

Polybrominated Diphenyl Ether (PBDE) Flame Retardants: Accumulation, Metabolism
and Disrupted Thyroid Regulation in Early and Adult Life Stages of Fish

by

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Dissertation submitted in partial fulfillment of requirements
for the degree of Doctor of Philosophy
in Environment in the Graduate School of
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ABSTRACT

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Abstract

Polybrominated diphenyl ethers (PBDEs) are a class of brominated flame retardant chemicals that are added to plastics, electronic components, furniture foam, and textiles to reduce their combustibility. Of the three commercial mixtures historically marketed, only DecaBDE, which is constituted almost entirely (~97%) of the fully brominated congener decabromodiphenyl ether (BDE-209), continues to be used in the U.S. today. While decaBDE is scheduled for phase-out in the U.S. at the end of 2013, exposures to BDE-209 and other PBDEs will continue into the foreseeable future as products that contain them continue to be used, recycled, and discarded. In addition, decaBDE use continues to be largely unrestricted across Asia, although restricted from use in electronic equipment in Europe.

Despite limits placed on PBDE uses, they are ubiquitous contaminants detected worldwide in humans and wildlife. Major health effect concerns for PBDEs come largely from evidence in laboratory rodents demonstrating neurotoxicity, reproductive and developmental impairments, and thyroid disruption. The potential for PBDEs, particularly BDE-209, to disrupt thyroid regulation and elicit other toxic outcomes in fish is less clear. Thus, the overall objective of this thesis research was to answer questions concerning how fish, as important indicators of overall environmental health, are metabolizing PBDEs and whether and how PBDEs are disrupting thyroid hormone

regulation. The central hypothesis was that PBDE metabolism in fish is mediated by iodothyronine deiodinase (dio) enzymes, which are responsible for activating and inactivating thyroid hormones, and that PBDE exposures are causing thyroid system dysfunction across fish life stages.

Under the first research aim, *in vitro* experiments conducted in liver tissues isolated from common carp (*Cyprinus carpio*) suggested a role for dio enzymes in catalyzing the reductive debromination of PBDEs. Carp liver microsomes efficiently debrominated BDE-99 to BDE-47, and enzymes catalyzing this reaction were associated predominantly with the endoplasmic reticulum (i.e., microsomal fraction) where dio enzymes are located. Competitive substrate experiments in carp liver microsomes also demonstrated that rates of BDE-99 debromination to BDE-47 were significantly inhibited upon challenges with 3,3',5'-triiodothyronine (rT3) and thyroxine (T4). This finding supported the hypothesis that enzymes involved in the metabolism of PBDEs may have high affinities for thyroid hormones. Indeed, experiments to determine apparent enzymatic kinetics (apparent V_{\max} and K_m values) of BDE-99 hepatic metabolism suggested that enzymes responsible for the catalytic activity appeared to have a higher affinity for native thyroid hormone than BDE-99.

The second and third research aims were focused on evaluating BDE-209 accumulation, metabolism, and thyroid toxicity in juvenile and adult life stages of fish using the fathead minnow (*Pimephales promelas*) as a model. BDE-209 bioaccumulated

and was debrominated to several reductive metabolites ranging from penta- to octaBDEs in both juvenile and adult fish exposed to BDE-209. In addition, thyroid hormone regulation in juvenile and adult male fathead minnows was severely disrupted by BDE-209 at low, environmentally relevant exposures. In juvenile minnows, the activity of dio enzymes (T4-outer ring deiodination; T4-ORD and T4-inner ring deiodination; T4-IRD) declined by ~74% upon oral doses of 9.8 ± 0.2 $\mu\text{g/g}$ wet weight (ww) food at 3% body weight (bw)/day for 28 days, compared to controls. Declines in dio activity were accompanied by thyroid follicle hypertrophy indicative of over-stimulation and injury. In addition to thyroid disruption, a distinctive liver phenotype characterized by vacuolated hepatocyte nuclei was measured in ~48% of hepatocytes from treated fish that was not observed in controls.

Under the third research aim, adult male fathead minnows received dietary treatments of BDE-209 at a low dose (95.3 ± 0.41 ng/g-food at 3% bw/day) and a high dose (10.1 ± 0.10 $\mu\text{g/g}$ -food at 3% bw/day) for 28 days followed by a 14-day depuration period to evaluate recovery. Compared to negative controls, adult male fish exposed orally to BDE-209 at the low dose tested for 28 days experienced a 53% and 46% decline in circulating total T4 and T3, respectively, while fish at the high BDE-209 dose tested had total T4 and T3 deficits of 59% and 62%, respectively. Depressed levels of plasma thyroid hormones were accompanied by a 45-50% decline in the rate of T4-ORD in brains of all treatments by day 14 of the exposure. The decreased T4-ORD continued in

the brain at day 28 with a ~65% decline measured at both BDE-209 doses. BDE-209 exposures also caused transient, tissue-specific upregulations of relative mRNA transcripts encoding dio enzymes (*dio1*, *dio2*), thyroid hormone receptors (*TR α* , *TR β*), and thyroid hormone transporters (*MCT8*, *OATP1c1*) in the brain and liver in patterns that varied with time and dose, possibly as a compensatory response to hypothyroidism. In addition, thyroid perturbations at the low dose tested generally were equal to those measured at the high dose tested, suggesting non-linear relationships between PBDE exposures and thyroid dysfunction in adult fish. Thus, mechanisms for BDE-209 induced disruption of thyroid regulation can be proposed in adult male minnows that involve altered patterns of thyroid hormone signaling at several important steps in their transport and activation.

A growing body of evidence describing PBDE toxicity in biota, including data generated here, along with studies showing continued and rising PBDE body burdens, raises concern for human and wildlife health. Long delays in removing PBDEs from the market, their ongoing presence in many products still in use, and their active use outside the U.S. and European Union will leave a lasting legacy of rising contamination unless more concerted regulatory and policy actions are taken to reduce future exposures and harm.

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List of Abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
ADME	Absorption, distribution, metabolism, and excretion
AhR	Aryl hydrocarbon receptor
ANOVA	Analysis of variance
BTEB	Basic transcription element-binding protein
BCA	bicinchoninic acid assay
BDE-28	2,4,4'-tribromodiphenyl ether
BDE-47	2,2',4,4'-tetrabromodiphenyl ether
BDE-49	2,2',4,5'-tetrabromodiphenyl ether
BDE-66	2,3',4,4'-tetrabromodiphenyl ether
BDE-99	2,2',4,4',5-pentabromodiphenyl ether
BDE-100	2,2',4,4',6-pentabromodiphenyl ether
BDE-101	2,2',4,5,5'-pentabromodiphenyl ether
BDE-153	2,2',4,4',5,5'-hexabromodiphenyl ether
BDE-154	2,2',4,4',5,6'-hexabromodiphenyl ether
BDE-155	2,2',4,4',6,6'-hexabromodiphenyl ether
BDE-179	2,2',3,3',5,6,6'-heptabromodiphenyl ether
BDE-183	2,2',3,4,4',5',6-heptabromodiphenyl ether
BDE-188	2,2',3,4',5,6,6'-heptabromodiphenyl ether
BDE-201	2,2',3,3',4,5',6,6'-octabromodiphenyl ether
BDE-202	2,2',3,3',5,5',6,6'-octabromodiphenyl ether
BDE-203	2,2',3,4,4',5,5',6-octabromodiphenyl ether
BDE-206	2,2',3,3',4,4',5,5',6-nonabromodiphenyl ether
BDE-207	2,2',3,3',4,4',5,6,6'-nonabromodiphenyl ether
BDE-208	2,2',3,3',4,5,5',6,6'-nonabromodiphenyl ether
BDE-209	Decabromodiphenyl ether
Br	Bromine
bw	Body weight
PentaBDE	Commercial PentaBDE mixture
OctaBDE	Commercial OctaBDE mixture
DecaBDE	Commercial DecaBDE mixture
CAR	Constitutive androstane receptor
cDNA	Complementary DNA
CDNB	Chlorinated dinitrobenzene
Cl	Chlorine

CI	Chemical ionization
CHO	Chinese hamster ovary
CRH	Corticotrophin releasing hormone
CSF	Cerebral spinal fluid
CYP450	Cytochrome P450
dpf	Days post fertilization
dph	Days post hatch
Dio	Iodothyronine deiodinase
DR-CALUX	Dioxin receptor-chemical activated luciferase gene expression
DTT	Dithiothreitol
ECNI	Electron capture negative ionization
EF1 α	Elongation factor1 α
EI	Electron impact
ESI	Electrospray ionization
EROD	Ethoxyresorufin-O-deethylase
EPA	Environmental Protection Agency
EU	European Union
F	Fluorine
FSH	Follicle stimulating hormone
GC/MS	Gas chromatography/mass spectrometry
GSH	Glutathione
GSH-Px	Glutathathione peroxidase
GR	Glutathione reductase
GSI	Gonado-somatic index
GST	Glutathione-S-transferase
HDT	Highest dose tested
HE	Hematoxylin and Eosin
hpf	Hours post fertilization
HPG	Hypothalamic-pituitary-gonadal
HPT	Hypothalamic-pituitary-thyroid
IaC	Iodoacetate
IC ₅₀	Half maximal inhibitory concentration
IDL	Instrument detection limit
IRD	Inner ring deiodination
I	Iodine
K _m	Michaelis constant (1/2 V _{max})
Kow	Octanol-water partition coefficient
LC/MS/MS	Liquid chromatography tandem mass spectrometry
LH	Luteinizing hormone

LM	Light microscopy
LOD	Limit of detection
LOQ	Limit of quantitation
MCT	Monocarboxylate transporter
MDL	Method detection limit
Mdr	Multidrug resistance protein
MeO-PBDE	Methoxylated PBDE
mRNA	Messenger RNA
MRM	Multiple reaction monitoring
Mrp	Multidrug resistance associated protein
MBP	Myelin basic protein
MW	Molecular weight
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NIS	Sodium/Iodide Symporter
OATP	Organic anion transport protein
OH-PBDE	Hydroxylated PBDE
ORD	Outer ring deiodination
Pa	Pascal
PBDE	Polybrominated diphenyl ether
PBDD	Polybrominated dibenzo- <i>p</i> -dioxin
PBDF	Polybrominated dibenzofuran
PBT	Persistent, bioaccumulative, and toxic
PCB	Polychlorinated biphenyl ether
PCR	Polymerase chain reaction
PTU	6-propyl-2-thiouricil
PTV	Pressure temperature variation
PXR	Pregnane X receptor
RT-qPCR	Quantitative real-time reverse transcription PCR
SULT	Sulfotransferase
T2	3,3'-diiodothyronine
T3	3,3',5-triiodothyronine
TBBPA	Tetrabromobisphenol A
rpl8	Ribosomal protein L8
rT3	3,3',5'-triiodothyronine
T4	Thyroxine
FT3	Free T3
FT4	Free T4
TBG	Thyroid binding globulin
TCDD	Tetrachlorodibenzo- <i>p</i> -dioxin

TCDF	Tetrachlorodibenzofuran
TEM	Transmission electron microscopy
Tg	Thyroglobulin
TR	Thyroid receptor
TRE	Thyroid response element
TRH	Thyrotropin releasing hormone
TSH	Thyroid stimulating hormone
TT3	Total T3
TT4	Total T4
TTR	Transthyretin
UGT	Uridine diphosphate glucuronosyl transferase
V_{\max}	Maximum enzyme velocity
Vtg	Vitellogenin
ww	Wet weight

1. Introduction

Fires took the lives of approximately 3,000 people annually in the U.S. from 1999-2008, injured many thousands more, and cost over \$10 billion/year in damages (USFA/FEMA 2009). Polybrominated diphenyl ethers (PBDEs) are a class of brominated flame retardant chemicals added to consumer and commercial products including textiles, carpeting, and electronics to reduce their combustibility. There are three main commercial mixtures of PBDEs: PentaBDE, OctaBDE, and DecaBDE. The PentaBDE and OctaBDE mixtures have been phased out in the U.S. and banned in the European Union due to concerns about persistence, bioaccumulation, and toxicity. The fully brominated congener, decabromodiphenyl ether (BDE-209) is the major PBDE congener in the DecaBDE. It is the only commercial PBDE mixture still used in the U.S. today with 2007 global consumption estimated at ~161 million pounds (Fink et al. 2008; Posner et al. 2011). Because PBDEs are not chemically bound but are rather added to plastics, they are susceptible to leaching as the products that contain them break down. In addition, PBDEs can enter the environment during their production. As a consequence, PBDEs are widespread contaminants in both living and non-living parts of the environment. Despite the increasing and far reaching contamination, our understanding of the toxicity

of PBDEs remains limited. PBDEs structurally resemble polychlorinated biphenyls (PCBs) and thyroid hormones (Figure 1). A growing body of evidence indicates that PBDEs perturb the endocrine system of vertebrates by impairing thyroid regulation, which plays an important role in the growth, development, reproduction, and metabolism of vertebrates, and by acting as reproductive, developmental, and neurological toxicants (Costa and Giordano 2007; Staskal and Birnbaum 2011). PBDE effects on fish and other wildlife continue to be poorly understood.

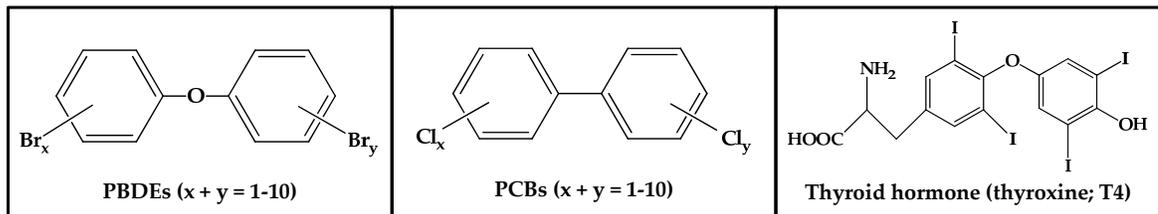


Figure 1: Structural comparison of PBDEs, PCBs, and T4.

1.1 Overview of Research

Fish have been shown to biotransform PBDEs to lower, persistent, and bioavailable congeners with fewer bromine atoms (Browne et al. 2009; Kierkegaard et al. 1999; Stapleton et al. 2004a; Stapleton et al. 2006; Tomy et al. 2004). However, enzyme

systems involved in this reductive debromination are unclear. In addition, while a few studies have shown that lower PBDEs can reduce thyroid hormone levels in fish and cause other thyroid perturbations (Lema et al. 2008; Tomy et al. 2004; Yu et al. 2011), less is known about whether and how the still widely used BDE-209 congener may be impacting thyroid system functioning of fishes (and other vertebrates). Given these ongoing gaps in our understanding of PBDE metabolism and thyroid toxicity, the overall objective of this doctoral research was to examine enzymatic mechanisms involved in PBDE metabolism and to elucidate the potential for PBDEs, particularly BDE-209, to adversely affect thyroid hormone regulation in fishes. The central hypothesis of this research is that PBDE exposures in fish lead to metabolism mediated by thyroid hormone regulating enzymes (i.e., deiodinases or dios), and furthermore that PBDE exposures cause thyroid perturbations across early and adult life stages. BDE-209 was the focus of most of this research given its ongoing widespread use and environmental contamination. Thus, specific research aims were:

- 1) To characterize enzymes involved in catalyzing the *in vitro* hepatic biotransformation of PBDEs to lower congeners with fewer bromine atoms in common carp [**Chapter 2**];

- 2) To measure the *in vivo* toxicokinetics of BDE-209 in fathead minnow juveniles and to examine BDE-209 effects on thyroid homeostasis and liver morphology [Chapter 3]; and
- 3) To measure the *in vivo* toxicokinetics of BDE-209 in fathead minnow adults and to investigate effects on thyroid hormone levels and regulation, including underlying mechanisms of thyroid dysfunction [Chapter 4].

The remainder of this introduction provides brief background information on classes of flame retardants, how/why they are used, and environmental exposure patterns. This summary is followed by a synopsis of current evidence of PBDE toxicity in human and wildlife, with special attention given to PBDE effects on fish and thyroid disruption. Finally, based on these lines of evidence, a more detailed summary of the experimental approach is outlined.

1.2 Why Flame Retardants?

Over the past 50 years, there has been a dramatic increase in the number and variety of electronic devices and plastic materials used in houses, offices, and public spaces. Computer use has become an integral part of the daily lives of most people. As of 2011, over three-fourths of all U.S. households (~77%) contained at least one computer

(ESA/DOC 2011). In 1984, only ~8% of U.S. households reported having a computer in the home (ESA/DOC 1991). By 1970, almost all U.S. households (96%) reported owning at least one television. A major difference today is that more than half (~55% as of 2010) of all U.S. households contain three or more television sets compared to ~11% in 1975 (NielsenCompany 2010). Similarly, European households are reported to contain an average of 22 electronic and electrical appliances (EFRA 2011).

As a result, in part, the global use of plastics has increased sharply over the past 60 years from ~1.5 million metric tons used in 1950 to ~245 million metric tons as of 2008, an increase of ~9% per year (Europe 2009). The dramatic increase in the use of plastics, electronics, and computers, coupled with stricter requirements for consumer fire protection, has helped to give rise to and promote the growth of the flame retardant industry. In the U.S. alone, fires killed ~3,000 people per year from 1999 to 2008, injured many thousands more, and caused over \$10 billion/year in property damage (USFA/FEMA 2009). The flame retardant industry is unique in that its existence is largely a function of and dependent on statutory and regulatory requirements designed to increase fire safety. As such, this industry has an active and expansive lobbying effort

that operates at all levels of government. Stricter legislative and fire safety standards are major forces that have propelled its growth.

Thus, flame retardant chemicals now represent a diverse variety of chemicals. They are classified into four main categories based on their chemical composition: inorganic, halogenated, organophosphorous, and nitrogen-based. Chemical-based flame retardants can also be either additive or reactive. Additive flame retardants, like the PBDEs, are incorporated into polymers typically after the polymerization process and so are not chemically bound to the polymer but are physically mixed into it. Therefore, PBDEs can enter the environment during production and may be released into the surrounding environment with the breakdown and volatilization of the parent polymer. Reactive flame retardants are added during the polymerization process and become integrally bound to the polymer (Posner et al. 2011).

1.3 How do Flame Retardants Work?

Flame retardant chemicals can act at any of a number of steps of the combustion process of plastics in a fire (Figure 2). The combustion process is a gas phase reaction that generally involves four major steps: preheating, volatilization/decomposition, combustion, and propagation (Troitzsch 1998).

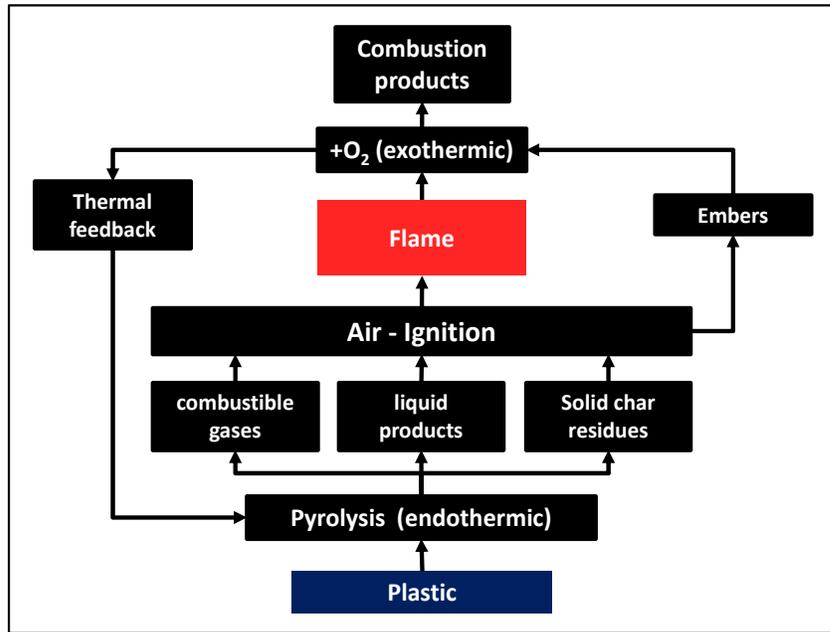


Figure 2: Combustion process of plastics (Alaee et al. 2003; Troitzsch 1998)

Endothermic heating and decomposition (pyrolysis) in the pre-ignition phase leads to the formation of flammable gases. These flammable gases mix with atmospheric oxygen to cause flame ignition that initiates an exothermic reaction, leading to fire propagation. Pyrolysis of the polymer is also strengthened by a thermal feedback that further fuels the flame to speed its spread over the decomposing polymer surface. Fire spread and diffusion is catalyzed by high energy free radicals, including highly oxidizing agents. These free radicals are essential for fire propagation. Flame retardant

chemicals work by interfering with one or more of these fundamental combustion processes, either chemically or physically. The halogenated flame retardants, including PBDEs, work by trapping high energy, free radicals that are produced during the combustion process, thereby slowing fire propagation. Halogenated organic molecules are effective materials for the storage and delivery of halogens in polymers. While all four halogens (iodine, bromine, chlorine, fluorine) are effective at binding to free radicals during combustion, bromine and chlorine are optimal due to their range of thermal stability and trapping efficiencies. Fluorine is very stable and decomposes at a higher temperature than polymers burn, while iodine is not very stable and decomposes at relatively low temperatures. Bromine is often preferred to chlorine because it has a higher trapping efficiency (larger) and is liberated from hydrogen bromide over a narrower temperature range than hydrogen chloride. Thus, bromine is available at higher concentrations than chlorine during fires (Alaee et al. 2003; Troitzsch 1998).

1.4 Flame Retardant Use Estimates

As of 2007, the amount of flame retardants used globally was estimated at ~1.8 million metric tons (Fink et al. 2008). While the phase-out in the U.S. and restrictions in Europe have limited the use of some brominated flame retardants, including PBDEs,

their consumption in Asia has grown substantially. There are four main manufacturers of brominated flame retardants: Albemarle Corporation, Chemtura, ICL Industrial Products, and Tosoh Corporation. Because there are only a limited number of manufacturers, information on production amounts are not often published. The Bromine Science and Environmental Forum (BSEF), which is an advocacy group representing the four producers, provided early estimates of use but have not updated these figures in over a decade. However, a number of market research firms have evaluated more recent sales (e.g., SRI Consulting). They estimated that brominated flame retardants accounted for only ~23% of the global market but almost half of the Asian consumption in 2007. The decaBDE commercial mixture, which is a main focus of research here, and tetrabromobisphenol A (TBBPA) are the dominant brominated flame retardants with 2007 global consumption estimated at ~73,000 metric tons (~161 million pounds) and ~230,000 metric tons (~507 million pounds), respectively (Fink et al. 2008; Posner et al. 2011). The U.S. EPA has published industry-reported figures of ~56,000 metric tons (83% decaBDE) consumed globally in 2003 (EPA 2010). The use of brominated flame retardants in Asia is projected to increase by ~7% per year based largely on increased usage in China; global increases in the use of all flame retardant

chemicals are projected at ~3-7% per year through the end of 2020 (BCC-Research 2009; Ceresana 2011; Fink et al. 2008).

1.5 PBDE Flame Retardants

PBDEs can have from 1-10 bromine atoms substituted on diphenyl ether (Figure 1). There are 209 PBDE congeners theoretically possible depending on the number and substitution patterns of bromine. In practice, the number of PBDE congeners formed is limited based on the commercial mixtures and its chemical properties (de Wit 2002). Three major PBDE commercial mixtures have been produced (Table 1): PentaBDE, OctaBDE, and DecaBDE (ICPS/WHO 1994). The PentaBDE product is a heterogeneous mixture of tetra-, penta-, and hexaBDEs that has been added mostly to polyurethane foams and textiles, and to a lesser extent in epoxy and phenolic resins and polyesters. The vast majority (~95%) of PentaBDE was used in the US in the manufacture of polyurethane foams in cushioning where it could constitute up to 30% of these products (Hale et al. 2002). The production and use of PentaBDE and OctaBDE were phased out in the U.S. and were banned in the European Union in 2004. In 2009, these products were also listed as persistent organic pollutants (POPs) under the Stockholm Convention (UNEP 2009).

The DecaBDE mixture (Saytex 102E) contains the fully brominated congener decabromodiphenyl ether (BDE-209; ~97%) with trace amounts of nonaBDEs. It is often used as an additive in high impact polystyrene, polyolefins, and polypropylene used in electronic equipment (e.g., plastic housing), automobiles, airplanes, and construction and building materials (e.g., wires, cables, pipes). It is also applied to textiles in upholstered furniture used in public spaces. To achieve U.S. fire safety standards for plastics (UL94¹ V-0 flammability standards), high impact polystyrene plastics usually contain ~10-15% of DecaBDE coupled with ~4-5% of the synergist antimony oxide (Weil and Levchik 2007). In the U.S., DecaBDE is scheduled for a voluntary production phase-out at the end of 2013. However, there continue to be concerns from end users (e.g., automobile manufacturers, U.S. Defense Department) about viable replacements. The states of Maine, Maryland, Oregon, Vermont, and Washington have also imposed restrictions on DecaBDE use. In the European Union, DecaBDE has been restricted from use in electrical and electronic equipment since 2008 under the EU Restriction of

¹ Industrial standards that refer to section 94 of Underwriter Laboratories, Inc., requirements covering tests for the flammability of plastic materials for use in electronic devices and appliances.

Hazardous Substances Directive (Eur.Parliament 2008). At present, DecaBDE is not subject to restrictions in any Asian countries.

Table 1: PBDE commercial mixtures (Alaee et al. 2003; La Guardia et al. 2006).

Congener (Br #)	% of Mixture	Typical Applications
DecaBDE Mixture (Saytex 102E)		
BDE 206 (9)	2.19	Plastics: high impact polystyrene (HIPS), polyolefins (PE), and polypropylene (PP) End products: electronics/computers, construction and building materials, automobiles, airplanes, textiles
BDE 207 (9)	0.24	
BDE 208 (9)	0.06	
BDE 209 (10)	96.8	
PentaBDE Mixture (DE-71)		
BDE 99 (5)	48.6	Plastic: Flexible polyurethane foam End products: Furniture foam, mattresses, automobile seats
BDE 47 (4)	38.2	
BDE 100 (5)	13.1	
BDE 153 (6)	5.44	
BDE 154 (6)	4.54	
BDE 85 (5)	2.96	
OctaBDE Mixture (DE-79)		
BDE 183 (7)	42.0	Plastic: Acrylonitrile butadiene styrene (ABS) End products: Office equipment, electronics
BDE 197 (8)	22.2	
BDE 207 (9)	11.5	
BDE 196 (8)	10.5	
BDE 153 (6)	8.66	
BDE 203 (7)	4.40	
BDE 171 (7)	1.81	
BDE 180 (7)	1.7	
BDE 206 (9)	1.38	
BDE 209 (10)	1.31	
BDE 154 (6)	1.07	

*Other commercial PBDE mixtures that contain different concentrations of BDEs include Bromkal 705DE (PentaBDE), Bromkal 79-8DE (OctaBDE), and Bromkal 820DE (DecaBDE).

PBDEs are highly persistent and lipophilic with a range of octanol-water partition coefficient (K_{ow}) values (e.g., tetraBDEs K_{ow} ~6-7; pentaBDEs K_{ow} ~7-9; decaBDE K_{ow} ~6-12) (de Wit 2002; Palm et al. 2002). They have low water solubilities ($\sim 10^{-3}$ - 10^{-8} g/m³) and vapor pressures ($\sim 10^{-5}$ - 10^{-11} Pa) and will strongly adsorb to soil, sediment, and biological matrices, as well as to dissolved and suspended particulates in air and water (Palm et al. 2002). The atmospheric half-lives ($t_{1/2}$) of BDE-47, BDE-99, and BDE-209 are estimated at 11, 19, and 318 days, respectively. The estimated half-lives of these oft-detected congeners in water and soils are 150 days and in sediment are estimated to be longer at 600 days (Palm et al. 2002).

1.6 Environmental Exposures to PBDEs

PBDEs are environmentally ubiquitous with ongoing and in many cases increasing trends of contamination detected in wildlife and people (Shaw and Kannan 2009). Like other persistent, hydrophobic chemicals, the most important route of uptake in aquatic animals appears to be by trophic transfer and consumption of PBDE-contaminated foods (Dominguez et al. 2011; Law et al. 2003; Shaw et al. 2009). This dietary exposure pathway in aquatic animals is distinguished from human uptake that

appears to depend on both dietary exposures and the incidental ingestion of PBDE-containing dust (Stapleton et al. 2012; Wu et al. 2007).

The presence of PBDEs in the environment has been studied recently by several research groups (Gauthier et al. 2008; Hale et al. 2006; Hites 2004; Klosterhaus et al. 2012; Law et al. 2008; Schechter et al. 2008; Voorspoels et al. 2006; Zhu and Hites 2004). Despite the U.S. phase-out of PentaBDE, its component congeners are still being detected in humans and the environment (Shaw et al. 2012; Trudel et al. 2011). These congeners frequently detected in humans and wildlife include: BDE-47 (2,2',4,4'-tetraBDE); BDE-99 (2,2',4,4',5-pentaBDE); BDE-100 (2,2',4,4',6-pentaBDE); BDE-153 (2,2',4,4',5,5'-hexaBDE); and BDE-154 (2,2',4,4',5,6'-hexaBDE). These congeners are consistently dominant congeners detected in biota worldwide despite the generally more limited use of PentaBDE outside the U.S. Potential sources of these congeners could be related to the ongoing use/recycling of products that contain PentaBDE, their high environmental persistence, and long-range transport (Law and Herzke 2011). It is also possible that the breakdown of DecaBDE in the environment to more bioaccumulative PBDE congeners is occurring. Finally, this pattern of ongoing exposure and persistence could be related to differences in species toxicokinetics, which is discussed in the next section.

In a meta-analysis of studies conducted from 1986-2003, Hites (2004) found that fish sampled from North American waters had six times higher concentrations of PBDEs than those sampled from European waters; the mean total PBDEs was 310 ng/g lipid in fish from North American waters and 49 ng/g lipid in fish from European waters. More recently, Shaw et al. (2009) measured Σ PBDE concentrations in fish that ranged from 18.3 ng/g lw (alewife; *Alosa pseudoharengus*) to 81.5 ng/g lw (Atlantic herring; *Clupea harengus*) in an analysis of several wild fish species from the Northwest Atlantic. These levels are consistent with bioaccumulation measured in wild fishes targeted at several other sites (Boon et al. 2002a; Jenssen et al. 2007; Johnson-Restrepo et al. 2005; Voorspoels et al. 2003).

Recent attention has focused on the potential for BDE-209 and other highly brominated PBDEs to bioaccumulate. BDE-209 is now the dominant PBDE congener measured in abiotic compartments, typically at $\mu\text{g/g}$ dw to low mg/g dw levels in dust (Stapleton et al. 2012; Wu et al. 2007), soils and sediments (Shaw et al. 2009; Sun et al. 2009; Webster et al. 2008) and biosolids (Hale et al. 2003; Peng et al. 2009). In addition, BDE-209 has been measured as the dominant PBDE in some human and wildlife populations (Bi et al. 2007; Chen et al. 2007; Christensen et al. 2005; Qu et al. 2007). BDE-

209 bioaccumulation has been measured in fishes (Johnson-Restrepo et al. 2005; Shaw and Kannan 2009; Shaw et al. 2012) and other wildlife species, including harbor seals (*Phoca fitulina*) (Shaw et al. 2012), kestrels (*Falco tinnunculus*) (Chen et al. 2007), red foxes (*Vulpes vulpes*) (Voorspoels et al. 2006), and grizzly bears (*Ursus arctos horribilis*) (Christensen et al. 2005). In addition to BDE-209, other highly brominated PBDEs are being detected more frequently in biota, including hexa- to nonaBDEs, which may reflect increasing use of BDE-209 and its environmental breakdown and metabolism by animals (Dominguez et al. 2011; La Guardia et al. 2007; Shaw et al. 2009; Xia et al. 2011). BDE-209 can undergo photolytic degradation (Soderstrom et al. 2004; Stapleton and Dodder 2008), microbial breakdown (Gerecke et al. 2005), and metabolic biotransformation (La Guardia et al. 2007; Stapleton et al. 2004a) to lower MW congeners. BDE-209 and the nona- and octaBDEs have been detected in eggs of herring gulls (Gauthier et al. 2008) and peregrine falcons (*Falco peregrines*) (Chen et al. 2008). For instance, a recent examination of herring gull eggs collected from 1981 to 2006 in the Great Lakes region showed that BDE-209, along with the nona- and octaBDEs, have been rising sharply with doubling times of 2-3 years, 3-11 years, and 2-5 years, respectively

(Gauthier et al. 2008). These rising levels of the higher PBDEs are also measured in some human populations (Thuresson et al. 2006).

With the protracted phase-out of PBDEs, exposures will continue for many years due to existing reservoirs from ongoing product use, disposal, and recycling. Moreover, ongoing PBDE uses in Asia will continue to serve as a source of PBDEs. For instance, people residing in areas of China with heavy E-waste recycling operations report some of the highest PBDE body burdens in the world, with BDE-209 concentrations in serum at up to 3,440 ng/g lipid and median concentrations that are 50-200 times higher than reported in other occupational settings (Bi et al. 2007; Qu et al. 2007).

1.7 Human Health Effects of PBDEs

PBDE toxicity has been examined in several informative reviews (Birnbaum and Staskal 2004; Costa and Giordano 2007, 2011; Darnerud 2008; Legler 2008; Staskal and Birnbaum 2011). The toxicity endpoints most frequently measured in epidemiology studies and laboratory experiments include neurotoxicity, reproductive and developmental impairments, and thyroid system disruption. Less is known about the toxicity of BDE-209 than the other environmentally persistent congeners. However, BDE-209 is the only PBDE that has been evaluated for carcinogenicity and has been

identified as having “suggestive evidence of carcinogenic potential” based on increased thyroid cell hyperplasia and thyroid adenomas/carcinomas in male mice and liver tumors in male rats (NTP 1986).

A limited number of epidemiological studies provide some evidence of neurotoxic outcomes, altered thyroid functioning, and reproductive impairments in the human population that are associated with PBDE exposures. For instance, PBDEs (BDE-47, -99, and -100) measured in umbilical cord blood were found to be correlated with reduced performance of gestationally-exposed children (aged 0-6) on mental performance tests (Herbstman et al. 2010). Consistent with these results, maternal prenatal and childhood PBDE exposures were associated with reduced attention, fine motor coordination, and cognition (declines in IQ scores) among a California cohort of Mexican-American children (Center for Health Assessment of Mothers and Children of Salinas; CHAMACOS) (Eskenazi et al. 2013). In terms of adverse reproductive outcomes, epidemiology studies have measured PBDE associations with: cryptorchidism (Main et al. 2007); early onset of menarche (Chen et al. 2011); decreased testosterone, luteinizing hormone (LH), and follicle stimulating hormone (FSH) in adult men (Meeker et al. 2009); increased estradiol in 3-month old boys (BDE-154) (Meijer et al. 2012); and decreased

sperm counts and testis size in young adults (BDE-153) (Akutsu et al. 2008). Several studies have also demonstrated associations between altered thyroid hormone homeostasis in adults and PBDE levels in serum and house dust (Bloom et al. 2008; Meeker et al. 2009; Stapleton et al. 2011; Turyk et al. 2008).

Results of PBDE toxicity testing in laboratory rodent models align well with the human health epidemiological evidence, lending it biological plausibility. Studies in rodents and *in vitro* assays have demonstrated that some PBDEs (PentaBDE, BDE-47, -99, -100, -209) have anti-androgenic effects (Kuriyama et al. 2005; Stoker et al. 2005; Tseng et al. 2006; van der Ven et al. 2008) and anti-estrogenic effects (Hamers et al. 2006; Lilienthal et al. 2006; Meerts et al. 2001). BDE-47 has been shown to alter reproductive development in gestationally exposed female rats by decreasing folliculogenesis and serum estradiol levels (Talsness et al. 2008). Canton and coauthors have also shown potential inhibitory effects of OH-PBDEs on steroidogenic enzymes, including CYP19 (aromatase) and CYP17 in human placental microsomes (Canton et al. 2008) and human adrenocortical carcinoma cells (Canton et al. 2005; Canton et al. 2006). Several studies have reported PBDE-induced disruption of thyroid homeostasis, including declines in circulating thyroid hormones (Lee et al. 2010; Rice et al. 2007; Richardson et al. 2008;

Tseng et al. 2008); altered thyroid hormone metabolizing enzymes (Butt et al. 2011; Richardson et al. 2008; Szabo et al. 2009), and competitive binding with plasma transporters (Marchesini et al. 2008; Meerts et al. 2000).

Studies in rodents, spanning different laboratories, have demonstrated that PBDEs elicit adverse neurobehavioral outcomes during early development. Erickson et al. (2001) showed that single doses of BDE-47 and BDE-99 at postnatal day (PND) 10 caused dose-dependent effects on Morris water maze learning and spontaneous behavior (locomotion, rearing, activity), with adverse habituation (initial hypoactivity followed by hyperactivity) that worsened with age. Studies by Viberg and coauthors have also shown long-lasting neurobehavioral effects in mice exposed to different PBDE congeners, including BDE-209 (Viberg et al. 2002; Viberg et al. 2003; Viberg et al. 2004, 2007; Viberg et al. 2008). PBDE-related neurological deficits have also been measured in other laboratories (Branchi et al. 2002; Gee and Moser 2008; Kuriyama et al. 2005; Rice et al. 2007; Rice et al. 2009; Suvorov et al. 2009).

Some neurological deficits measured in rodents have been accompanied by reductions in circulating T4 (Kuriyama et al. 2007; Rice et al. 2007). While mechanisms of PBDEs neurotoxicity are unclear, thyroid hormone disruption during development may

be a contributing factor as thyroid hormone deficits and surpluses during early development can cause severe cognitive and motor impairments (Anderson 2008; Oppenheimer and Schwartz 1997). However, neurotoxicity from PBDEs has been observed absent impacts on thyroid hormone regulation, suggesting other mechanistic pathways (Gee et al. 2008). Indeed, a growing body of evidence suggests that PBDE mechanisms of neurotoxicity may operate by several pathways that include disrupted thyroid signaling, altered cholinergic neurotransmissions (Dufault et al. 2005; Johansson et al. 2008); impaired neuronal proliferation/plasticity (Dingemans et al. 2010; Ibhazehiebo et al. 2011; Xing et al. 2009), and oxidative stress (Huang et al. 2010; Tagliaferri et al. 2010).

1.8 PBDE Toxicokinetics

Levels of PBDEs in biota are determined predominantly by their absorption, distribution, metabolism, and excretion (ADME) kinetics. As described below, PBDE toxicokinetic patterns have been shown to vary depending on the PBDE congener, species, life-stage, and route of exposure. PBDE toxicokinetic and toxicity studies conducted to date in fish are summarized in Appendix A.

1.8.1 Absorption

Our understanding of PBDE absorption in fishes is limited to just a small number of studies. Two studies in Northern Pike (*Esox lucius*) by Bureau et al. (2000, 1997) found that BDE-47 was readily absorbed with measured uptake efficiencies of ¹⁴C-BDE-47 at 90-100%, which is consistent with uptake measured in rodents. This group also measured pike uptake efficiencies of BDE-99 and BDE-153 at ~60% and ~40%, respectively, slightly less than uptake detected in rodents. Studies in rodents have measured absorption of BDE-47, -99, and -100 in the range of ~75-90% upon oral administration (Chen et al. 2006; Hakk et al. 2006; Sanders et al. 2006; Staskal et al. 2005) with absorption of BDE-153 and BDE-154 at ~70-80% (Hakk et al. 2009; Sanders et al. 2006).

In contrast, BDE-209 has been found to be less bioavailable in fish than other lower MW PBDEs detected in the environment. For instance, a dietary exposure study in juvenile rainbow trout receiving 7.5-10 mg/kg bw-day of BDE-209 measured bioavailability at 0.2-0.13% and rapid excretion (Kierkegaard et al. 1999). Higher BDE-209 bioavailability was measured at 3.2% in juvenile rainbow trout receiving dietary exposures of BDE-209 at 940 ng/g ww food at 1% bw/day for 5 months (Stapleton et al.

