyzing PBDE debromination in fish (Browne *et al.*, 2009; Noyes *et al.*, 2010; Stapleton *et al.*, 2004). DI enzymes, of which three isoforms have been identified in fish (types 1, 2, and 3), are membrane-bound proteins that are associated with the endoplasmic reticulum and regulate intracellular thyroid hormone (TH) homeostasis (Eales *et al.*, 1999). Transferase enzymes, such as glutathione S-transferases (GSTs), which frequently catalyze xenobiotic metabolism, have also been hypothesized to be involved in mediating PBDE

and TH metabolism by conjugation processes. For example, American kestrels (*Falco sparverius*) exposed to PBDEs exhibited depressed circulating thyroxine (T4) and altered glutathione (GSH) homeostasis, suggesting a possible mediating role for GSTs (Fernie *et al.*, 2005). However, in contrast with these findings, GSTs did not catalyze pentaBDE-99 debromination in cytosolic fractions from either Chinook salmon (*Oncorhynchus tshawytscha*) or common carp (*Cyprinus carpio*), implying that they may not be involved in PBDE metabolism in fishes (Browne *et al.*, 2009).

PBDEs closely resemble the structure of THs, and increased scrutiny has focused on the potential for these contaminants to cause thyroid disruption. THs are essential for vertebrate growth, development, and reproduction. Early fish life stages may be uniquely susceptible to thyroid-perturbing xenobiotics because their thyroid systems are incompletely formed but are nonetheless crucial to development. Circulating levels of THs have been shown to decline in adult fathead minnows (*Pimephales promelas*) and lake trout (*Salvelinus namaycush*) exposed to tetraBDE-47 and a PBDE congener mix, respectively (Lema *et al.*, 2008; Tomy *et al.*, 2004). The PentaBDE commercial mixture and BDE-47 have also been shown to impair embryonic and larval fish development (Lema *et al.*, 2007; Timme-Laragy *et al.*, 2006).

Informative reviews by Browne and Eales, among others, propose that understanding xenobiotic effects on the fish thyroid system requires examination at the central hypothalamic-pituitary-thyroid (HPT) axis and in peripheral tissues (Brown *et al.*, 2004; Eales and Brown, 1993). T4 is the prohormone of the more biologically active hormone 3,3',5-triiodothyronine (T3), and its biosynthesis and regulation are under negative feedback control by the central HPT axis. In peripheral tissues, T4 is converted to T3 by outer ring deiodination (T4-ORD) mediated by DI enzymes. The fish thyroid, unlike the mammalian thyroid, is thought to be only a negligible source of circulating T3. Moreover, research has shown that plasma T3 levels in fish may not provide a meaningful index of thyroid status as some tissues (e.g., liver and gill) mostly use locally generated T3 for nuclear receptor-mediated activity (Maclatchy and Eales, 1992). These thyroidal aspects that are unique to fishes make it important to examine peripheral intracellular activity, in addition to changes at the central HPT axis, when evaluating xenobiotic effects on the fish thyroid system.

Given ongoing gaps in our understanding of PBDE metabolism and toxicity among fishes, objectives of this study were to measure PBDE accumulation and metabolism in juvenile fathead minnows (*P. promelas*) receiving dietary exposures of BDE-209 and to examine effects of these exposures on thyroid structure and function (as measured by DI activity) and liver morphology. BDE-209 effects on DI and GST activities were both evaluated in this study because results continue to be mixed as to their involvement in PBDE metabolism. Histological and morphometric evaluations of

the thyroid and liver were performed to better understand the potential for PBDEs to impart structural changes that might be indicative of altered functioning of these key organ systems.

MATERIALS AND METHODS

Materials. BDE-209 (98% pure) was purchased from Sigma-Aldrich (St Louis, MO). Unlabeled THs (T4, T3, rT3, 3,3'-T2, and 3,5-T2), reduced GSH, 1-chloro-2,4-dinitrobenzene (CDNB), and dithiothreitol (DTT) were also purchased from Sigma-Aldrich. Internal and surrogate standards included: ^{13}C -2,2',3,4,5,5'-hexachlorodiphenylether (CDE 141) and ^{13}C -decabromodiphenyl ether (^{13}C -BDE-209) (Wellington Labs, Guelph, Canada); 4'-fluoro-2,3',4,6-tetrabromodiphenyl ether (F-BDE-69) (Chiron, Trondheim, Norway); and ^{13}C -3,3'-diiodothyronine (^{13}C -T4), ^{13}C -T3, ^{13}C -rT3, and ^{13}C -T3 (Isotec, Miamisburg, OH). All solvents used were of high performance liquid chromatography grade.

Animals. Approximately 750 seven-day-old fathead minnows (*P. promelas*) were purchased from Aquatox, Inc. (Hot Springs, AR) and randomly distributed into six 10-gallon glass tanks (approximately 125 fish per tank). Fish were 28 days old (DO) at the start of the study and were acclimated for 21 days prior to study commencement to a control diet of preserved brine shrimp (*Artemia salina*) nauplii (Drs. Foster and Smith, Rhinelander, WI). Further information concerning animal care can be found in the Supplementary data.