2006). Nyholm et al. (2009) measured uptake efficiencies in zebrafish adults receiving dietary exposures to BDE-209, BDE-183, and BDE-28 at 100 nmol/g ww diet (2% bw/day feeding rate). They found that <1% of BDE-209 and ~10% of BDE-183 were bioavailable, while all (~100%) of the BDE-28 was estimated to be bioavailable. Similar to fishes, the absorption of BDE-209 is relatively low in rodents with a range of results from ~7-26% based on oral and intravenous dosing studies (Huwe and Smith 2007; Morck et al. 2003). Taken together, studies suggest that PBDE absorption efficiency in the vertebrate gastrointestinal system may depend on a combination of passive diffusion (lower MW congeners), facilitated transport, and uptake with lipids and other macromolecules. The extent of the latter two pathways of facilitated transport (e.g., by p-glycoproteins) and uptake with lipids are still not well understood for PBDEs.

1.8.2 Tissue Distributions

The dominant PBDEs measured in humans and wildlife (BDE-47, -99, -100, -153, and -154) are deposited and stored in lipophilic tissue compartments and appear to be generally persistent and differentially metabolized depending on the species and bromine substitution pattern. For instance, as described in the previous exposure discussion, these congeners are detected frequently in human serum, breast tissue and

milk (Petreas et al. 2011; Schechter et al. 2003) and in adipose tissues of a variety of wildlife species, including marine mammals (Law et al. 2003; Shaw et al. 2012). Furthermore, PBDEs have been shown to cross the blood–brain barrier to accumulate in the central nervous system of some birds of prey (Naert et al. 2007). A study of toxicokinetic patterns of BDE-47, -99, -100, and -153 in mice measured higher levels of BDE-153 in lipid tissues than the other congeners tested, suggesting that BDE-153 was more persistent than the other congeners tested (Staskal et al. 2006). These authors also found that BDE-47 disposition patterns in developing mice were similar to those of adults but that bioaccumulation was higher in young mice because of their reduced capacity to excrete BDE-47.

In addition to accumulation in lipid-rich tissues, the liver is an important target organ of disposition and toxicity. The U.S. EPA National Toxicology Program has published findings showing hepatotoxicity, including elevated liver enzyme activity and histopathology (i.e., hepatic hypertrophy and vacuolizations) in mice exposed orally to the PentaBDE commercial mixture for 13-weeks (Dunnick and Nyska 2009). This study and many others (Kuriyama et al. 2007; Lee et al. 2010; Richardson et al. 2008; Staskal et al. 2006; Szabo et al. 2009; Zhou et al. 2001) have demonstrated that the liver is a primary

target organ of PBDEs. The liver also appears to be the target organ of PBDE accumulation in fish. The previously mentioned research by Burreau et al. (2000) in pike measured bioaccumulation of parent ¹⁴C-BDE-47 and putative hydrophobic metabolites in the liver, as well as in lipid rich tissues, perivisceral (abdominal) adipose tissues and vertebrate surrounding tissues. In rainbow trout exposed orally to BDE-209, the highest concentrations of BDE-209 were measured in the liver on both a lipid normalized and wet weight basis, followed by accumulation in the serum, with less lipid normalized accumulation in the intestine and carcass (Stapleton et al. 2006).

The high distribution of BDE-209 to adipose tissues might also be expected given its very low water solubility (<0.1 µg/l) and high log Kow (6-12) (Hardy 2002). However, studies have shown that this pattern of preferential deposition to lipid depots does not occur for BDE-209. Rather, evidence in humans, rodents, and fish points to BDE-209 preferentially distributing to plasma and blood-rich tissues, including the liver, kidney, heart, and intestinal wall (Huwe et al. 2008a; Jin et al. 2009; Morck et al. 2003; Sjodin et al. 2001; Stapleton et al. 2004a). The exact reason for this distribution pattern of BDE-209 is not well understood, but the large size and high MW of BDE-209 are thought to play a

role in its more limited diffusion into muscle tissues and interaction with adipocytes than the other lower MW congeners (Huwe et al. 2008a).

1.8.3 Metabolism in Mammals

Rodent studies that have examined PBDE metabolism have shown the formation of hydroxylated and methoxylated PBDEs in tissues and fecal matter (Chen et al. 2006; Hakk et al. 2009; Huwe et al. 2008b; Malmberg et al. 2005; Marsh et al. 2006; Staskal et al. 2006). BDE-209 is also metabolized by oxidative reactions although to a lesser extent than the lower PBDEs frequently tested (BDE-47,-99, -100, -153) (Huwe and Smith 2007; Morck et al. 2003; Sandholm et al. 2003). Results from these BDE-209 studies suggest that a first step in BDE-209 metabolism in rats may be reductive debromination to form nona- and octaBDEs that is then followed by arene epoxide reactions leading to the formation of OH- and MeO-PBDE intermediates. The formation of brominated phenol intermediates has also been demonstrated that may be attributable to hydrolysis of the PBDE ether linkage between the two phenyl rings. For instance, *in vitro* testing in our laboratory (Stapleton et al. 2009) using human hepatocytes incubated with 10 μ M of BDE-99 showed the formation of 2,4,5-tribromophenol, two mono-OH-pentaBDEs, and an unknown tetraBDE in extracts isolated from hepatocytes treated with BDE-99. The

2,4,5-tribromophenol metabolite was also identified in rats exposed to BDE-99 (Chen et al. 2006). This metabolite formation was accompanied by an increase in relative mRNA transcript abundance of genes encoding CYP1A2 and CYP3A4, *dio 1*, and glutathione-S-transferase M1 (Stapleton et al. 2009).

Consistent with these *in vitro* human results, the rodent literature supports a PBDE metabolic pathway that has two major reactions: (1) a cytochrome P450 (CYP)-mediated epoxidation of the phenyl ring catalyzed predominantly by CYP2B (via constitutive androstane receptor (CAR) inductions) and by CYP3A (via pregnane X receptor (PXR) inductions); and (2) debromination or Phase II conjugation of an OH-intermediate with glucuronides catalyzed by uridine diphosphate glucuronosyl transferases (UGTs) and with sulfates by sulfotransferases (SULTs) (Chen et al. 2006; Letcher et al. 2001; Marsh et al. 2006; Qiu et al. 2007; Staskal et al. 2006; Szabo et al. 2009; Zhou et al. 2001). Glutathione conjugates have also been detected in the Phase II metabolism of BDE-47, -99, and -154 in laboratory rodents, suggesting a role for glutathione-S-transferases (GSTs) in the Phase II metabolism of PBDEs in mammals (Chen et al. 2006; Hakk et al. 2002; Hakk et al. 2009; Sanders et al. 2006). Reductive

debromination reactions appear to be only minor pathways of PBDE metabolism in mammals (e.g., BDE-209 debromination to octa- and nonaBDEs).

Indeed, results of quantitative PCR targeting CYP expression in rodents have demonstrated that CAR and PXR induction leading to *CYP2B* and *CYP3A* expression are favored in the hepatic oxidation of PBDEs (Fery et al. 2009; Pacyniak et al. 2007; Richardson et al. 2008; Sanders et al. 2005; Staskal et al. 2006; Szabo et al. 2009). For instance, elevated mRNA expression of *CYP2B* isoforms has been observed in rodents exposed to BDE-47 (Richardson et al. 2008). In further evidence, BDE-153 elicited a dose-dependent increase in *CYP2B* expression in the liver of rats with relative *CYP2B* mRNA transcripts increased 20- to 30-fold at the highest dose tested (64 mg/kg-day) (Sanders et al. 2005). This study also measured a 6-fold increase in relative *CYP3A* mRNA transcripts at the high dose and only weak upregulation of *CYP1A1*, further supporting that PBDE metabolism and toxicity appear to proceed through non-AhR dependent pathways. Findings in rat pups exposed perinatally to the PentaBDE mixture demonstrated enhanced activity and relative mRNA transcript abundances of *CYP1a1* along with *CYP2b1/2* and *CYP3a1* (Szabo et al. 2009). However, the commercial PBDE mixtures contain small amounts of polybrominated dibenzo-*p*-dioxin (PBDD) and

dibenzofurans (PBDF) impurities that have been shown to mediate most of the AhR/CYP1A1 induction observed (Chen and Bunce 2003; Kuiper et al. 2006; Wahl et al. 2008). Szabo et al. (2009) also measured an upregulation in relative transcript abundances of Phase II metabolizing enzymes, including those encoding *SULT1b1*, *UGT1a6*, *UGT1a7*, and *UGT2b*, as well as an increase in UGT-T4 activity, suggesting potentially enhanced metabolism and elimination of thyroid hormones that are also conjugated by UGTs and SULTs (Visser 1996).

1.8.4 Metabolism in Fish

PBDE metabolism in fishes appears to be substantially different from that observed in mammals. As outlined in Appendix A, several *in vivo* and *in vitro* studies have demonstrated that reductive debromination of PBDEs appears to be the primary route of metabolism in a limited subset of cyprinid and salmonid species, including common carp (*Cyprinus carpio*) (Stapleton et al. 2004a), rainbow trout (*Oncorhynchus mykiss*) (Kierkegaard et al. 1999; Stapleton et al. 2006), lake trout (*Salvelinus namaycush*) (Tomy et al. 2004), Chinook salmon (*Oncorhynchus tshawytscha*) (Browne et al. 2009; Roberts et al. 2011), and zebrafish (*Danio rerio*) (Kuiper et al. 2006).

1.8.4.1 Pathways of Reductive Debromination

While reductive debromination of PBDE congeners appears to be the major metabolic pathway in fish, the role of specific enzyme systems is unclear. A novel pathway that has been hypothesized to mediate reductive metabolism of PBDEs in fish is by the activity of deiodinase enzymes (Stapleton et al. 2004a; Tomy et al. 2004). Deiodinases (dios) are membrane-bound enzymes that are located in the endoplasmic reticulum and regulate thyroid hormones in all vertebrates (Blanton and Specker 2007). There are three known dio isoforms in fish, Types 1, 2, and 3 (dio 1, dio 2, and dio 3, respectively), that share functional homology with mammalian dio forms.

As depicted in Figure 3, the activation of T4 to 3,3',5-triiodothyronine (T3) is catalyzed by the cleavage of iodine from the *meta*-position of the outer phenyl ring of T4. The reductive debromination of PBDEs in fishes has also been shown to be dominated by *meta*-cleavages of bromine, suggesting a possible role for this thyroid hormone metabolizing enzyme in PBDE debromination (Stapleton et al. 2004a; Stapleton et al. 2004b; Stapleton et al. 2004c; Tomy et al. 2004). Other studies have also shown that the *in vitro* debromination of PBDEs is substantially reduced when dithiothreitol (DTT), which

is required for the *in vitro* activation of dio enzymes, is withheld from incubation media (Benedict et al. 2007; Browne et al. 2009; Roberts et al. 2011).

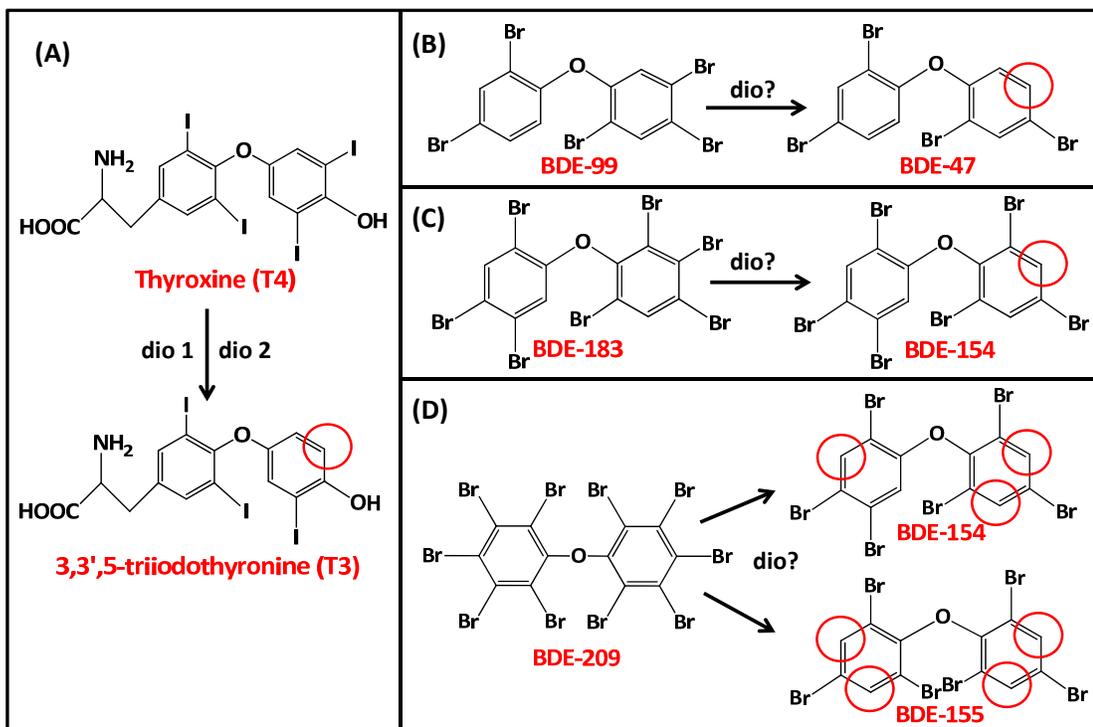


Figure 3: Outer-ring deiodination of thyroxine (A) catalyzed by dio 1 and 2 enzymes resulting in meta-cleavage of iodine from T4. Reductive metabolites and meta-position debromination measured in common carp (*C. carpio*) exposed orally to (B) BDE-99, (C) BDE-183, and (D) BDE-209 (Stapleton et al. 2004a; Stapleton et al. 2004b; Stapleton et al. 2006).

1.8.4.2 Potential Oxidative Metabolism

Laboratory *in vivo* and *in vitro* studies in fish have demonstrated mixed results with regards to the formation of hydroxylated PBDE metabolites. For instance, no OH-

PBDEs were detected in the serum of common carp receiving dietary exposures of a mixture of BDE-28, -47, -99, and -153 (Stapleton et al. 2004c), and no OH-PBDEs were measured in common carp, rainbow trout, or Chinook salmon microsomes incubated with various PBDE congeners, including BDE-209 (Browne et al. 2009; Roberts et al. 2011). *In vivo* and *in vitro* studies in fish have shown both weak induction (Chen et al. 2010; Kuiper et al. 2006) and inhibition (Kuiper et al. 2008; Olsvik et al. 2009) of EROD activity (biomarker for CYP1A activity) upon exposure to individual PBDE congeners.

However, a larger number of studies with individual PBDEs or mixtures of congeners, including results in rodents, have shown no AhR/CYP1A activation (Boon et al. 2002b; Kuiper et al. 2004; Timme-Laragy et al. 2006; Tomy et al. 2004). Thus, the current body of evidence does *not* appear to support that PBDEs are agonists for the AhR or are debrominated by CYP1A1. Moreover, microsomal CYP activation requires reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor for activation. Previous research has demonstrated that PBDE reductive debromination is not dependent on the presence of NADPH in liver and intestine microsomal incubations in common carp, Chinook salmon, and rainbow trout, suggesting that PBDE metabolism

(reductive or oxidative) may not be CYP-mediated in teleosts (Benedict et al. 2007; Browne et al. 2009; Roberts et al. 2011).

In contrast, Zeng et al. (2012) identified 11 hydroxy PBDEs in the serum of juvenile common carp exposed to the PentaBDE mixture but no OH-PBDEs in DecaBDE exposed fish. Munschy et al. (2010) also measured OH-PBDE metabolites in juvenile common sole exposed to a mixture of PBDE congeners (BDE-28, -47, -99, -100, -153, -209). Finally, Feng et al. (2012) measured substantial OH- and MeO-BDE metabolite formation in rainbow trout exposed orally to BDE-209. It is notable that the GC/MS injection techniques used for the PBDE analysis with positive OH-PBDE results were either not specified (Zeng et al., 2012) or employed split/splitless injection (Feng et al., 2012; Munschy et al., 2010). GC/MS splitless injection techniques for PBDE analysis can lead to thermal degradation of parent PBDEs and the formation of byproducts in the GC/MS inlet that may confound identification of methoxy PBDEs (GC/MS derivatives of OH-PBDE metabolites) (Stapleton 2006). Although potential analytical confounders were not addressed in these studies, if oxidative metabolism does occur in fish, it appears to be probably a minor metabolic pathway compared to reductive debromination.

1.8.4.3 Do PBDEs Activate CAR/PXR in Fish?

In rodent models, evidence supports PBDE oxidative metabolism by CYP2B and CYP3A catalyzed by CAR and PXR inductions, respectively. However, similar pathways have not been measured in fish to date. The expression of CYP2B genes in fish and regulatory mechanisms involved in their induction are not well understood. In mammals, phenobarbital (PB) and halogenated aromatic hydrocarbons (e.g., ortho-Cl-substituted PCBs) are strong inducers of CYP2B through activation of the CAR (Honkakoski et al. 1998; Kawamoto et al. 1999). In fish, however, the induction of CYP2B in the presence of PB-type inducers has not been observed, although this isoform has been shown to be mammalian CYP2B-like (Elskus et al. 1994; Iwata et al. 2002; Schlenk et al. 2008). Thus, there appear to be important functional differences between piscivorous and mammalian CAR and CYP2B that may play a role in its lack of induction in PBDE-exposed fish. However, the role of CYP2B in PBDE metabolism has not been evaluated in fish to date.

The lack of clear evidence of oxidative metabolism of PBDEs in fish is contrasted by results with PCBs that share similar chemical structures whereby apparent CYP-mediated metabolism of ortho-Cl-substituted PCBs (i.e., non-coplanar PCBs) and

formation of OH-PCBs has been detected in rainbow trout and some deep-sea species (Buckman et al. 2004; Koenig et al. 2012). The observed oxidative metabolism of non-coplanar PCBs in fish was postulated to be consistent with degradation by CYP2B-like isoforms, although these authors acknowledged that CYP2B inducibility in fish remained an open question. With regard to other AhR-independent mechanisms, the function, role, and tissue distribution of enzymes in the CYP3 gene family are unclear in fish, although the CYP3A isozymes are thought to be among the most versatile with broad substrate affinities (Celandier et al. 1996; McArthur et al. 2003; Schlenk et al. 2008). The potential role of CYP3A isoforms in PBDE metabolism in fishes is unknown.

1.8.4.4 Metabolic Conjugation of PBDEs

The role of Phase II conjugating enzymes in the metabolism of PBDEs by fish also is not well understood. While BDE-glutathione metabolites have been measured in rodents (Hakk et al. 2002) and avian species (Fernie et al. 2005), GSTs have not been found to be involved in the reductive debromination of BDE-99 to BDE-49 in Chinook salmon and of BDE-99 to BDE-47 in common carp, suggesting that they may not play a role in PBDE debromination in fish (Benedict et al. 2007; Browne et al. 2009). This finding was later repeated and confirmed by Roberts et al. (2011) in Chinook salmon,

rainbow trout, and common carp liver microsomes exposed to several PBDE congeners. There has been little research to date to examine the role of UGTs and SULTS in the metabolism of PBDEs in fishes, although there is considerable evidence that these enzymes are important catalytic drivers of Phase II metabolism in fishes (Schlenk et al. 2008). The UGTs are major conjugating enzymes that catalyze the glucuronidation of an array of endogenous and exogenous substrates, including OH-PBDEs in rodents, to more polar, water soluble compounds for elimination. The SULTs catalyze the transfer of the sulfonate from 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to hydroxylated and amine substituents on numerous exogenous and endogenous substrates.

In zebrafish, several UGT and SULT gene isoforms have been characterized with prototypical substrates such as bilirubin, thyroid hormone, estradiol, testosterone, and phenolic xenobiotics (Clarke et al. 1991; Leaver et al. 2007; T Liu et al. 2010; Mortensen and Arukwe 2007). As many as 10 different UGTs are present in zebrafish, with nucleotide similarities to some mammalian UGT1 and 2 gene families (George and Taylor 2002). Two studies have shown a decrease in the relative mRNA abundance of genes encoding *UDPGT1ab* in zebrafish larvae exposed to BDE-209 (Q Chen et al. 2012) and *UGT1* in juvenile Atlantic cod (*Gadus morhua*) exposed to BDE-47 (Olsvik et al. 2009).

This observation may be a compensatory response to reduced thyroid hormone levels in these fish as UGTs are involved in the metabolism of thyroid hormones and maintenance of thyroid hormone homeostasis. The role of UGTs in PBDE metabolism is not understood in fish but a reduction in their expression or activity could play a role in reducing the biotransformation and elimination of PBDEs in fish. Over a dozen SULT isoforms have been cloned and characterized in zebrafish a few of which share limited (<60%) homology with mammalian SULT1 isoforms (T Liu et al. 2010; Ohkimoto et al. 2003; Sugahara et al. 2003). The role of UGTs and SULTs in the metabolism of PBDEs in piscivores is unknown and warrants further study given their broad substrate affinities.

1.8.5 Elimination

Table 2 provides whole body elimination half-lives ($t_{1/2}$) calculated and reported in humans, rodents, and fish for several of the environmentally relevant PBDEs. It is notable that Geyer et al. (2004) estimated apparent half-lives in humans for the tetra-hexaBDEs that are substantially longer than those values reported in rodents and some of the fish data. While these values may reflect species differences, additional research is needed as results by Geyer et al. (2004) were published as an extended abstract as part of the 2004 Dioxin symposium and have not been published to date in a peer reviewed

journal. Conversely, some data measured in rodents suggest relatively short half-lives (e.g., BDE-99) that are incongruous with the human and fish data. Thus, there continues to be a great deal of uncertainty about PBDE elimination half-lives in biota with substantial species variability.

The major route of elimination of PBDEs in rodents is by the fecal route with low levels of excretion in the urine and bile depending on the PBDE congener (Hakk et al. 2002; Hakk et al. 2006; Hakk et al. 2009; Staskal et al. 2005; Staskal et al. 2006). Few studies have examined PBDE elimination in fishes. In one early study, examination of the disposition and metabolism of ¹⁴C-BDE-47 in pike found high levels of radioactivity remained in lipid rich tissues (abdominal and vertebrate surrounding adipose). Even after a 65-day exposure, no detectable clearance of BDE-47 was measured in pike, suggesting high persistence and bioaccumulation potential (Burreau et al. 2000). Elimination half-lives and depuration rates were also highly variable in juvenile lake trout exposed orally to a mixture of 13 PBDE congeners, including BDE-209, over a 56-day exposure and 112-day depuration (Tomy et al. 2004). No clear trend with treatment, log Kow, or the bromine number and position could be identified, suggesting that reductive debromination was a confounder.

Table 2: Whole body elimination half-lives ($t_{1/2}$) of environmentally relevant PBDEs measured and estimated in humans, laboratory rodent models, and fish species.

Congener	Human half-life	Rodent half-life (days)	Teleost half-life (days)
tetraBDE-47	1.5-2.5 years ^a	25 ^c (mouse) 19-30 ^e (rat, tetraBDE)	18-42 (Common carp) ^h 173-519 (Lake trout) ⁱ 12 (Japanese medaka) ^j
pentaBDE-99	1.8-3.4 years ^a	6 ^d (rat)	173-519 (Lake trout) ⁱ
pentaBDE-100	1.3-1.8 years ^a	42-52 ^e (rat, pentaBDE)	46-80 (Lake trout) ⁱ
hexaBDE-153	3.6-12.4 years ^a	50-105 ^e (rat, hexaBDE)	4-23 (Common carp) ^h 154-308 (Lake trout) ⁱ
hexaBDE-154	2.3-4.3 years ^a	undetermined	17-53 (Common carp) ^h 70-208 (Lake trout) ⁱ
heptaBDE-183	94 days ^b	undetermined	173-519 (Lake trout) ⁱ 10-15 (Zebrafish) ^k
BDE-209	11-18 days ^b	2.5 ^f , 8.6 ^g (rat)	21-34 (Lake trout) ⁱ 6.5 (Zebrafish) ^k

^aGeyer et al. 2004; ^bThuresson et al. 2006; ^cStaskal et al. 2005; ^dHakk et al. 2002; ^eUncharacterized tetra-, penta-, and hexaBDEs by Von Meyerinck et al. 1990; ^fSandholm et al. 2003; ^gHuwe and Smith 2007; ^hStapleton et al. 2004c; ⁱTomy et al. 2004; ^jMuirhead et al. 2006; ^kNyholm et al. 2009

1.9 PBDE Toxicity in Fishes

There continues to be only a limited understanding of PBDE biological effects on fish and other wildlife. However, PBDE-related adverse effect studies published to date in fish and wildlife share important similarities with effects measured in human epidemiology and laboratory rodent models, supporting common pathways of toxicity across vertebrates. In particular, a growing body of evidence in fish (Appendix A)

supports that exposures to PBDEs cause neurodevelopmental malformations, thyroid system perturbations, and reproductive impairments, among other potential toxicities (oxidative stress, immune system perturbations).

1.9.1 PBDE Neurotoxicity

Several recent studies have shown impaired neurodevelopment and developmental malformations in zebrafish larvae exposed to PBDEs. Impaired normal motor behavior, inhibited neuronal growth and differentiation, and developmental deformities have been measured in zebrafish (*D. rerio*) larvae exposed to: PentaBDE (L Chen et al. 2012b; Yu et al. 2010); a mixture of BDE-47, -99, -100, -153, and -183 (Usenko et al. 2011); BDE-47 (XJ Chen et al. 2012; Chou et al. 2010; Lema et al. 2007), BDE-49 (McClain et al. 2012); and BDE-209 (He et al. 2011). Lema et al. (2007) noted delayed hatching, neural defects, and cardiac arrhythmias at 168 hours post fertilization in zebrafish embryos exposed to 5 mg/l of BDE-47. In this study, findings of cardiac and developmental toxicity in BDE-47 dosed larvae were coupled with reduced flow rates of cerebrospinal fluid in neural tubes and brain ventricles of the hindbrain. The reduced flow of cerebrospinal fluid was postulated to be caused by the observed deformities (i.e., dorsal curvatures) and is potentially relevant to the etiology of neurotoxicity measured

in this and other studies. In another study, neurodevelopmental impairments in zebrafish larvae exposed maternally to BDE-209 included delayed hatching and motor neuron development, loose muscle fiber deformities and slow locomotion, and hyperactivity with a light/dark photostimulation test (He et al. 2011). Only one study has examined neurotoxicity endpoints in fish species beyond zebrafish. This study, conducted in early life stages of mummichogs (*Fundulus heteroclitus*) detected hindered behavior and learning ability, as well as subtle morphological deformities, including abnormal dorsal curvatures at later life-stages in fish exposed as embryos to the PentaBDE commercial mixture (Timme-Laragy et al. 2006).

1.9.2 PBDE Reproductive Toxicity

PBDE impacts on fish reproduction and reproductive development have been evaluated in a limited number of studies. Reduced survival and fecundity have been measured in zebrafish larvae exposed orally to a purified (no PBDDs/PBDFs) PentaBDE commercial mixture (Kuiper et al. 2008). Reduced spawning was observed in adult fathead minnow male/female pairs exposed orally to ~14 µg/fish of BDE-47 for 25 days, with reproduction completely ceased within 10 days of exposure (Muirhead et al. 2006). The impaired reproduction measured may have been attributable to selective toxicity in

male fathead minnows as they were noted to have decreased mature spermatozoa and reduced condition factors. Sex-specific differences in reproductive responses to PBDEs have been observed in other studies as well. Lema et al. (2008) measured a decline in mature spermatozoa, but no effect on fecundity, in adult male fathead minnows exposed orally to ~6 µg/fish of BDE-47 for 21 days. Similarly, a decline in spermatogenesis was measured in male Chinese rare minnows (*Gobiocypris rarus*) exposed aqueously to BDE-209 (Li et al. 2011). Related to this evidence of PBDE impacts on male gametogenesis, a dose-dependent increase in relative mRNA transcripts of genes encoding vitellogenin (Vtg; egg yolk precursor protein) and zona radiata protein (eggshell protein) were measured in hepatocytes of juvenile male Atlantic salmon (*Salmo solar*) exposed to BDE-47 and a mixture of congeners (BDE-47, 153, -154) (Softeland et al. 2011). These *in vitro* results in Atlantic salmon suggest a possible role for PBDEs in estrogenicity and feminization of male fish. However, some of these *in vitro* findings have not been observed in *in vivo* studies of juvenile Atlantic salmon exposed to the PentaBDE commercial mixture (Boon et al. 2002b) or in zebrafish larvae exposed to BDE-47 (Chen et al. 2010), whereby no changes in the relative expression of mRNA transcripts encoding Vtg protein were detected. Taken together, studies that have examined PBDE

effects on fish reproduction and reproductive development have presented mixed evidence of reduced fecundity and survival in offspring as well as impaired fertility and reproduction, particularly among male fish, with PBDE-induced declines in sperm counts and feminization playing potential roles.

While a growing body of evidence suggests that PBDE exposures may be impacting the reproductive health of fish (and other vertebrates), there have been few studies that have examined interactions between thyroid and reproductive functioning in fishes. Thus, important questions remain as to whether PBDE effects on reproduction are being mediated directly and/or indirectly via altered thyroid hormone homeostasis. In mammals, for instance, both hypothyroidism and hyperthyroidism have been shown to impair reproductive physiology and lower fertility (Hatsuta et al. 2004; Krassas et al. 2002; Krassas and Pontikides 2004). An early review by Cyr and Eales (1996) described important interactions between thyroid hormone regulation and reproductive physiology in fishes. More recently, studies in goldfish and zebrafish suggest that thyroid hormones may have important inhibitory effects on teleost reproductive functioning at different levels of the hypothalamic-pituitary-gonadal (HPG) axis, including by: inhibiting pituitary luteinizing hormone (LH) and follicle stimulating

hormone (FSH); and reducing steroidogenesis and gonadal aromatase expression (Habibi et al. 2012; CS Liu et al. 2010; Nelson and Habibi 2009). The limited research to date that has examined PBDE effects on fish reproduction has revealed reduced spermatogenesis in exposed males (Lema et al. 2008; Li et al. 2011; Muirhead et al. 2006). Less is known about the role of PBDE-induced hypothyroidism in potential reproductive toxicity in fishes. Thus, it remains unclear whether PBDE effects on reproduction are mediated by directly impairing HPG functioning or alternatively whether these effects are mediated indirectly or in cross-talk with perturbations of the hypothalamic-pituitary-thyroid (HPT) axis.

1.10 PBDE Effects on Thyroid Regulation

Because PBDEs, particularly the OH-PBDEs, are similar in structure to thyroid hormones, including T4, T3, and 3,3',5'-triiodothyronine (reverse T3), concerns have been raised about their effects on thyroid system functioning (Birnbaum and Staskal 2004; Darnerud 2003; Hakk and Letcher 2003). The thyroid system is regulated by the HPT axis in all vertebrates and acts permissively to regulate numerous physiological processes, including development, growth, metabolic homeostasis, and reproduction

(Anderson 2008; Blanton and Specker 2007; Gereben et al. 2008b; Kapoor et al. 2012; Kohrle et al. 2005; Zhang and Lazar 2000).

1.10.1 Thyroid Regulation in Fish

Informative reviews by Browne and Eales, among others, propose that understanding xenobiotic effects on the fish thyroid system requires examination of perturbations at both the central HPT axis and in peripheral tissues (Brown et al. 2004; Eales and Brown 1993). T4 is the prohormone of the genomically active hormone T3, and its biosynthesis and regulation are under negative feedback control (Figure 4).

Reductions in circulating thyroid hormone are compensated for by release of thyroid-releasing hormone from the hypothalamus, which in turn increases thyroid stimulating hormone (TSH) release from the pituitary, ultimately stimulating thyroid hormone production.

Thyroid hormones are synthesized in thyroid follicles of vertebrates with secretion in fish thought to be largely limited to T4 (Eales et al. 1999). Thyroid hormones circulate in plasma bound to thyroid hormone binding proteins, including albumin, thyroid binding globulin (TBG), and transthyretin (TTR). Plasma lipoproteins are also important plasma transporters of thyroid hormones in fishes (Eales and Brown 1993).

Most thyroid hormone is bound to protein with only a small amount (<1%) thought to be free and available for uptake into cells. The transport of thyroid hormones into and out of cells is mediated largely by membrane bound transporters, including the high affinity monocarboxylate transporter 8 (MCT8) and organic anion transporter proteins (e.g., OATP1c1), among others, depending on the species and tissue type (Arjona et al. 2011; Popovic et al. 2010; van der Deure et al. 2008; Visser et al. 2008; Visser et al. 2011).

Once in the cell, T4 can be deiodinated to the bioactive T3 hormone or inactivated to rT3 or 3,3'-diiodothyronine (T2) (Figure 5). Deiodination is catalyzed by iodothyronine deiodinase (dio) enzymes of which three isoforms (dio 1, dio 2, and dio 3) have been identified in fish that appear to be functionally homologous to mammalian dios. Dio enzymes are mostly associated with the endoplasmic reticulum and therefore can be isolated in the microsomal fraction of cells (Eales et al. 1999). In all vertebrates, dio 1 and dio 2 enzymes catalyze T4-ORD to produce the active T3 hormone, while dio 1 and dio 3 catalyze T4-IRD to inactive rT3. Thus, dio 1 can be involved in both ORD and IRD of T4 (Figure 5). In addition, T3-IRD and rT3-ORD pathways can further inactivate hormone to T2 isoforms (Gereben et al. 2008a; Kohrle 2002).

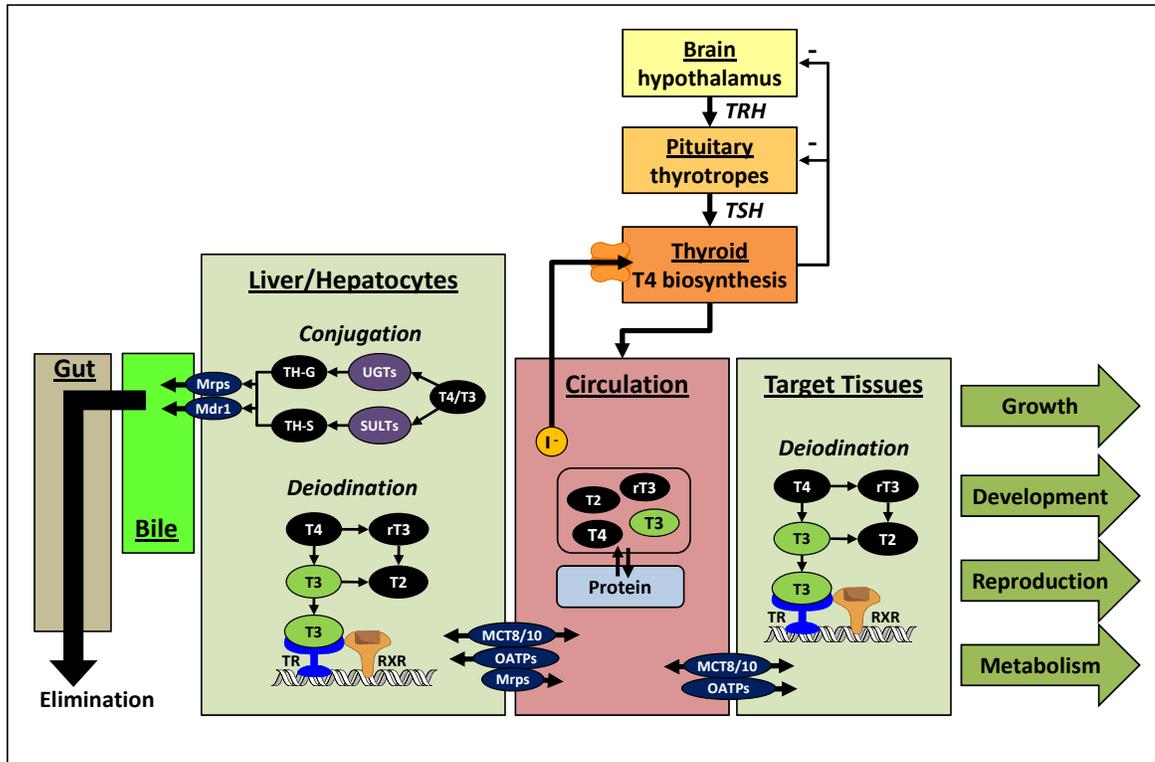


Figure 4: Overview of thyroid hormone regulation and signaling in piscivores. (TRH = thyrotropin releasing hormone; TSH = thyroid stimulating hormone; T4 = thyroxine; T3 = 3,3',5-triiodothyronine; rT3 = 3,3',5'-reverse T3; T2 = 3,3'-diiodothyronine; UGT = uridine diphosphate glucuronosyl transferase; SULT = sulfotransferase; TH-G = glucuronidated thyroid hormone; TH-S = sulfated thyroid hormone; Mrp = multidrug resistance associated protein; Mdr1 = multidrug resistance protein 1 or P-glycoproteins; MCT = monocarboxylate transporter; OATP = organic anion transport protein; TR = thyroid receptor; RXR = retinoic x receptor (Brown et al. 2004; Eales and Brown 1993).

While dio enzymes in fish and mammals are believed to share many functional features, their relative tissue distributions vary, which may have implications for their role in thyroid hormone regulation (Eales et al. 1999; Gereben et al. 2008b; Kohrle 2002; Mol et al. 1998; Orozco and Valverde-R 2005; Orozco et al. 2012). Dio 1 has been localized in the kidneys and liver of both fish and mammals (as well as in the mammalian thyroid gland) (Kohrle 2000; Orozco et al. 2003). However, hepatic T4-ORD activity is thought to be catalyzed mostly by dio 1 in mammals and dio 2 in fish (Gereben et al. 2008b; Orozco and Valverde-R 2005).

Dio 2 is not thought to play as important role in T4-ORD in the mammalian liver but rather it is found to be most active in the brain, pituitary, and brown adipose tissue (Kohrle 2002). A notable characteristic of dio activity patterns in fish is the low T4-ORD activity observed in the fish brain. Early studies that measured deiodination in rainbow trout attributed reduced brain T4-ORD in fish to a lack of or only negligible dio 2 expression, suggesting that T3 may be delivered to the fish brain largely by blood circulation (Eales et al. 1999; Frith and Eales 1996). More recent evidence using quantitative real-time PCR techniques, however, has localized mRNA expression of

genes encoding dio 2 in brains of fishes, although at relatively lower levels than in other tissues (liver) (Johnson and Lema 2011; Sutija et al. 2003; Wambiji et al. 2011).

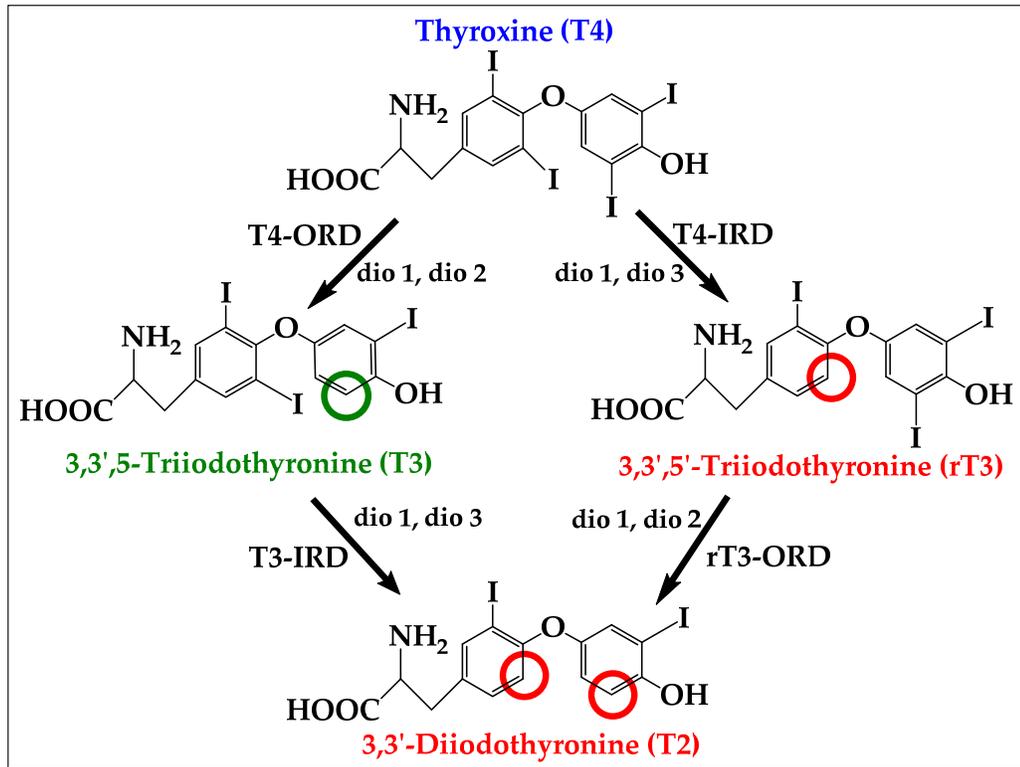


Figure 5: Pathways of outer and inner ring deiodination of thyroid hormones in extrathyroidal peripheral tissues of vertebrates (Kohrle 2002).

In fish, the homeostatic activation and inactivation of thyroid hormone (Figure 5) is thought to occur largely in peripheral tissues as opposed to being mediated at the

central HPT axis. The central HPT of fishes is believed to function largely to maintain T4 homeostasis by a negative feedback mechanism (Brown et al. 2004; Eales et al. 1999). Thyroid hormones can also be further conjugated in the liver to glucuronides or sulfates catalyzed by UGTs and SULTs, respectively, and secreted through the bile and urine (Finsson et al. 1999). The genomic action of T3 is mediated by complexing with nuclear thyroid receptors (TRs) that act as ligand-bound transcription factors that bind to thyroid hormone response elements (TREs) of hormone responsive genes (Yen 2001; Zhang and Lazar 2000).

1.10.2 PBDE Thyroid Toxicity

Laboratory studies in fish have shown declines in thyroid hormones and altered thyroid hormone signaling in some species upon exposure to PBDEs (Lema et al. 2008; Tomy et al. 2004). In one of the earlier studies, Tomy et al. (2004) observed decreased circulating levels of free T4 and T3 in lake trout (*Salvelinus namaycush*) dosed with 13 PBDE congeners (e.g., BDE-28, -47, -99, -100, -153, -154, -209) at 2.5 ng/g and 25 ng/g per congener for 56 days.

More recently other laboratory fish studies have observed PBDE-induced declines in circulating thyroid hormones accompanied by altered thyroidal gene

expression. Fathead minnows that received dietary exposures of BDE-47 at 2.4 and 12.3 µg/breeding pair-day for 21 days showed decreased total T4 (TT4) but not total T3 (TT3) that was accompanied by elevated mRNA transcripts encoding TSH β in the pituitary of low dose fish (Lema et al. 2008). In addition, elevated and reduced levels of mRNA transcripts encoding *TR α* and *TR β* , respectively, were detected in the brain but not the liver, suggesting that the adult fish brain may be a sensitive target of BDE-47 (Lema et al. 2008). Results by Lema et al. (2008) and Tomy et al. (2004) are consistent with observations in European flounder (*Platichthys flesus*) whereby declines in circulating TT4 with no change in TT3 were detected after a 101 day exposure to the PentaBDE commercial mixture purified of PBDDs/PBDFs (Kuiper et al. 2008).

In early life-stages of fish, BDE-209 exposures have been reported to reduce circulating levels of free T4 and T3 in juvenile rainbow trout (Feng et al. 2012). Declines in whole fish T4 have also been reported in zebrafish larvae exposed aqueously to BDE-209 (Q Chen et al. 2012) and the PentaBDE mixture (Yu et al. 2010). In a subsequent study, however, Yu et al. (2011) showed the opposite results with elevated whole fish T4 and T3 in zebrafish offspring exposed to PentaBDE at the same dose as their previous study (Yu et al., 2010) but by a different route (maternal) and longer duration (5

months). The opposing effects of PentaBDE on thyroid hormone levels that was measured in the Yu et al. (2010-11) zebrafish work demonstrate potentially important differences in PBDE impacts on thyroid signaling that may depend on exposure pathway and duration (e.g., aqueous, short-term vs. maternal, chronic). Chen et al. (2012) also measured an increase in whole-fish T3 but this increase was accompanied by a decline in whole-fish T4 in zebrafish larvae exposed aqueously to BDE-209 exposed fish.

BDE-209 and the PentaBDE mixture also have been found to enhance relative mRNA expression of genes encoding *dio 1* and *dio 2* in zebrafish larvae (Q Chen et al. 2012; Yu et al. 2010). In the Chen et al. (2012) study, an upregulation in relative mRNA abundances was measured in several genes involved in thyroid functioning, including those encoding the sodium iodide symporter (NIS), thyroglobulin (TG) and other transcription factors regulating NIS and TG expression (Nkx2.1a, Pax8). Li et al. (2011) also measured an increase in *dio 2* transcripts in the larvae of Chinese rare minnows exposed to BDE-209, but a decrease in *dio 2* transcripts in the brains of BDE-209 exposed adult rare minnows. Thus, taken together, studies in fish provide evidence that some PBDE congeners cause depressed thyroid hormone levels and altered expression of

genes that encode proteins involved in thyroid hormone signaling (e.g., CRH, TSH β , TR α , dio1, dio2) possibly as a compensatory response to hypothyroidism. However, results of studies conducted to date are limited with mixed results and additional work is needed to clarify PBDE effects on the thyroid system, particularly with regard to BDE-209 for which less thyroid-related research has been undertaken.

1.10.3 Mechanisms of Thyroid Toxicity

While not well understood, several mechanisms have been posited to play a role in the perturbed thyroid hormone regulation measured in fish (and other vertebrates) exposed to PBDEs including: interferences with dio enzyme activity and expression; enhanced metabolism and elimination of thyroid hormones; altered expression and activity of plasma transporters and membrane bound transporters; and altered genomic signaling.

1.10.3.1 Interference with Dio Enzymes

The relative distribution and induction of dio enzymes are pivotal to the maintenance of thyroid hormone homeostasis. Moreover, since PBDEs closely resemble thyroid hormones, and because iodine and bromine are both halogens, it is reasonable to deduce that PBDEs could alter the activity of these enzymes. As described in the

previous sections on PBDE metabolism, studies in our laboratory (Roberts et al. 2011; Stapleton et al. 2006; Stapleton et al. 2009) and by others (Benedict et al. 2007; Browne et al. 2009) implicate dio enzymes in PBDE fish metabolism. Thus, one of the mechanisms that might be contributing to thyroid perturbations in fish (and other vertebrates) could be interference of PBDEs with the expression and activity of dio enzymes. PBDEs may be acting as competitive substrates for dio enzymes or otherwise altering the expression and activity of these enzymes. However, it remains unclear whether PBDEs (or OH-PBDEs) can bind directly to dio enzymes or whether they may elicit other allosteric effects on these enzymes that affect their capacity to mediate thyroid hormone regulation. In addition, PBDE interferences with deiodinases may occur indirectly as compensatory responses to other thyroid perturbing events caused by PBDEs, such as increased thyroid hormone elimination by UGT inductions in rodents.

1.10.3.2 Induced Thyroid Hormone Metabolizing Enzymes

Indeed, another hypothesis for PBDE-induced thyroid dysfunction is that exposures catalyze the metabolism and excretion of thyroid hormones due to enhanced activity of phase II metabolizing enzymes. Declines in plasma thyroid hormones may be attributable to PBDE detoxification responses inducing elevated expression and activity

of thyroid hormone catabolizing enzymes. In support of this hypothesis, studies have demonstrated increased mRNA expression of thyroid hormone-conjugating UGT and SULT transferase enzymes in rodents exposed to BDE-47 and the PentaBDE commercial mixture (Richardson et al. 2008; Szabo et al. 2009; Zhou et al. 2002). Declines in circulating levels of TT4 with PBDE exposures have been linked to enhanced glucuronidation associated with the induction of UGTs (Skarman et al. 2005; Szabo et al. 2009; Zhou et al. 2001; Zhou et al. 2002). Other studies, however, have shown little to no change in UGT levels in rodents following exposure to PBDEs despite decreased T4 levels (Hallgren et al. 2001; Richardson et al. 2008), although increased mRNA expression of UGT transcripts has been observed in some of this work (Richardson et al. 2008). In contrast to some of the rodent evidence, a limited number of studies in fish have shown declines in relative *UGT* mRNA transcript levels suggesting that a compensatory response of these thyroid hormone metabolizing enzymes could be occurring in PBDE exposed fish (Q Chen et al. 2012; Olsvik et al. 2009).

1.10.3.3 Altered Thyroid Hormone Transport

Another mechanism hypothesized to be underlying PBDE-related reductions in thyroid hormones is that these xenobiotics are affecting thyroid hormone and hepatic

cellular transport proteins. The OH-PBDE metabolites produced in rat liver microsomes enriched with CYP2b (i.e., phenobarbital-induced) have been found to compete with thyroid hormones for binding to the blood transport protein transthyretin, potentially leading to greater elimination of thyroid hormone (Meerts et al. 2000). Likewise in teleosts, several parent PBDEs (BDE-28, -49, -47, -99) have been shown to be potent inhibitors of T3 binding to transthyretin in recombinant sea bream transthyretin whereas 6-OH-BDE-47 had less affinity for sea bream TTR than T3 or T4 (Morgado et al. 2007).

The mechanisms by which PBDEs enter the liver (and other target tissues) are not well understood but are *not* thought to be attributable to passive diffusion given their relatively high MWs (485-1000 Da). Recent research has shown that some Phase III transporter proteins may play important roles in the cellular transport of PBDEs. For instance, organic anion transport proteins (OATPs) have been shown to be potentially important transporters responsible for the accumulation of PBDEs in the liver. OATPs mediate sodium independent uptake of numerous substrates, including xenobiotics, bile acids, and thyroid hormones. BDE-47, -99, and -153 have been shown to be effective substrates for human OATP1B1, OATP1B3, and OATP2B1 expressed in Chinese hamster ovary (CHO) cells (Pacyniak et al. 2010). OATP1B1 and OATP1B3 were found to

transport BDE-47 with the highest affinity, while OATP2B1 was found to transport all three tested congeners with similar affinities. This same research group has also studied the affinity of some PBDE congeners for mouse hepatic OATPs by using human embryonic kidney cells transiently expressing individual OATPs to quantify uptake of BDE-47, -99, and -153. OATP1a4, OATP1b2, and OATP2b1 transported all three tested PBDEs with BDE-47 at the highest affinity; OATP1a1 was found to transport none of the congeners tested (Pacyniak et al. 2011). Consistent with work by Pacyniak et al. (2010), Szabo et al. (2009) also measured an upregulation in the relative mRNA expression of genes encoding OATP1a4 in rat pups exposed to PentaBDE. OATP1a4 belongs to the ABC cassette superfamily, is regulated by CAR and PXR, and transports bile acids, xenobiotics, and thyroid hormones (Hagenbuch and Gui 2008).

PBDEs have also been shown to affect the Phase III hepatic efflux transporters, P-glycoproteins (i.e., Pgp; Mdr1) and multidrug resistance-associated proteins (Mrps). Pgp and Mrp transporters also are members of the ABC binding cassette superfamily, are regulated by the AhR, CAR, and PXR, and play important roles in the efflux of xenobiotics and thyroid hormones into the bile for elimination. The study by Szabo et al. (2009) with PentaBDE commercial mixture in young rats measured an increase in

relative mRNA transcripts encoding Mdr1, Mrp2, and Mrp3. The increase in transcription of Mdr and Mrp transporters was postulated in this study to have been a detoxification response to PBDE exposures. In contrast, Richardson et al. (2008) detected a significant decrease in relative mRNA transcript levels for Mdr1a and no change in transcription for Mdr1b in adult female mice exposed to BDE-47. Authors here postulated that the decline in Mdr1a transcripts in adults could have been a compensatory response to declines in circulating TT4, which were also measured.

1.10.3.4 PBDEs as Thyroid Receptor Agonists/Antagonists

The genomic activity of T3 is regulated by its binding to TRs that then act transcriptionally with nuclear TREs (Flamant et al. 2006). A handful of studies in fish and other vertebrates have provided compelling evidence that PBDE exposures could be mediating effects on TR signaling. This altered signaling may be playing a role in other PBDE toxicological outcomes, including the measured neurotoxicity in vertebrates. As mentioned, thyroid hormone receptors are encoded by two primary genes, α and β , which can result in different functional TR isoforms that vary with species and developmental life-stage (Bradley et al. 1989; Flamant et al. 2006; Lebel et al. 1993; Weinberger et al. 1986). Two genetically distinct receptors $TR\alpha$ and $TR\beta$ have been

identified in fathead minnows with additional subtypes identified in teleosts that may be attributable to gene duplication or alternative mRNA splicing (Filby and Tyler 2007; Lema et al. 2009; Nelson and Habibi 2009). In rats, three functional TRs (TR α 1, TR β 1, and TR β 2) have been characterized that are capable of binding T3 (Flamant et al. 2006).

Questions continue as to whether xenobiotics can bind directly to TRs with mixed results concerning whether PBDEs and OH-PBDEs may competitively bind to TRs (Crofton 2008; Ren and Guo 2013). For instance, several PBDEs and OH-PBDEs were examined in a rat pituitary cell-line to assess their competitive binding with TRs (Kitamura et al. 2008). In this study, 4-OH-BDE-90 and 3-OH-BDE-47 inhibited T3-binding to the TR, while BDE-138, BDE-209, 4'-OH-BDE-49, 4-OH-BDE-42, 4'-OH-BDE-17, and 3'-OH-BDE-7 showed no TR affinity. This study by Kitamura et al. (2008) suggested that hydroxy moieties in the 3 or 4 position of the phenyl ring along with two adjacent bromine substituents were necessary structural requirements for OH-PBDE to bind to TRs. These structural attributes were somewhat confirmed in a QSAR model (Li et al. 2010) that demonstrated that hydrogen bonding characterized interactions between OH-PBDEs and TR β with OH-PBDEs exhibiting significantly higher thyroid hormone activities than parent PBDEs. In addition, consistent with results by Kitamura et al.

(2008), 4-OH-BDE-90 was also shown to be a weak TR antagonist using a reporter gene assay in CHO cells (Kojima et al. 2009). Other studies in rat pituitary GH3 cell proliferation assays showed that BDE-127 and BDE-185 were TR agonists while BDE-206 was a TR antagonist (Hamers et al. 2006; Hamers et al. 2008; Schriks et al. 2006). Frietas et al. (2011) also showed that 4-OH-BDE-69 and 4-OH-BDE-121 were TR agonists. Another recent reporter gene assay study using CV-1 monkey fibroblast-derived cells showed TR antagonistic activity for BDE-209, -153, -154, -100, and the PentaBDE commercial mixture (Ibhazehiebo et al. 2011). In this study, none of the OH-PBDEs tested (6-OH-BDE-47, 4'-OH-BDE-49, 2'-OH-BDE-68) affected TR-mediated gene expression. These results are contrasted by work published around the same time showing binding between TR β 1 and 18 OH-PBDEs in a yeast two-hybrid assay (Li et al. 2010) and other studies described here showing competitive binding of OH-PBDEs to TRs (Freitas et al. 2011; Kitamura et al. 2008; Kojima et al. 2009). However, consistent with results by Ibhazehiebo et al. (2011), none of these studies have demonstrated that the OH-PBDE binding has altered TR-mediated gene transcription. Thus, taken together, the PBDEs and OH-PBDEs have demonstrated both TR agonistic and antagonistic activities as well as no interaction with the TR pathway.

In fish, Lema et al. (2008) showed a decline in relative mRNA expression of *TR β* in brains of adult fathead minnows exposed to BDE-47. Declines in TR transcript expression in brains of fathead minnows could have been a response to PBDE-induced declines in circulating T4, which was also measured, and/or could have been related to interactions between BDE-47 and TRs or their corepressors (Lema et al. 2008). In addition, a decline in thyroid hormone responsive gene expression was observed in this study with reduced relative abundance of brain transcription element binding protein (BTEB), which is a transcription factor involved in neurogenesis. Similarly, in primary rat cerebellar granule cell cultures, BDE-99 was found to disrupt *TR α 1* and *TR α 2* mRNA transcript levels and functioning by disrupting T3-responsive gene transcription (e.g., brain-derived neurotrophic factor) and increasing the production of reactive oxygen species (Blanco et al. 2011). Finally, a study with CV-1 cell cultures measured suppressed TR binding with TREs through the DNA binding domain (versus between THs and TRs) upon exposure to several PBDEs and their hydroxylated metabolites, with BDE-209 showing the greatest suppression at the lowest dose (Ibhazehiebo et al. 2011). The suppressed TR-TRE binding was then shown to inhibit TH-dependent dendrite arborization of cerebellar Purkinje cells, suggesting TR-TRE mediated impacts on PBDE

neurotoxicity. While limited in number, these studies demonstrate potentially important mechanistic pathways that start with altered nuclear thyroid regulation that then produce downstream impacts on neurological functioning and development.

1.11 Thesis Objective and Experimental Approach

The overall objective of this thesis research was to answer fundamental questions concerning how fish, as important indicators of overall environmental health, are metabolizing the PBDEs and how these metabolic pathways might be disrupting thyroid hormone regulation. The central hypothesis of this research was that PBDE metabolism in fish is mediated by dio enzymes and that PBDE exposures will cause thyroid system dysfunction across early and adult life stages of fish. To test this hypothesis, the following three study aims and experimental approaches were designed and performed:

Specific Aim 1: Characterizes the *in vitro* hepatic biotransformation of BDE-99 to congeners with fewer bromine atoms in common carp [Chapter 2]. A series of *in vitro* experiments was conducted using carp liver microsomes and cytosolic fractions to determine the primary metabolic location, biotransformation kinetics, and potential enzyme systems involved in PBDE biotransformation.

Specific Aim 2: Measures the *in vivo* toxicokinetics of BDE-209 in juvenile fathead minnows; and examines BDE-209 impacts on thyroid regulation and liver morphology [Chapter 3]. This study measured PBDE accumulation and metabolism in juvenile fathead minnows treated with BDE-209 by the diet. In addition, several *in vitro* assays were conducted to evaluate BDE-209 impacts on *dio* activity. Finally, BDE-209 related effects on thyroid and liver morphology were examined histologically.

Specific Aim 3: Measures the *in vivo* toxicokinetics of BDE-209 in adult fathead minnows; and examines BDE-209 impacts on thyroid hormone regulation and underlying mechanisms of thyroid dysfunction [Chapter 4]. Very little is known about how PBDEs cause thyroid toxicity in fishes. Therefore, the purpose of this study was to evaluate BDE-209 dietary treatments, at two environmental relevant exposures, on the central and peripheral thyroid systems of adult male fathead minnows. BDE-209 bioaccumulation and reductive metabolites were measured along with: effects on circulating thyroid hormone levels; relative *dio* mRNA expression and activity in the brain and liver; and mRNA expression of genes encoding the nuclear thyroid receptors and several membrane-bound transporters from the MCT and OATP families. Because previous studies have demonstrated that PBDEs may impair vertebrate reproduction,

BDE-209 effects on fish gonadal size (via gonado-somatic index; GSI) was measured as an initial metric of reproductive fitness.

Chapter 5 discusses major findings and limitations of this research thesis and provides ideas for future research directions. It focuses special attention on how the data generated fit into findings from the peer reviewed literature and advance our understanding of PBDE effects on biota.

2. Characterizing the *In Vitro* Hepatic Biotransformation of the Flame Retardant BDE 99 by Common Carp²

Polybrominated diphenyl ethers (PBDEs) are a class of persistent and bioaccumulative flame retardant chemicals that are known to biomagnify in aquatic foodwebs. However, significant biotransformation of some congeners via reductive dehalogenation has been observed during *in vivo* and *in vitro* laboratory exposures, particularly in fish models. Little information is available on the enzyme systems responsible for catalyzing this metabolic pathway in fish. This study was undertaken to characterize the biotransformation of one primary BDE congener, 2,2',4,4',5-pentabromodiphenyl ether (BDE-99), using *in vitro* techniques. Hepatic sub-cellular fractions were first prepared from individual adult common carp (*Cyprinus carpio*) to examine metabolism in both microsomal and cytosolic sub-cellular fractions. Debromination rates (i.e. BDE-99 biotransformation to BDE-47) were generally higher in the microsomal fraction than in the cytosolic fraction, and some intra-species variability was observed. Further experiments were conducted to determine the biotransformation

² Noyes PD, Kelly SM, Mitchelmore CL, Stapleton HM. 2010. Characterizing the *in vitro* hepatic biotransformation of the flame retardant BDE-99 by common carp. *Aquatic Toxicology* 97(2): 142-150.

kinetics and the influence of specific co-factors, inhibitors and competitive substrates on metabolism using pooled carp liver microsomes. The estimated K_m and V_{max} values were 24.7 μM and 1,140 $\text{pmol hr}^{-1} \text{mg protein}^{-1}$, respectively. However, substrate saturation may not have been achieved due to limitations in BDE 99 availability and solubility. Iodoacetate (IaC) and the two thyroid hormones, reverse triiodothyronine (rT3) and thyroxine (T4), significantly inhibited the debromination of BDE-99 in microsomal sub-cellular fractions with IC_{50} values of 2.2 μM , 0.83 μM , and $>1.0 \mu\text{M}$, respectively. In addition, debromination appears to be dependent on the presence of the co-factor dithiothreitol (DTT), suggesting a cysteine residue at the enzyme(s) active site(s), requiring a reducing environment to maintain activity. These results support our hypothesis that deiodinase enzymes may be catalyzing the metabolism of PBDEs in fish liver tissues. Further studies are needed to evaluate metabolic activity in other species and tissues that contain these enzymes.

2.1 Introduction

Polybrominated diphenyl ethers (PBDEs) are flame retardant chemicals applied to numerous types of polymers and resins to reduce their flammability. Because PBDEs are added and not chemically bound to these matrices, they can leach from products and

accumulate in indoor and outdoor environments (Alaee et al. 2003; de Wit et al. 2006; Hale et al. 2006). PBDEs encompass as many as 209 different congeners, containing from one to ten bromine atoms substituted around a diphenyl ether backbone, similar to polychlorinated biphenyls (PCBs). PBDEs have different physical and chemical properties dependent largely on the number and substitution of bromine atoms. Generally, BDE congeners with six or fewer bromine atoms are more bioaccumulative and persistent than the higher brominated congeners (Birnbaum and Staskal 2004; Burreau et al. 1997; Stapleton et al. 2004c; Tomy et al. 2004).

Several studies have investigated the bioaccumulation, metabolism and disposition of BDE congeners in different animal models (Burreau et al. 2000; Hakk et al. 2002; Morck et al. 2003; Staskal et al. 2006). Metabolism of BDE congeners in rodents appears to occur primarily through oxidative, cytochrome P450-mediated pathways (CYPs) that generate hydroxylated metabolites (e.g. OH-BDEs) similar to the metabolism of some PCB congeners (Letcher et al. 2001; Marsh et al. 2006; Qiu et al. 2007). In fish, however, studies that have examined the accumulation and metabolism of BDE congeners suggest that metabolism primarily occurs by a reductive dehalogenation pathway (Kierkegaard et al. 1999; Stapleton et al. 2004b; Stapleton et al. 2004c; Tomy et

al. 2004). No hydroxylated metabolites have been observed in PBDE- exposed fish. Recently, we confirmed with *in vivo* and *in vitro* testing that debromination of BDE congeners does occur through metabolic pathways in common carp (*Cyprinus carpio*) (Benedict et al. 2007; Stapleton et al. 2006). Recent studies have also found evidence to support reductive debromination of BDEs in rats (Huwe and Smith 2007), lactating cows (Kierkegaard et al. 2007), and European starlings (Van den Steen et al. 2007), suggesting that this metabolic pathway also occurs in mammals and birds, albeit likely as a minor pathway compared to oxidative routes.

While reductive debromination of BDE congeners is a major metabolic pathway in fish, the involvement and role of specific enzyme systems are unknown. Previous work demonstrated that debromination of an environmentally relevant BDE congener, 2,2',4,4'-pentabromodiphenyl ether (BDE-99), occurs in carp and forms 2,2',4,4'-tetrabromodiphenyl ether (BDE-47), which accumulates in carp tissues (Stapleton et al. 2004c). A follow-up study confirmed that this metabolic pathway occurs in carp liver and intestinal tissues, was not dependent upon the presence of the co-factor NADPH, and verified that debromination was not a function of gut microfauna (Benedict et al. 2007). However, no information is available on the sub-cellular location of PBDE

debromination in fish hepatocytes (e.g. endoplasmic reticulum, cytosol, etc.). This is often a useful first step in identifying enzyme system(s) responsible for xenobiotic metabolism.

Major enzyme systems often responsible for the metabolism of xenobiotics include the CYPs that catalyze a majority of Phase I reactions and some isoforms of glutathione-S-transferases (GSTs), which are major Phase II conjugating enzymes (Di Giulio et al. 1995). Studies have shown induction of CYP1A (Stapleton et al. 2009; Timme-Laragy et al. 2006; Wahl et al. 2008) while others have shown no response or inhibition of CYP1A in PBDE-exposed animals (Boon et al. 2002b; Kuiper et al. 2004). Moreover, induction of other CYP isoforms has been observed in rodents, including CYP2B (Richardson et al. 2008). Hepatic microsomal CYPs require NADPH and have broad substrate specificity allowing for oxidative and reductive metabolism of a spectrum of endogenous and xenobiotic agents. However, as stated previously, Benedict et al (2007) observed that carp debromination of BDE 99 was not dependent upon the presence of NADPH in the incubation buffer. Thus the probability that PBDE debromination is catalyzed by CYPs seems unlikely. BDE-glutathione metabolites have been observed in rodents (Hakk and Letcher 2003) and avian species (Ferne et al. 2005),

suggesting a possible role for GSTs, which are a supergene family that protect organisms from a variety of endogenous and xenobiotic agents. GSTs act by conjugating reduced glutathione to electrophilic centers on endogenous and exogenous compounds, and are also involved in transporting compounds through the cytoplasm (Di Giulio et al. 1995; Schlenk et al. 2008). They can act by oxidative and reductive pathways and are widely distributed in cytosolic, microsomal, and mitochondrial loci with several different isoforms.

Because the metabolism of BDE 99 is not well characterized, additional approaches are needed to elucidate enzymes involved in PBDE debromination. Alternate approaches include identifying the sub-cellular locations of metabolism, withholding specific enzyme co-factors, and adding enzyme inhibitors and substrate competitors. This study was undertaken to further characterize the metabolism of BDE-99 in common carp liver tissue using *in vitro* techniques to help differentiate the roles of suspected enzyme systems, including the GSTs and deiodinases (dios). Our previous research has implicated dio enzymes in catalyzing debromination of BDE congeners in carp and rainbow trout (Benedict et al. 2007; Stapleton et al. 2004c; Stapleton et al. 2006). The three known isoforms of dio (dio 1, dio 2, and dio 3) are typically associated with

the microsomal fraction and regulate intracellular levels of thyroid hormones, such as thyroxine (T4) and reverse triiodothyronine (rT3), by catalyzing deiodination (Baqui et al. 2000). This process parallels the metabolism of BDE-99 (structurally similar to T4; Figure 6) to BDE-47 observed by Stapleton et al. (2004b) whereby a bromine atom is selectively removed (i.e. debromination) from the meta-position of the diphenyl ring.

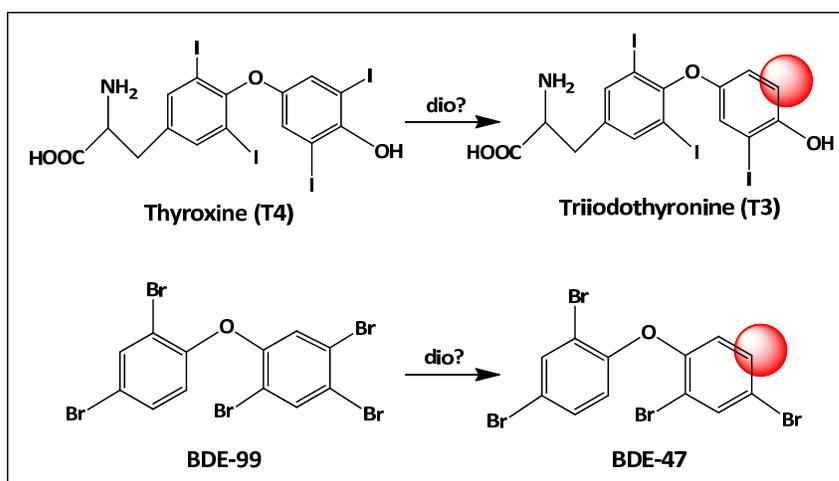


Figure 6: Thyroxine (T4) conversion to T3 by deiodinase (DI) enzymes in cells, and similarities to the reductive dehalogenation of BDE-99 to BDE-47 observed *in vitro* and *in vivo* in common carp hepatic tissue

The specific objectives of this study were: to compare the metabolism of BDE-99 in carp liver microsomal and cytosolic sub-cellular fractions; to determine the maximum velocity (V_{max}) and the Michaelis constant (K_m) to describe the biotransformation kinetics

of BDE-99 metabolism in carp; and to characterize enzyme(s) modulating metabolism by comparing the influence of specific co-factors, enzyme inhibitors, and putative competitive substrates (e.g. thyroid hormones) on the biotransformation rate of BDE-99.

2.2 Materials and Methods

2.2.1 Animals

Common Carp (*Cyprinus carpio*) were purchased as juveniles from Hunting Creek Fisheries (Thurmont, MD) and reared at the Chesapeake Biological Laboratory in Solomons, MD for over a year prior to this study. Using approved animal care guidelines, carp were reared in tanks with flow-through ambient water (18-22 °C) and a 12-hour photoperiod, and were fed 3-5% of their body weight daily with standard pelleted Koi food. Seven adult male carp were randomly selected for this study. The fork length of the animals ranged from 243-297 mm and their body masses ranged from 445-673 g. Fish were sacrificed and their livers dissected, washed in a phosphate buffer, and immediately frozen in liquid nitrogen for transport (on dry ice) to Duke University.

2.2.2 Materials

Internal and surrogate standards (¹³C labeled 2,2',3,4,5,5'-chlorinated diphenyl ether (¹³C-CDE-141) and 4'-fluoro-2,3',4,6-tetrabromodiphenyl ether (F-BDE-69) used in

this study were purchased from Chiron (Trondheim, Norway). PBDE quantification standards were purchased from Accustandard (New Haven, CT). All solvents used throughout this study were High Performance Liquid Chromatography (HPLC) grade.

2.2.3 Hepatocyte sub-cellular fraction preparations

Following mincing and thorough rinsing in ice cold 0.150 M KCl (pH 7.4), liver tissue was homogenized on ice using a Potter-Elvehjem tissue grinder in 4x (w/v) of washing buffer (0.25 M sucrose, 0.1 M Tris-hydrochloric acid (HCl), 1 mM Ethylenediaminetetraacetic acid (EDTA), 10 mM dithiothreitol (DTT) (pH 7.4)). In one experiment, described below, DTT was excluded from the washing buffer to evaluate its role in BDE-99 metabolism. Homogenates were centrifuged at 10,000 x g for 20 minutes at 4 °C. The resulting supernatant (or S9 fraction) containing both cytosolic and microsomal proteins was either further processed or flash frozen in liquid nitrogen and stored at -80 °C until use. To separate the microsomal pellet and cytosolic supernatant, the S9 fraction was centrifuged at 105,000 x g for one hour at 4 °C. Cytosolic fractions were immediately flash frozen in liquid nitrogen and stored at -80 °C, while the microsomal pellet was resuspended in ice cold 0.15 M Tris base buffer (pH 8.0) and centrifuged again at 105,000 x g for one hour at 4 °C to remove remaining cytosolic

proteins. The final microsomal pellet was homogenized in the tissue washing buffer plus 20% glycerol (pH 7.4). The amount of re-suspension buffer added to the microsomal pellet was equivalent to 1 mL/g of liver tissue. The resulting microsomal fraction was flash frozen and stored at -80 °C until use.

As quality control to verify the viability of all three types of sub-cellular fractions, an assay was conducted for measuring GST enzyme activity toward 1-chloro-2,4-dinitrobenzene (CDNB). As various isoforms of GST exist in both microsomal and cytosolic fractions, CDNB is a relatively nonspecific reference substrate for most GST isoforms. The spectrophotometric method of Habig and Jakoby (1981) was employed using a 96-well microplate reader, performed at 25 °C in a reaction buffer containing 1 mM of reduced glutathione (GSH) (pH 6.5). Following addition of 1 mM CDNB, absorbance was measured at 340 nm for 5 min. GST activity toward CDNB ranged from 140 - 236 nmol min⁻¹ mg protein⁻¹ in microsomes, 148 - 448 nmol min⁻¹ mg protein⁻¹ in cytosol, and from 278 - 441 nmol min⁻¹ mg protein⁻¹ in S9 fractions. These values are all within normal ranges for carp, suggesting the fractions were viable (Taysse et al. 1998).

2.2.4 BDE-99 incubations with sub-cellular fractions

All incubations were conducted in glass test tubes and contained either 900 μL or 950 μL of incubation buffer, 100 μL or 50 μL of the appropriate sub-cellular fraction, and 354 pmol of BDE-99 for a total volume of 1 mL/incubation. The enzyme kinetic experiment used varying substrate concentrations. The buffer used for all the incubations consisted of 0.1 M potassium phosphate (K_2HPO_4), 0.1 M sodium phosphate (NaH_2PO_4), and 100 μM NADPH (pH 7.4). In addition, all incubation buffers contained 10 mM DTT, with the exception of one experiment investigating DTT's role in BDE-99 debromination. All incubations were conducted for 60 minutes at 25°C in a water bath with continuous shaking at 140 rpm. The timing of all incubations began with the addition of sub-cellular fractions. Non-enzymatic controls used in the experiments included inactivated sub-cellular fractions (i.e. immersed in boiling water for 10 min) treated with BDE-99 and incubated alongside the active sub-cellular fractions. At the conclusion of the incubation period, 1 mL of ice cold methanol was added to halt the reactions. Samples were stored at 4°C until extraction for analysis. Protein concentrations of all the carp hepatocyte fractions ranged from 10.86 – 21.54 mg/mL and were determined with a bicinchoninic acid assay (BCA) kit (Pierce, Rockford, IL) using

bovine serum albumin to generate a standard curve. Concentrations of metabolites formed were normalized to time and protein concentration.

Three different sets of incubations were undertaken. In the first experiment, microsomal and cytosolic fractions isolated from the liver tissue of 7 juvenile carp were incubated with BDE-99 to evaluate the sub-cellular site of metabolic debromination and intra-species variability. For each specimen, 50 μL of microsomes or cytosol were incubated in 950 μL of incubation buffer solution and 354 pmol of BDE-99. This BDE-99 treatment was selected based on incubation conditions from our previous *in vitro* work in carp hepatocytes and to minimize exposure to the acetone diluents used (Stapleton et al. 2006).

In the second set of incubations, pooled carp liver microsomes (from the specimens in the first experiment) were exposed to a range of substrate conditions in triplicate to determine the V_{max} and K_{m} values of BDE-99 biotransformation to BDE-47. Substrate concentrations ranged from 1 – 250 μM of BDE-99. In addition, BDE-99 incubations at the 50 μM treatment were extended to 16 and 24 hours in triplicate to evaluate catalytic debromination of BDE-99 over time. The exact concentration of individual treatment solutions of BDE-99 were confirmed using gas chromatography

mass spectrometry operated in electron capture negative ionization mode (GC/ECNI-MS).

In the final set of incubations, hepatic microsomes from the pooled carp livers were challenged with and without enzyme inhibitors, substrate competitors, and *in vitro* DI cofactors to gain insight into the identity of metabolizing enzymes. Each incubation contained 900 μL of incubation buffer and 100 μL of hepatic microsome incubated with 354 pmol of BDE-99. The enzyme inhibitors used in the challenge were 10 mM of propylthiouracil (PTU) and 0.1 – 100 mM of iodoacetate (IaC). The substrate competitors used were 0.001 – 2.0 μM of reverse triiodothyronine (rT3) and 0.0001 – 1.0 μM of thyroxine (T4). The *in vitro* DI cofactor used was 10 mM of DTT. We also examined the possibility that GSTs may be involved in the biotransformation of PBDEs in carp liver. Since purified GST enzymes from carp are not available, we investigated the effect of rT3 on GST catalytic activity in carp liver microsomes to determine whether rT3 may inhibit its activity, similar to the inhibition of BDE-99 metabolism observed. Using the CDNB assay described in Section 2.3, GST activity was measured in buffers containing rT3 in concentrations ranging from 0 - 2.0 μM .

2.2.5 Sample Extractions

All samples were spiked with 50 ng of a recovery standard (F-BDE-69) and subjected to a liquid-liquid extraction procedure using hexane and sulfuric acid. Three mL of hexane was added to each glass test tube containing the aqueous sample. Test tubes were vortexed thoroughly and then centrifuged at 2000 rpm for 10 minutes to facilitate separation of the aqueous (bottom) and organic (top) layers. The organic layer was transferred to another clean glass tube and the extraction repeated twice more. To remove lipids and other biogenic material from the samples, the organic extractions were then treated with 3 mL of concentrated sulfuric acid followed by vortexing and centrifugation at 2000 rpm for 10 minutes. The organic layer was removed and the sulfuric acid was back-extracted with an additional 3 mL of hexane. The final combined extract was concentrated to a volume of 0.5 mL using a rapid evaporation system with ultra-high grade nitrogen gas and 50 ng of an internal recovery standard (¹³C-CDE-141) was added in preparation for analysis.

2.2.6 Sample Analysis

All samples were analyzed using GC/ECNI-MS. Extracts were analyzed for a suite of 12 BDE congeners ranging from tri- to penta-BDE. A 0.25 mm (I.D.) x 15 m fused silica capillary column coated with 5% phenyl methylpolysiloxane (0.25 µm film

thickness) was used for the separation of BDE congeners. Pressurized temperature vaporization (PTV) injection was employed in the GC. The inlet was set to a temperature of 50°C for 0.3 minutes and then a 700°C/min ramp to 275 °C was employed to efficiently transfer samples to the head of the GC column. The oven temperature program was held at 40°C for 1 min with the following temperature ramping parameters: 18°C/min to 250°C; 1.5°C/min to 260 °C; and 25 °C/min to 300 °C, which was held for an additional 20 min. The transfer line temperature was maintained at 300°C and the ion source was held at 200°C. The tri- through penta-BDE congeners were quantified by monitoring bromide ions (m/z 79 and 81).

2.2.7 Quality Assurance

Recovery of F-BDE-160 averaged $80.5 \pm 20\%$. BDE-99 and BDE-47 were detected in laboratory blanks; however, levels were low enough to avoid blank correction of samples. Limits of detection (LOD) were determined by taking three times the standard deviation of laboratory blanks. For congeners not detected in blanks, the LOD was set at the instrumental detection limit (IDL).

2.2.8 Data Analysis

The rate of BDE-47 metabolite formation was determined in all treatments and the data are presented as units of pmol of BDE-47 produced hour⁻¹ mg protein⁻¹. Data were analyzed using StatView 5.0 (SAS, Inc., Cary, NC) and Microsoft Excel (2007) with statistical significance defined at the $p < 0.05$ level. Differences in the formation rate of BDE-47 among individual carp were evaluated using a single factor ANOVA and Fisher's Protected Least Significant Difference (PLSD) post hoc test for multiple pairwise comparisons. We also used t-tests to test for significant differences among active and inactive fractions, including those fractions incubated with inhibitors, cofactors, and competitive substrates. V_{\max} and K_m values in pooled liver microsomal fractions were calculated using SigmaPlot 9.0 (Point Richmond, CA) and non-linear and linear regression analysis, and ligand binding models with additional confirmatory line-fitting analysis using Microsoft Excel (2007).

2.3 Results

2.3.1 Sub-cellular biotransformation among carp

Significant ($p < 0.05$) debromination was observed among all the individual specimen incubations of BDE-99 with microsomal and cytosolic fractions relative to heat

inactivated fractions (Figure 7). No BDE-99 debromination was observed in heat inactivated incubations. Biotransformation rates in the microsomal fractions ranged from 39.6 ± 6.5 to 167.03 ± 17.23 pmol BDE-47 hour⁻¹ mg protein⁻¹ (mean \pm SEM; n=3). In the cytosolic fraction, biotransformation rates ranged from 9.34 ± 0.58 to 89.3 ± 21.46 pmol BDE-47 hour⁻¹ mg protein⁻¹ (mean \pm SEM; n=3).