BDE-209 dietary exposure. Fish in three tanks received a dietary exposure of 9.8 ± 0.16 μg of BDE-209/g wet weight (ww) of food at 5% of their body weight per day for 28 days. Untreated fish in the remaining three tanks received a control diet containing no BDE-209 at the 5% body weight per day feeding regimen. To monitor recovery, the 28-day exposure was followed by a 14-day depuration period in which all fish received control food containing no BDE-209. The BDE-209 amended food was prepared by dissolving BDE-209 in cod liver oil (TwinLab, UT) and spiking the cod oil solution onto preserved brine shrimp nauplii. To confirm dosages, BDE-209 amended and control diets were analyzed by gas chromatography mass spectrometry operated in electron capture negative ionization mode (GC/ECNI-MS). Ten fish were weighed from each tank at the start of the experiment and then weekly over the course of the study. The average mass per tank was used to calculate the feeding rate, and the feeding rate was adjusted to account for growth. Fish from treatment and control tanks were sampled on experimental days 0 (28-day-old fish), 14 (42-day-old fish), 28 (56-day-old fish), and 42 (70-day-old fish). Fish were euthanized using an overdose of MS-222, immediately frozen in liquid nitrogen, and stored at -80°C until further analysis.

PBDE extractions and analysis. PBDEs were extracted from whole-body samples of fish collected from BDE-209-treated and control replicate tanks on days 0, 14, and 28 (Table 1). Procedures used to isolate PBDEs from fish tissues are described in the Supplementary data. All samples were analyzed using GC/ECNI-MS (Agilent models 6890N and 5975). Extracts were analyzed for a suite of 32 PBDE congeners ranging from tri- to decaBDE. The operating conditions for the GC/MS have been described previously (Stapleton *et al.*, 2008). The homologue groups of PBDE metabolites were confirmed by analyzing some extracts by gas chromatography/electron impact-mass spectrometry (GC/EI-MS) and monitoring the molecular ion fragment M-2Br. Tri- through octaBDE congeners were quantified by monitoring bromide ions (m/z 79 and 81). The nonaBDEs and BDE-209 were quantified by monitoring m/z responses of 486.6 and 484.6, and the 13C-BDE-209 was quantified using m/z responses of 494.6 and 496.6.

TABLE 1
Sampling Regimen for Study End Points Evaluated

Study end point	Sample size	Sampling times
Measurements of PBDE bioaccumulation and metabolism	$n = 3$ tanks (12–15 fish pooled per tank)	Days 0, 14, and 28
Preparation of microsomes and cytosol for enzymatic assays	$n = 3$ tanks (12–15 fish pooled per tank)	Days 0, 14, 28, and 42
DI activity in microsomes	$n = 3$ fish pools	Days 0, 14, 28, and 42
GST activity in microsomal and cytosolic fractions	$n = 3$ fish pools	Days 0, 14, 28, and 42
Histological examinations	$n = 3$ (one individual per tank; three tanks)	Day 28

In vitro DI assay. A series of *in vitro* assays were undertaken whereby exogenous T4 and 3,3',5'-triiodothyronine (rT3) were incubated with whole-fish microsomes prepared from BDE-209-treated and control fathead minnows to examine changes in intracellular DI activity and TH metabolism. Whole-fish microsomal preparation procedures are described in the Supplementary Data. Fish were randomly pooled from each tank (within a treatment) across each sample time point to provide approximately 0.5 g of tissue per tank for microsome preparation (Table 1). Microsomes from BDE-209-treated and control groups ($n = 3$ fish pools; 12–15 fish per pool) across the four sampling time points (days 0, 14, 28, and 42) were incubated in glass test tubes with either 0.64 μ M of T4 or 0.77 μ M of rT3 to measure rates of ORD and IRD. All incubations contained 950 μ l of incubation buffer and 50 μ l of the appropriate microsomal fraction diluted to 10 mg/ml. The buffer used for all incubations consisted of 0.1 M potassium phosphate (K_2HPO_4) buffer with 10mM of DTT and 100 μ M of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) (pH 7.4). Incubations were conducted for 1.5 h in glass test tubes in a water bath at 25°C and 140 revolutions per minute oscillations. Negative controls ($n = 3$ pools) consisted of microsomes incubated with no exogenous TH. Buffer controls contained TH alone with no microsomal protein to correct for any substrate impurities and abiotic degradation. At the conclusion of the incubation period, 1 ml of ice-cold methanol was added to halt the reaction.

TH analysis. TH formation rates mediated by endogenous DIs were determined using a liquid-liquid solid-phase extraction procedure and liquid chromatography tandem mass spectrometry (LC/MS/MS) analytical methodology recently developed in our laboratory (Wang and Stapleton, 2010). Rates of T3, rT3, 3,3'-T2, and 3,5-T2 production were measured using LC/MS/MS operated in positive electrospray ionization mode with recently published multiple reaction monitoring transitions and run parameters (Butt *et al.*, 2011). Labeled internal standards, ^{13}C -T4, ^{13}C -rT3, ^{13}C -T3, ^{13}C -3,3'-T2 (Cambridge Isotope Laboratories, Andover, MA and Accustandard, New Haven, CT), were added to each standard and sample to quantify levels of T4, rT3, T3, and T2 hormones, respectively. Concentrations of THs were normalized to time and protein concentration to determine ORD and IRD rates.

Cytosolic and microsomal GST activity. GST activity was determined in cytosolic and microsomal fractions of BDE-209-treated and control fish (Table 1) using previously published methods to measure the conjugation of GSH to CDNB to form glutathione-2,4-dinitrobenzene (Habig and Jakoby, 1981). Assay procedures and conditions are detailed in the Supplementary data.

Histological examination. The thyroid follicles and livers of BDE-209-treated and control intact fish ($n = 3$; one individual per tank), euthanized on day 28, were examined histologically for morphological alterations. Methods used to prepare tissues for histological examination are outlined in the Supplementary Data. Frontal sections (2.5 μ m thicknesses) of whole fish, selected at 50 μ m intervals, were mounted on glass slides and stained with hematoxylin and eosin. Imaging and examinations were performed with a Nikon Eclipse E600 light microscope, Nikon DXM 1200 digital camera, and NIS-Elements 3.1 imaging software (Nikon, Melville, NY).