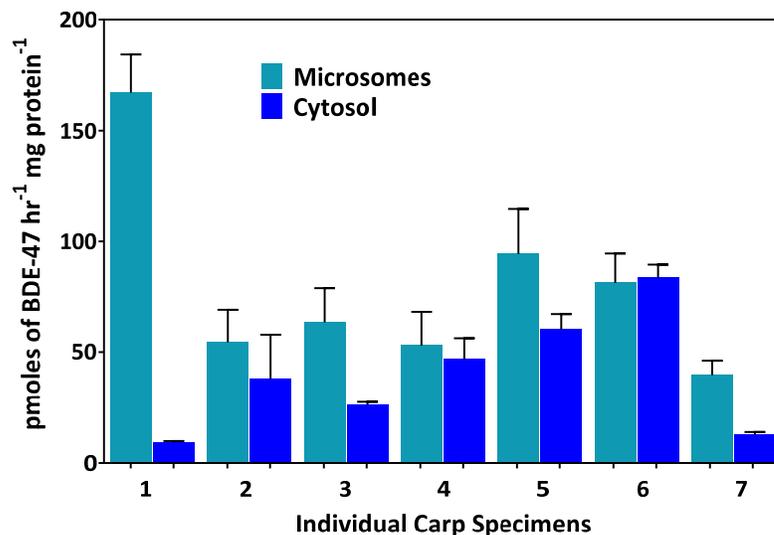


Figure 7: *In vitro* biotransformation rates of 354 pmol of BDE-99 incubated for 60 minutes at 25°C with microsomal and cytosolic sub-cellular fractions prepared from livers of 7 adult carp (mean \pm SEM; n=3).

The rate of BDE-47 formation was significantly higher ($p < 0.05$) in the microsomal fraction than in the cytosolic fraction of fish 1, 3, 5, 7, whereas no differences were detected in the other three specimens. The biotransformation rate of BDE-99 was 15X

higher in the microsomal fraction relative to the cytosolic fraction in specimen 1, and was 1.5X – 3.5X higher in the other specimens. Significant ($p < 0.05$) intra-species variability in debromination rates was observed among some of the carp specimens (e.g., fish 1 and 7), although there was also no difference in BDE-47 formation among some individuals (e.g., fish 2 and 4).

2.3.2 Enzyme Kinetics

The V_{\max} and K_m values for BDE-47 formation were at least $1,140 \pm 72.7$ pmol BDE-47 hour^{-1} mg protein^{-1} and 24.7 ± 6.13 μM BDE-99, respectively, in pooled carp microsomes incubated with BDE-99 at concentrations ranging from 1 – 250 μM (the highest concentration we could test given the material available). As seen in Figure 8, the observed biotransformation rates increased with BDE-99 concentrations following first-order kinetics, which was potentially followed by zero-order kinetics upon enzyme saturation of the substrate. However, it is possible that we did not achieve full substrate saturation as the linear and nonlinear regression analyses of the data were equally robust.

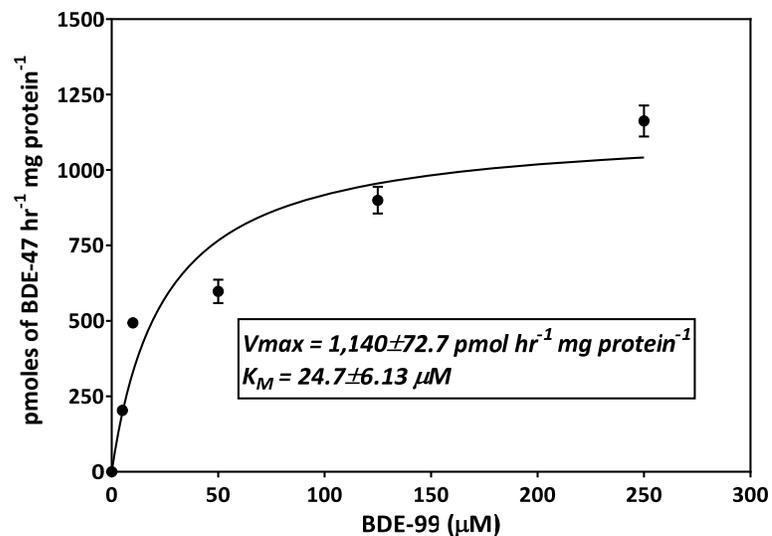


Figure 8: *In vitro* biotransformation rates of BDE-47 in pooled carp liver microsomes incubated in 1 - 250 μM of BDE-99 for 60 minutes at 25°C.

It is notable that other tetraBDE congeners, BDE-66 (2,3',4,4'-tetraBDE) and BDE-49 (2,2',4,5'-tetraBDE), as well as small amounts BDE-28/-33 were measured in incubations containing $>125 \mu\text{M}$ of BDE-99. In the 250- μM treatment group, approximately 23% and 6% of the debromination products by mass were BDE-66 and BDE-49, respectively, while approximately 4% was either BDE-28 or BDE-33 (these congeners co-elute using our GC/ECNI-MS method) (Figure 9). In the 125- μM treatment group, approximately 14% and 5% of the metabolites measured were BDE-66 and BDE-49, respectively. These results have been blank corrected to account for small amounts

(<1%) of these congeners observed in our dosing solutions; however, it is important to note that these congeners were not observed in our heat-inactivated controls, suggesting they were formed via metabolism. At lower substrate concentrations (1 – 50 μM of BDE-99), BDE-47 dominated nearly all the metabolite profile with only negligible amounts of these other congeners detected. Sustained velocity of BDE-47 formation was also measured at approximately $600 \text{ pmol hr}^{-1} \text{ mg protein}^{-1}$ in the $50\text{-}\mu\text{M}$ treatment group subjected to extended incubations for up to 24 hours (maximum time) (Figure 9).

2.3.3 Effects of Inhibitors, Substrate Competitors, and Cofactors

IaC had a significant ($p<0.05$) effect on the biotransformation rate of BDE-99 in pooled liver microsomes (Figure 10). The concentration that inhibited the debromination by 50% (half-maximal inhibitory concentration or IC_{50}) was approximately 2 mM IaC. No inhibition of BDE-47 was observed in pooled hepatic microsomal fractions challenged with 10 mM PTU (data not shown). However, BDE-99 metabolism dropped significantly ($p<0.05$) when DTT was removed from the incubation buffer and only retained in the homogenization buffer of pooled hepatic microsomes. Specifically, with DTT in the homogenization and incubation buffers of pooled microsomal fractions, the rate of BDE-47 formation was $120 \pm 7 \text{ pmol hr}^{-1} \text{ mg protein}^{-1}$, whereas removing DTT

from the incubation buffer but retaining it in the homogenization buffer reduced the BDE-47 formation rate to 5.3 ± 1.1 pmol hr⁻¹ mg protein⁻¹.

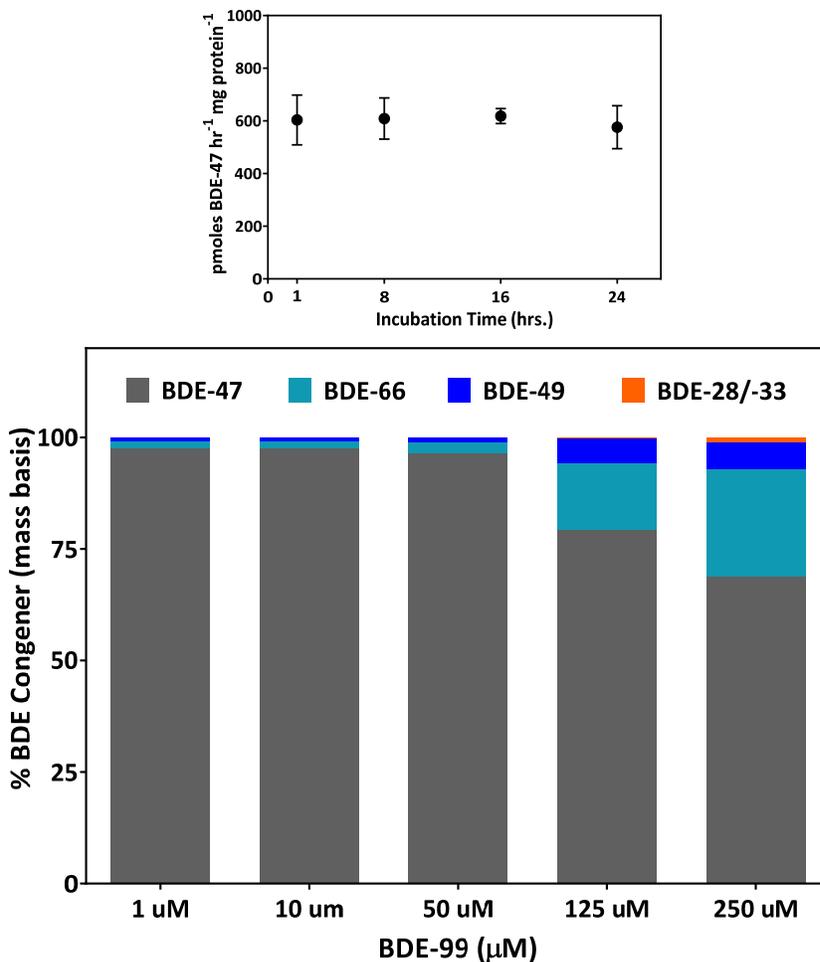


Figure 9: Percent composition of major metabolites measured in pooled carp liver microsomes incubated for 60 minutes at 25°C in 1 - 250 μM of BDE-99. [Inset: Biotransformation rates (mean ± SEM; n=3) of 50 μM of BDE-99 incubated for 1, 8, 16, and 24 hours at 25°C in pooled carp liver microsomes.]

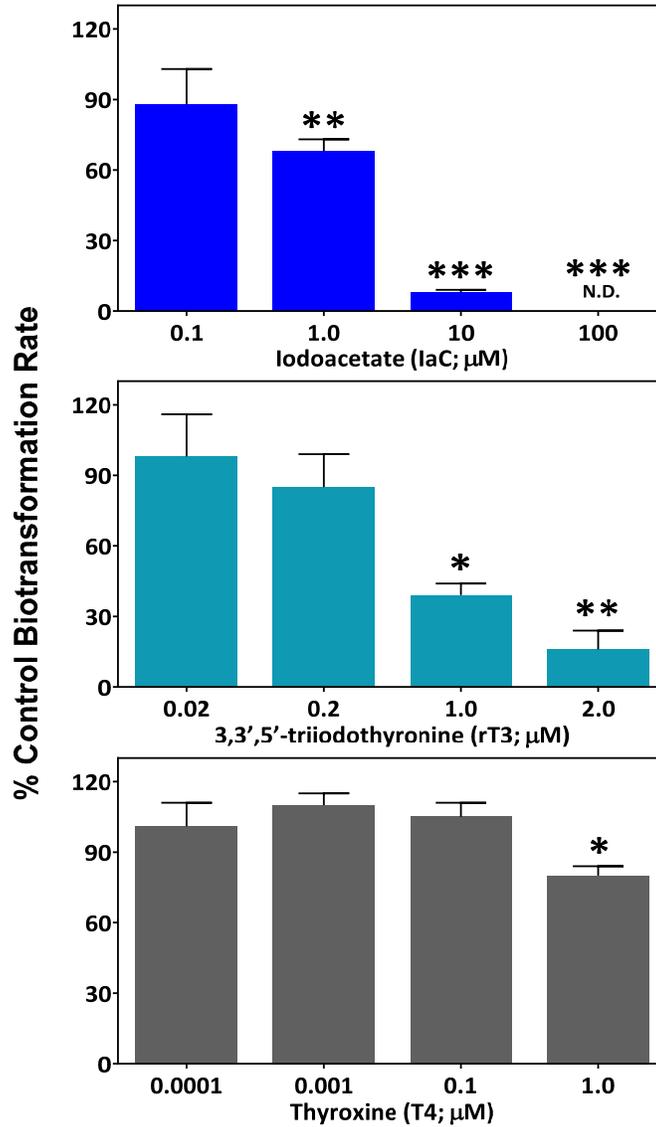


Figure 10: BDE-99 biotransformation rates (% control) in pooled carp microsomes co-incubated in the presence of IaC, rT3, or T4 (mean ± SEM; n=3). Asterisks indicate data points that were significantly different than controls ($p < 0.05$).

As shown in Figure 10, both T4 and rT3 substrate competitors had significant ($p < 0.05$) effects on the BDE-99 biotransformation rates when added to the incubation buffer. Specifically, incubations with 200 nM of rT3 reduced the metabolite formation rate, but only incubations with 1.0 and 2.0 μM rT3 were significant ($p < 0.05$). In the T4 treatment, only the highest level tested, 1.0 μM , had a significant ($p < 0.05$) effect on the metabolite formation rate. Based on these assays, the IC_{50} value calculated for rT3 was 0.83 μM , whereas the value for T4 was greater than 1.0 μM . Finally, microsomal GST activity (as measured by the CDNB assay) was found to be relatively consistent among all rT3 treatments tested in pooled carp liver microsomal fractions and no statistically significant ($p > 0.05$) differences were observed.

2.4. Discussion

2.4.1 Sub-cellular metabolism

Our data suggest that there is some variability in metabolic activity among individual carp and among sub-cellular sites in hepatocytes. We cannot rule out, however, the possibility that our sub-cellular separation steps (e.g. centrifugation) did not completely isolate soluble and membrane-bound enzymes. Overall, these data

suggest the enzymes catalyzing the metabolism are more prevalent in the microsomal fraction, and thus are likely bound and/or associated in part with the endoplasmic reticulum. Xenobiotic metabolizing enzymes systems known to be present in microsomal fractions that may be capable of catalyzing PBDE metabolism include the CYP enzymes, uridine diphosphate glucuronosyl transferases (UGTs), and some isoforms of GSTs. CYP enzymes have been known to catalyze reductive reactions, but these typically occur under low oxygen conditions (Di Giulio et al. 1995). Furthermore, previous work has shown that BDE-99 metabolism in carp liver microsomal fractions is not dependent on NADPH in the incubation buffer (Benedict et al. 2007). As such, the likelihood that CYPs are involved in PBDE metabolism in carp seems unlikely.

The phase II metabolizing enzymes, UGTs and GSTs, have also been proposed to play a role in PBDE biotransformation via conjugation processes (Hallgren and Darnerud 2002; Stapleton et al. 2009; Zhou et al. 2002). While we did not examine the UGTs directly, since our incubation buffer does not contain the co-factor, uridine diphosphate glucuronic acid, it seems unlikely that UGTs are modulating BDE-99 metabolism in carp. GSTs may catalyze the debromination of BDE-99 as there are microsomal (MAPEGs) and cytosolic isoforms, as well as some background GSH present

in our sub-cellular fractions. As described in the methods section, a common assay used to measure GST activity in sub-cellular fractions involves a nucleophilic substitution reaction whereby GST catalyzes the conjugation of GSH to CDNB after removing a chlorine atom, resulting in a glutathione-2,4-dinitrobenzene product. Thus, GSTs appear to be capable of dehalogenation reactions. However, Browne et al (2009) observed no change in BDE-47 debromination rates in dialyzed carp cytosol (i.e. cytosol containing no endogenous GSH) when exogenous GSH was excluded or included in their incubations. This suggests that GSTs may not be important enzymes modulating BDE-99 debromination in carp.

Other enzymes that we have previously hypothesized as being involved in BDE-99 metabolism are dios. Work by Baqui et al. (2000) has shown that some dio isoforms have an NH₂-terminus that remains in the endoplasmic reticulum while the COOH-terminus of the enzyme is in the cytosol. Therefore, the variability we observed between microsomal and cytosolic fractions may be due to the trans-membrane nature of dio enzymes, which may result in their presence in both microsomal and cytosolic fractions. This postulate requires more study. Additionally, a study using cerebral cortex tissue from hypothyroid rats observed deiodinase activity in both microsomal and cytosolic

fractions (Leonard et al. 1982), as did a study on human thyroid tissues (Molnar et al. 2002) suggesting some dio activity can be found in the cytosol. Thus, our results suggest that the BDE metabolism by carp may be catalyzed by dios or some combination of microsomal and cytosolic GSTs.

2.4.2 Enzyme Kinetics

It is difficult to compare the enzyme kinetic rates we observed in carp hepatic microsomes incubated with BDE-99 to literature values as enzyme kinetics can vary quite significantly within and among species, different enzymes systems, and between and across endogenous and xenobiotic substrates. While we cannot be certain that substrate saturation was achieved, the K_m and V_{max} values ($24.7 \pm 6.13 \mu\text{M}$ BDE-99 and $1,140 \pm 72.7 \text{ pmol BDE-47 hr}^{-1} \text{ mg protein}^{-1}$, respectively) we measured are generally consistent with studies exploring hepatic biotransformation rates and affinity among other xenobiotic metabolizing enzymes (Fitzsimmons et al. 2007). Additional examination of the enzymatic kinetics of BDE-99 in other species is needed to evaluate the magnitude of the values we measured.

We observed a dose-response formation of two other tetraBDE congeners of note, BDE-66 and BDE-49 (Figure 9). While the congener pattern was dominated by BDE-47,

akin to T4 to T3 conversions via dios, at higher substrate concentrations BDE-66 and BDE-49 constituted appreciable percentages of the metabolite profile. The formation of BDE-49 has been observed in other teleosts, notably salmon (Browne et al. 2009). Figure 11 proposes a metabolic debromination pathway for BDE-99 in carp at these higher substrate concentrations. While the majority of BDE-99 metabolism in carp is dominated by the meta-substitution of bromine to produce BDE-47, at higher substrate concentrations, we observe the potential for cleavage of ortho- and para-substituted bromines to produce BDE-66 and BDE-49, respectively. Finally, the significant ($p < 0.05$) albeit small amounts of the triBDE congeners BDE-28 or BDE-33 (these congeners co-elute with our analytical method) formed may be suggestive of further meta-, ortho-, and/or para- cleavage of bromine from diphenyl ether. The underlying enzymatic pathways driving this biotransformation at elevated substrate concentrations require further characterization, and may point to the enhanced induction of other enzymatic systems, in addition to dios.

The enzyme systems catalyzing debromination of BDE-99 in carp liver microsomes also sustained high in vitro activity (approximately $600 \text{ pmol hr}^{-1} \text{ mg protein}^{-1}$) for an extended period lasting at least 24 hours (Figure 9). This may provide

further support that CYPs are not responsible for catalyzing BDE-99 biotransformation as CYP1A activity in human hepatic microsomes maintained at 25 °C was observed to cease after 6 hours (Yamazaki et al. 1997). While this result was observed in human, non-induced microsomes, a number of other studies involving PBDE exposures in fish have presented evidence that the CYPs are probably not catalyzing PBDE debromination (Benedict et al. 2007; Browne et al. 2009; Raldua et al. 2008).

2.4.3 Influence of co-factors, inhibitors, and substrate competitors

Because BDE-99 is metabolized in carp through a reductive dehalogenation pathway, and since our previous work has implicated involvement of dios, we examined the effects of enzyme inhibitors, substrate competitors, and DTT *in vitro* co-factor on BDE-99 metabolism. The model thyroid hormone reducing agent, 6-propyl-2-thiouracil (PTU) is a specific inhibitor of dio 1 that works well in mammalian liver tissues and is often used to differentiate the role of dio 1 and dio 2 (Kohrle 1999). While we observed no effects in our carp hepatic microsomes challenged with PTU (i.e., no inhibition of BDE-47), studies have demonstrated that dio 1 in teleosts can be resistant to PTU (Frith and Eales 1996; Mol et al. 1993; Orozco et al. 2000; Orozco and Valverde-R 2005). For example, 1 mM of PTU has been observed to cause only weak inhibition of dio

1 in tilapia (Mol et al. 1993), trout (Frith and Eales 1996), and killifish (Orozco et al. 2000). However, IaC is also an inhibitor of DI activity and we found significant ($p < 0.05$) inhibition of BDE-99 biotransformation in the presence of IaC. This compound has been shown to inhibit dio 1 more effectively than dio 2 or dio 3 (Sanders et al. 1997). Our results with IaC demonstrate a possible role for dio 1 in BDE-99 metabolism. IaC inhibitory action occurs via the alkylation of cysteine residues at enzyme active centers. Thus, it is not a specific dio inhibitor but probably a promiscuous inhibitor of many enzyme systems that contain cysteine residues. For example, IaC would likely inhibit GST activity by alkylating cysteine residues at GSH binding sites (Board and Anders 2007). Nonetheless, previous studies have measured a dose dependent inhibition of rT3-ORD, T4-ORD, and T3-IRD among several fish species exposed to IaC, with IC_{50} values in liver tissues ranging from 0.6 mM to $\gg 1$ mM (Mol et al. 1998). Thus the IC_{50} value we observed of approximately 2 mM is in the range reported for inhibition of dio activity.

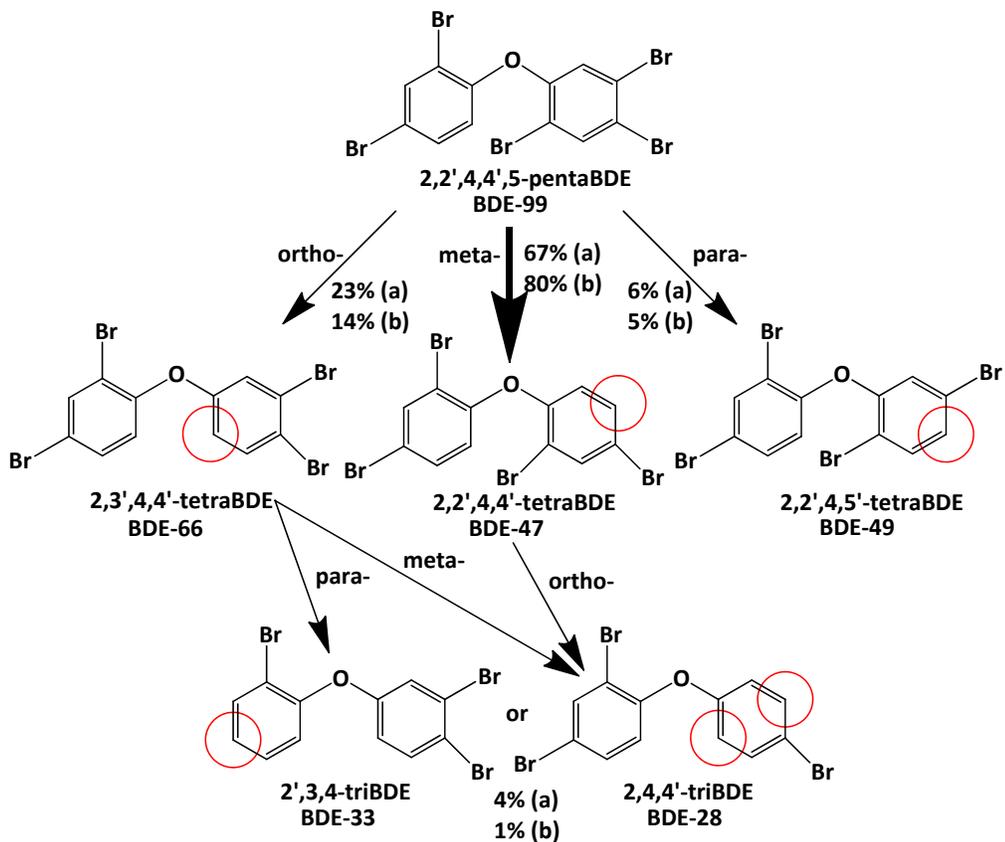


Figure 11: Proposed *in vitro* biotransformation pathway of meta-, ortho-, and para-cleavage of bromine observed in pooled carp microsomes exposed to (a) 250 μM of BDE-99 or (b) 125 μM of BDE-99.

DTT is a potent reducing agent and is used to reduce disulfide bonds in proteins (e.g. dios), and specifically to prevent disulfide bonds from forming between active site selenocysteine residues. DTT is generally regarded as an essential thiol co-factor for investigating dio activity in *in vitro* assay systems (Kohrl 1999). Studies examining dio

activity in fish hepatic microsomes have found that DTT is required to maintain dio activity and that increasing the amount of DTT can often increase the level of dio activity (Frith and Eales 1996; Orozco et al. 1997). In a previous study, BDE-99 biotransformation in carp hepatic and intestinal microsomal fractions was found to not be dependent on the presence of NADPH, suggesting that CYP enzymes were not mediating the reaction (Benedict et al. 2007). Therefore, in the present study we investigated the effect of DTT in the tissue homogenization buffer and in the incubation buffer of pooled carp hepatic microsomes. While further study is needed to fully evaluate the influence of DTT on BDE-99 metabolism, our results of enhanced activity in incubation buffers containing DTT and depressed activity in buffers without DTT suggest that this co-factor is important to BDE-99 catalytic activity in carp. This provides further evidence suggesting the involvement of dio enzymes in BDE biotransformation in fish.

To further investigate the role of dios in BDE-99 biotransformation, several competitive substrate incubations were conducted using T4 and rT3. In a previous study, 0.77 μ M of rT3 was found to significantly inhibit the BDE-99 debromination reaction by almost 50% in both intestinal and hepatic carp liver microsomal fractions (Benedict et al. 2007). Because the dio-catalyzed K_m value for T4 is typically low (0.5-1.0

nM) relative to rT3 (8-180 nM) in fish liver tissues (Orozco et al. 1997; Orozco et al. 2000), we selected different test concentrations for T4 and rT3. The T4 ranges were selected to bracket known K_m values for dio activity, and thus ranged from 0.1 nM to 1.0 μ M, whereas the range of rT3 levels used ranged from 20 nM to 2.0 μ M. We found that T4 and rT3 appeared to act as competitive substrates and/or inhibit the catalytic activity of the mediating biotransformation enzyme, with rT3 acting as a more potent inhibitor ($IC_{50} = 0.83 \mu$ M) than T4 ($IC_{50} >1$).

Dio 1 and dio 2 are known to be present and active in fish hepatocytes (Mol et al. 1998). Dio 1 catalyzes both outer and inner ring deiodination of T4 and has a substrate preference for rT3. Dio 2 catalyzes only outer ring deiodination of T4 so has a substrate preference for T4 (Kohrle 1999). Our finding that rT3 and T4 both inhibited BDE-99 biotransformation points to the potential activity of dio enzymes in catalyzing this activity. This is supported by findings that IaC inhibited the biotransformation with DTT acting as a potentially necessary cofactor. Moreover, rT3 appeared to be a stronger inhibitor of BDE-99 biotransformation than T4, suggesting a potentially more prominent role for dio 1 in catalyzing this reaction. However, we cannot rule out the involvement of GSTs due to the high metabolic activity observed in carp cytosolic fractions and given

that IaC could also inhibit this class of enzymes. Previous studies have also demonstrated an inhibitory effect of thyroid hormones on GST expression and activity in rat liver tissue (Coecke et al. 2000; Vanhaecke et al. 2001). However, this reduced GST activity seemed to occur from downregulation of specific GST subunits, *in vivo*, and not necessarily through competitive or noncompetitive interactions between GSTs and thyroid hormones.

Table 3: GST activity in pooled carp liver microsomes incubated with increasing concentrations of rT3.

<i>rT3 (nM)</i>	<i>Activity (nmol CDNB min⁻¹ mg protein⁻¹)</i>
0	55.7 ± 6.3
20	70.1 ± 2.8
200	64.4 ± 2.8
1000	60.6 ± 3.9
2000	65.1 ± 1.9

We might expect that if rT3 inhibits microsomal GST (MAPEG) activity, then this inhibition may be responsible for the observed decrease in BDE-99 metabolism observed with increasing rT3. However, we observed no decrease in MAPEG activity from co-incubation with rT3 (Table 3), so this metabolic pathway in fish microsomes seems unlikely. This conclusion also seems to be supported in a recent study that found no

change in BDE-47 debromination rates when exogenous GSH was added or withheld from carp hepatic cytosol dialyzed to remove endogenous GSH (Browne et al. 2009).

2.5. Conclusions

This study has confirmed that carp hepatocytes can efficiently debrominate BDE-99 via a reductive pathway and that the enzymes catalyzing this reaction are likely associated with the endoplasmic reticulum (e.g. microsomal fraction). However, cytosolic debromination was also observed, making it difficult to fully pinpoint the sub-cellular sites of biotransformation. This outcome is distinguished from results observed in human liver hepatocytes whereby no reductive debromination was observed (Stapleton et al. 2009). Debromination of BDE-99 was significantly inhibited by the presence of iodoacetate (IaC) and thyroid hormones (rT3 and T4). Inhibition by IaC suggests the involvement of dios and GSTs, which both contain cysteine residues at their active sites and are generally associated with microsomal fractions. GST isoforms are also active in hepatic cytosol, which fits well with our observations of significant metabolism in this fraction. However, the inhibition by rT3 and T4 seems to implicate a greater role for DIIs in catalyzing the reaction. Stronger inhibition by rT3 as opposed to T4 would also appear to implicate a role for dio 1 in carp hepatocytes. Further studies

are needed to confirm the influence of PBDEs on hepatic dio activity in carp tissues and other teleosts to conclusively identify the specific enzyme systems involved in PBDE debromination.

3. Accumulation and debromination of BDE-209 in juvenile fathead minnows induces thyroid disruption and liver alterations³

Polybrominated diphenyl ether (PBDE) flame retardants are known to affect thyroid hormone regulation. The thyroid hormone regulating deiodinases have been implicated in this metabolism; however, PBDE effects on the fish thyroid system are largely unknown. Moreover, the liver as a potential target organ of PBDE toxicity has not been explored in young fish. This study measured BDE-209 effects on thyroid hormone regulation by measuring deiodinase activity in juvenile fathead minnows (*Pimephales promelas*). Dietary accumulations and debromination of BDE-209 were also measured, and morphology of thyroid and liver tissues were 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/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

³ Noyes PD, Hinton DE, Stapleton HM. 2011. Accumulation and debromination of decabromodiphenyl ether (BDE-209) in juvenile fathead minnows (*Pimephales promelas*) induces thyroid disruption and liver alterations. *Toxicological Sciences* 122(2): 265-274.

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, although further testing is needed. All together, these results suggest that juvenile fish may be uniquely susceptible to thyroid disruptors like PBDEs.

3.1 Introduction

Decabromodiphenyl ether (BDE-209) is the fully brominated 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 (HIPS) 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 phase-out in the U.S. 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. 2004a). This piscine metabolic pathway is distinguished from that of rodent and human metabolism whereby oxidative, cytochrome P450 (CYP)-mediated pathways dominate to produce hydroxylated PBDEs (Germer et al. 2006; Richardson et al. 2008; Stapleton et al. 2009).

While 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 (dio) enzymes in catalyzing PBDE debromination in fish (Browne et al. 2009; Noyes et al. 2010; Stapleton et al. 2004a). Dio enzymes, of which three isoforms have been identified in fish (dio 1, dio 2, and dio 3), are membrane-bound proteins that are associated with the endoplasmic reticulum and regulate intracellular thyroid hormone 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 catalyzing PBDE and TH metabolism by conjugation processes. For example, American kestrels (*Falco sparverius*) exposed to PBDEs exhibited depressed circulating T4 and altered glutathione (GSH) homeostasis, suggesting a possible mediating role for GSTs (Ferne et al. 2005). However, in contrast with these findings, GSTs did not catalyze pentaBDE-99 debromination in cytosolic fractions from either Chinook salmon (*Onchorhynchus tshawytscha*) or common carp (*Cyprinus carpio*), implying that they may not be involved in PBDE metabolism (Browne et al. 2009).

PBDEs closely resemble the structure of thyroid hormones, and increased scrutiny has focused on the potential for these contaminants to cause thyroid disruption.

thyroid hormones are essential for growth, development, and reproduction of vertebrates. 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 thyroid hormones 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). Thyroxine (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 dio 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, 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 (*Pimephales promelas*) receiving dietary exposures of BDE-209 and to examine effects of these exposures on thyroid structure and function (as measured by dio activity) and liver morphology. BDE-209 effects on dio 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.

3.2 Materials and Methods

3.2.1 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 glutathione, 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 High Performance Liquid Chromatography (HPLC) grade.

3.2.2 Animals

Approximately 750 seven day old (DO) fathead minnows (*Pimephales promelas*) were purchased from Aquatox, Inc. (Hot Springs, AK) and randomly distributed into six 10-gallon glass tanks (approximately 125 fish/per tank). Fish were 28 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,

Rhineland, WI). Fish were maintained under static renewal conditions with 12 L of pre-conditioned water added every three days to all tanks. Temperature was monitored daily and maintained at 24.5-25°C, and a 16-8 hour light/dark photoperiod was implemented.

3.2.3 BDE-209 Dietary Exposure

Fish in three tanks received a dietary exposure of 9.8 ± 0.16 µg of BDE-209/g ww of food at 5% of their body weight (bw)/day for 28 days. Untreated fish in the remaining three tanks received a control diet containing no BDE-209 at the 5% bw/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 pools were sampled on experimental days 0 (28-DO fish), 14 (42-DO fish), 28 (56-DO fish), and 42 (72-DO fish). Fish were euthanized using an overdose of MS-222, immediately frozen in liquid nitrogen, and stored at -80°C until further analysis.

3.2.4 PBDE Extractions

PBDEs were extracted from whole body samples of fish collected from BDE-209 treated and control replicate tanks on days 0, 14, and 28. Approximately 0.5 g of pooled fish were homogenized with sodium sulfate and spiked with two PBDE surrogate standards (F-BDE-69 and ¹³C-BDE-209). PBDEs were isolated from fish homogenates using Soxhlet extractors operated for 24 hours with 50 mL of a 1:1 mixture of dichloromethane and hexane. Lipid content was determined in extracts using a gravimetric analysis. Extracts were then cleaned using an acid-silica chromatography procedure whereby each sample was eluted with a 1:1 mixture of dichloromethane and hexane in a chromatography column containing 4 g of silica deactivated with 40% sulfuric acid. Extracts were transferred to hexane and reduced in volume to approximately 0.5 mL using a rapid evaporation system with purified nitrogen. An internal standard (CDE-141) was added prior to analysis.

3.2.5 PBDE Analysis

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 deca-BDE. 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 octa-BDE congeners were quantified by monitoring bromide ions (m/z 79 and 81). The nona-BDEs 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.

3.2.6 Whole-fish microsome preparations

Following rinsing in ice cold 0.150 M KCl (potassium chloride; pH 7.4), pooled whole fish (n=3) from each replicate tank and sampling day were homogenized separately using a Bullet Blender with 0.5 mm stainless steel beads (Next Advance, Averill Park, NY) and twice the amount of homogenization buffer (0.25 M sucrose, 0.1 M Tris-hydrochloric acid (HCl), 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM dithiothreitol (DTT)) to fish tissue (pH 7.4). The remaining methods used to prepare

microsomal fractions have been described previously (Noyes et al. 2010). The amount of re-suspension buffer added to the microsomal pellet was equivalent to 750 $\mu\text{L/g}$ of whole tissue. Protein concentrations were determined using a bicinchoninic acid (BCA) assay (Pierce, Rockford, IL) and ranged from approximately 20-26 mg/mL. Resulting microsomal fractions were stored at $-80\text{ }^{\circ}\text{C}$ until use.

3.2.7 *In vitro* Deiodinase (DI) Assay

A series of *in vitro* assays were undertaken whereby exogenous T4 and rT3 were incubated with microsomes prepared from BDE-209 treated and control fathead minnows to examine changes in intracellular dio activity and thyroid hormone metabolism. Specifically, microsomes from BDE-209 treated (n=3) and control (n=3) groups across the four sampling time points (days 0, 14, 28, 42) were incubated in glass test tubes with either 0.64 μM of T4 or 0.77 μM of rT3 to measure rates of outer ring deiodination (ORD) and inner ring deiodination (IRD). All incubations contained 950 μL of incubation buffer and 50 μL 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 10 mM of DTT and 100 μM of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) (pH 7.4). Incubations were conducted for 1.5 hours in

glass test tubes in a water bath at 25°C and 140 rpm oscillations. Negative controls (n=3) consisted of microsomes incubated with no exogenous thyroid hormone. Buffer controls contained thyroid hormones 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.

3.2.8 Thyroid Hormone Analysis

thyroid hormone formation rates mediated by endogenous DIs were determined using a liquid-liquid solid phase extraction (SPE) procedure and 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 (ESI+) mode with multiple reaction monitoring (MRM) transitions and under recently published run parameters (Butt et al. 2011). Labeled internal standards, ¹³C-T4, ¹³C-rT3, ¹³C-T3, ¹³C-3,3'-T2 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.

3.2.9 Cytosolic and Microsomal GST Activity

GST activity was determined in cytosolic and microsomal fractions of BDE-209 treated and control fish using previously published methods to measure the conjugation of glutathione to 1-chloro-2,4-dinitrobenzene (CDNB) to form glutathione-2,4-dinitrobenzene (Habig and Jakoby 1981). Reactions were performed at 25 °C in a reaction buffer containing 1 mM of reduced glutathione (GSH) (pH 6.5) with 1.0 mg/mL microsomal protein. Absorbance was measured at 340 nm for 5 min using a 96-well plate reader (FluoStar Optima, BMG Labtech, Cary, NC) after adding 1 mM of CDNB.

3.2.10 Histological Examination

BDE-209 treated and control intact fish (n=3), sacrificed on day-28, were fixed in 10% neutral-buffered formalin for 72 hours at 4°C and stored in a 10% sucrose solution at 4°C until processing. Due to their small body size, fixative penetration was achieved without surgical opening of abdominal cavity. Fish were decalcified using EDTA, dehydrated in a graded series of ethanol solutions, and oriented in ventral recumbency. To ensure increased resolution, fish were embedded in the plastic monomer glycol methacrylate (GMA) using a Technovit 7100 GMA Kit from EBSciences (East Granby, CT). Infiltration and hardening followed the manufacturer's recommendations. A Leica

2065 Supercut Microtome (Leica Microsystems Inc., Bannockburn, IL) with a knife holder for triangular glass knives was used for sectioning. Processing and microtomy were performed by the Histopathology Laboratory, College of Veterinary Medicine, North Carolina State University. Frontal sections (2.5 μm thicknesses) of whole fish, selected at 50 μm intervals, were mounted on glass slides and stained with hematoxylin and eosin (HE). 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).

3.2.11 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 degrees 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 non-overlapping fields of 40 μm^2 per field at 400x magnification. The mean ratio (\pm std dev) 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).

3.2.12 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 standard deviation of laboratory blanks and were typically measured at < 0.3 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, method detection limits (MDLs) were calculated as three times the standard

deviation 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⁻¹ mg protein⁻¹; T3 and rT3 = 0.21 pmol hr⁻¹ mg protein⁻¹; 3,3'-T2 and 3,5-T2 = 0.57 pmol hr⁻¹ mg protein⁻¹. Differences in thyroid hormone 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). 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, La Jolla, CA). Statistical significance was defined at the p<0.05 level and high statistical significance was defined at the p<0.005 level.

3.3 Results

3.3.1 Growth

Fish growth was monitored throughout the experiment by taking weekly measurements of the mass of approximately 25 fish randomly collected from each tank. Fish mass increased from ~20 mg/fish to ~50 mg/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/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 were observed between BDE-209 treated and control individuals. Growth Rates were $27.6 \pm 1.4 \mu\text{g/day}$ among treated fish and $25.7 \pm 1.7 \mu\text{g/day}$ among control fish. Mortalities in the BDE-209 treated and control tanks averaged 2.9% and 2.6%, respectively. Lipid content in whole fish did not exceed 1.1% among either treated or control individuals, and no significant differences in lipid content were measured between treatment and control groups.

3.3.2 BDE-209 Accumulation and Metabolism

Figure 12 displays PBDE concentrations measured in fathead minnows exposed for 28-days to BDE-209 spiked *Artemia* sp. at a concentration of 9.8 ± 0.16 $\mu\text{g/g}$ ww of food at 5% of their bw/day. In addition to BDE-209 accumulation (488 ± 46 ng/g ww by day 28 of the exposure) (Figure 12a), 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 (Figure 12b and 12c). PentaBDE-101 was the lowest molecular weight congener detected, and hexaBDE-154 was the metabolite measured at the highest concentration at approximately 215 ± 11 ng/g ww of tissue by the end of the 28-day exposure. In addition to BDE-155, two additional hexaBDE congeners were detected but could not be identified with available PBDE standards. Two hepta- and octaBDE congeners (BDE-179, -188, -201, and -202) along with the three nonaBDE congeners (BDE-206, -207, and -208) were also measured. 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 ng/g ww of food.

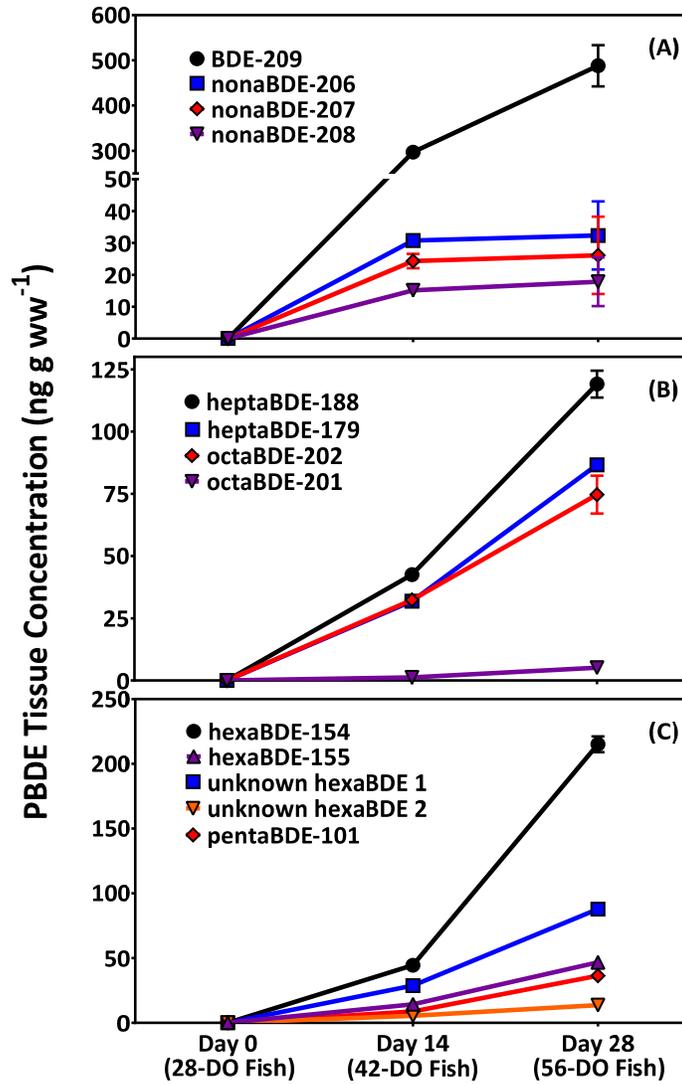


Figure 12: Concentrations (ng/g wet weight) of BDE-209 and major metabolites measured in whole carcasses of fathead minnows receiving a 28-day dietary exposure of BDE-209 at $9.8 \pm 0.2 \mu\text{g/g ww}$ of *Artemia* sp. at 5% body weight(bw)/day ($n=3$; mean \pm SE). Note differences in scale of y-axis.

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 predicted to be present in pooled fathead minnows at day 28 of the exposure would not exceed 0.35 ng (i.e., 0.15 ng/g ww • 0.075 g of food/fish/day • 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:

$$\% \text{ assimilated/bioavailable} = \frac{\Sigma [\text{BDE-101} + \text{BDE-154} + \text{hexaBDE 1} + \text{hexaBDE2} + \text{hexaBDE 3} + \text{BDE-179} + \text{BDE-188} + \text{BDE-201} + \text{BDE-202 (nmol)}]}{\text{cumulative 28-day BDE-209 exposure (nmol)}} \quad (2)$$

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

3.3.3 Intracellular Deiodinase (DI) Activity

Figure 13 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 ~27% and ~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 ~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 ~45% compared to 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).

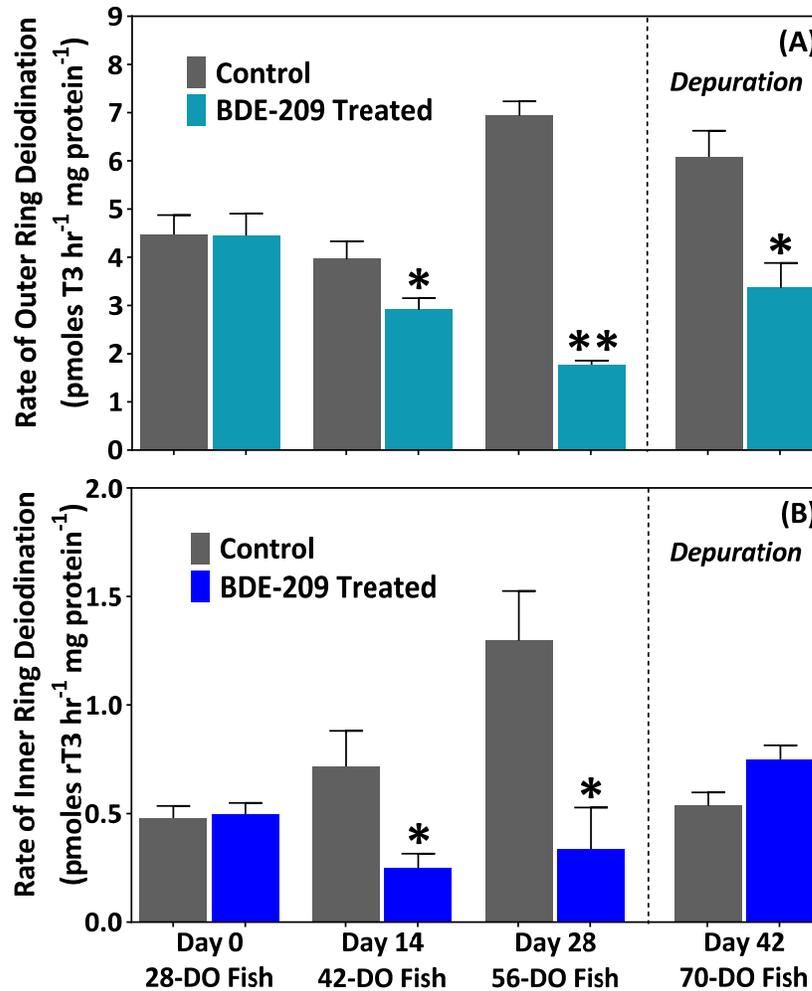


Figure 13: Outer ring deiodination (A) and inner ring deiodination (B) in microsomes from BDE-209 treated and control fish incubated with 0.64 μ M of thyroxine (T4) for 90 minutes at 25°C (n=3; mean \pm SE). One asterisk denotes activity significantly different from controls (p<0.05); two asterisks indicate high statistical significance (p<0.01). Note differences in y-axis scales.

Microsomes from BDE-209 treated and control groups were also incubated with 0.77 μM of rT3 as fathead minnows have three different dio isoforms that have different affinities for different TH substrates. Microsomes from treated individuals showed statistically significant increases (~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 ~56% reduction in rT3-ORD at the conclusion of the 14-day depuration. Age-specific differences in T4-ORD and T4-IRD among controls were observed. A one-way ANOVA followed by a Bonferroni Multiple Comparison 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.

3.3.4 Glutathione-S-transferase (GST) Activity

No significant ($p > 0.05$) differences in GST activity were measured in either cytosolic or microsomal fractions prepared from BDE-209 treatments relative to controls (Figure 14). Specifically, the cGST activity measured in day-0 treatment and control individuals was 96 ± 2 nmol CDNB conjugated hr^{-1} mg protein $^{-1}$ and 94 ± 3 nmol CDNB conjugated hr^{-1} mg protein $^{-1}$, respectively. The cGST activity among treatments and

controls sampled on days 14, 28, and at the 14-day depuration ranged from 115 ± 4 - 125 ± 9 nmol CDNB conjugated $\text{hr}^{-1} \text{mg protein}^{-1}$.

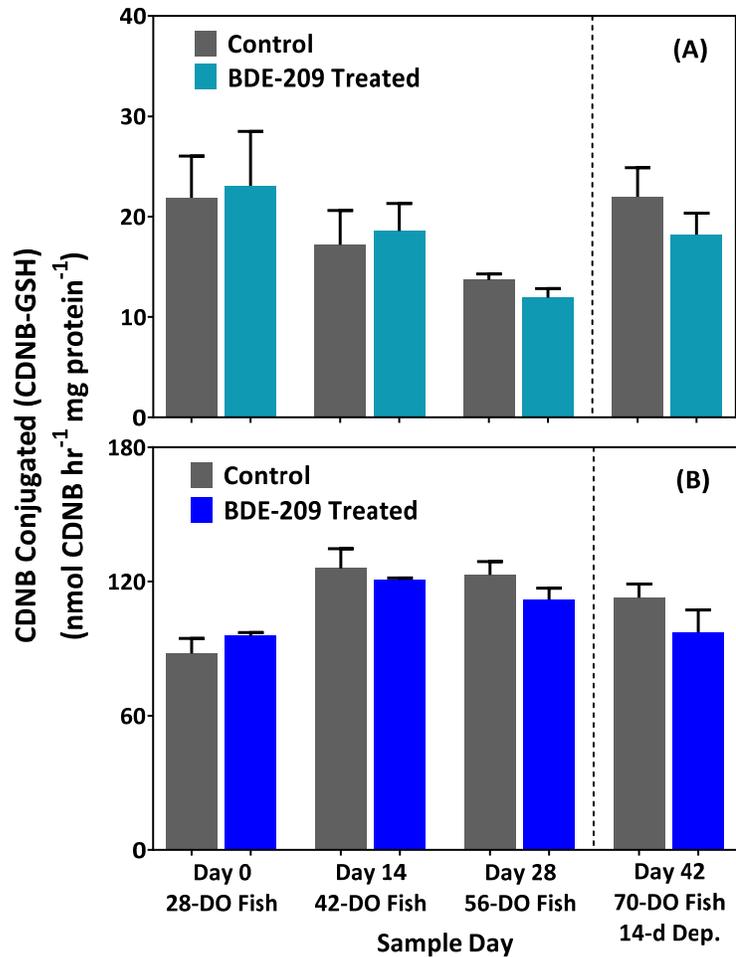


Figure 14: GST activity measured by CDNB assay in juvenile fathead minnow microsomes (A) and cytosol (B) treated orally with BDE-209 at 9.8 ± 0.2 mg/g ww of food for 28 days followed by a 14-day depuration ($n=3$; mean \pm SE). Statistical significance determined at $p < 0.05$. Note differences in y-axis scales.

Microsomal GST (mGST) activity in treated and control individuals ranged from 12 ± 1 - 23 ± 5 nmol CDNB conjugated hr^{-1} mg protein $^{-1}$ (n=3; mean \pm SE). Similar to results in cytosol, potential growth-related changes in mGST activity were also observed. A decreasing trend in mGST activity was measured among treatments and controls over the 28 day exposure.

3.3.5 Morphological Alterations

Results of high resolution microscopy and morphometric analysis (Figures 15 and 16) show that juvenile fathead minnows exposed to BDE-209 for 28 days exhibited increased thyroid follicular epithelial cell height ($p < 0.001$) and colloid depletions relative to controls. Most teleosts, including fathead minnows, do not have individual thyroid glands but rather have several non-encapsulated follicles dispersed predominantly at the base of the branchial arches near the ventral aorta. Whereas follicles from control individuals had predominantly squamous epithelium with well-formed colloids (Figure 15a), follicles from BDE-209 treated individuals presented a cuboidal to low columnar epithelium (Figure 15c). 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 (Figure 15b) 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 (Figure 15d). A quantitative analysis of 20 non-overlapping fields among BDE-209 treated individuals revealed that vacuolated nuclei constituted $48 \pm 12\%$ (mean \pm std dev) 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.

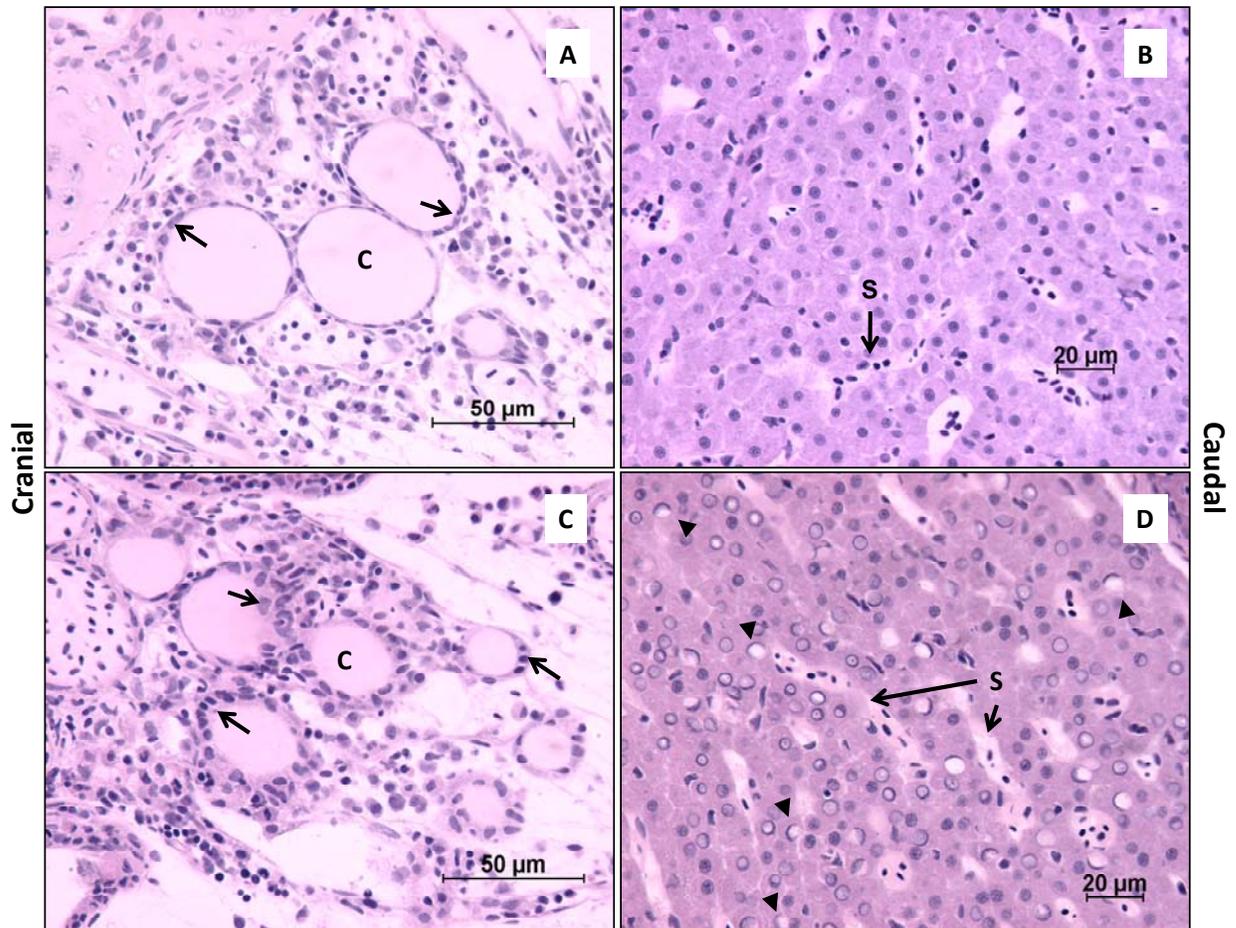


Figure 15: Light micrographs (oil immersion, 600x, HE stain) of juvenile fathead minnow thyroid follicles and liver tissue from control and BDE-209 treated individuals at exposure day 28 (n=3). (A) Thyroid follicles from a control fish; (B) Liver tissue from a control fish; (C) Thyroid follicles from 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.

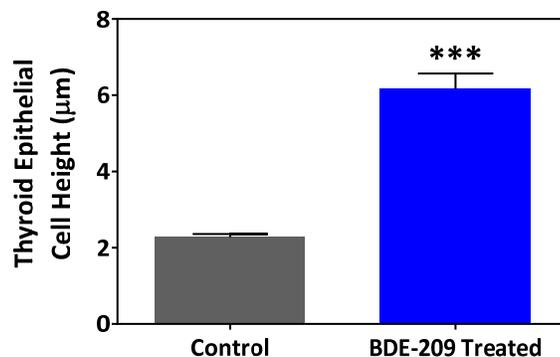


Figure 16: Epithelial cell height of thyroid follicles from control and BDE-209 treated juvenile fathead minnows exposed orally for 28 days (n=3; mean \pm SE). Three asterisks indicate very high statistical significance ($p < 0.001$).

3.4 Discussion

3.4.1 BDE-209 Accumulation and Metabolism

BDE-209 accumulated in juvenile fathead minnows and was readily debrominated to PBDE congeners with fewer bromine atoms (Figure 12). Based on the major metabolites detected, Figure 17 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 (*Cyprinus carpio*) exposed to BDE-209 via the diet, whereby the dominant metabolites detected were also penta- to octaBDEs (Stapleton et al. 2004a). 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, while 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. 2004a) 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/fish) and percent 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).

3.4.2 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 -IRD were measured. Types 1 and 2 isoforms of dio (dio 1 and dio 2) catalyze T4-ORD to produce the active T3 hormone, while dio 1 and Type 3 isoforms of dio (dio 3) catalyze T4-IRD to inactive rT3. Thus, dio 1 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 down-regulations of mRNA expression of genes encoding dios. 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 thyroid hormone mimics, the depressed dio activity measured in this study could also be linked to increased activity of thyroid hormone metabolizing enzymes, such as uridine diphosphate glucuronosyl transferases (UGTs) and sulfotransferases (SULTs). TH conjugating enzymes, and other classes of enzymes involved in thyroid hormone 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 accompanied by decreased expression and activity of dios (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, dios 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 thyroid hormones 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.

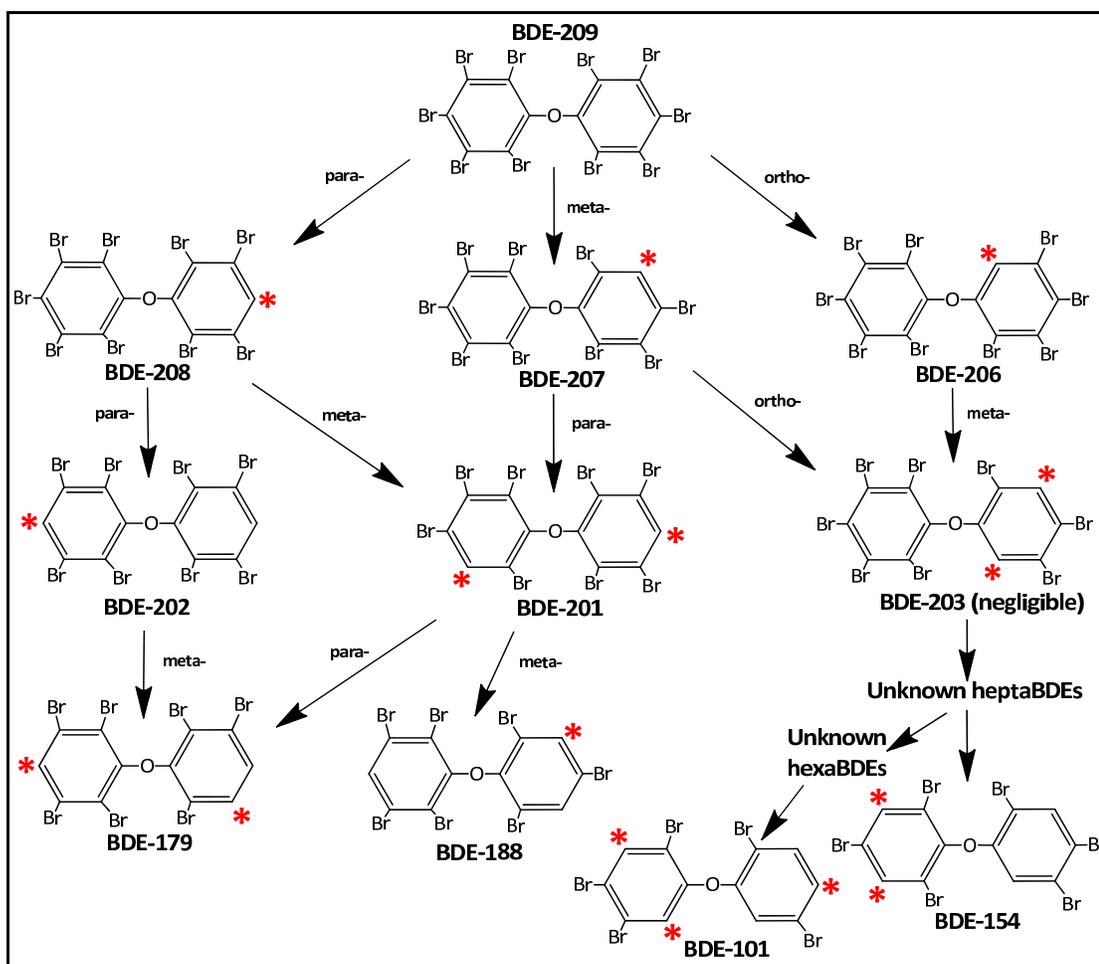


Figure 17: Predicted debromination pathway of BDE-209 in 28-day old fathead minnows (*P. promelas*) exposed to $9.8 \pm 0.16 \mu\text{g/g ww}$ of *Artemia* sp. at 5% of their bw/day for 28 days. Asterisks indicate the predicted site of debromination.

An analysis of dio 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. It is also noteworthy that 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 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). All together, marked perturbations in dio activity observed among 56- and 70-DO fathead minnows exposed to BDE-209, in relation to increases in dio 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, reproduction, and growth.

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 dio 1 isoforms in fathead minnows potentially altered by exposure to BDE-209. Dio 1 has been shown to have a substrate preference for rT3, while dio 1 has been shown to have a substrate preference for T4 (Mol et al. 1998). It is possible that dio 1 was up-regulated and dio 1 was down-regulated in BDE-209 exposed fish. The up-regulation of dio 1 could be a compensatory response to marked inhibition of dio 2, but is insufficient to fully compensate for the dio 2 inhibition in treated individuals. Additional work will be needed to better understand relative responses of dio isoforms in fish exposed to PBDEs.

3.4.3 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 over-stimulation 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 hypothyroidism 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 (Ferne 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 thyroid stimulating hormone (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 (ANIT), 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. Specifically, studies with transmission electron microscopy (TEM) are needed to substantiate these interesting aspects of fathead minnow responses to PBDE exposures.

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 thyroid hormone efflux transporters, leading to increased hormone excretions. Previous studies have shown that mRNA expression of multidrug resistance-associated proteins 2 and 3 (Mrp2, Mrp3), which are major efflux transporters of glucuronides out of cells, was increased in rodents exposed to the pentaBDE commercial mixture DE-71 (Szabo et al. 2009). In addition, multidrug resistance protein (Mdr1a) protein, which encodes P-glycoproteins (Pgps) that are efflux transporters of glucuronides and thyroid hormones into the bile, was decreased (Richardson et al. 2008) and increased (Szabo et al. 2009) in rodents exposed to BDE-47 and DE-71, respectively. Little is known about the activity of these membrane bound transporters in PBDE-exposed fish and further investigation is warranted.

3.5 Conclusion

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, while the morphological findings observed in this study are noteworthy, they will require further examination with increased resolution, at different doses of BDE-209, over more exposure time points, 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. All together, these results suggest that BDE-209 may be affecting the thyroid system of fishes at multiple levels, including in peripheral tissues and at the central HPT axis, and that juvenile fish may be uniquely susceptible to developmental and reproductive abnormalities when exposed to thyroid disruptors like PBDEs.

4. Low dietary exposures to BDE-209 reduce thyroid hormone levels and disrupt thyroid hormone signaling in fathead minnows

Polybrominated diphenyl ether (PBDE) flame retardants have been shown to reduce plasma thyroid hormone levels in some animals. However, effects of the most widely used PBDE, decabromodiphenyl ether (BDE-209), on thyroid signaling are largely unknown. This study examined effects of dietary exposures of BDE-209 on plasma thyroid hormone levels, peripheral brain and liver deiodination activity, and the relative mRNA abundance of genes encoding deiodinase enzymes (*dio1*, *dio2*, *dio3*), thyroid receptors (*TR α* , *TR β*), monocarboxylate transporters (*MCT8*, *MCT10*), and several organic anion transporter proteins (*OATPs*). Adult male fathead minnows (*Pimephales promelas*) received dietary treatments of BDE-209 at a low dose (95.3 ± 0.41 ng/g-food·3% body weight (bw)/day) and high dose (10.1 ± 0.10 μ g/g-food·3% bw/day) for 28 days followed by a 14-day depuration period to evaluate recovery. The model thyroid hormone reducing agent 6-propyl-2-thiouracil (PTU) was used as a positive control. Compared to controls, adult fish exposed to BDE-209 at the low dose for 28-days experienced a 53% and 46% decline in plasma thyroxine (T4) and 3,5,3'-triiodothyronine (T3), respectively, while fish at the high BDE-209 dose showed T4 and T3 deficits at 59%

and 62%, respectively. Depressed circulating thyroid hormone levels were accompanied by a 45-50% decline in the rate of T4-outer ring deiodination (ORD) in the brain of all treatment groups by day 14 of the exposure. This decreased rate of T4-ORD worsened in the brain by day 28 with a 65% decline measured at both BDE-209 doses that was consistent with the PTU positive control (75% decline in T4-ORD). BDE-209 exposures also caused transient, tissue-specific upregulation of *dio1*, *dio2*, *TR α* , *TR β* , *MCT8*, *OATP1c1*, and *OATP2a1* mRNA transcripts in the brain and liver in patterns that varied with time and dose, possibly as a compensatory response to hypothyroidism. Results here provide strong evidence of disrupted thyroid signaling at both the central HPT axis and in peripheral tissues of adult fish by BDE-209 at low, environmentally relevant exposures. Thyroid perturbations at the low dose were largely equal to and in some cases more pronounced than those measured at the high dose, suggesting non-linear relationships between PBDE exposures and thyroid dysfunction.

4.1 Introduction

The decabromodiphenyl ether commercial mixture, which is composed almost entirely of the fully brominated PBDE congener decabromodiphenyl ether (BDE-209), is the only PBDE mixture used in the U.S. today, mostly as a flame retardant in the plastics

of computer/electronic casings, wire/cable insulation, and textile backcoatings. The widespread use of decaBDE, which is composed almost entirely of BDE-209, has resulted in rising levels of BDE-209 in humans and wildlife (Bi et al. 2007; Chen et al. 2007; Gauthier et al. 2008; Johnson-Restrepo et al. 2005; Qu et al. 2007; Shaw et al. 2012; Stapleton et al. 2012). BDE-209 is detected increasingly as the dominant PBDE congener in abiotic matrices, including the atmosphere, sediments, soils, and indoor dust (Klosterhaus et al. 2012; Moller et al. 2012; Salvado et al. 2012; Stapleton et al. 2012; Wang et al. 2011). These reservoirs of contamination serve as sources of PBDE exposure and bioaccumulation as BDE-209 can undergo photolytic degradation (Soderstrom et al. 2004; Stapleton and Dodder 2008), microbial breakdown (Gerecke et al. 2005), and metabolic biotransformation (La Guardia et al. 2007; Stapleton et al. 2004a; Van den Steen et al. 2007) to lower, bioactive PBDE congeners. While decaBDE is scheduled for phase-out in the U.S. at the end of 2013, exposures to BDE-209 and other PBDEs are expected to continue into the coming decades as products that contain them continue to be used, recycled, and disposed. In addition, decaBDE use continues to be largely unrestricted across Asia, but restricted from use in electronic equipment in Europe (Eur.Parliament 2008).

Despite the widespread use of BDE-209, we continue to have limited information on its potential thyroid toxicity in vertebrates, although DecaBDE caused thyroid tumors in adult mice in a 2-year carcinogenicity study (NTP 1986). Exposures to lower PBDE congeners have been shown to alter thyroid hormone regulation among early life stages of vertebrates and cause neurodevelopmental impairments, among other adverse effects (Kuriyama et al. 2007; Lema et al. 2007; Rice et al. 2007; Tseng et al. 2008; Viberg et al. 2008). While our understanding of the mechanisms underlying PBDE effects on early life stages of animals continues to grow, PBDE effects on thyroid signaling among adult animals are poorly understood. The importance of thyroid hormones in the early development of vertebrates has been well established (Anderson 2008). The thyroid in mature animals also plays critical, permissive roles in regulating a variety of physiological and systemic functions, including organ system functioning, basal metabolism, and reproduction (Blanton and Specker 2007; Kapoor et al. 2012). It is notable that PBDEs have been shown to impair reproductive functioning among fishes (He et al. 2011; Lema et al. 2008; Muirhead et al. 2006) and elicit anti-androgenic effects in developing rodents (Kuriyama et al. 2005; Stoker et al. 2005; Tseng et al. 2006). Thus, the potential for direct and indirect effects of BDE-209 on the thyroid and reproductive

systems of adult animals warrants further study. Moreover, the vertebrate thyroid system maintains normal physiological functioning by responding to endogenous and exogenous perturbations both via changes in hormone production from thyroid follicles as well as through changes in the capacity and sensitivity of peripheral tissues. Such integrated compensatory responses at the thyroid axis and target tissue levels make it difficult to evaluate the mechanisms of action for thyroid disruptors like PBDEs.

Thyroid hormones are synthesized in thyroid follicles of vertebrates with secretions from the fish thyroid possibly being limited to thyroxine (T4) (Eales et al. 1999). Thyroid hormones circulate in plasma bound to proteins, including albumin, thyroid binding globulin, and transthyretin (Eales and Brown 1993). Most thyroid hormone is bound to plasma proteins with only a small amount (<1%) free and available for uptake into cells. The transport of thyroid hormones into and out of cells is mediated largely by membrane bound transporters, including the high affinity monocarboxylate transporter 8 (MCT8) and organic anion transporter proteins (e.g., OATP1c1), among others and depending on the tissue type (Arjona et al. 2011; Visser et al. 2011). Once in the cell, T4 can be deiodinated to the bioactive 3,3',5-triiodothyronine (T3) or inactivated to 3,3',5'-reverse T3 (rT3) or 3,3'-diiodothyronine (T2). Deiodination is catalyzed by

iodothyronine deiodinase (dio) enzymes of which three isoforms (dio 1, dio 2, dio 3) have been identified in fish that appear to be functionally homologous to mammalian dios. Thus, in fish, the homeostatic activation and inactivation of thyroid hormones appears to occur predominantly in peripheral tissues as opposed to being mediated at the hypothalamic-pituitary-thyroid (HPT) axis. The central HPT of fishes is believed to function largely to maintain T4 homeostasis via negative feedback. Thyroid hormones can also be further conjugated as glucuronides or sulfates in the liver and secreted through the bile and urine (Finnsen et al. 1999). The genomic action of thyroid hormones is mediated by nuclear thyroid receptors (TRs) that act as ligand-bound transcription factors that bind to thyroid hormone response elements (TREs) to induce or repress the expression of hormone responsive genes (Yen 2001; Zhang and Lazar 2000).

Given our limited understanding of BDE-209 effects on these thyroid signaling pathways in animals, the purpose of this study was to evaluate effects of BDE-209 dietary treatments, at two environmentally relevant doses, on the central and peripheral thyroid systems of adult male fathead minnows. The bioaccumulation of BDE-209 and its reductive metabolites were measured along with effects on circulating thyroid hormone levels, relative dio mRNA levels and activity in the brain and liver, and the

transcript abundances of genes encoding encoding thyroid receptors (TR α , TR β) and several membrane bound transporters from the MCT and OATP families. Because previous studies have demonstrated that PBDEs may impair vertebrate reproduction, and given the permissive interactions of the thyroid on the vertebrate reproductive system, we also evaluated BDE-209 effects on fish gonadal size by use of a gonadosomatic index (GSI) as an initial metric of reproductive fitness. The thyroid system is substantially conserved both structurally and functionally across vertebrate taxa, and so increasing our knowledge of BDE-209 effects in adult fish can inform our understanding of effects on the thyroid systems of other vertebrates.

4.2 Materials and Methods

4.2.1 Animals and Care

Approximately 600 adult male fathead minnows (*Pimephales promelas*; 9 months old) were obtained from Aquatic BioSystems (Fort Collins, CO). Fish were maintained in 151 liter glass aquaria under a 16:8 hr light/dark photoperiod at 24-26°C and 7.8-8.2 pH. Animal care and welfare procedures were implemented in accordance with Duke University's Institute for Animal Care and Use Program.

4.2.2 Dietary Exposures to BDE-209

Fish were randomly distributed across 12 exposure tanks (50 fish/tank; 151 L tanks) and assigned to the following treatment groups: three BDE-209 high dose tanks; three BDE-209 low dose tanks; three positive control tanks with 6-propyl-2-thiouracil (PTU); and three negative control tanks. Fish in the high dose treatments received dietary exposures of BDE-209 at 10.1 ± 0.10 $\mu\text{g/g}$ wet weight (ww) of food at 3% body weight (bw)/day. Low dose groups were exposed to BDE-209 at 95.3 ± 0.41 ng/g ww of food at 3% bw/day. The model thyroid hormone reducing agent PTU was used as a positive control at 0.5 mg/g ww of food at 3% bw/day. BDE-209 (~97% pure; ~3% nonaBDEs measured; Appendix B.1) and PTU were purchased from Sigma-Aldrich (St Louis, MO). Negative control fish received clean food containing cod liver oil with no BDE-209. The BDE-209 amended food was prepared by dissolving BDE-209 in 10 ml of cod liver oil by stirring it covered for 12 hrs, and spiking 1 ml of this solution into an Omnivore Gel Diet (Aquatic Ecosystem, Inc., Apopka, FL). 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; See Appendix B.1; Table 8) using methods outlined below for the fish tissue analysis.

Fish were exposed to the BDE-209 and control treatments daily for 28 days followed by a 14 day depuration period in which all fish received clean food containing no test chemical to evaluate thyroid recovery. Fish were sampled on days 0, 14, 28, and 42 (8-12 fish sampled/sample day). Twenty percent of fish from each tank were weighed at the start of the experiment and then weekly over the study to measure weight changes and maintain target feeding rates. In addition, fish mass and fork length were measured at necropsy. Table 4 outlines the sampling regimen for the study endpoints evaluated.

Table 4: Sample Size and Tissue/Blood Pooling for Endpoints Evaluated.

Study Endpoint	Sample Size	Tissue/Blood Pooling
PBDE bioaccumulation and metabolism	n = 3 fish/treatment	None
Plasma thyroid hormone levels	n = 3 replicate tanks/treatment/sampling day	Blood pooled from 8-12 fish/replicate ¹
Microsome preparation for DI assay	n = 3 replicate tanks/treatment/sampling day	Livers or brains pooled from 6 fish /replicate
DI activity assay	n = 3 tissue pools (brain or liver)/treatment/sampling day	None
mRNA expression in brain and liver	n = 6 brains or livers/treatment/sample day	None
Gonado-somatic Index (GSI)	n = 3 replicate tanks/treatment/sampling day	8-12 fish measured/replicate ¹

¹ Variations in the number of fish pooled resulted from differences in mortality across treatments and time.

4.2.3 PBDE Extractions and GC/ECNI-MS Analysis

PBDEs were extracted from one fish randomly selected from each BDE-209 treated and negative control replicate across each sampling day. The PBDE extraction methods used in this study have been previously described (Noyes et al. 2011). Two PBDE surrogate standards, ^{13}C -BDE-209 (Wellington Labs, Guelph, Canada), and 4'-fluoro-2,3',4,6-tetrabromodiphenyl ether (F-BDE-69; Chiron, Trondheim, Norway), were added to each sample at the start of the extraction, and an internal standard ^{13}C -2,2',3,4,5,5'-hexachlorodiphenylether (^{13}C -CDE-141) was added prior to GC/ECNI-MS analysis to measure recovery of the surrogate standard.

All samples and the food 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 deca-BDE. The operating conditions for the GC/MS have been described previously (Stapleton et al. 2008). Tri- through octa-BDE congeners were quantified by monitoring bromide ions (m/z 79 and 81). The nona-BDEs and BDE-209 were quantified by monitoring m/z responses of 486.6 and 484.6, and the ^{13}C -BDE-209 was quantified using m/z responses of 494.6 and 496.6.

Recoveries of F-BDE-69 and ^{13}C -BDE-209 averaged $68 \pm 9.1\%$ and $98 \pm 9.7\%$, respectively. BDE-209, BDE-99, and BDE-47 were detected in laboratory blanks at 12.0 ± 1.18 ng, 5.8 ± 0.6 ng, and 4.4 ± 0.5 ng, respectively (n=3). All samples were blank-corrected by subtracting the average blank values. Method detection limits (MDLs) were determined as three times the standard deviation of laboratory blanks. For congeners not detected in blanks, the MDL was set at the laboratory instrumental detection limit (IDL) normalized to the average mass of tissue extracted. Lipid content was determined in extracts using a gravimetric analysis.

4.2.4 Measurements of Circulating T4 and T3

Plasma concentrations of total T4 and T3 (TT4 and TT3, respectively) were measured using newly developed extraction methods (described in Appendix C) and our published LC/MS/MS analytical methodology with some modification (Wang and Stapleton 2010). The LC/MS/MS was operated in positive electrospray ionization (ESI+) mode with multiple reaction monitoring (MRM) transitions and modified LC/MS/MS conditions that are described in Appendix C. Blood was drawn from the caudal vein of euthanized fish using heparin-coated 75 mm capillary tubes, and centrifuged at $3,000 \times G$ for five min to isolate the plasma fraction. Plasma was pooled from fish collected at

each replicate (n=3; 8-12 fish/replicate). All replicates were extracted and measured by LC/MS/MS in triplicate across all treatments and sampling times. Samples were extracted in batches based on sampling day. The stable isotopes $^{13}\text{C}_{12}$ -T4 and $^{13}\text{C}_6$ -T3 (50 μL ; 10 ng/ml; Cambridge Isotope Laboratories, Andover, MA; Accustandard, New Haven, CT) were added to each sample at the start of the extraction and were used to quantify levels of T4 and T3, respectively. These values were normalized to the plasma volume extracted (50 μl). Blank controls that contained 50 μl of ^{13}C -T4 and ^{13}C -T3 alone were used to correct for trace levels (~0.5%) of unlabeled hormones present as commercial impurities in the ^{13}C -labeled standards.

Method detection limits (MDLs) were calculated as three times the standard deviation of thyroid hormones detected in blanks containing no plasma. Average MDLs normalized to the amount of plasma extracted over the four batches were: T4 = 0.24 ± 0.07 ng/ml and T3 = 0.12 ± 0.02 ng/ml. Intra-assay coefficients of variation (CVs) within a single batch run were $9.2 \pm 2.0\%$ and $9.8 \pm 2.0\%$ for TT3 and TT4, respectively. These intra-assay CVs represent variations in TT4 and TT3 (mean \pm SE) measured in triplicate extractions of each replicate pool of plasma. Inter-assay CVs, representing the reproducibility of values across batch runs, was $11 \pm 1.9\%$ for TT3 and $12 \pm 2.0\%$ for TT4.

These inter-assay CVs reflect the TT4 and TT3 measured in repeated extractions and LC/MS/MS measurements of the same samples in triplicate across different batches.

4.2.5 Deiodinase Activity Assays

To examine changes in the activity of deiodinase enzymes in BDE-209 treated and control fish, a series of *in vitro* assays were undertaken whereby microsomes (1 mg protein) from pooled brains and livers of fish were incubated with 0.64 μ M of T4. Briefly, brain and liver microsomes were prepared using previously published methods by pooling tissues from six fish per replicate (n=3; 6 organs/replicate) for each treatment group and sampling time point (Noyes et al. 2010). All incubations contained 900 μ l of 0.1 M potassium phosphate buffer (pH 7.4), 10 mM of dithiothreitol (DTT; Sigma-Aldrich), and 100 μ l of the appropriate microsomal fraction diluted to 10 mg/ml. Incubations were undertaken for 1.5 h in a water bath operated at 25°C and 140 rpms. Negative controls (n=3) consisted of microsomes incubated with no exogenous T4.