Morphometric analysis. Ten unique follicles, randomly identified and selected in three treated and control fish, were measured. Specifically, epithelial cell heights (i.e., distance from basal to apical plasma membrane) were determined for four individual cells per fish at the widest diameter of each follicle. By design, the four cells were located approximately 90° from each other encompassing a total of 40 cells per fish. Mean cell heights were then calculated using the direct method established by Kalisnik *et al.* (1977). This method overcomes possible errors due to tangential planes through the epithelium. Morphometric analysis of livers from BDE-209-treated and control individuals ($n = 3$) involved capturing 20 nonoverlapping fields of 40 μ m² per field at $\times 400$ magnification. The mean ratio (\pm SD) of vacuolated to total hepatocyte nuclei was established among these fields for each individual. After survey of hepatic structure, an array of specific alterations was selected for further analysis, including hepatocyte necrosis, glycogen/lipid inclusions, biliary passageway alterations, and inflammation sites (Hinton *et al.*, 2008).

Quality assurance and data analysis. Recoveries of F-BDE-69 and ^{13}C -BDE-209 averaged $90 \pm 1.4\%$ and $71 \pm 1.1\%$, respectively, during the GC/MS analysis. Small amounts of BDE-209 (3.9 ± 0.7 ng), BDE-207 (1.3 ± 0.3 ng), and BDE-47 (2.33 ± 0.1 ng) were detected in laboratory blanks ($n = 3$). Samples were blank corrected by subtracting mean blank values from fish sample results. Levels of the remaining tri- to nonaBDEs targeted under our method were below method detection limits (MDLs) in laboratory blanks and negative control samples. MDLs were defined as three times the SD of laboratory blanks (i.e., mean blank values + $3 \times$ SD) and were typically measured at < 0.5 ng/g ww of tissue. For congeners not detected in blanks, the MDL was set at the laboratory instrumental detection limit (IDL). For the LC/MS/MS analysis, MDLs were calculated as three times the SD of T4 detected in negative controls and of T3 and rT3 in buffer controls. No 3,3'-T2 or 3,5-T2 hormones were detected in control samples, so the IDL was used as the MDL for these hormones. Trace quantities of T3 and rT3 were consistently detected in buffer control vials at levels of -0.5% and -0.1% , respectively, relative to T4 concentrations. These contaminants originated from T4 commercial material impurities; no abiotic transformation was observed during incubations. MDLs normalized to incubation conditions were as follows: T4 = 2.59 pmol/hr/mg protein; T3 and rT3 = 0.21 pmol/hr/mg protein; 3,3'-T2 and 3,5-T2 = 0.57 pmol/hr/mg protein. Differences in TH formation rates in T4- and rT3-incubated microsomes from BDE-209-treated versus control fish were analyzed for statistical significance using a Student's *t*-test (Graphpad Prism 5.0, La Jolla, CA). Thyroid follicle measurements of treated and control fish were also evaluated using *t*-tests. Intergroup comparisons of changes in DI activity over fish age were determined using a one-way ANOVA analysis followed by a Bonferroni Multiple Comparison *post hoc* test (Graphpad Prism 5.0). Statistical significance was defined at the $p < 0.05$ level with high and very high statistical significance defined at $p < 0.005$ and $p < 0.0005$, respectively.

RESULTS

Growth

Fish growth was monitored throughout the experiment by taking weekly measurements of the mass of 10 fish randomly collected from each tank. Fish mass increased from ~20 to ~50 mg per fish over the course of the study. Fish growth rates for each tank were determined by fitting body weight measurements to the exponential model:

$$\ln(\text{fish mass}) = b \cdot t + a, \quad (1)$$

where b is the growth rate (slope; fish mass per time), t is the time in days, and a is a constant (Fisk *et al.*, 1998). Concentrations of PBDEs in whole fish were corrected for growth dilution by multiplying fish concentrations by a factor of $1 + b \cdot \text{time}$. No significant differences in growth rates or lipid content were observed between BDE-209-treated and control groups. Additional results of growth and lipid measurements can be found in the Supplementary data.

BDE-209 Accumulation and Metabolism

Figure 1 displays PBDE concentrations measured in fathead minnows exposed for 28 days to BDE-209-spiked *Artemia* sp. at a concentration of $9.8 \pm 0.16 \mu\text{g/g}$ ww of food at 5% of their body weight per day. In addition to BDE-209 accumulation ($488 \pm 46 \text{ ng/g}$ ww by day 28 of the exposure) (Fig. 1a), several lower PBDE congeners, ranging from pentaBDE-101 to octaBDEs, were formed via debromination of BDE-209 and increased in concentration over the exposure period (Figs. 1b and 1c). PentaBDE-101 was the lowest molecular weight congener detected, and hexaBDE-154 was the metabolite measured at the highest concentration at approximately $215 \pm 11 \text{ ng/g}$ ww of tissue by the end of the 28-day exposure. Three additional hexaBDE congeners were also detected but could not be identified with available PBDE standards. Two hepta- and octaBDE congeners (BDE-179, BDE-188, BDE-201, and BDE-202) along with the three nonaBDE congeners (BDE-206, BDE-207, and BDE-208) were also detected. DecaBDE contains small amounts of the nonaBDEs as impurities, and GC/ECNI-MS analysis of the BDE-209-treated food showed levels of the nonaBDEs at approximately 1.6% of the BDE-209-spiked food. No other congeners were detected in the BDE-209-treated food; MDLs for the penta- to octaBDEs were $< 0.2 \text{ ng/g}$ ww of food.

To ensure that metabolites detected were not due to low-level accumulations of minor impurities in the BDE-209-treated food, the maximum mass that could be accumulated in fish was calculated assuming they were present in food at concentrations equal to detection limits. For example, the MDL for BDE-154 in the BDE-209-treated food was 0.15 ng/g ww of food, so if BDE-154 was in the treated food at levels just below detection limits, the maximum mass that would be

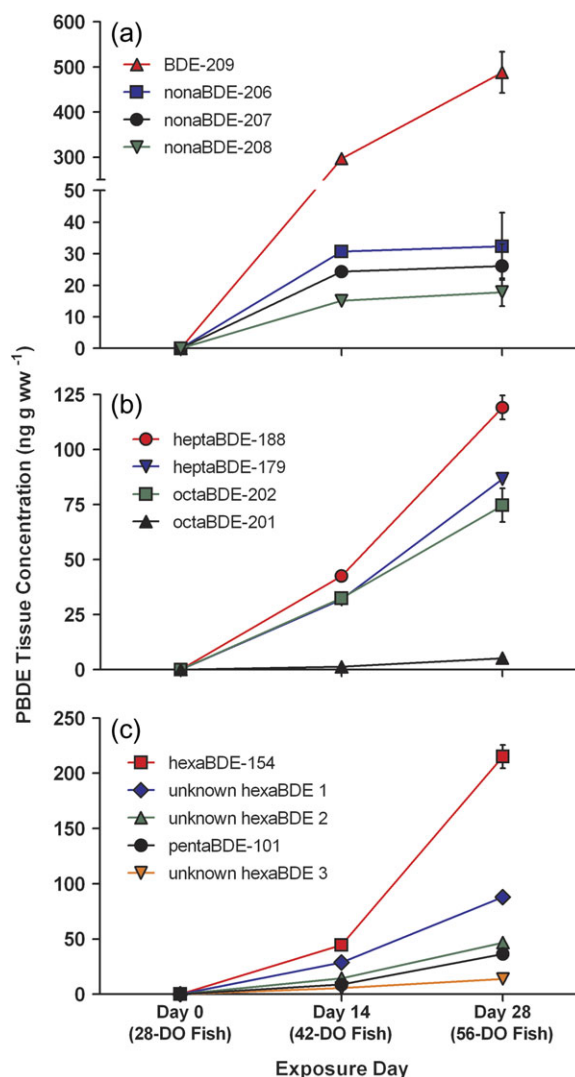


FIG. 1. Concentrations (nanograms per gram wet weight) of BDE-209 and major metabolites measured in whole carcasses of fathead minnows receiving a 28-day dietary exposure of BDE-209 at $9.8 \pm 0.16 \mu\text{g/g}$ ww of *Artemia* sp. at 5% of fish body weight per day ($n = 3$; mean \pm SEM). Approximately 12–15 fish were pooled from three tanks ($n = 3$; mean \pm SEM) across the exposure period (days 0, 14, and 28).

predicted to be present in pooled fathead minnows at day 28 of the exposure would not exceed 0.32 ng (i.e., 0.15 ng/g ww $\cdot 0.075 \text{ g}$ of food per fish pool per day $\cdot 28$ day exposure). The total mass of BDE-154 detected in BDE-209-treated fish ($n = 3$ pools) exceeded 60 ng after the 28-day exposure, making it infeasible for these accumulations to be attributable to low-level food contaminations.

Based on the suite of metabolites identified, approximately 5.8% of the BDE-209 exposure was estimated to be bioavailable to juvenile fathead minnows in this study. This percentage was calculated by estimating the average body burden of metabolites in fathead minnows at the end of the 28-day exposure as follows:

$$\begin{aligned} \% \text{assimilated/bioavailable} = & \sum [\text{BDE-101} + \text{BDE-154} \\ & + \text{hexaBDE1} + \text{hexaBDE2} + \text{hexaBDE3} + \text{BDE-179} \\ & + \text{BDE-188} + \text{BDE-201} + \text{BDE-202}(\text{nmol})] \\ & / \text{cumulative 28-day BDE-209 exposure}(\text{nmol}). \end{aligned} \quad (2)$$

The cumulative BDE-209 exposure over the 28-day exposure was estimated to be ~ 0.45 nmol per fish (or ~ 429 ng of BDE-209 per fish), and the summed metabolites detected at day 28 were ~ 0.026 nmol per fish.

Intracellular DI activity

Figure 2 displays rates of T4-ORD and T4-IRD measured in microsomes prepared from BDE-209-treated and control individuals. At day 14 of the BDE-209 exposure, T4-ORD and T4-IRD activities were significantly inhibited among treated individuals by $\sim 27\%$ and $\sim 66\%$, respectively. By day 28 of the exposure, deiodination rates were substantially inhibited among BDE-209-treated individuals with highly significant ($p < 0.005$) and significant ($p < 0.05$) reductions of $\sim 74\%$ in both T4-ORD and T4-IRD, respectively. Some apparent recovery of T4-ORD was observed over the 14-day depuration period, although activity was still significantly inhibited by $\sim 45\%$ compared with controls. Significant increases in T4-IRD activity were also detected in treatment groups over the 14-day depuration period. Formation rates of 3,3'-T2 (from the sequential loss of two iodine atoms from T4) were measured with no statistically significant differences observed between treatments and controls (data not shown).

Microsomes from BDE-209-treated and control groups were also incubated with $0.77\mu\text{M}$ of rT3 as fathead minnows have three different DI isoforms that have different affinities for different TH substrates. Microsomes from treated individuals showed statistically significant increases ($\sim 41\%$) in rT3-ORD activity at day 28 only. This significant increase in rT3-ORD at day 28 of the exposure was followed by a $\sim 56\%$ reduction in rT3-ORD at the conclusion of the 14-day depuration period. Age-specific differences in T4-ORD and T4-IRD among controls were observed. A one-way ANOVA followed by a Bonferroni Multiple Comparison *post hoc* test revealed significant increases ($p < 0.05$) in T4-ORD activity among control individuals starting at day 28 of the exposure that continued over the 14-day depuration period. T4-IRD was also significantly ($p < 0.05$) increased among controls at day 28 relative to the other sampling time points.