Formation rates of T3, rT3, 3,3'-T2, and 3,5-T2 catalyzed by the activity of deiodinase enzymes were measured using our published LC-ESI+/MS/MS method (Wang and Stapleton 2010) with some modifications to run parameters (Butt et al. 2011). The labeled internal standards $^{13}\text{C}_{12}$ -T4, $^{13}\text{C}_6$ -rT3, $^{13}\text{C}_6$ -T3, $^{13}\text{C}_6$ -3,3'-T2 (100 μ l; 250 ng/ml) were added

to each sample to quantify levels of T4, rT3, T3, and 3,3'-T2 hormones, respectively. Concentrations of thyroid hormones were normalized to time and protein concentration to determine rates of ORD and IRD. Blank controls containing buffer alone were used to correct for trace levels (~0.5%) of unlabeled hormones present as commercial impurities in the ¹³C-labeled internal standards. Method detection limits (MDLs) were calculated as three times the standard deviation of thyroid hormones detected in negative control samples containing no T4 buffer solution. No 3,3'-T2, or rT3 hormones were detected in control samples so the IDL was used as the MDL for these hormones. MDLs normalized to incubation conditions were as follows: T4 = 0.26 pmol hr⁻¹ mg protein⁻¹; T3 = 0.24 pmol hr⁻¹ mg protein⁻¹; rT3 = 0.04 pmol hr⁻¹ mg protein⁻¹; 3,3'-T2 = 0.05 pmol hr⁻¹ mg protein⁻¹.

4.2.6 Taqman Real-Time RT-PCR

Quantitative real-time reverse transcription PCR (RT-qPCR) was performed in collaboration with Dr. Sean Lema, California Polytechnic State University and is described in Appendix B.2 to B.6. The following genes were targeted: dio enzymes (*dio1*, *dio2*, *dio3*); thyroid receptors (*TRα*, *TRβ*); monocarboxylate transporters (*MCT8*, *MCT10*), and several organic anion transporter proteins (*OATP1c1*, *OATP1f1*, *OATP1f2*, *OATP2a1*, *OATP2b1*, *OATP3a1*, *OATP4a1*, and *OATP5a1*). Total RNA was extracted from livers and

brains of treated and control fish across each sampling time point using previously described methods that are outlined in Appendix B.2 (Johnson and Lema 2011). Primers and hydrolysis (Taqman) probes were designed to the amino acid coding regions of partial cDNA sequences encoding *dio1*, *dio2* and *dio3*, as well as three reference genes *beta-actin*, elongation factor 1alpha (*EF1a*) and ribosomal protein L8 (*rpl8*) from the fathead minnow (See Appendix B.5, Tables 9 and 10). In addition, primers and probes were designed to *TRα* and *TRβ* (GenBank accession nos. DQ074645, AY533142; Lema et al. 2009). When possible, assays were designed to span intron boundaries. All primers and probes were synthesized by Integrated DNA Technologies (Coralville, IA). The RT-qPCR procedures and conditions are described in Appendix B.4. For each gene, a standard curve was made from a pool of total RNA from samples representing all treatment groups and sampling time points. Each standard was serially diluted and assayed in triplicate while samples were either run in duplicate or individually. The intra-plate variability did not exceed 4% for any of the PCR reactions. In addition, five duplicates were run between every plate, and this inter-plate variability did not exceed 6% for any of the genes evaluated. DNA contamination was assessed for each gene by analyzing RNA samples that were not reverse transcribed. Each RT-qPCR run also

included two samples with no cDNA template to further control for contamination. No amplification was observed in these samples. In the liver, *rpl8* and *EF1a* mRNA expression was affected by the BDE-209 treatment so *beta-actin* was selected as the normalizing reference gene. In the brain, *rpl8* fold change was the least variable across treatment and time making it an optimal brain reference gene. For each target gene, correlation coefficients (R^2) for the standard curves ranged from 0.98 to 1.00. PCR efficiencies for each gene are provided in Appendix B.5, Tables 9 and 10 and were calculated using the equation: efficiency = $[10^{(-1/\text{slope})} - 1]$ (Rasmussen 2000). Relative mRNA levels were calculated for each gene using a serially diluted standard curve and were expressed relative to mRNA levels of the reference gene (Pfaffl 2001). These normalized values from treatments were then normalized to mean values of the negative control group at each sampling day.

4.2.7 Reproductive Status

The gonado-somatic index (GSI) was used as a metric of reproductive status in adult male fathead minnows exposed to BDE-209. Approximately 8-12 fish were included in the analysis from each replicate tank and at each sampling day and treatment. After fish were euthanized, spermatic ducts were severed proximal to the

genital pore, and testes were dissected from the abdominal cavity in a caudal to cranial direction. Left and right testes were positioned in a tared cassette for future histological analysis and weighed. The GSI was calculated as the testes mass (to nearest 0.01 mg) divided by the body mass (mg), multiplied by 100 (%). The GSI value for a given replicate tank was derived by taking the average GSI of 8-12 fish per sampling day and treatment. Data were evaluated within sampling day using a one-way ANOVA and Tukey's test (n=3; mean \pm SEM).

4.2.8 Statistical Analyses

For the dio activity assays, differences in thyroid hormone formation rates in T4-incubated microsomes across BDE-209 treated and control fish were analyzed for statistical significance within sampling day using a one-way ANOVA and Tukey's post hoc test (Graphpad Prism 6.0, La Jolla, CA). For the plasma thyroid hormone analysis, the average of three extractions was used for each replicate across all treatments and sampling days. Differences in circulating TT4 and TT3 levels were analyzed for statistical significance within sampling day using a one-way ANOVA and Tukey's test. Gene expression changes among treated and control fish were also evaluated within sampling day using a one-way ANOVA and Tukey's test to evaluate effects of BDE-209

and PTU treatments relative to negative controls. Data were evaluated for normality using a Kolmogorov-Smirnov test, and non-conforming data were square root transformed. Statistical significance was defined at the $p < 0.05$ level with high and very high statistical significance defined at $p < 0.01$ and $p < 0.005$, respectively. For the mortality evaluation, survival curves were analyzed using a log-rank test (or Mantel-Cox test); statistical significance was established using a Bonferroni correction factor to correct for multiple survival curve comparisons.

4.3 Results

4.3.1 Mortality

A multi-comparison survival curve showed a statistically significant increase in percent cumulative mortality among both the high and low dose BDE-209 treatments (Figure 18). Specifically, $13 \pm 3.1\%$ and $12 \pm 2.9\%$ of fish from the high and low BDE-209 treatments, respectively, had died by the conclusion of the 42-day study (28-day treatment + 14-day depuration). We observed $< 1\%$ mortality in negative controls. Mortality in the PTU positive control group was increased but was not statistically significant.

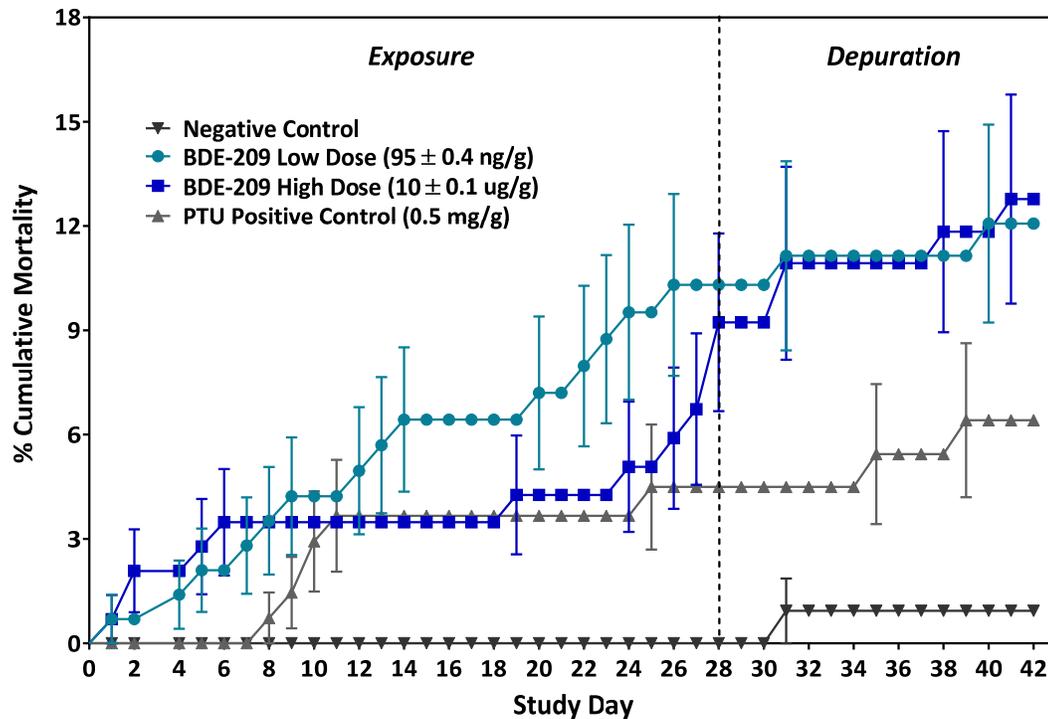


Figure 18: Percent cumulative mortality observed among fish exposed to BDE-209 or 6-propyl-2-thiouricil (PTU; positive control) by the diet for 28 days followed by a 14-day depuration period in which fish were fed food containing no BDE-209 or PTU (n=3; mean ± SE; 50 fish/replicate).

No significant changes in fish mass, fork length, or condition factors (i.e., fish mass (g)/fork length (cm) x 100) were observed in any treatment group at any sampling day. Lipid content (Appendix B.7) in fish carcasses was highly variable, averaging 1.3 ± 1.1%, but no significant differences were observed between treatment and controls. However, a non-significant decrease in fish lipid content was measured at sampling day

14 in BDE-209 low dose carcasses ($0.6 \pm 0.1\%$) and high dose carcasses ($0.6 \pm 0.2\%$) compared to controls (1.8 ± 0.7). This non-significant decrease in percent lipid was also measured at day 28 in BDE-209 high dose fish ($0.4 \pm 0.03\%$) compared to controls (1.8 ± 1.0).

4.3.2 Reproductive Status and Gonado-Somatic Index

Significant decreases in the GSI were measured in adult male fish exposed to BDE-209 at all sampling time points, including after the 14-day depuration (Figure 19). Moreover, the magnitude of the GSI decline was similar across both the BDE-209 low and high doses. Specifically, at day 14 of the exposure the GSI declined ~41% to $1.0 \pm 0.1\%$ ($p < 0.01$) at the BDE-209 low dose and ~24% to $1.3 \pm 0.1\%$ ($p < 0.05$) at the BDE-209 high dose, relative to negative controls ($1.7 \pm 0.01\%$). At day 28 of the exposure, GSI levels remained significantly diminished relative to negative controls ($1.7 \pm 0.01\%$) at the BDE-209 low dose ($1.3 \pm 0.1\%$) and high dose ($1.2 \pm 0.03\%$). The reduced GSI in BDE-209 treated fish continued over the 14-day depuration with GSI levels decreased at the BDE-209 low dose by ~29% ($1.2 \pm 0.2\%$) and ~35% at the high dose ($1.1 \pm 0.1\%$), relative to negative controls ($1.7 \pm 0.1\%$). Dietary exposures to the PTU positive control had no effect on the GSI.

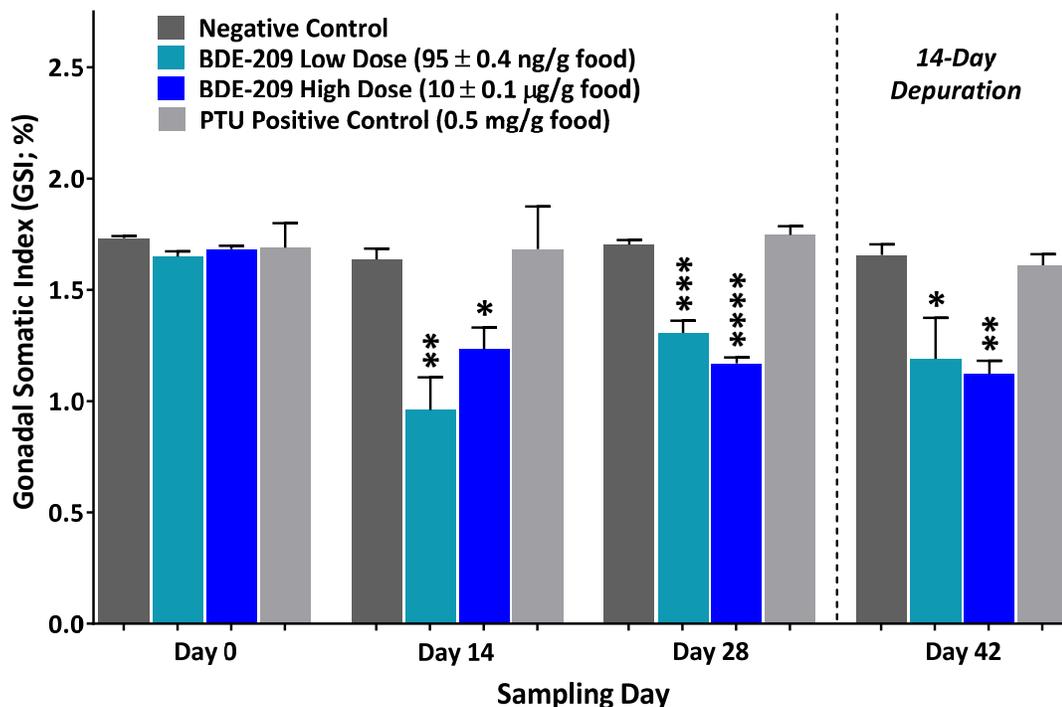


Figure 19: Gonado-somatic index (GSI) measured in adult male fathead minnows (n=3; mean ± SE; 8-12 fish/replicate) exposed to BDE-209 via the diet for 28 days followed by a 14-day depuration period. Statistical significance was evaluated with a one-way ANOVA and Tukey's test within sampling day (* $p < 0.01$, ** $p < 0.01$, *** $p < 0.0005$, **** $p < 0.0001$).

4.3.3 PBDE Bioaccumulation and Metabolism

Figures 20 and 21 (Table 5) display BDE-209 and reductive metabolite concentrations (ng/g ww and ng/g lw) measured in BDE-209 low and high dose groups. In addition, detailed data sheets outlining results of the PBDE quantitative analysis are

provided in Appendix B.7. Accumulations of BDE-209 and several metabolites, ranging from penta- to octa-BDEs, were measured in both dose groups. In low dose fish (Figure 20A), BDE-209 concentrations increased to 1.4 ± 0.5 ng/g ww at sampling day 14 followed by generally sustained levels at 1.1 ± 0.2 ng/g ww at sampling day 28 and 1.0 ± 0.2 ng/g ww over the 14-day depuration. In high dose fish, BDE-209 concentrations increased from 6.1 ± 1.0 ng/g ww at sampling day 14 to 10 ± 5.4 ng/g ww at sampling day 28 after which levels decreased to 3.1 ± 1.0 ng/g ww over the 14-day depuration (Figure 21A).

HexaBDE-154 (2,2',4,4',5,6'-hexaBDE) was the metabolite detected at the highest concentration in both dose groups and pentaBDE-101 (2,2',4,5,5'-pentaBDE) was the lowest molecular weight (MW) congener detected (Figure 20F, 21F). Specifically, in the low dose group, BDE-154 increased initially to 6.6 ± 3.0 ng/g ww at sampling day 14 and then declined by sampling day 28 and over the depuration (Figure 20E-F). At the high dose, hexaBDE-154 was measured at 51 ± 7.3 ng/g ww at the conclusion of the 28-day exposure (Figure 21E). HexaBDE-155 and hexaBDE-153, as well as two hexaBDEs that could not be identified with available PBDE standards, were also detected at the high dose (Figure 21E) and at low levels in the low dose group (Figure 20E). Two hepta- and

octaBDE congeners (BDE-179, -188, -201, and -202) were measured (Figure 20C-D; 21C-D). Finally, decaBDE contains small amounts of nonaBDEs as impurities (Figure 20B, 21B), and GC/ECNI-MS analysis of the high and low dose BDE-209 treated food showed levels of the nonaBDEs at approximately 2.8% of BDE-209 concentrations (Appendix B2).

Negligible amounts of BDE-47, BDE-99, and BDE-154 were detected in the BDE-209 treated food (Appendix B.1; Table 8). These congeners were also detected at similar levels in the negative control food, suggesting their presence as background contaminants in the commercial diet or cod liver oil used. No other congeners were detected in the food. Over the 14-day depuration, average concentrations of three reductive metabolites increased in the high dose (BDE-188, BDE-202, BDE-155) while others declined (BDE-154, unk. hexaBDE-1) or did not change (BDE-201, BDE-179, unk. hexaBDE 2, BDE-101, unk. pentaBDE) (Figure 21C-F). In the low dose group, levels of reductive metabolites were generally less than or did not change from levels measured at the exposure conclusion. Based on the suite of metabolites identified, approximately 3.8% and 1.3% of the BDE-209 exposure was estimated to be bioavailable to adult fathead minnows at the low and high doses tested, respectively. These percentages were

calculated by estimating the average body burden of BDE-209 and metabolites in fathead minnows at the end of the 28-day exposure as follows:

$$\begin{aligned} \% \text{ bioavailable} = & \Sigma [\text{BDE-101} + \text{BDE-153} + \text{BDE-154} + \text{BDE-155} + \text{hexaBDE 1} + \\ & \text{hexaBDE2} + \text{BDE-179} + \text{BDE-188} + \text{BDE-201} + \text{BDE-202} + \text{BDE-209 (nmol)}] / \\ & \text{cumulative 28-day BDE-209 exposure (nmol)} \end{aligned} \quad (2)$$

The daily and cumulative BDE-209 exposure over the 28-day low dose exposure was estimated to be ~7.1 pmol/fish-day (or ~6.8 ng of BDE-209/fish-day; 2.37 g fish × 3% bw × 95.4 ng/g food) and ~200 pmol/fish-28 days (or ~190 ng of BDE-209/fish-28 days; 2.37 g fish × 3% bw × 95.4 ng/g food × 28 d), respectively. The summed level of PBDEs (Σ PBDEs; BDE-209 + PBDE reductive metabolites) detected at day 28 of the low dose treatment were 7.81 ± 2.44 pmol/fish (or 5.50 ± 1.69 ng/fish).

At the BDE-209 high dose, the daily and cumulative exposure over the 28-day treatment was estimated to be ~0.8 nmol/fish-day (or ~0.72 µg of BDE-209/fish-day; 2.37 g fish × 3% bw × 10.1 µg/g food) and ~21 nmol/fish/28 days (or ~20.1 µg of BDE-209/fish-28 days; 2.37 g fish × 3% bw × 10.1 µg/g food × 28 d), respectively. The Σ PBDEs (BDE-209 + PBDE reductive metabolites) detected at day 28 of the high dose treatment were 265 ± 55.9 pmol/fish (or 180 ± 39.2 ng/fish).

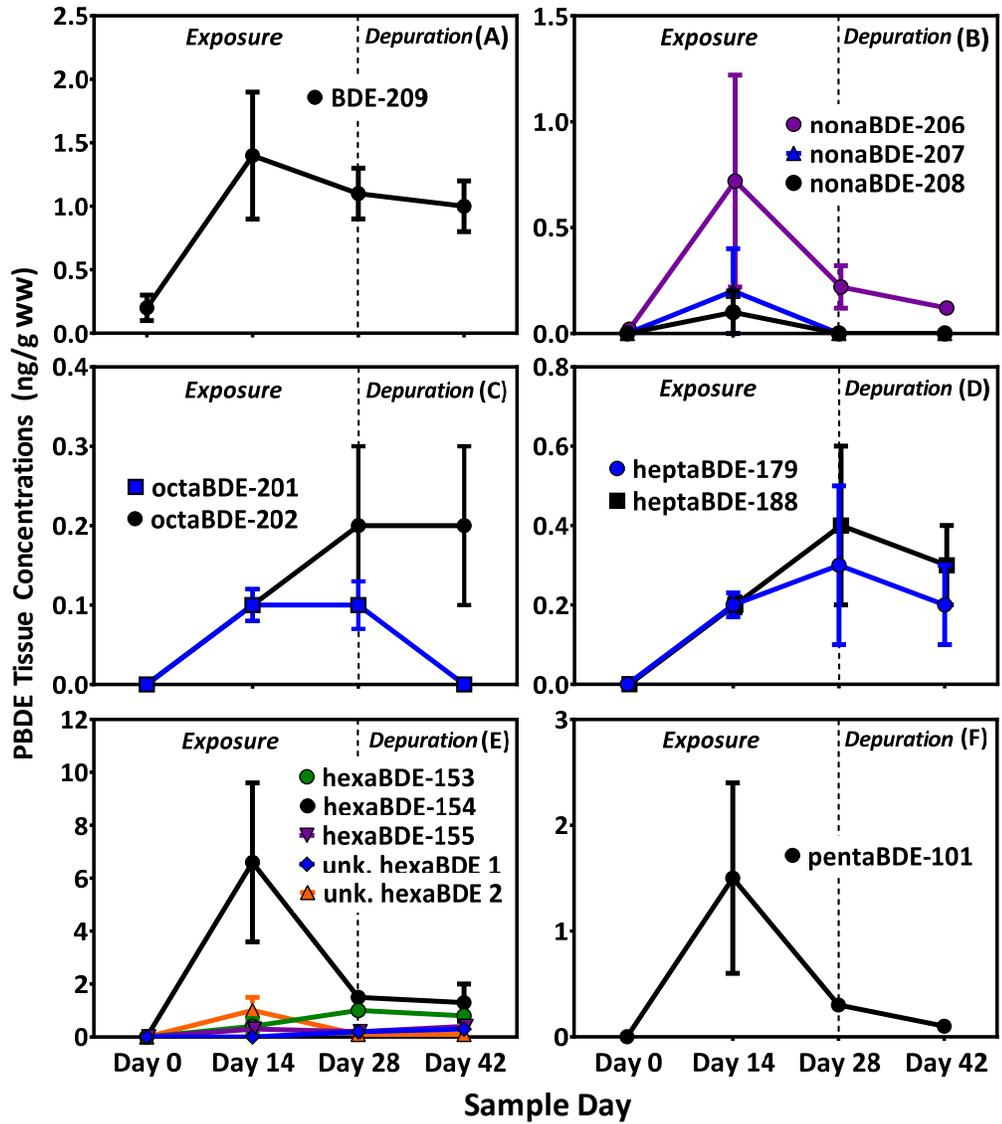


Figure 20: BDE-209 Low Dose - Concentrations (ng/g ww) of BDE-209 (A) and major reductive metabolites (B-F) measured in carcasses of fathead minnow adult dosed orally with BDE-209 at 95.3 ± 0.41 ng/g-food \cdot 3% bw/day (n=3; mean \pm SE). Note differences in y-axis scales.

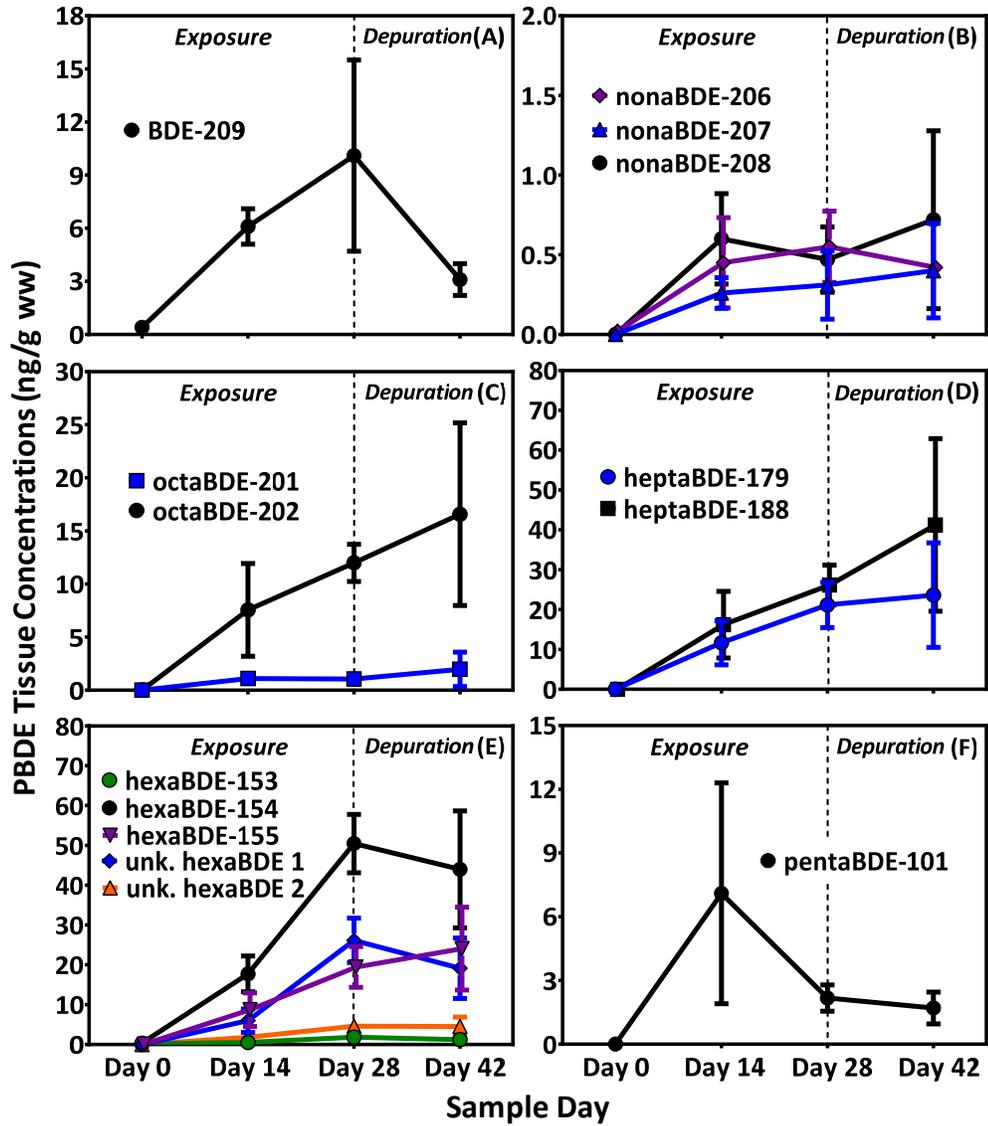


Figure 21: BDE-209 High Dose - Concentrations (ng/g ww) of BDE-209 (A) and major reductive metabolites (B-F) measured in carcasses of fathead minnow adult males dosed orally with BDE-209 at $10 \pm 0.1 \mu\text{g/g food} \cdot 3\% \text{ bw/day}$ ($n=3$; mean \pm SE). Note differences in y-axis scales.

Table 5: PBDE concentrations (n=3; mean ± SE) normalized to body weight (BW; ng/g bw) and lipid weight (LW; ng/g lw) in adult male fathead minnows exposed orally to a BDE-209 (A) low dose at 95 ± 0.4 ng/g ww of food; and (B) high dose at 10.1 ± 0.10 µg/g ww food. nd= not detected.

(A) Low Dose	Day 0		Day 14		Day 28		Day 42 (Depuration)	
	BW	LW	BW	LW	BW	LW	BW	LW
BDE-209	<0.1	<10	1.4±0.5	250±67	1.1±0.2	100±14	1.0±0.2	195±119
nonaBDE-208	nd	nd	nd	nd	nd	nd	nd	nd
nonaBDE-207	nd	nd	nd	nd	nd	nd	nd	nd
nonaBDE-206	nd	nd	<0.1	<20	<0.2	<15	<0.1	<44
octaBDE-202	nd	nd	<0.2	<30	<0.4	<25	<0.3	<50
octaBDE-201	nd	nd	<0.2	<20	<0.1	<6.0	<0.1	<2
heptaBDE-188	nd	nd	<0.3	<45	<0.6	<36	<0.5	<110
heptaBDE-179	nd	nd	<0.3	<36	<0.6	<32	<0.4	<74
hexaBDE-155	nd	nd	<0.5	67±24	<0.3	<21	<0.5	<76
hexaBDE-154	nd	nd	6.6±3.0	1500±860	1.5±0.1	140±30	1.3±0.7	130±48
hexaBDE-153	nd	nd	0.4±0.3	67±48	1.0±0.2	91±13	0.8±0.2	150±79
unk hexaBDE 1	nd	nd	<0.1	<10	<0.3	<20	<0.6	<57
unk hexaBDE 2	nd	nd	1.1±0.5	230±140	<0.2	<8	<0.2	<52
pentaBDE-101	nd	nd	1.5±0.9	290±150	<0.5	<50	<0.2	<11

(B) High Dose	Day 0		Day 14		Day 28		Day 42 (Depuration)	
	BW	LW	BW	LW	BW	LW	BW	LW
BDE-209	<0.2	<36	6.1±1.0	2300±1100	10±5.4	2700±1200	3.1±1.0	310±92
nonaBDE-208	nd	nd	0.6±0.3	280±190	0.5±0.2	130±43	0.7±0.6	37±11
nonaBDE-207	nd	nd	0.3±0.1	110±64	0.3±0.2	78±43	0.4±0.3	20±6.0
nonaBDE-206	nd	nd	0.5±0.2	150±47	0.6±0.2	170±44	0.4±0.1	62±27
octaBDE-202	nd	nd	9.4±4.4	3800±2000	12±1.7	3400±220	17±8.6	1300±300
octaBDE-201	nd	nd	1.1±0.5	500±330	1.1±0.4	290±80	3.0±1.8	130±13
heptaBDE-188	nd	nd	16±8.4	6100±3000	26±5.0	7400±690	41±22	2900±280
heptaBDE-179	nd	nd	12±5.6	4600±2400	21±5.7	5900±1000	24±13	1700±190
hexaBDE-155	<0.1	5.1±2.1	8.7±4.2	3200±1500	20±5.1	5400±1000	24±10	1600±910
hexaBDE-154	<0.4	27±9.0	18±4.5	6000±1900	51±7.3	14000±1500	44±15	4700±2300
hexaBDE-153	<0.1	6.6±1.0	0.5±0.3	128±51	1.9±0.3	530±26	1.2±0.3	200±110
unk hexaBDE 1	nd	nd	6.0±2.9	2200±1100	26±5.6	7500±1400	19±7.6	2100±1300
unk hexaBDE 2	nd	nd	1.8±0.8	720±380	4.6±1.0	1300±160	4.5±2.3	350±190
pentaBDE-101	nd	nd	7.1±5.2	1600±930	6.3±0.9	1800±300	5.2±2.6	570±430

4.3.3 Plasma Thyroid Hormone Levels

Levels of circulating TT4 and TT3 decreased in BDE-209 exposed fish at both the low and high dose tested over the 28-day exposure (Figure 22A-B). In BDE-209 low dose fish, significant ($p<0.05$) declines in circulating TT4 and TT3 were measured at day 14 and 28, as well as at the 14-day depuration, compared to negative controls. Specifically, by sampling day 14, TT3 and TT4 concentrations in the low dose group had declined $53 \pm 4.1\%$ (1.71 ± 0.26 ng/ml) and $57 \pm 6.2\%$ (1.41 ± 0.35 ng/ml), respectively, compared to negative controls (TT3 = 3.67 ± 0.77 ng/ml; TT4 = 3.27 ± 0.41 ng/ml).

The magnitude of the decline in plasma TT3 and TT4 among low dose fish continued into sampling day 28 and after the 14-day depuration. At sampling day 28, TT3 and TT4 levels in low dose fish were decreased $46 \pm 3.7\%$ (1.62 ± 0.19 ng/ml) and $53 \pm 3.6\%$ (1.77 ± 0.23 ng/ml), respectively, compared to negative controls (TT3 = 2.98 ± 0.25 ng/ml; TT4 = 3.73 ± 0.35 ng/ml). Over the 14-day depuration (day 42), circulating levels of thyroid hormones in low dose fish remained depressed with TT3 reduced $46 \pm 3.7\%$ (1.62 ± 0.19 ng/ml) and TT4 reduced $52 \pm 2.8\%$ (1.42 ± 0.14 ng/ml). At the high dose, highly significant ($p<0.01$) declines in plasma TT3 ($62 \pm 8.2\%$; 1.13 ± 0.43 ng/ml) and TT4 ($59 \pm 11\%$; 1.55 ± 0.73 ng/ml) were measured at the end of the 28-day exposure relative to

negative controls (TT3 = 2.98 ± 0.25 ng/ml; TT4 = 3.73 ± 0.35 ng/ml). After the 14-day depuration, TT3 levels were not different from negative controls, but further very highly significant ($p < 0.005$) reductions in TT4 of $66 \pm 3.0\%$ (0.99 ± 0.15 ng/ml) were measured at the BDE-209 high dose. In the PTU positive control group, very highly significant ($p < 0.005$) deficits in TT3 ($50 \pm 10\%$; 1.49 ± 0.50 ng/ml) and TT4 ($52 \pm 10\%$; 1.81 ± 0.63) were measured at sampling day 28 relative to negative controls. After the 14-day depuration, circulating TT3 levels returned to normal among PTU-treated fish but TT4 continued to be reduced by $59 \pm 10\%$ (1.21 ± 0.49 ng/ml).

4.3.4 Deiodinase Activity in the Brain and Liver

By day 14 of the BDE-209 exposure, significant ($p < 0.05$) declines in the rate of T4-ORD were detected in brains of adult minnows at both the low and high dose (Figure 22C). The BDE-209 low dose elicited impacts on rates of T4-ORD that were similar to effects measured at the high dose and PTU positive control. Specifically, at day 14, T4-ORD was reduced by $49 \pm 15\%$ (1.37 ± 0.39 pmol T3 hr⁻¹ mg protein⁻¹), $46 \pm 12\%$ (1.44 ± 0.32 pmol T3 hr⁻¹ mg protein⁻¹), and $44 \pm 11\%$ (1.51 ± 0.29 pmol T3 hr⁻¹ mg protein⁻¹) in brains of BDE-209 low dose, BDE-209 high dose, and positive control fish, respectively, compared to negative controls (2.69 ± 0.19 pmol T3 hr⁻¹ mg protein⁻¹).

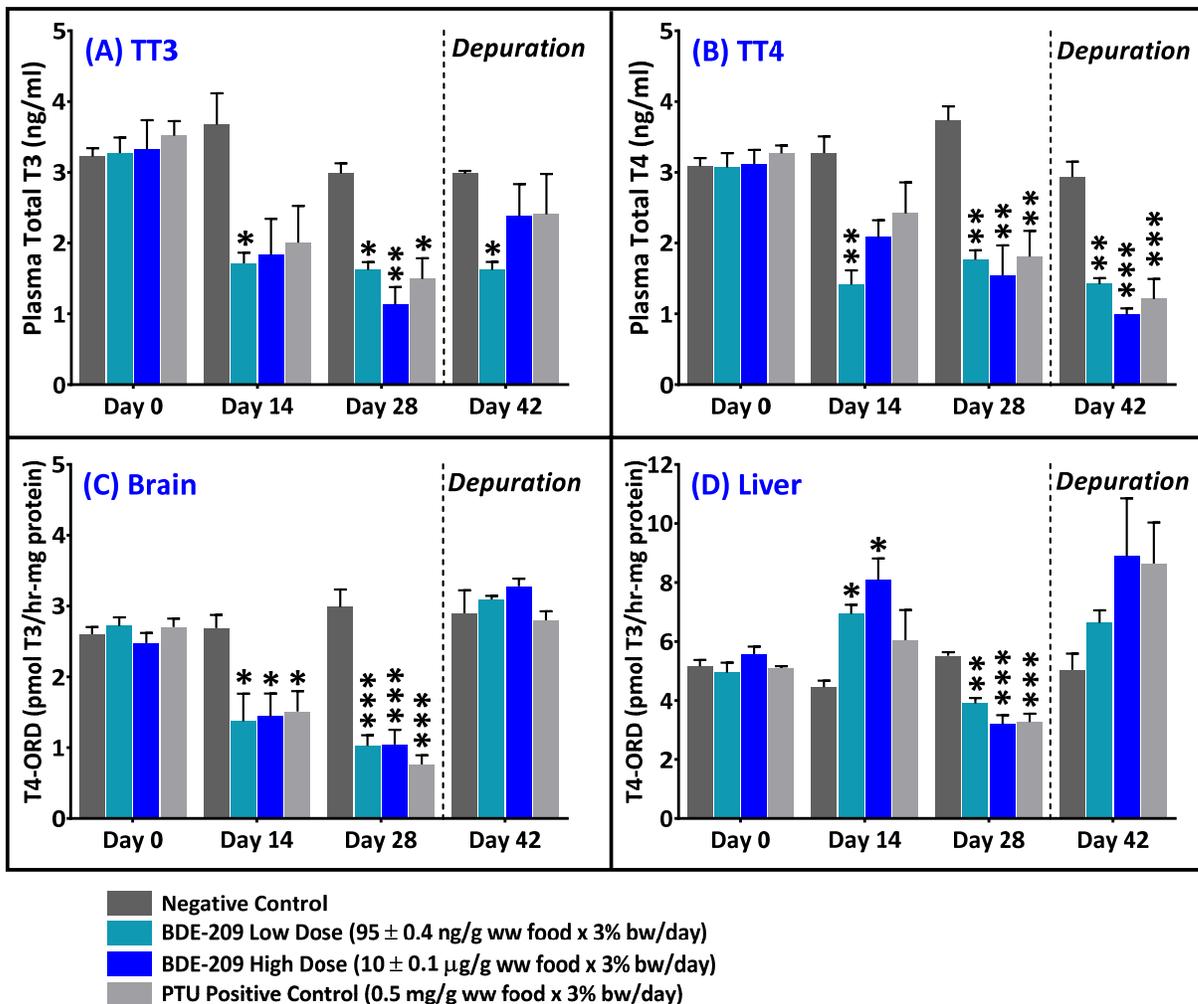


Figure 22: Levels of (A) plasma total T3; (B) plasma total T4; and T4-outer ring deiodination in (C) brains and (D) livers of adult male fathead minnows exposed orally to BDE-209 for 28 days followed by a 14-day depuration. Data analyzed within sampling day with a one-way ANOVA and Tukey's test with statistical significance measured at the * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. Note differences in T4-ORD y-axis scale.

Compared to negative controls (2.99 ± 0.43 pmol T3 hr⁻¹ mg protein⁻¹), by day 28 of the BDE-209 exposure, declines in T4-ORD in the brain (Figure 22C) had advanced with T4-ORD decreased in the brain by $65 \pm 6.9\%$ and $66 \pm 5.0\%$ ($p < 0.005$) at the BDE-209 low dose (1.03 ± 0.26 pmol T3 hr⁻¹ mg protein⁻¹) and high dose (1.04 ± 0.36 pmol T3 hr⁻¹ mg protein⁻¹), respectively. T4-ORD was also significantly depressed by $74 \pm 4.3\%$ (0.76 ± 0.22 pmol T3 hr⁻¹ mg protein⁻¹) in the PTU positive control. After the depuration, T4-ORD in brains of BDE-209 and PTU exposed fish returned to negative control levels.

In liver microsomes (Figure 22D), a significant ($p < 0.05$) increase in the rate of T4-ORD was measured at day 14 of the BDE-209 exposure that was followed by a highly significant ($p < 0.01$) drop at sampling day 28. Specifically, at sampling day 14, liver T4-ORD had risen by $56 \pm 7.0\%$ (6.93 ± 0.31 pmol T3 hr⁻¹ mg protein⁻¹) at the low dose and $81 \pm 16\%$ (8.08 ± 0.73 pmol T3 hr⁻¹ mg protein⁻¹) at the high dose compared to negative controls (4.46 ± 0.22 pmol T3 hr⁻¹ mg protein⁻¹). T4-ORD was also increased in the PTU positive control group at day 14, but this increase was not statistically significant. In contrast, by exposure day 28, the rate of T4-ORD in the liver had declined by $29 \pm 3.3\%$ (3.90 ± 0.32 pmol T3 hr⁻¹ mg protein⁻¹) at the low dose and $42 \pm 5.6\%$ (3.20 ± 0.53 pmol T3 hr⁻¹ mg protein⁻¹) at the high dose relative to negative controls (5.52 ± 0.43 pmol T3 hr⁻¹ mg protein⁻¹).

mg protein⁻¹). Similar to the BDE-209 high dose, rates of T4-ORD in PTU positive control fish also declined by 41 ± 5.1% (3.27 ± 0.49 pmol T3 hr⁻¹ mg protein⁻¹) at day 28. After the depuration, T4-ORD in livers of treated animals was not significantly elevated from negative controls. Changes in T4-IRD were not detected (<MDL) and no changes in 3,3'-T2 production (T3-IRD/rT3-ORD) were measured (Appendix B.8; Figure 30).