GST Activity

No significant ($p > 0.05$) differences in GST activity were measured in either cytosolic or microsomal fractions prepared from BDE-209 treatment groups relative to controls over the exposure period. Further results of the GST analysis can be found in the Supplementary data.

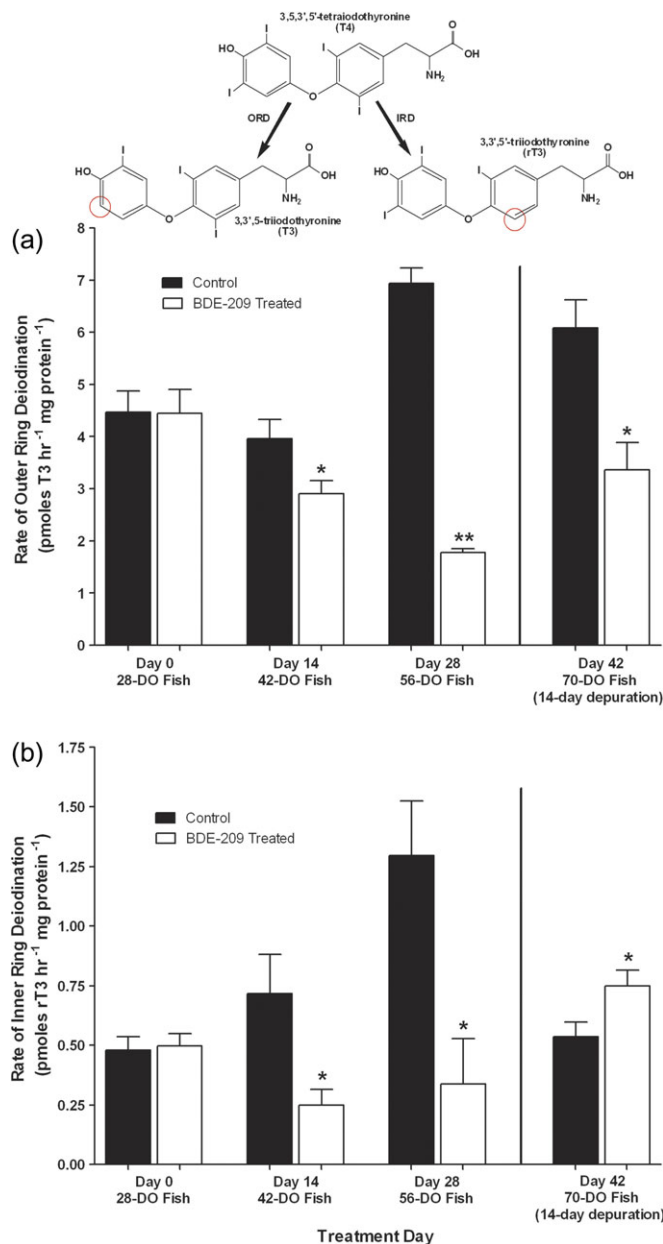


FIG. 2. ORD (a) and IRD (b) measured in microsomes from BDE-209-treated and control fish incubated with $0.64\mu\text{M}$ of thyroxine (T4) for 90 min at 25°C ($n = 3$; mean \pm SEM). Fish that were 28 DO were exposed to 9.8 ± 0.16 $\mu\text{g/g}$ of BDE-209 in their food at 5% of their body weight per day for 28 days followed by a 14-day depuration period to monitor recovery. Approximately 12–15 fish were pooled from three tanks ($n = 3$; mean \pm SEM) across each sampling time point (days 0, 14, 28, and 42). One asterisk denotes deiodination activity that was significantly different from controls ($p < 0.05$), and two asterisks indicate high statistical significance ($p < 0.005$).

Morphological Alterations

Results of high-resolution microscopy (Figs. 3 and 4) show that juvenile fathead minnows exposed to BDE-209 for 28 days exhibited increased thyroid follicular epithelial cell height ($p < 0.0005$) and colloid depletions relative to controls. Most teleosts, including fathead minnows, do not have individual

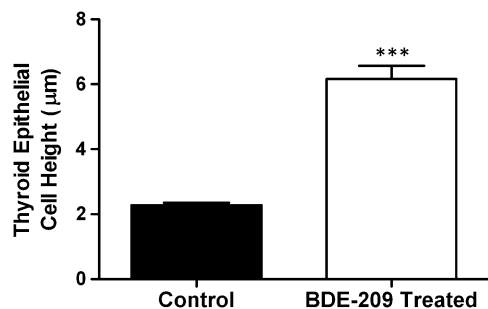


FIG. 3. Epithelial cell height of thyroid follicles from juvenile fathead minnows exposed to BDE-209 for 28 days and in control individuals receiving clean food ($n = 3$; one individual/tank; mean \pm SEM). Measurements were made by identifying 10 unique follicles in each of the three treated and control fish. Cell height determinations were made at the widest follicle diameter by measuring the height of four cells located approximately 90° from each other for a total of 40 cells per fish. Three asterisks indicate very high statistical significance ($p < 0.0005$).

thyroid glands but rather have several nonencapsulated follicles dispersed mainly at the base of the branchial arches near the ventral aorta. Whereas follicles from control individuals had predominantly squamous epithelium with well-formed colloids (Fig. 4a), follicles from BDE-209-treated individuals presented a cuboidal to low columnar epithelium (Fig. 4c). In addition, qualitative examination of thyroid sections indicated that increases in epithelial cell height in BDE-209-treated individuals were accompanied by varying degrees of irregularity in follicle outlines and decreasing colloid. No changes in tissue vascularity were apparent, and colloid vacuoles were not detected among treated individuals. However, substantial increases in inflammatory cells were observed in tissue surrounding follicles of treated fish.

Hepatocytes of control individuals (Fig. 4b) presented a normal phenotype in which cytoplasm contained large areas of eosinophilic staining that contrasted with smaller perinuclear cuffs of basophilia overlying rough endoplasmic reticulum. Nuclei of hepatocytes in controls stained with uniform basophilia (i.e., were dark purple). In contrast, a readily apparent altered phenotype was observed in livers of juvenile fish exposed to BDE-209. Hepatocyte nuclei from treated individuals contained white vacuolated regions and peripheral basophilic areas as rings or crescents (Fig. 4d). A quantitative analysis of 20 non-overlapping fields among BDE-209-treated individuals revealed that vacuolated nuclei constituted $48 \pm 12\%$ (mean \pm SD) of total hepatocyte nuclei counted. No vacuolated nuclei were observed in control individuals. Hepatocytes of treated individuals appeared structurally intact and well defined with no indication of apoptosis or nuclear lysis. Moreover, despite the chronic duration of the study, no foci of inflammation were observed.

DISCUSSION

BDE-209 Accumulation and Metabolism

BDE-209 accumulated in juvenile fathead minnows and was readily debrominated to PBDE congeners with fewer bromine

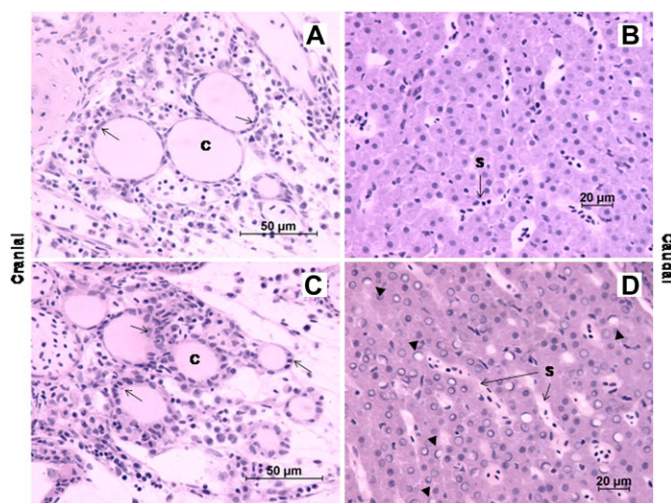


FIG. 4. Light micrographs (oil immersion, $\times 600$, hematoxylin and eosin staining) of juvenile fathead minnow thyroid follicles and liver tissue from control and BDE-209-treated individuals at exposure day 28 ($n = 3$; one individual per tank). (A) Thyroid follicles from a control fish; (B) liver tissue from a control fish; (C) thyroid follicles from a fish exposed to BDE-209 via the diet for 28 days; (D) liver tissue from a fish exposed to BDE-209 via the diet for 28 days. C = follicular colloid; arrows = follicular epithelium; S = sinusoid containing nucleated red blood cells; black triangles = ringed or crescent-shaped nuclei with apparent continuity to interhepatic biliary passages.

atoms (Fig. 1). Based on the major metabolites detected, Figure 5 presents a predicted pathway of reductive debromination. An analysis of this pathway suggests that reductive debromination was dominated by cleavage of bromine atoms from meta- and para-substituted positions, which is consistent with debromination patterns observed in incubations of carp liver microsomes with PBDEs (Roberts *et al.*, 2011). Debromination patterns in treated individuals were also consistent with results observed in common carp (*C. carpio*) exposed to BDE-209 via the diet, whereby the dominant metabolites detected were also penta- to octaBDEs (Stapleton *et al.*, 2004). This suggests possible family-specific commonality in PBDE metabolism as both carp and fathead minnows are members of the family Cyprinidae. However, we detected BDE-209 accumulation in fathead minnows, whereas no BDE-209 bioaccumulation was observed in juvenile carp.

Formation of hexaBDEs and pentaBDE-101 appeared to occur rapidly, given the negligible levels of predicted intermediate metabolites measured. These data suggest that BDE-209 metabolism in cyprinids is relatively rapid but may stop at the pentaBDEs with no appreciable metabolism to lower-molecular weight congeners. Longer exposure periods would be needed to fully evaluate this hypothesis as it does not appear that steady-state conditions were reached in this study. With regard to BDE-209 bioavailability, whereas the percent bioavailable in fathead minnows was limited to 5.8%, it was substantially higher than BDE-209 bioavailability measurements in common carp (Stapleton *et al.*, 2004) and rainbow trout (Kierkegaard *et al.*, 1999), which were less than 0.5%. In addition, the BDE-209 cumulative exposure over the 28-day treatment (~ 429 ng per fish) and the percent