4.3.5 Deiodinase mRNA Expression

At day 14, relative *dio2* mRNA levels were elevated ~12-fold in the brain and ~4-fold in the liver of fish exposed to the BDE-209 high dose. At day 14 in the low dose, a ~5-fold ($p<0.01$) increase in relative *dio2* transcript levels was observed in the liver (Figure 23). By day 28, *dio2* transcripts in livers and brains of treated fish had returned to negative control levels. Relative *dio1* transcripts levels in the brain were significantly ($p<0.05$) increased ~4-fold at day 28 in both low and high dose fish. After the depuration, relative *dio1* and *dio2* transcripts were again significantly ($p<0.05$) increased ~3-fold in livers of BDE-209 low dose fish. PTU did not significantly affect *dio* transcript levels in either the brain or liver. No significant changes in *dio3* transcription were detected in either brains or livers of treated fish. Table 6 provides a summary of BDE-209 effects on relative mRNA expression.

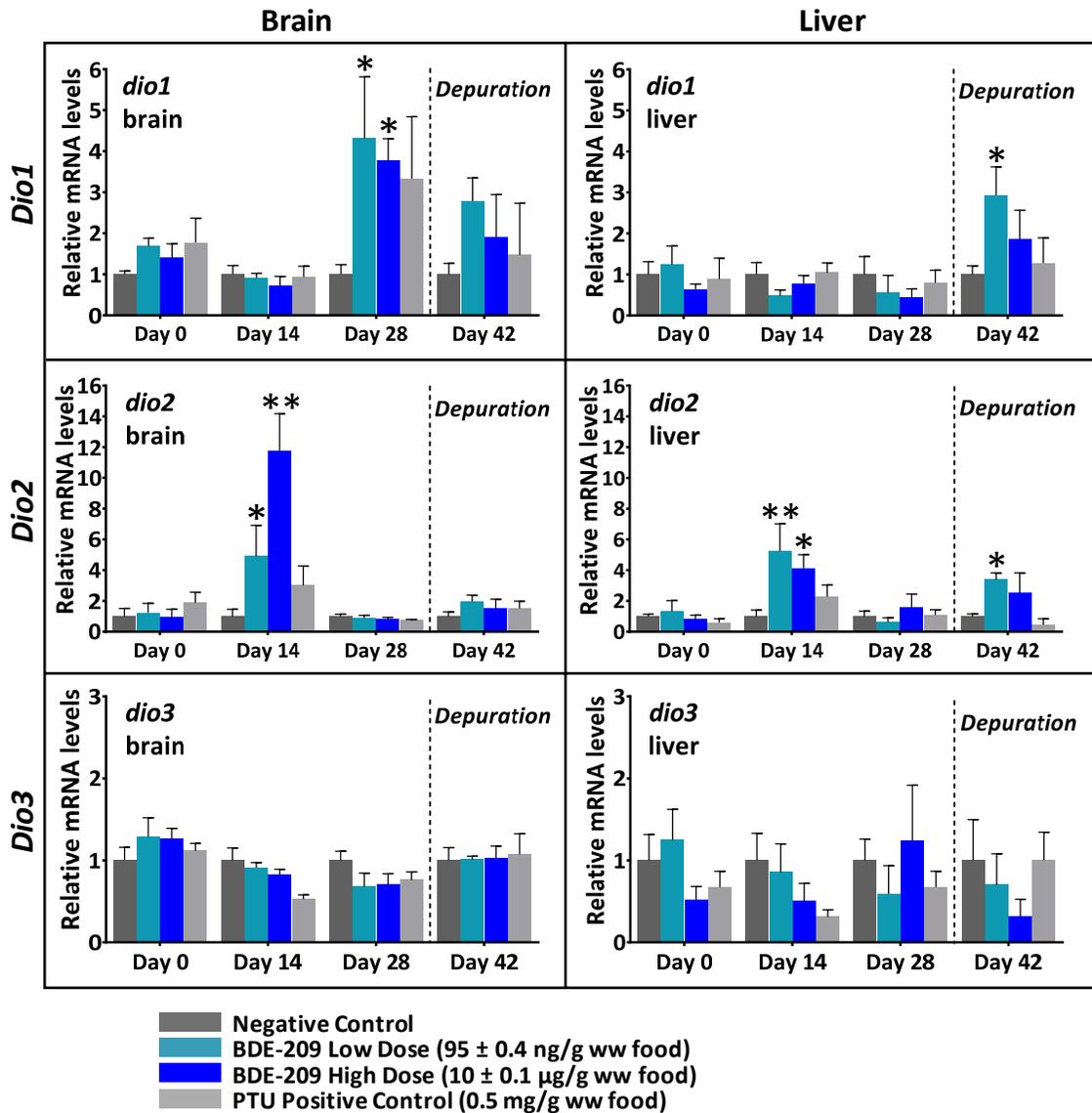


Figure 23: Relative expression of deiodinase (*dio*) mRNA levels (n=6; mean ± SE) in brains and livers of adult male fathead minnows exposed to BDE-209 for 28 days followed by a 14-day depuration. Statistical significance evaluated within sampling day with one-way ANOVA and Tukey's test (*p<0.05, **p<0.01).

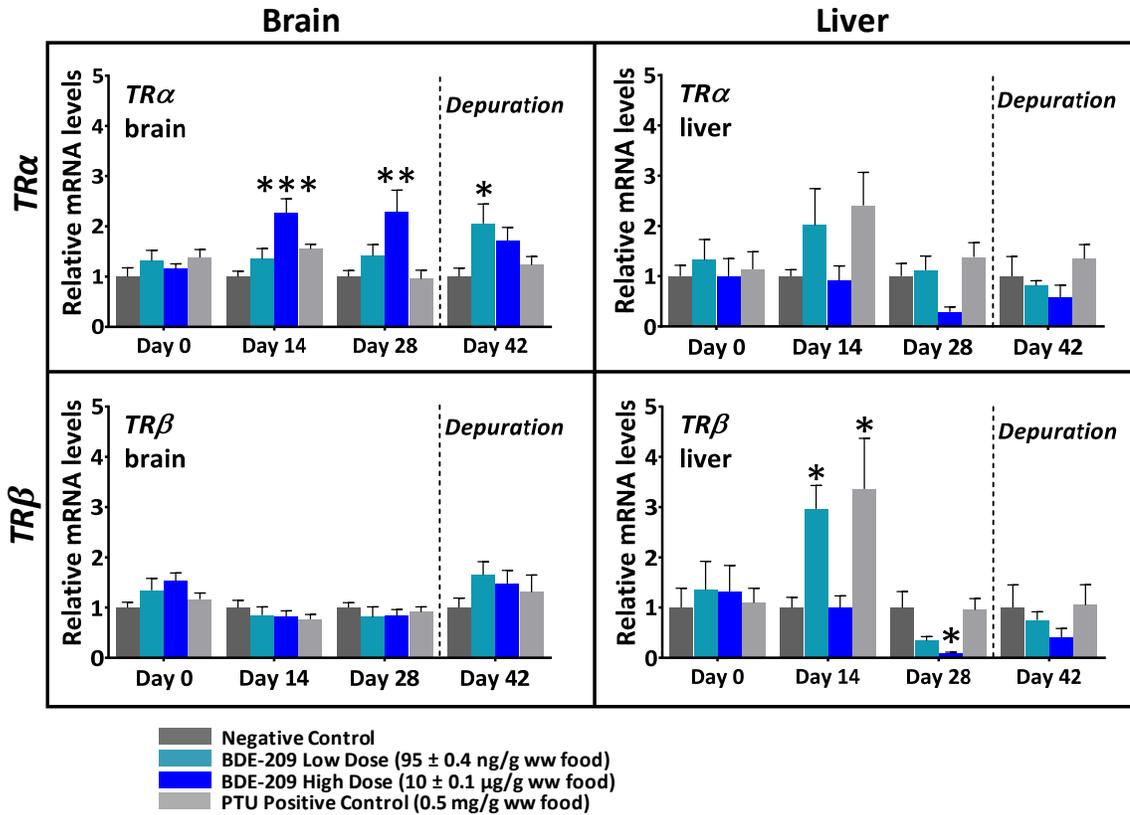


Figure 24: Relative expression of thyroid receptors ($TR\alpha$, $TR\beta$) mRNA levels ($n=6$; mean \pm SE) in brains and livers of adult male fathead minnows exposed orally to BDE-209 for 28-days followed by a 14-day depuration. Statistical significance evaluated within sampling day using a one-way ANOVA and Tukey's test (* $p<0.05$, ** $p<0.01$, *** $p<0.005$).

4.3.6 Thyroid Receptor mRNA Expression

Relative *TRα* mRNA levels were significantly ($p<0.01$, $p<0.005$) elevated ~2-fold in brains of BDE-209 high dose fish at both days 14 and 28 of the exposure (Figure 24). Moreover, BDE-209 caused a significant ($p<0.05$) ~2-fold increase in relative *TRα* mRNA abundance in the brains of low dose fish at the 14 day depuration time point. *TRβ* transcription in the brain was not affected by BDE-209 at either dose tested. However, in the liver, *TRβ* transcripts significantly ($p<0.05$) increased ~3-fold at the BDE-209 low dose and similarly to the PTU positive control. Non-significant increases in *TRα* were also measured at day 14 in the liver of BDE-209 low dose and PTU dosed fish. Transcripts for *TRβ* also significantly ($p<0.05$) declined ~3-fold at sampling day 28 in the livers of fish treated at the BDE-209 high dose.

4.3.7 Transport Protein mRNA Expression

At sampling day 14, relative *MCT8* mRNA levels were significantly ($p<0.05$, $p<0.0001$) increased ~2-fold to ~3-fold in brains of fish treated with BDE-209 and the PTU positive control (Figure 25). *MCT8* transcription returned to negative control levels in the brain at sampling day 28 among BDE-209 low dose fish, but it was again significantly ($p<0.01$) elevated ~2-fold after the 14-day depuration. Relative *MCT8*

transcript levels were also significantly ($p < 0.05$) elevated ~3-fold in livers of BDE-209 low dose fish at sampling day 14. No significant changes in *MCT10* transcription were observed. Of the *OATP* isoforms tested, only *OATP2a1* (brain and liver) and *OATP1c1* transcription (liver only) were altered by the BDE-209 exposure (Figure 26). Relative expression levels of mRNA encoding *OATP1c1* were significantly ($p < 0.05$) increased ~7-fold in livers of both BDE-209 low and high dose fish. For the PTU positive control, relative transcript levels of *OATP1c1* were increased over 12-fold in the liver. No other changes in *OATP* transcription was observed among PTU positive control fish. Relative mRNA transcript abundances for the other *OATP* isoforms tested are provided in Appendix B (Figure 31).

In the brain, the BDE-209 high dose significantly ($p < 0.01$) increased relative *OATP2a1* transcript levels ~2-fold. Likewise, significant ($p < 0.05$), ~2-fold increases of relative *OATP2a1* mRNA levels were also measured in brains of BDE-209 low dose fish after the 14-day depuration. In the liver and in some contrast to the brain, we measured reduced levels of *OATP2a1* transcripts at sampling day 14 at the BDE-209 high dose. Table 6 provides a summary of BDE-209 effects on relative mRNA expression.

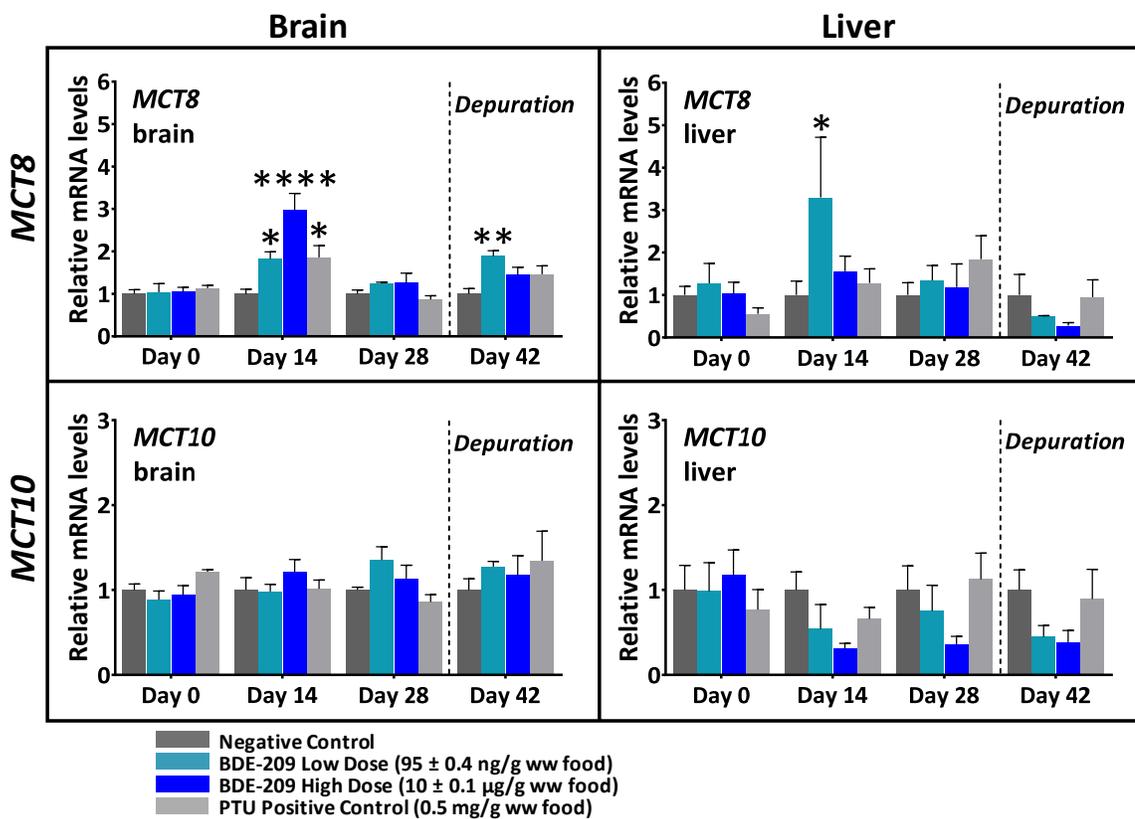


Figure 25: Relative expression of monocarboxylate transporters, *MCT8* and *MCT10*, mRNA levels (n=6; mean ± SE) in brains and livers of adult male fathead minnows exposed orally to BDE-209 for 28-days followed by a 14-day depuration. Statistical significance evaluated within sampling day with one-way ANOVA and Tukey's test (*p<0.05, **p<0.01, ***p<0.001).

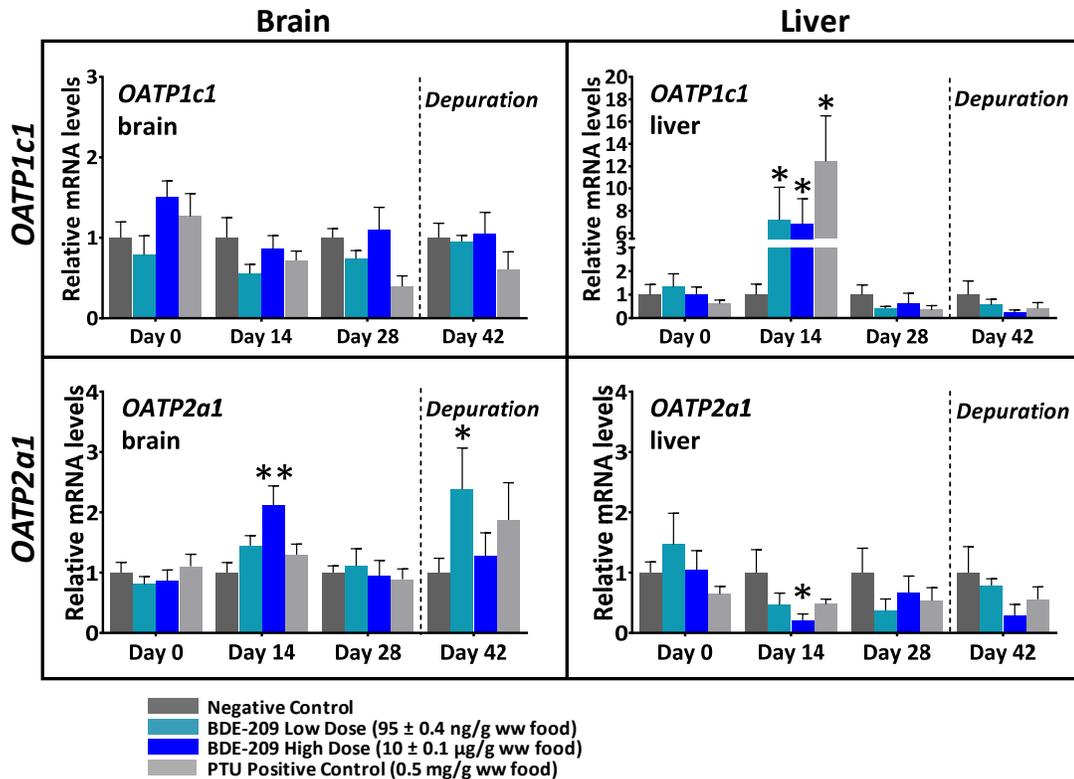


Figure 26: Relative expression of organic anion transport proteins, *OATP1c1* and *OATP2a1*, mRNA levels (n=6; mean \pm SE) in brains and livers of adult male fathead minnows exposed orally to BDE-209 for 28-days followed by a 14-day depuration. Statistical significance evaluated within sampling day with one-way ANOVA and Tukey's test (*p<0.05, **p<0.01). Note differences in y-axis scales.

Table 6. Summary of BDE-209 and PTU positive control effects on the relative mRNA expression of targeted genes*

<i>Endpoint</i>	<i>Treatment</i>	<i>Day 14</i>		<i>Day 28</i>		<i>Day 42</i>	
		<i>Brain</i>	<i>Liver</i>	<i>Brain</i>	<i>Liver</i>	<i>Brain</i>	<i>Liver</i>
Dio1	BDE-209 Low Dose	—	—	↑	—	—	↑
	BDE-209 High Dose	—	—	↑	—	—	—
	PTU Pos Ctrl	—	—	—	—	—	—
Dio2	BDE-209 Low Dose	↑	↑↑	—	—	—	↑
	BDE-209 High Dose	↑↑	↑	—	—	—	—
	PTU Pos Ctrl	—	—	—	—	—	—
TR α	BDE-209 Low Dose	—	—	—	—	↑	—
	BDE-209 High Dose	↑↑↑	—	↑↑	—	—	—
	PTU Pos Ctrl	—	—	—	—	—	—
TR β	BDE-209 Low Dose	—	↑	—	—	—	—
	BDE-209 High Dose	—	—	—	↓	—	—
	PTU Pos Ctrl	—	↑	—	—	—	—
MCT8	BDE-209 Low Dose	↑	↑	—	—	↑↑	—
	BDE-209 High Dose	↑↑↑↑	—	—	—	—	—
	PTU Pos Ctrl	↑	—	—	—	—	—
OATP1c1	BDE-209 Low Dose	—	↑	—	—	—	—
	BDE-209 High Dose	—	↑	—	—	—	—
	PTU Pos Ctrl	—	↑	—	—	—	—
OATP2a1	BDE-209 Low Dose	—	—	—	—	—	—
	BDE-209 High Dose	↑↑	↓	—	—	—	—
	PTU Pos Ctrl	—	—	—	—	—	—

*Relative mRNA transcript abundances of genes that were not affected by BDE-209 or PTU included: *dio3*, *MCT10*, *OATP1f1*, *OATP1f2*, *OATP2b1*, *OATP3a1*, *OATP4a*, and *OATP5a1*. One arrow= $p<0.05$; two arrows = $p<0.01$; three arrows = $p<0.005$; four arrows = $p<0.001$.

4.4 Discussion

4.4.1 Mortality

The percent cumulative mortality was increased in fish exposed to BDE-209 at both the low and high dose tested compared to negative and positive controls (Figure 18). PBDE-induced mortality has not been frequently reported in the literature nor

observed in the previous *in vivo* fish studies conducted in our laboratory (Noyes et al. 2011; Stapleton et al. 2006). Recently, He et al. (2011) reported a significant increase in mortality (~44%) in adult zebrafish exposed to 1- μ M concentrations of BDE-209 for five months relative to negative controls (~38%). A limited number of toxicity studies have targeted PBDE-induced mortality as an endpoint in young fish (Usenko et al. 2011) and amphibians (Van Schmidt et al. 2012). Usenko et al. (2010) measured the acute toxicity of six PBDE congeners in developing zebrafish (*Danio rerio*) receiving aqueous exposures and found that BDEs-28, -47, -99, and -100 induced acute mortality by an unknown mechanism but that the higher PBDEs studied (BDE-153, -183) caused no mortality. Thus, it is possible that some of the lower reductive metabolites formed here could have contributed to the increased mortality. For instance, fathead minnow adult males (not females) were found to have declines in body condition factors upon dietary exposure to BDE-47 (Muirhead et al. 2006). While no changes in body condition factors were measured in this study, a non-significant decrease in lipid content was measured at day 14 and 28 of the BDE-209 exposure. Finally, it is notable that tanks containing male minnows exposed to BDE-209 in this study displayed increased aggressive behaviors and territoriality (e.g., fighting, chasing, head-butting) than did negative control and

PTU-treated fish tanks. Thus, while not a specifically targeted endpoint in this study, it is possible that stress-related responses potentially related to the BDE-209 exposure could have contributed to the increased mortality. It is also possible that adult male fathead minnows are more sensitive to PBDEs and thyroid toxicity than other species, although species sensitivities to PBDEs and other organohalogenes are not well understood.

4.4.2 Bioaccumulation and Metabolism

BDE-209 bioaccumulation and reductive debromination was measured in adult fathead minnows exposed to both BDE-209 doses (Figure 20-21; Table 5). The suite of reductive metabolites measured here in adult minnows was nearly identical to metabolites detected in juvenile fathead minnows (Figures 12 and 17) (Noyes et al. 2011). One difference is that small amounts of hexaBDE-153 were measured in adult minnows that were not observed in juveniles. Work in our laboratory has shown that carp liver microsomes debrominate BDE-153 to BDE-101 (Roberts et al. 2011). Thus, a predicted pathway of reductive debromination is described in Figure 27 based on the metabolites measured. Like with juvenile minnows, this pathway suggests rapid reductive debromination as predicted intermediate metabolites were not measured in many

instances. In addition, several metabolites (BDE-202, -179, -188, and -101) measured are not found in any commercial mixtures supporting their presence as metabolites. The data generated here suggest that while BDE-209 metabolism in Cyprinids is rapid, it may stop at the pentaBDEs as no appreciable reductive metabolism to lower MW congeners has been detected to date.

Results here also support our previous observations of preferential debromination dominated by cleavage of bromine atoms from *meta*-substituted positions. The reductive metabolism observed in adult minnows is consistent with metabolism observed *in vitro* and *in vivo* in common carp (*Cyprinus carpio*), another Cyprinid species, suggesting family-specific patterns of debromination (Roberts et al. 2011; Stapleton et al. 2004a). However, BDE-209 did not bioaccumulate in carp as observed here in fathead minnows. The daily exposures to BDE-209 at both the high and low doses of ~0.7 µg of BDE-209/fish-day and ~6.8 ng of BDE-209/fish-day, respectively, were environmentally relevant given that BDE-209 levels in sediments have been measured at thousands of ng/g dry weight (Mai et al. 2005; Sellstrom et al. 1998; Vane et al. 2010; Vigano et al. 2011).

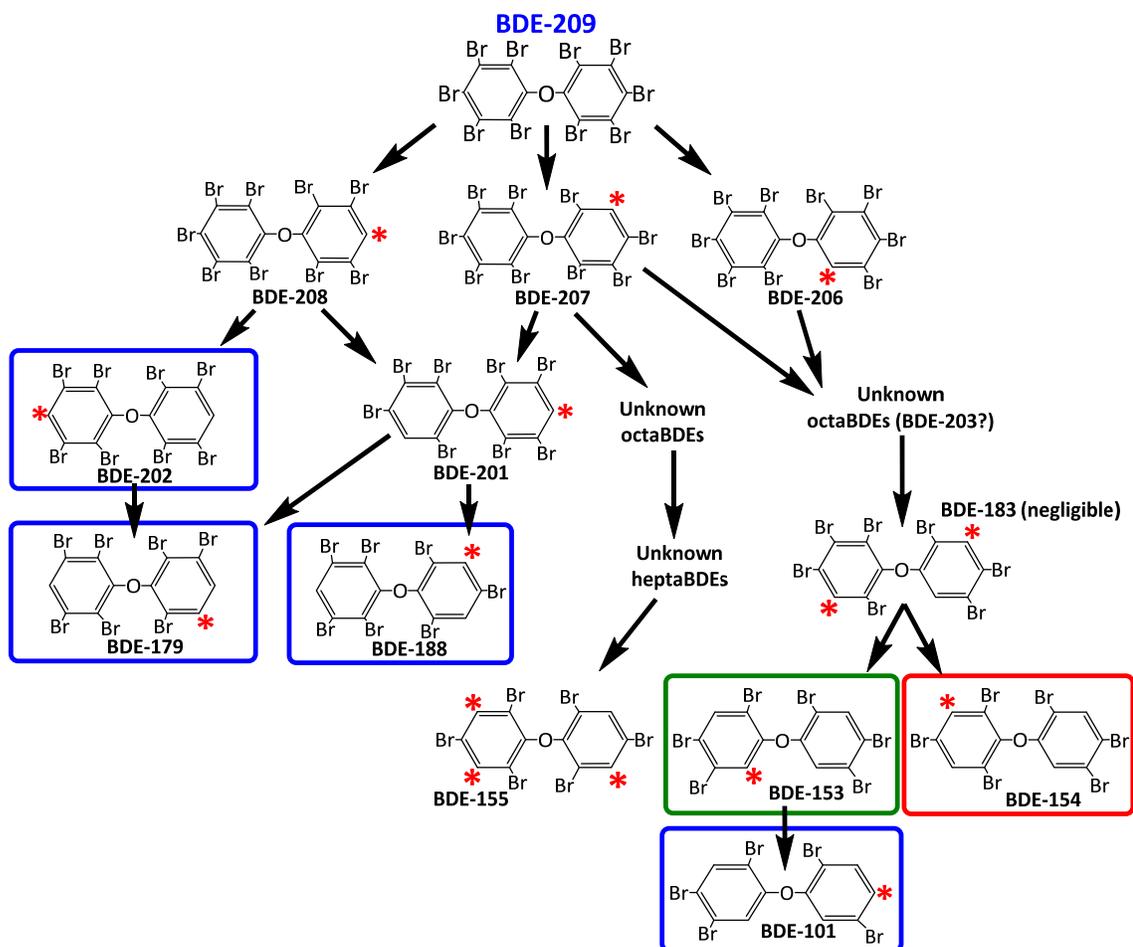


Figure 27: Predicted debromination pathway of BDE-209 in adult male fathead minnows exposed to $10.1 \pm 0.10 \mu\text{g/g}$ ww food at 3% bw/day for 28 days. Asterisks indicate predicted sites of debromination. Congeners in blue boxes represent PBDEs not found in any commercial mixtures. BDE-154 (red box) was measured at the highest concentration. BDE-153 (green box) was not detected in juvenile fathead minnows receiving similar exposures (Figures 12 and 17; Noyes et al. 2011).

The higher BDE-209 bioavailability measured at the lower dose tested (3.8%) versus at the BDE-209 high dose (1.3%) is unclear but may be attributable to individual sampling and feeding variability. In addition, there could be dose-related differences in BDE-209 uptake from the intestine and enhanced metabolic detoxification and elimination at higher doses. The 1.3% of BDE-209 bioavailable in adult minnows at the BDE-209 high dose was lower than the percent bioavailability (5.8%) measured in juvenile minnows receiving equivalent treatments (Noyes et al. 2011). Nonetheless, BDE-209 assimilation was higher in adult fathead minnows than the <0.5% bioavailability measured in juvenile common carp (Stapleton et al. 2004a) and juvenile rainbow trout (Kierkegaard et al. 1999). However, it is notable that the dose rates in this study (low dose = 2.9 ng/g bw-day; high dose = 0.3 µg/g bw-day) were lower than doses tested in the Kierkegaard study with rainbow trout (7.5-10 µg/g bw-day). Moreover, additional research in our laboratory (Stapleton et al. 2006) in juvenile rainbow trout at lower doses (940 ng/g ww food at 1% bw/day for five months) than the Kierkegaard study found that 3.2% of BDE-209 exposures were assimilated, which aligns more closely with observations here in adult minnows. Taken together, these data support that BDE-209 uptake appear to be generally low in fishes, but that young fathead minnows appear to

assimilate higher percentages of BDE-209 than adult minnows. Additional research is needed to better understand the dose-related differences in BDE-209 bioavailability observed here and across other species and life-stages.

4.4.3 Reduced Plasma Thyroid Hormones

The significant deficits in plasma TT3 (low dose = $46 \pm 3.7\%$; high dose = $62 \pm 8.2\%$) and TT4 (low dose = $53 \pm 3.6\%$; high dose = $59 \pm 11\%$) measured here in adult male fathead minnows after a 28-day exposure were consistent with thyroid hormone reductions (TT3 = $50 \pm 10\%$; TT4 = $52 \pm 10\%$) induced by the PTU positive control treatment. While this is the first study to examine BDE-209 effects on circulating thyroid hormone levels in adult fish, PBDE effects on thyroid hormone regulation have been observed among other species, life-stages, and PBDE congeners. For instance, a 56-day exposure to a mixture of 13 PBDE congeners, including BDE-209, at ~ 2.5 and ~ 25 ng/g dw food/PBDE congener at a feeding rate of 1.5% bw/day was shown to reduce free T4 ($\sim 35\%$ at low dose; $\sim 45\%$ at high dose) and free T3 ($\sim 50\%$ at low dose) in juvenile lake trout (*Salvelinus namaycush*) (Tomy et al. 2004). Studies in developing rodents exposed by the diet and maternally have also shown that BDE-209 can induce dose-dependent reductions in serum T4 and T3 levels (Fujimoto et al. 2011; Rice et al. 2007; Tseng et al.

2008). In addition, the significant declines in plasma thyroid hormone levels observed here in adult minnows are consistent with reductions in plasma thyroid hormones observed in fish exposed to other PBDE congeners. For instance, a significant ($p < 0.05$) decline (35-45%) in TT4 relative to controls was measured in adult male fathead minnows exposed orally to BDE-47 at 12.4 $\mu\text{g}/\text{breeding pair-day}$ for 21 days (Lema et al. 2008). However, in contrast to our results, BDE-47 had no effect on TT3 in exposed fish. In addition, the BDE-47 doses administered by Lema et al. (2008) at $\sim 6.2 \mu\text{g}/\text{fish-day}$ were higher than the BDE-209 doses tested in this study (high dose = 0.7 $\mu\text{g}/\text{fish-day}$; low dose = 6.8 ng/fish-day).

One leading hypothesis for thyroid hormone disruption in mammals exposed to PBDEs is by the induction of Phase II liver enzymes that enhance the metabolism and biliary elimination of thyroid hormones. In support of this hypothesis, laboratory rodent studies have demonstrated increased gene transcript abundances and activity of thyroid hormone conjugating UGT and SULT enzymes in rats and mice exposed to BDE-47 and DE-71 (Richardson et al. 2008; Szabo et al. 2009; Zhou et al. 2002). However, in contrast to work in mammals, a limited number of studies in piscivores have shown down-regulation of *UGT* mRNA expression, suggesting that a compensatory response of these

PBDE and thyroid hormone metabolizing enzymes could be occurring in PBDE exposed fish (Q Chen et al. 2012; Olsvik et al. 2009). Another mechanism that has been hypothesized to play an important role in PBDE-related thyroid dysfunction is through thyroid hormone mimicry. OH-PBDE metabolites produced in rat liver microsomes enriched with CYP2b (phenobarbital-induced) have been found to compete with thyroid hormone for binding to the blood transport protein transthyretin (Meerts et al. 2000). Similarly, several parent PBDEs (BDE-28, -49, -47, -99) were shown to be potent inhibitors of T3 binding to transthyretin in a recombinant sea bream transthyretin assay, potentially leading to greater elimination of thyroid hormone, whereas 6-OH-BDE-47 had less affinity for sea bream TTR than T3 or T4 (Morgado et al. 2007).

PBDEs may also alter thyroid hormone regulation by interfering with dio enzymes. For instance, *in vitro* data generated in carp liver microsomes provide evidence that thyroid hormones inhibit the reductive metabolism of PBDEs in carp liver microsomes, suggesting that PBDEs may interfere with dio enzymes and alter thyroid hormone homeostasis (Figure 10) (Noyes et al. 2010). However, it remains unclear whether PBDEs (or OH-PBDEs) can bind directly to dio enzymes or whether they may elicit other allosteric effects on these enzymes that affect their capacity to mediate

thyroid hormone regulation. In addition, PBDE interferences with deiodinases may occur indirectly as compensatory responses to other thyroid perturbing events caused by PBDEs (e.g., competitive binding to plasma transporters).

4.4.4 Altered Expression and Activity of Dio Enzymes

The significantly reduced dio enzyme activity measured in adult minnows (Figure 22) generally aligns with findings in juvenile fathead minnows (Noyes et al. 2011). In particular, declines in the rate of T4-ORD measured in brains of adult male minnows ($65 \pm 6.9\%$) exposed to the BDE-209 high dose were consistent with declines in whole-fish T4-ORD (~74%) in juvenile minnows receiving approximately equivalent dietary treatments of BDE-209 (Figure 13). This highly altered brain T4-ORD in adult minnows suggests that thyroid regulation in brains of adult fish may be particularly sensitive to BDE-209. Specifically, BDE-209 exposures in adult male minnows altered the expression and activity of dio enzymes in a time and tissue dependent manner. Notable differences in T4-ORD activity between the brain and liver were measured at sampling day 14 in BDE-209 treated fish. T4-ORD was depressed in brains of minnows at both the low and high dose but was increased in the liver at day 14. This dichotomy suggests differences in local signaling in peripheral tissues in response to BDE-209. The increased

T4-ORD in the liver may be attributable to a local response of this organ to reductions in circulating thyroid hormones. Although little is known about dio activity in fish brain tissues compared to the liver, the measured reductions of dio activity in brain microsomes of treated fish may be a demonstration of the brains inability to compensate locally to depressed levels of hormone in circulation.

While it is necessary to use caution when comparing changes in transcriptional and post-transcriptional activity, altered mRNA expression can inform our understanding of tissue-specific effects of BDE-209 on thyroid signaling. This study is one of only a few that has measured PBDE-induced changes in mRNA transcripts encoding dio enzymes and TRs in fish. Results measured here largely align with these other studies, although there were notable differences. Consistent with our findings of upregulated *dio 1* and *dio 2* mRNA expression in the brain and liver (low dose only; Figure 23), BDE-209 and the commercial PentaBDE mixture (DE-71) have also been found to increase the mRNA expression of *dio 1* and *dio 2* in zebrafish larvae (Q Chen et al. 2012; Yu et al. 2010). Li et al. (2011) also measured an increase in *dio 2* transcripts in the larvae of Chinese rare minnows (*Gobiocypris rarus*) exposed to BDE-209, but in contrast to our results, they measured a decrease in *dio 2* transcripts in brains of adult

rare minnows. One distinguishing characteristic of these other studies is that the BDE-209 exposures in these studies were aqueous. Given the large size (mw=959) and hydrophobic character of BDE-209 (Kow 6-12), and its propensity to readily bind to sediments, surfaces, and dissolved organic matter, the dietary exposures here reflect a more environmentally relevant exposure. In mammals, PBDEs have been shown to increase and decrease the expression of *dio* mRNA transcripts. For example, BDE-99 increased relative mRNA transcripts encoding *dio 1* in human hepatocytes, consistent with results here (Stapleton et al. 2009). In contrast, however, *dio 1* transcripts were downregulated in livers of rat pups exposed to the commercial PentaBDE mixture DE-71 (Szabo et al. 2009). However, it is difficult to compare and interpret differences in *dio* mRNA expression patterns in PBDE studies conducted to date given major differences in dosing routes, exposure timing, and PBDE congeners targeted.

4.4.5 Compensatory Responses of Thyroid to BDE-209

The functional and biochemical properties of *dio* enzymes can provide insights into differences in the postulated compensatory responses of *dio* mRNA expression measured here in adult male minnows exposed to BDE-209. In all vertebrates, *dio 1* and *dio 2* catalyze the T4-ORD pathway to produce the genomically active T3 hormone.

Thus, it is possible that the overall directional change here of elevated *dio* mRNA abundance among BDE-209 treated fish could be attributable to a localized compensatory response of the peripheral thyroid system to a hypothyroid state, as indicated by depressed levels of plasma TT3 and TT4. This response is consistent with other studies in which chemically-induced hypothyroidism increased and exogenous thyroid hormone reduced relative *dio 1* and *dio 2* mRNA levels in livers and brains of fishes (Garcia et al. 2004; Johnson and Lema 2011; Van der Geyten et al. 2001; Walpita et al. 2007). Another aspect that cannot be overlooked is that studies in mammals have also measured substantial variability in dio activity and expression in the presence of thyroid hormone challenge, suggesting substantial post-transcriptional regulation that may include dio degradation/preservation by ubiquitination/deubiquitination and assembly by dimerization (Burmeister et al. 1997; Gereben et al. 2008a).

The increased rate of T4-ORD measured at day 14 in livers of BDE-209 treated fish, along with the increase in *dio 2* mRNA abundance, is consistent with studies in methimazole-treated tilapia (*Oreochromis niloticus*) where hypothyroidism increased both hepatic T4-ORD and *dio 2* transcripts (Mol et al. 1999; Van der Geyten et al. 2001). Dio 2 enzyme has demonstrated substantial physiological plasticity in vertebrates,

making it a sensitive regulator of T4-ORD and intracellular T3 homeostasis. Work in laboratory rodent models supports that *dio 2* regulation occurs at both a transcriptional and post-transcriptional level (Burmeister et al. 1997). *Dio 2* has been shown to be highly sensitive to thyroid hormone with a relatively short half-life in mammals of ~40 min (St Germain 1988). Thus, the early upregulation of *dio 2* mRNA expression measured at day 14 in livers and brains of BDE-209 treated fish may be attributable to the unique biochemical properties and rapid homeostatic behavior of *dio 2* in response to depressed plasma thyroid hormones. It is notable that T4-ORD activity was not increased in brains of BDE-209 treated fish, suggesting that this compensatory response may not occur in the adult male fathead minnow brain.

Indeed, early studies have questioned whether *dio 2* is expressed in brains of piscivores because of the lack of or only negligible T4-ORD activity measured in the fish brain (Fines et al. 1999; Frith and Eales 1996; Mol et al. 1998; Sambroni et al. 2001). However, in accordance with our results, more recent studies using RT-qPCR techniques have localized *dio 2* transcripts to brains of fish (Johnson and Lema 2011; Sutija et al. 2003; Wambiji et al. 2011). Still though, normalized *dio 2* mRNA levels were about six times lower in the male fathead minnow brain than in livers of negative controls,

supporting that *dio 2* expression in the fish brain may be low. This also comports with our measurements of relatively low T4-ORD in the fish brain compared to the liver. The relatively low *dio 2* expression and activity in the fathead minnow brain may be contributing to the potentially absent homeostatic compensation of the brain to BDE-209.