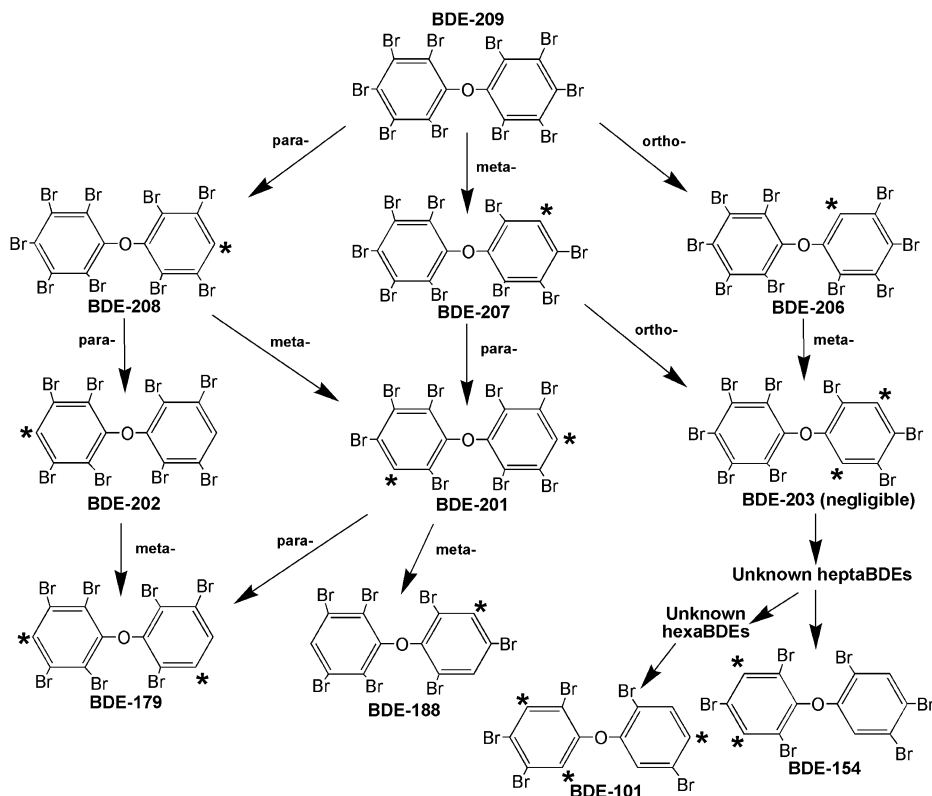


FIG. 5. Predicted debromination pathway of BDE-209 in 28-day-old fathead minnows (*Pimephales promelas*) exposed to $9.8 \pm 0.16 \mu\text{g/g}$ ww of *Artemia* sp. at 5% of their body weight per day for 28 days. Asterisks indicate the predicted site of debromination.

bioavailability measured here are environmentally relevant. For example, BDE-209 levels in river, estuarine, and marine sediments have been measured at thousands of ng/g dry weight (Mai *et al.*, 2005; Sellstrom *et al.*, 1998; Vane *et al.*, 2010).

Alterations in Deiodination Activity

It is possible that some or all three DI isoforms were inhibited in treated fathead minnows as both T4-ORD and T4-IRD declined, especially by day 28, where severely depressed T4-ORD and T4-IRD were measured. Type 1 and 2 isoforms of DI (D1 and D2) catalyze T4-ORD to produce the active T3 hormone, whereas D1 and type 3 isoforms of DI (D3) catalyze T4-IRD to inactive rT3. Thus, D1 can be involved in both ORD and IRD of T4. A 14-day recovery period in which fish received control food containing no BDE-209 resulted in some possible recovery of T4-ORD activity, although it was still significantly less than activity in controls. The decreased T4-ORD and T4-IRD observed in treated fish may be attributable to PBDEs mimicking THs, triggering direct downregulations of messenger RNA (mRNA) expression of genes encoding DIs. Recent *in vitro* testing in our laboratory using microsomal fractions prepared from carp liver tissue provides evidence of a possible competitive binding interaction between THs and PBDEs for DIs (Noyes *et al.*, 2010).

If PBDEs are acting as TH mimics, the depressed DI activity measured in this study could also be linked to increased activity

of TH-metabolizing enzymes, such as uridine diphosphate glucuronosyl transferases and sulfotransferases. TH-conjugating enzymes, and other classes of enzymes involved in TH metabolism, have not been examined in detail in fish. However, there is evidence of alterations of conjugating enzymes in mammals exposed to PBDEs (Richardson *et al.*, 2008; Szabo *et al.*, 2009; Zhou *et al.*, 2002), and in one study, these alterations were concomitant with decreased expression and activity of DIs (Szabo *et al.*, 2009). Further testing is needed to examine BDE-209 effects on transferase enzymes in fish.

In addition to BDE-209 potentially acting as a competitive substrate, DIs could have distinctive substrate specificities for the various reductive metabolites measured in this study, thereby differentially competing with THs to affect deiodination rates. Very little is known about the deiodination kinetics of THs in fishes in the presence of PBDEs. In addition, widespread differences in Michaelis-Menton kinetics (K_m and V_{max} values) have been shown across teleosts further complicating interpretations (Leatherland *et al.*, 1990). More work is needed to better understand the potential for differential competitive interactions of PBDE congeners on TH deiodination.

An analysis of DI activity among control individuals sampled over the course of the study revealed significant ($p < 0.05$) increases in both T4-ORD and T4-IRD at day 28 of the study when fish were 56 DO and that the elevated T4-ORD continued over the 14-day depuration period. The increase in

T4-ORD activity measured at this age coincided with substantial inhibitions of T4-ORD and altered T4-IRD among BDE-209-exposed fish. The juvenile fish phase is conventionally considered a period of fish growth and gonadal maturation, and TH disturbances have been shown to impair reproductive functioning (Cyr and Eales, 1996; Lanno and Dixon, 1994; Timmermans *et al.*, 1997). Previous results have shown that tetraBDE-47 caused reductions in mature spermatozoa and spawning among adult fathead minnows (Lema *et al.*, 2008; Muirhead *et al.*, 2006), and these impairments were concomitant with depressed plasma T4 and elevated thyroid-stimulating hormone (TSH) β mRNA expression (Lema *et al.*, 2008). In contrast to these findings, no changes in gonadal development were indicated in juvenile zebrafish exposed to tetraBDE-47, although hypoactivity was measured that could be related to neurodevelopmental impairments (Chou *et al.*, 2010). Altogether, marked perturbations in DI activity observed among 56- and 70-day-old fathead minnows exposed to BDE-209, in relation to increases in DI activity across control groups of the same age, suggest that BDE-209 and/or its metabolites could alter important developmental pathways in juvenile fish, particularly those linked to gonadal maturation and reproduction.

The statistically significant increases in rT3-ORD, resulting in elevated formation rates of 3,3'-T2, stands in some contrast to effects in T4-incubated microsomes where declines in ORD were observed. The purpose for choosing T4 and rT3 as incubation substrates was to help delineate DI isoforms in fathead minnows potentially altered by exposure to BDE-209. D1 has been shown to have a substrate preference for rT3, whereas D2 has been shown to have a substrate preference for T4 (Mol *et al.*, 1998). It is possible that D1 was upregulated and D2 was downregulated in BDE-209-exposed fish. The upregulation in D1 could be a compensatory response to marked inhibition of D2 but is insufficient to fully compensate for the D2 inhibition in treated individuals. Additional work will be needed to better understand relative responses of DI isoforms in fish exposed to PBDEs.

Morphological Alterations

The thickening of the follicular epithelium and qualitative changes detected in thyroid tissues (i.e., irregular follicle outlines, colloid depletions, and increase in inflammatory cells) of BDE-209-treated fish are hallmarks of thyroid overstimulation and injury (Eales and Brown, 1993). The follicular epithelium hypertrophy observed may have resulted from reduced levels of circulating T4 over the exposure period. This hypothesis of depressed plasma TH will require further verification by measuring circulating T4 levels, which was not possible during this study. However, previous studies have shown that dietary exposures to PBDEs depress circulating T4 in fish (Lema *et al.*, 2008; Tomy *et al.*, 2004), rodents (Kuriyama *et al.*, 2007; Rice *et al.*, 2007; Richardson *et al.*, 2008), and birds (Fernie *et al.*, 2005).

Unlike the mammalian thyroid system, the fish thyroid system may not be centrally driven through the HPT axis. Rather, the HPT of fishes may govern predominantly circulating T4 homeostasis through TSH stimulation from the pituitary (Eales and Brown, 1993; Maclatchy and Eales, 1992). Depressions in plasma T4 will stimulate the pituitary to release TSH that then activates the thyroid to compensate for depressed TH levels. This feedback condition can lead to thickening of the follicular epithelium with extended stimulation. Depressed circulating T4 among BDE-209-treated individuals could have contributed to decreased levels of T4 in peripheral tissues that subsequently produced declines in intracellular T4-ORD and T4-IRD observed in our DI assay through the 28-day exposure.

In addition to notable changes in the thyroid, a profoundly altered liver phenotype characterized by a large number of vacuolated hepatocellular nuclei was measured among fish exposed to BDE-209. We hypothesize that these vacuolated nuclei, which were not observed in controls, may be attributable to impingements caused by enlarged "intrahepatic" biliary passageways. In fish, hepatocytes are arranged as tubules clustered around an interhepatic biliary passageway with their apices directed toward the central biliary canaliculus and/or bile preductules (Hinton *et al.*, 2008). Cyprinids, like fathead minnows, also have finger-like indentations, conventionally termed *intrahepatic* canaliculi that extend into hepatocytes, acting as continuous extensions of the interhepatic canaliculi (Vogt and Segner, 1997; Yamamoto, 1965). A careful review of these high-resolution images showed continuity between the vacuole detected in the nuclei of BDE-209-treated fish and interhepatic biliary passageways, suggesting that these areas are connected. These types of biliary dilations and enlargements have also been observed in Japanese medaka (*Oryzias latipes*) exposed to aqueous α -naphthylisothiocyanate, a well-described hepatotoxicant (Hardman *et al.*, 2008). The possible relationship of vacuoles observed in the present study to canaliculi, both intercellular and so-called intracellular, requires higher-resolution examination with transmission electron microscopy.

Further examination of hepatotoxicity among fishes exposed to PBDEs is merited, given the profound structural abnormalities observed here. For example, enlarged biliary passageways, which may be a BDE-209 clearance mechanism, could be accompanied by increased expression or activity of TH efflux transporters, leading to increased hormone excretions. Previous studies have shown that mRNA expression of multidrug resistance-associated proteins 2 and 3 (Mrp2 and Mrp3), which are major efflux transporters of glucuronides out of cells, was increased in rodents exposed to tetraBDE-47 and the pentaBDE commercial mixture DE-71 (Richardson *et al.*, 2008; Szabo *et al.*, 2009). In addition, multidrug resistance protein (Mdr1a), which encodes P-glycoproteins (P-gps) that are efflux transporters of glucuronides and THs into the bile, was also increased in rodents exposed to DE-71 (Szabo *et al.*, 2009). Little is known about the activity of these membrane-bound transporters in PBDE-exposed fish and further investigation is warranted.

CONCLUSIONS

Results of this study provide a more integrated understanding of the potential for BDE-209 to impair thyroid systems of juvenile fathead minnows and other fish species. BDE-209 was readily metabolized by juvenile fish to lower PBDE congeners dominated by penta- to octaBDEs. Marked perturbations of intracellular TH formation rates among treated fish suggest impaired deiodination activity with only limited recovery after a 14-day depuration period. These perturbations in deiodination were accompanied by significant thyroid follicle damage and liver alterations. However, although the morphological findings observed in this study were pervasive, they will require further examination with increased resolution, across multiple doses of BDE-209, and with greater sample sizes to verify and elucidate effects observed here. Further study is also needed to describe toxicological mechanisms that underly PBDE-related thyroid disruptions. Altogether, these results suggest that BDE-209 may be affecting the fish thyroid system at multiple levels, including in peripheral tissues and central HPT axis, and that juvenile fish may be uniquely susceptible to developmental and reproductive abnormalities when exposed to thyroid disruptors like PBDEs.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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