It is also possible that the upregulation of *dio1* mRNA measured in the brain of fathead minnows at day 28 may be indicative of a secondary regulatory mechanism that could be associated with longer periods of thyroid hormone depression. Specifically, no changes in *dio 1* mRNA expression were detected in either brains or livers of parrotfish with experimentally elevated T3 or depressed T4 (via methimazole) after a three-day treatment (Johnson and Lema 2011). In contrast, however, increased relative *dio 1* mRNA transcripts were reported in two species of tilapia after a 90-day treatment with methimazole (Van der Geyten et al. 2001), suggesting the possibility that compensatory mechanisms of the thyroid may change over time as BDE-209 exposures continue chronically.

BDE-209 effects on plasma thyroid hormone levels and T4-ORD were largely consistent with and in some cases worse than effects measured in the PTU positive

control. PTU is a model thyroid hormone reducing agent that acts primarily at the central HPT by inhibiting the activity of thyroid peroxidase iodination of tyrosine residues in thyroglobulin. PTU also acts in peripheral tissue of mammals by inhibiting dio 1 and is therefore an effective compound in delineating relative dio activity profiles (Visser et al. 1983). However, in fishes, effects of PTU on peripheral dio activity is less clear with some studies suggesting that dio 1 in some fish species may be resistant to PTU (Frith and Eales 1996; Mol et al. 1993; Orozco et al. 2000; Sanders et al. 1997). Conversely, early work from the Eales group also reported that PTU treatments decreased dio 1 activity in trout liver hepatocytes (Shields and Eales 1986). The possible resistance of PTU to fish dio 1 is also consistent with our work in carp liver microsomes whereby no change in BDE-99 metabolism to BDE-47 was measured upon challenge with PTU while thyroid hormone co-incubations reduced this BDE-99 metabolism (Noyes et al. 2010). Unlike effects measured in BDE-209 treated fish, the PTU positive control treatment had no effect on *dio* mRNA expression in the brain or liver, suggesting that its effects on minnows were mediated predominantly at the central HPT rather than by inhibiting dio 1. Nonetheless, post-transcriptional effects of PTU on *dio 1* expression in fish cannot be ruled out.

Taken together, despite the early upregulation of *dio 2* mRNA and increased T4-ORD activity in the liver as well as the substantial *dio 2* and *dio 1* upregulation in the brain, by day 28 of the exposure, rates of T4-ORD were very significantly reduced ($p < 0.01$ to $p < 0.005$) in both tissues and across all treatments, including the PTU positive control. Moreover, in the brain, T4-ORD was significantly reduced at sampling day-14, suggesting that the brain of adult animals may be uniquely susceptible to BDE-209 exposures. Data here suggest that peripheral brain and liver tissues were unable to fully compensate for the reduced levels of plasma thyroid hormones from the low BDE-209 exposures tested. However, apparent recovery in peripheral tissue T4-ORD was observed after the 14-day depuration in both the brain and liver. The return of T4-ORD to negative control levels suggested recovery of peripheral tissues after the 14-day depuration, but possibly ongoing disruption of the central HPT axis given the reduced plasma TT4 and TT3 (low dose only) after the 14-day depuration. Moreover, a significant increase in the relative expression of *dio 1* and *dio 2* mRNA transcripts was observed in low dose fish after the depuration period (Figure 23). This trend of upregulated *dio* transcription could be an ongoing response of fish to low levels of

thyroid hormone in circulation, which could involve the removal of T4 from circulation and conversion to T3 to try to maintain appropriate levels of T3 in circulation.

4.4.6 Altered Expression of Thyroid Receptors

In addition to changes in *dio* transcript abundances, results here support that the altered thyroid hormone signaling from BDE-209 affected the relative expression of TR mRNA transcripts in a tissue-specific manner (Figure 24). Two genetically distinct receptors *TR α* and *TR β* have been identified in fathead minnows with additional subtypes identified in teleosts that may be attributable to gene duplication or alternative mRNA splicing (Filby and Tyler 2007; Lema et al. 2009; Nelson and Habibi 2009). While limited in number, other PBDE studies have measured changes in TR expression that both align with and differ from results here. Similar to the increase in TR transcripts measured here, BDE-209 also increased *TR α* and *TR β* mRNA expression in zebrafish larvae (Q Chen et al. 2012). Aqueous exposures to the PentaBDE, in contrast, had no effect on TRs of zebrafish larvae (Yu et al. 2010). While these results are informative, it is necessary to use caution when comparing expression patterns of TRs from early life stages to adults as TR expression and functionality can vary substantially depending on fish life-stage (Filby and Tyler 2007; Forrest et al. 1990; Lema et al. 2009; Nelson and

Habibi 2009). For instance, during zebrafish embryonic development, *TR α* mRNA is more highly expressed than *TR β* mRNA, but in zebrafish adults *TR β* mRNA is greater than *TR α* in the brain while *TR α* mRNA is more abundant in the liver and ovary (Liu et al. 2000).

The increased relative TR mRNA transcript levels measured in BDE-209 and PTU dosed fish reveals an apparent contradiction with studies in hyperthyroid fish. Specifically, an increase in relative *TR α* and *TR β* transcript levels has also been measured in brains and livers of adult fathead minnows treated with T3 (Lema et al. 2009). One hypothesis for the increase in TR transcript levels in hyperthyroid fish treated with T3 is because TRs themselves contain TREs and can be auto-induced by T3 (Krain and Denver 2004; Liu et al. 2000; Manchado et al. 2009). Thus, based on this positive relationship between TR transcription and T3-induced hyperthyroidism, a decrease in the expression of TR mRNA expression might be predicted in PTU and BDE-209 treated fish in response to hypothyroidism.

However, as shown in Figure 24 an increase in relative *TR α* mRNA levels was measured in brains of BDE-209 high dose (days 14, 28) and low dose (14 day depuration

only) fish while an upregulation in *TRβ* transcripts was measured in livers of BDE-209 low dose and PTU treated fish at day 14. While this transcriptional upregulation of TRs in response to BDE-209 and PTU may seem contradictory to the evidence in T3-treated animals, both hypothyroidism (via thyroidectomy) and hyperthyroidism (T3-induced) have been shown to increase TR receptors in brains of adult rats (Hamada and Yoshimasa 1983). In addition, an increase in the relative abundance of *TRα* transcripts was measured in the blubber of free ranging harbor seals that was correlated with PCB exposures and depressed levels of circulating T4 (Tabuchi et al. 2006). Moreover, adding further complexity to these relationships, the expression of TRs and their response to thyroid perturbations has even been shown to vary within tissues. For instance, hypothyroidism (via methimazole) has been shown to alter the expression of TRs in brains of adult rats in a region-specific manner by, for example, increasing relative levels of *TRα1* transcripts in the hippocampus and cerebral cortex, but eliciting no effect on TR expression in the cerebellum (Constantinou et al. 2005).

The only investigation that we are aware of that has looked at PBDE effects on mRNA transcripts encoding TRs in fish was conducted by our co-authors with BDE-47 in adult fathead minnows (Lema et al. 2008). In this study, *TRα* transcripts were

significantly ($p < 0.005$) elevated in brains of female minnows exposed to BDE-47 but not males, while *TR β* transcripts were significantly ($p < 0.05$) depressed in brains of both sexes. We also measured a significant ($p < 0.01$) increase in *TR α* transcripts in adult minnows exposed to the BDE-209 high dose, starting at sampling day 14, and also in the BDE-209 low dose after the 14-day depuration. However, in contrast to our results, Lema et al. (2008) only measured elevated *TR α* mRNA expression in the female minnow brain. Moreover, no changes in TR transcription in livers of BDE-47 treated fish were measured whereas we measured an increase in relative *TR β* transcript levels in BDE-209 low dose fish and a decrease at the high dose.

In addition to sex- and tissue-specific differences, the response of gene transcripts encoding TRs may also vary over time. A time-course study in rat cerebellar granular neurons observed depressed *TR α 1* and *TR α 2* mRNA levels within only a few hours of exposure to BDE-99, whereas significant increases in *TR β* transcripts were observed but not until after 12 hours (Blanco et al. 2011). Thus, a general trend of PBDE effects on gene transcripts encoding the TRs is difficult to determine and may vary depending on fish life stage and sex, tissue-type, time, and PBDE congener. In addition, while beyond the scope of this study, there continue to be questions about whether

xenobiotics like PBDEs can actively bind to TRs as agonists or whether they exert effects on the receptor as antagonists. Recent evidence suggests that some OH-PBDEs with hydroxyl moieties located at para- and meta- positions may bind with TRs as antagonists (Freitas et al. 2011; Kitamura et al. 2008; Kojima et al. 2009), while parent PBDEs tested to date (BDE-47) have not shown an affinity for binding with TRs allosterically or as competitive substrates (Li et al. 2010; Suvorov et al. 2011).

Indeed, the increase in TR expression could reveal alternative mechanisms of TR-responses to PTU and BDE-209 that have yet to be fully described. For instance, it may be that the enhanced expression of *MCT8* in the brain and liver (Figure 25) and *OATP1c1* in the liver (Figure 26) is facilitating the increased transport of thyroid hormones into the brain and liver to maintain T3 homeostasis that is in turn leading to the upregulation of TRs by autoinductive mechanisms. Another hypothesis for the TR induction in hypothyroid animals is that there may be functional heterogeneity of some thyroid receptors that allows them to act as carrier proteins to concentrate hormone in the nucleus to maintain thyroid homeostasis (Hamada and Yoshimasa 1983). It is also notable that a significant decrease in relative *TR β* transcript levels was measured in

livers of BDE-209 high dose fish at day 28, which could be linked to reduced T3 levels measured and inhibition of TR auto-inductive mechanisms.

4.4.7 Membrane Bound Transporters

This study is the first to explore effects of PBDEs on the MCT and OATP membrane-bound transporters in fish. We measured an upregulation in mRNA transcripts encoding *MCT8* and *OATP1c1* in brains and livers of fish exposed to BDE-209 (Figures 25-26). *MCT8* and *OATP1c1* are important, high affinity transporters of thyroid hormones into and out of cells (Visser et al. 2011). *MCT8* has been structurally and functionally characterized as a specific and active transporter of thyroid hormones in mammals and fish (Arjona et al. 2011; Friesema et al. 2003). It has also been shown to have broad tissue distributions, with the highest expression in zebrafish found in the brain followed by the liver and kidneys (Arjona et al. 2011). The significant upregulation (~2- to 3-fold increase) of *MCT8* in brains of BDE-209 treated fish and the PTU positive control at day 14 and after the 14-day depuration (low dose only) supports a tissue-specific response of the brain to hypothyroidism (Figure 25). The significant ($p < 0.05$) ~3-fold increase in *MCT8* mRNA transcript levels measured in livers of BDE-209 low dose fish is also consistent with a compensatory response of the liver to hypothyroidism.

OATPs mediate the cellular uptake of a range of amphipathic organic molecules, including thyroid hormones and xenobiotics. Only a limited number of these transporters have been characterized in vertebrates, with recent efforts in zebrafish to clarify their tissue distribution and function (Popovic et al. 2010). We targeted a number of OATPs for RT-qPCR analysis in livers and brains of fish treated with BDE-209 and PTU (Appendix B, Table 10). Of the several OATPs evaluated, BDE-209 was found to increase the relative levels of *OATP1c1* mRNA transcripts (Figure 26). In addition, an increase and decrease in *OATP2a1* mRNA expression was measured in the brain and liver, respectively, of BDE-209 treated fish.

OATP1C1 transporters have been cloned in humans with the highest expression measured in the brain and testes (Pizzagalli et al. 2002). In humans, *OATP1C1* has been found to have relative narrow substrate specificity, transporting thyroid hormones with high affinity (T4 $K_m = 90$ nM; rT3 $K_m = 130$ nM). An investigation by Sugiyama et al. (2003) showed that *OATP1c1* mRNA and protein expression was upregulated in hypothyroid rat brains and downregulated in hyperthyroid rat brains, similar to findings here in the liver. It remains to be confirmed whether *OATP1c1* in fish is functionally homologous to *OATP1C1* in humans. We did not measure an increase of

OATP1c1 transcripts in the brains of BDE-209 treated fish, nor the PTU positive control, despite the substantial hypothyroid status of these fish. This finding raises further questions about the functional equivalency of *OATP1c1* in fish and mammals and/or the capacity of the fish brain to maintain homeostasis under BDE-209 induced hypothyroidism.

The significant ($p < 0.05$ to $p < 0.01$) increase in *OATP2a1* mRNA expression measured in brains of BDE-209 high dose fish at sampling day 14 and among BDE-209 low dose fish after the 14-day depuration was contrasted by the significant ($p < 0.05$) decrease detected in livers of the high dose treatment at sampling day 14. *OATP2a1* has been found to be ubiquitously distributed across a variety of tissues in zebrafish, including in the liver and brain (Popovic et al. 2010). It has also been found to have widespread distribution in other vertebrates (Hagenbuch and Gui 2008). *OATP2a1* is predominantly involved in the uptake of prostaglandins and other eicosanoids into cells. Prostaglandins are autocrine signaling molecules that are derived from fatty acids and produced during the activation of inflammatory and immunity responses of tissues, among other functions. A recent *in vitro* study using alveolar macrophages showed that DE-71 suppressed the release of prostaglandin products involved in innate and adaptive

immune system responses (Hennigar et al. 2012). While this study is not directly applicable to fish as it was targeted toward lung macrophages, these results suggest that more work is needed to understand PBDE impacts on the vertebrate immune system. A recent study in juvenile sub-yearling Chinook salmon found that a PBDE mixture of BDE-47, -99, -100, -153, and -154 increased the susceptibility of young fish to the marine pathogen *Listonella anguillarum* (Arkoosh et al. 2010).

Finally, there continue to be questions about the extent to which PBDEs may act as effective substrates of OATPs allowing for their cellular uptake. A study that used human kidney cells, which transiently expressing several OATPs, demonstrated the uptake of BDE-47, BDE-99, and BDE-153 into these cells (Pacyniak et al. 2011). OATP1a4, OATP1b1, and OATP1b2 were found to transport all three PBDEs whereas OATP1a1 transported none. While the extent to which PBDEs are substrates for OATPs was beyond the scope of this study, further analysis of the role of these transporters in the uptake and disposition of PBDEs in fish and other vertebrates is merited.

4.4.8 Reproductive Endpoints of Concern

Significant declines in the GSI were measured in male minnows exposed to BDE-209 with no differences measured between the low and high dose tested (Figure 19).

Significant ($p < 0.05$ to $p < 0.01$) deficits in the GSI were observed early in the exposure at sampling day 14, suggesting that BDE-209 impacts on the testes were rapid. Moreover, the GSI continued to be significantly depressed even after the 14-day depuration. The ongoing depressed GSI suggests the possibility that BDE-209 impacts on the reproductive system of male fish may be permanent or may otherwise recover only slowly with the cessation of exposure. Additional depuration time points would be needed to evaluate recovery more fully.

PBDEs have been shown to affect the reproduction of fishes. A 5-month, aqueous exposure of zebrafish to BDE-209 caused dose dependent impairments of testicular development, including decreased GSIs and reduced sperm counts and motility (He et al. 2011). In other evidence, reduced spawning was observed in fathead minnows exposed to approximately 14 $\mu\text{g/g}$ of BDE-47 in spiked food daily for 25 days, with reproduction completely stopped within 10 days of exposure (Muirhead et al. 2006). The impaired reproduction was attributed to selective toxicity in male fathead minnows as they had reduced numbers of mature spermatozoa. Likewise, another dietary study measuring BDE-47 effects on fathead minnows (2.4 μg and 12 μg of BDE-47/ breeding pair-day for 21 days) measured reduced mature spermatozoa coupled with elevated

spermatocytes and spermatids (Lema et al. 2008). Studies in early life stages of laboratory rodents have also shown that PBDEs can elicit anti-androgenic effects that impair reproductive development (Kuriyama et al. 2005; Stoker et al. 2005; Tseng et al. 2006). Taken together, declines in GSI measured here over the BDE-209 exposure and depuration periods suggest the potential for BDE-209 and/or its metabolites to impair male fish reproduction.

Important questions remain as to whether PBDE effects on reproduction are being mediated directly by impacts on the hypothalamic-pituitary-gonadal (HPG) axis and/or indirectly via altered thyroid hormone homeostasis. It is notable that PTU had no effect on GSI values of treated fish given that thyroid hormones have been demonstrated to affect reproductive functioning. This dichotomy suggests that BDE-209 may be impacting adult male fathead minnow reproduction by non-thyroidal mechanisms of action. However, to date, there has been only limited examination of the cross-talk between the HPT and HPG axes of fish. Early reviews by Cyr and Eales described important interactions between thyroid hormone regulation and reproductive physiology in fishes. More recently, studies in goldfish and zebrafish have provided evidence supporting that thyroid hormones may have important inhibitory effects on

teleost reproductive functioning at different levels of the HPG axis including by: inhibiting pituitary luteinizing hormone (LH) and follicle stimulating hormone (FSH); and reducing steroidogenesis and gonadal aromatase expression (Habibi et al. 2012; CS Liu et al. 2010; Nelson and Habibi 2009). For instance, reduced levels of T4 and T3 (via PTU) in adult female zebrafish were correlated with increased circulating levels of plasma testosterone, LH, and FSH, and increased expression of steroidogenic genes in the ovaries (StAR, 3 β HSD, and 17 β HSD). Male fish were not studied by Liu et al. (2010).

Effects of PBDEs on these HPG/HPT endpoints have been mixed with only limited work in fishes. PBDE exposures have been associated with depressed levels of LH and FSH accompanied by associations with increased T4 in men (Meeker et al. 2009). However, in contrast, increased LH has been measured in male rats exposed to the PentaBDE mixture (Stoker et al. 2005). The work by Stoker et al. (2005) in rats suggested anti-androgenic effects associated with direct competitive inhibition of the androgen receptor by PBDEs, in contrast to indirect modulation by thyroidal perturbations. Thus, whether PBDE effects on reproduction are mediated by direct modifications to physiological functioning of the HPG or alternatively whether these effects are mediated indirectly or in combination with impacts on the HPT remain unclear.

4.4.9 Low Dose Effects

The BDE-209 low dose tested in this study ($95 \text{ ng/g-food} \times 3\% \text{ bw/day}$) elicited impacts on thyroid signaling and reduction in the GSI at a similar level to the high dose ($10 \text{ } \mu\text{g/g-food} \times 3\% \text{ bw/day}$). Moreover, the BDE-209 low dose caused significant reductions in plasma TT3 early in the exposure at day 14 that continued after the 14-day depuration (Figures 22A). Neither the BDE-209 high dose tested nor the PTU positive control was found to significantly affect TT3 at either sampling day 14 or after the 14-day depuration. Studies in developing rodents have also demonstrated that BDE-209 can decrease circulating TT4 and TT3 (Fujimoto et al. 2011; Rice et al. 2007; Tseng et al. 2008). However, the BDE-209 doses tested in rodents have been generally higher than doses administered in this study (BDE-209 high dose = $0.3 \text{ } \mu\text{g/g bw-day}$; BDE-209 low dose = 2.9 ng/g bw-day). For instance, significant declines in serum TT3 ($\sim 21\%$) relative to controls were measured in adult male mice exposed maternally to BDE-209 at 10 and 1500 mg/kg-day ($\mu\text{g/g-day}$) from gestational days 0-17. While the doses tested by Tseng et al. (2008) were greater than those in this study, it is notable that TT3 was not affected in mice at the mid-dose tested (500 mg/kg-day), suggesting a u-shaped dose-response of TT3 to BDE-209 in developing mice. Further study is needed to determine whether

similar non-monotonic dose-responses are occurring in fish exposed to BDE-209. However, findings here in adult male minnows suggest the possibility of non-linear relationships between PBDE exposures and effects on thyroid signaling. These types of non-linear dose-response relationships could extend into reproduction based on the altered GSI status measured and have been detected with other endocrine disrupting compounds (Vandenberg et al. 2012).

4.5 Conclusions

This study provides insights into mechanisms underlying BDE-209 effects on thyroid regulation in adult male fish that appear to involve altered signaling at several important steps in the transport and activation of thyroid hormones (Figure 28). BDE-209 bioaccumulated in tissues and was reductively debrominated to congeners ranging from penta- to octaBDEs. Biological effects of BDE-209 and/or its metabolites on fish thyroid functioning appeared to proceed through multiple pathways that involve: declines in circulating levels of thyroid hormone eliciting altered patterns of T4 activation to T3 in peripheral brain and liver tissues; and altered expression of genes involved in the production and transport of thyroid hormones. In support of this mechanism of action, fish exposed to BDE-209 at both the low and high dose

experienced profound deficits in plasma thyroid hormones that extended into the 14-day depuration for TT4 and at the low dose for TT3. BDE-209 also significantly depressed deiodination of T4 to T3 in peripheral liver and brain tissues with recovery observed after a 14-day depuration. The elevated relative expression of genes encoding *dio 1*, *dio 2*, *TR α* , *TR β* , *MCT8*, and *OATP1c1* appeared to be a response to BDE-209 induced hypothyroidism.

The initiating events that lead to the multitude of thyroid signaling perturbations observed here in adult male minnows remain unclear. One hypothesis for the thyroid dysfunction is that PBDEs (or OH-PBDEs) can act as thyroid hormone mimics and compete for binding with plasma transporter proteins, leading to the elimination of free unbound thyroid hormone. Another leading hypothesis suggests that increased biliary elimination of thyroid hormones could be occurring as a result of PBDE inductions of Phase II and Phase III hepatic metabolizing enzymes (i.e., UGTs, SULTS, Mdr1, Mrps) as has been observed in rodents (Richardson et al. 2008; Szabo et al. 2009; Zhou et al. 2001). Additional study will be needed to elucidate the role of these Phase II and III enzymes in PBDE metabolism and hypothyroidism in fishes.

The apparent compensatory responses of the adult fish thyroid to BDE-209 appeared to be transient in the liver and absent in the brain, suggesting that brains of adult fish may be particularly sensitive to BDE-209. The hypothyroid status of BDE-209 treated fish was sustained and extended into the 14-day depuration. Thus, taken together, apparent compensatory responses of the peripheral thyroid system appeared to have been unable to overcome the disrupted thyroid signaling elicited by BDE-209 at either dose tested. Finally, the GSI of BDE-209 treated fish also was reduced and may be indicative of reproductive impairments. Further histological analysis will be undertaken to determine whether and how dietary treatments of BDE-209 are causing structural alterations of the testes.

The thyroid system is highly conserved both structurally and functionally across vertebrate taxa, and so the BDE-209 effects measured here in adult minnows may have broad implications for thyroid dysfunction in other species and life stages. While BDE-209 levels measured in humans appear to be generally less than average values measured here at the lower dose tested (i.e., 100 ± 14 ng/g lw), these BDE-209 levels in fish may be comparable to those measured in children with upper end detections of BDE-209 in their blood (Fischer et al. 2006; Lunder et al. 2010; Stapleton et al. 2012). For

instance, work in our laboratory, in collaboration with the Centers for Disease Control (CDC) and Boston University, measured BDE-209 levels in the serum of a North Carolina cohort of toddlers (3 years old) ranging from <6 – 68 ng/g lw (Stapleton et al. 2012). In addition, studies in occupationally exposed E-waste workers report some of the highest BDE-209 body burdens measured in people in the world with BDE-209 levels detected at greater than 3000 ng/g lw in some individuals (Bi et al. 2007; Qu et al. 2007), which are well within the BDE-209 levels detected in this study.

The BDE-209 bioaccumulation measured in adult minnows is also comparable to levels measured in wild fish and other wildlife species (Chen et al. 2007; Johnson-Restrepo et al. 2005; La Guardia et al. 2007; Shaw et al. 2008; Shaw and Kannan 2009; Shaw et al. 2012). For instance, concentrations of BDE-209 in sunfish were measured at 2,880 ng/g lw downstream of a waste water treatment facility (La Guardia et al. 2007). In further evidence, three species of shark collected off the Florida coast were found to have BDE-209 body burdens ranging from 17 – 778 ng/g lw (Johnson-Restrepo et al. 2005). Thus, taken together, although BDE-209 continues to be seldom targeted in PBDE bioaccumulation studies, BDE-209 levels measured here in adult fathead minnows were

comparable to BDE-209 body burdens measured in some human and wildlife populations, raising further concerns about the toxicity observed.

5. Discussion

Polybrominated diphenyl ether (PBDE) flame retardants have been shown to reduce circulating thyroid hormone levels in animals. However, effects of the most widely used PBDE, decabromodiphenyl ether (BDE-209), on thyroid hormone signaling are largely unknown. Results from research conducted under the three studies here provide important insights into mechanisms involved in the metabolism of PBDEs and a clearer understanding of BDE-209 effects on early and adult life stages of fish, including possible mechanisms of action.

5.1 BDE-209 Body Burdens in Fathead Minnows vs. Other Species

BDE-209 bioaccumulated and was reductively debrominated to several lower PBDE congeners ranging from penta- to octaBDEs in both juvenile and adult fathead minnows. The composition of reductive metabolites measured in adult fish was nearly identical to those measured in juvenile fish with hexaBDE-154 being the dominant metabolite measured at both life stages. However, BDE-209 bioavailability was higher at the lower dose tested (3.8%) than the high dose (1.3%). The underlying reasons for these differences are unclear but may be attributable to individual sampling and feeding variability. There also could have been dose-related differences in BDE-209 uptake from

the intestine and enhanced metabolic detoxification and elimination at higher doses. Juvenile fathead minnows assimilated a higher percentage (5.8%) of the BDE-209 exposure than adults (1.3%) receiving equivalent doses. Thus, higher levels of parent BDE-209 and reductive metabolites were measured in juvenile minnows than in adult minnows relative to the cumulative BDE-209 exposure. This outcome could be attributable to greater uptake and metabolism and/or less elimination in young fish. Taken together, data generated here support that BDE-209 uptake appears to be generally low in fishes, but that young fathead minnows appear to assimilate higher percentages of BDE-209 than adult minnows. Additional research is needed to better understand the underlying mechanisms and dose-related differences in BDE-209 bioavailability observed here and across other species and life-stages.

While BDE-209 levels in tissues of humans and wildlife are seldom targeted, an increasing number of biomonitoring studies are focusing on BDE-209. Many of these studies have reported BDE-209 body burdens that are similar to and higher than BDE-209 levels measured here in adult fathead minnows, particularly at the lower dose tested (Table 5). As can be seen from Table 7, a range of BDE-209 body burdens are reported in biota but are limited by the relatively finite sample size. The U.S. Centers for Disease

Control (CDC) has estimated BDE-209 levels in the U.S. population (aged ≥ 12 years) at ~ 2 ng/g lw, based on testing under its National Health and Nutrition Examination Survey (NHANES 2001-02) (Sjodin et al. 2008). In a more recent study of a North Carolina toddler cohort (aged 1-3 years) that was conducted in our laboratory, in collaboration with researchers from the CDC and Boston University, serum levels of BDE-209 measured in toddlers ranged from $< 6 - 6.8$ ng/g lw (Stapleton et al. 2012). These BDE-209 accumulations are consistent with BDE-209 body burdens measured in a case study of a California family (Fischer et al. 2006). In another recent multi-state mother/child cohort, median concentrations of BDE-209 were measured in the serum of toddlers (3 years old) at 1.7 ng/g lw with upper end accumulations at 19 ng/g lw and 35% of child participants having BDE-209 body burdens that exceeded the 2 ng/g lw value estimated under the NHANES program (Lunder et al. 2010). Thus, taken together, BDE-209 levels (100 ± 14 ng/g lw) measured in adult male fathead minnows at the lower dose tested may be comparable to children with upper end detections of BDE-209 in their blood.

Table 7: BDE-209 body burdens measured in humans, fish and other wildlife.

Species	Tissue	Location	Year	BDE-209 Body Burdens (ng/g lw) ¹
Adult Male Fathead Minnows (Study Aim 3; Sampling day 28)				
BDE-209 low dose	carcass	na	2012	100 ± 14
BDE-209 high dose	carcass	na	2012	2700 ± 1200
Humans				
Children (≥12 yrs) ²	serum	US NHANES	2001-02	2 (est.)
Toddlers (3 yrs); mothers ³	serum	US multi-state	2006-07	nd-19 (1.7); nd-3.2 (1.4)
Toddlers (1-3 yrs) ⁴	serum	US North Carolina	2009-10	<6 - 68
Adult (mid 30s) ⁵	serum	US California	2004	7.3 (fem); 9.3 (male)
Child; infant (5, 1.5 yrs) ⁵	serum	US California	2004	51; 93
Teenagers (14 yrs) ⁶	serum	Nicaragua ⁷	2002	(8.9)
Adults (18-45 yrs) ⁸	serum	China, Guangdong ⁹	2005	nd-3436 (83.5)
Adults (18-45 yrs) ⁸	serum	China, Guangzhou	2005	nd-63.2 (5.7)
Adults (18-81 yrs) ¹⁰	serum	China, Guiyu ⁹	2005	nd-3100 (310)
Adults (18-81 yrs) ¹⁰	serum	China, Haojiang	2005	nd-370 (86)
Fish				
Sunfish ¹¹	carcass	US North Carolina	2002	[2880]
Atl. Mackerel ¹²	carcass	US Gulf of Maine	2006	0.21 - 3.9 [1.6]
American Plaice ¹²	carcass	US Gulf of Maine	2006	[1.8]
Atl. sharpnose shark ¹³	muscle	US Florida coast	2004	[514]
Spiny dogfish ¹³	muscle	US Florida coast	2004	[17]
Bull shark ¹³	muscle	US Florida coast	2004	[778]
Other Wildlife				
Harbor seal ^{14, 15}	liver; blubber	US Gulf of Maine	2006	nd - 40; nd - 8
Kestrel ¹⁶	liver; muscle	China north	2004-06	2870 ± 1930; 2150 ± 1040
Peregrine falcon ¹⁷	eggs	US northeast	1996-06	29 - 175
Gull sp. ¹⁸	eggs	Canada	2008	0.5 - 137
Glaucus gull ¹⁹	eggs	Great Lakes	1982-06	4.5 - 20 ²⁰
Grizzly bear ²¹	adipose	Canada, BC	2003	0.74 - 41.7
Red fox ²²	liver; muscle	Belgium south	2003-04	<9-290 (2.4); <4-290 (3.4)

1) Median values in parenthesis and mean values bracketed when reported; 2) Sjodin et al. 2008; 3) Lunder et al. 2010; 4) Stapleton et al. 2012; 5) Fischer et al. 2006; 6) Athanasiadou et al. 2008; 7) Children working and living at Managua MSW site; 8) Qu et al. 2007; 9) Electronic waste dismantling workers; 10) Bi et al. 2007; 11) La Guardia et al. 2007; 12) Shaw et al. 2009; 13) Johnson-Restrepo et al. 2005; 14) Shaw et al. 2012; 15) Shaw et al. 2008; 16) Chen et al. 2007; 17) Chen et al. 2008; 18) Chen et al. 2012; 19) Gauthier et al. 2008; 20) Reported as ng/g ww; 21) Christensen et al. 2005; 22) Voorspoels et al. 2006.

An increasing number of studies have targeted occupationally exposed individuals, especially those working at E-waste recycling facilities. Body burdens of BDE-209 in workers from E-waste facilities represent some of the highest levels of BDE-209 measured in people in the world to date with accumulations detected at greater than 3000 ng/g lw in some individuals (Bi et al. 2007; Qu et al. 2007). Moreover, in referent groups representing the general population far from these facilities (i.e., residing in Guangzhou, Haojiang; Table 7), BDE-209 body burdens are also elevated suggesting broader PBDE contamination in parts of Asia. The BDE-209 body burdens in occupationally-exposed and residential populations residing in regions with heavy E-waste recycling operations are comparable to levels of BDE-209 measured in adult fish under this research at both the high and low dose tested.

In addition to biomonitoring evidence in humans, bioaccumulation of BDE-209 measured in a limited sampling of wildlife species also suggests BDE-209 levels that are consistent with levels measured here in fathead minnows. In harbor seals residing in the North Atlantic, BDE-209 concentrations in the liver were measured at up to 40 ng/g lw, and in Atlantic mackerel (seal food source) collected from the same area, median BDE-209 concentrations were reported at 7.9 ng/g lw (Shaw et al. 2009; Shaw et al. 2012).

Several species of sharks collected off the Florida coast also have shown elevated levels of BDE-209 with median values ranging from 17 – 778 ng/g lw (Johnson-Restrepo et al. 2005). High levels of BDE-209 detected in predatory species may suggest the potential for biomagnification. Indeed, while additional study is needed of these processes for the higher PBDEs, some studies suggest biomagnification of lower PBDEs in aquatic and terrestrial food chains (Burreau et al. 2004; Voorspoels et al. 2007). There was also an examination undertaken of PBDE bioaccumulation and metabolism by aquatic species residing downstream of a wastewater treatment facility in Roxboro, North Carolina that reported high levels of PBDEs in its effluent under the U.S. EPA Toxics Release Inventory (La Guardia et al. 2007). In this study, BDE-209 concentrations in sunfish (*Lepomis gibbosus*) and crayfish (*Cambarus puncticambarus*) were measured at 2,880 ng/g lw and 21,600 ng/g lw, respectively. There were also several highly brominated PBDEs (BDE-179, -184, -188, -201, -202) measured in sunfish that were not present in any commercial mixtures or biosolids, suggesting metabolic debromination. It is notable that similar metabolites were measured here in fathead minnow juveniles and adults exposed to BDE-209. In the La Guardia study, BDE-209 was not detected in Creek chubs residing in the path of this wastewater effluent, although elevated levels of several high

MW PBDE congeners were detected. The Creek chub (*Semotilus atromaculatus*) is a member of the family Cyprinidae and was hypothesized to be reductively debrominating BDE-209 in this riverine system. This hypothesis is consistent with research here in fathead minnows and earlier findings in our laboratory in common carp (Stapleton et al. 2004a), both of which are also cyprinids. Additional study of more wild species and populations is needed to better understand how the highly brominated PBDEs are being metabolized and contributing to biological burdens of PBDEs in the environment.

5.2 Mechanisms of PBDE Metabolism in Fish

Under the first research aim, *in vitro* experiments conducted in the carp liver suggest a role for dio enzymes in catalyzing the reductive debromination of PBDEs in fish. These experiments demonstrated that carp liver microsomes readily debrominated BDE-99 by a reductive pathway and that enzymes catalyzing this reaction were associated predominantly with the endoplasmic reticulum (e.g. microsomal fraction) where dio enzymes are located. In addition, a series of experiments in carp liver microsomes co-incubated with BDE-99 and competitive substrates also suggested a role for dio enzymes in catalyzing PBDE debromination in fish. In particular, the rate of

debromination of BDE-99 to BDE-47 in carp liver microsomes was significantly inhibited by the presence of rT3 and T4 (IC_{50} values = 0.83 μ M; >1 μ M, respectively), suggesting that enzymes involved in the PBDE metabolism appeared to have high affinities for thyroid hormones. Indeed, experiments to determine the apparent enzymatic kinetics (apparent V_{max} and K_m values) of BDE-99 hepatic metabolism in carp suggested that enzymes responsible for the BDE-99 catalytic activity appeared to have a higher affinity for native thyroid hormone than BDE-99. In addition, DTT, which is a reducing agent required for the *in vitro* deiodination of thyroid hormones, was required to catalyze the debromination of BDE-99 to BDE-47 in carp liver microsomes. Finally, IaC, which is a potent inhibitor of enzymes with cysteine residues such as dios, also reduced the debromination of BDE-99 to BDE-47.

Earlier studies by other laboratories have suggested that GSTs might be involved in the metabolism of PBDEs as PBDE-glutathione metabolites have been identified in rodents (Hakk et al. 2002) and birds (Ferne et al. 2005). Moreover, because GST enzymes also contain cysteine residues, it is possible that they were inhibited by IaC that in turn could have mediated the reduced BDE-99 metabolism observed. However, co-incubations of rT3-treated microsomes with CDNB, which is a common substrate for

many GST isoforms, showed that GST activity was not inhibited by rT3 (Table 3). Thus, GSTs appeared to not have been involved in the reduced rate of BDE-99 debromination observed in rT3 co-incubated microsomes. This finding was reaffirmed in juvenile fathead minnows under research aim two where no changes in GST activity (via CDNB assay) were detected in BDE-209 dosed fish despite substantial reductive debromination (Figure 13). Results from other studies in carp, rainbow trout, and Chinook salmon also provide no evidence that GSTs are involved in PBDE reductive metabolism in carp, rainbow trout, and salmon intestinal and liver microsome (Benedict et al. 2007; Browne et al. 2009; Roberts et al. 2011).

One limitation of this study is that it implicates dio enzymes in the reductive debromination of PBDEs by a line of indirect evidence. Direct measurements, using cell cultures with transfected fish dios and/or with gene knockdown approaches (e.g., siRNA), for example, might be useful in directly measuring the involvement of dio enzymes in PBDE metabolism. Some of this dio transfection work is being investigated in our laboratory and by other Duke tox-investigators. In addition, it might be instructive to test the feasibility of using a morpholino approach to transiently knockdown expression of dio enzymes in developing zebrafish embryos (or another

species with larger eggs). Fathead minnows might be a candidate because partial sequences for the dios are already known from research here that may facilitate morpholino design. While survival might be an issue, a dechorionated egg might further allow for PBDE interactions with the dio enzyme machinery of the developing embryo. With this approach it might be possible to examine the role of dio enzymes in mediating PBDE debromination in embryos and early larval forms.

In addition, it would be useful to further examine whether oxidative metabolism is catalyzing PBDE breakdown as studies are mixed as to whether fish oxidatively metabolize PBDEs. While there has been some *in vitro* testing in our laboratory and elsewhere to evaluate the formation of hydroxylated PBDE metabolites, it would be informative to investigate whether these products form *in vivo* in fish. There are adult male fathead minnow carcasses available from the study conducted under specific aim three that could be used to test for these oxidative metabolites. However, it might be expected that these OH-PBDEs, if they are being formed, might not concentrate at high levels in fish carcasses, but would rather be expected to occur at higher concentrations in the blood and highly perfused tissues like the liver where they are formed. The OH-PBDEs closely resemble the structure of thyroid hormones and appear to be retained in

blood as a consequence of their high affinity for binding with thyroid hormone transport proteins, especially transthyretin (Hamers et al. 2008; Meerts et al. 2000; Morgado et al. 2007). Nevertheless, carcasses are available and could be useful as an initial screen.

5.3 BDE-209 Effects on Thyroid Hormone Regulation

The thyroid systems of both juvenile and adult fathead minnows were severely disrupted by BDE-209 at low, environmentally relevant exposures. In juvenile fathead minnows, the activity of dio enzymes (T4-ORD and T4-IRD) declined by ~74% in fish dosed with 9.8 µg/g ww food at 3% bw/day for 28 days compared to controls. The decline in dio activity was accompanied by thyroid follicle hypertrophy indicative of over-stimulation and injury. In addition to thyroid disruption, a distinctive liver phenotype characterized by vacuolated hepatocyte nuclei was measured in ~48% of hepatocytes counted from treated fish and has not been observed previously in fish exposed to PBDEs. We hypothesize that these vacuolated nuclei, which were not observed in controls, may be attributable to impingements caused by enlarged biliary passageways perhaps as a physiological response to facilitate elimination of PBDEs. Although thyroid hypertrophy and liver alterations were pervasive across all fishes examined, the analysis was limited by the number of fish evaluated and doses tested.

Additional histological examinations of the thyroid, liver, and gonad are planned for adult male fathead minnows exposed to BDE-209 (study aim 3) that may provide further insights into morphological impairments observed in juvenile fish.

Under the third research aim, substantial thyroid disruption was measured in adult fathead minnows exposed to BDE-209. Compared to negative controls, fish exposed to BDE-209 at the low dose (95 ± 0.4 ng of BDE-209/g ww food at 3% bw/day) for 28 days experienced a 53% and 46% decline in plasma TT4 and TT3, respectively, while fish at the BDE-209 high dose (10.1 ± 0.1 μ g/g ww food at 3% bw/day) had TT4 and TT3 deficits at 59% and 62%, respectively (Figure 22). Depressed levels of thyroid hormones were accompanied by a 45-50% decline in the rate of T4-ORD in brains of all treatments by day 14 of the exposure. This decreased rate of T4-ORD worsened in the brain by day 28 with a 65% decline measured at both BDE-209 doses that was consistent with the PTU positive control (75% decline in T4-ORD). BDE-209 exposures also caused transient, tissue-specific upregulation of *dio1*, *dio2*, *TR α* , *TR β* , *MCT8*, *OATP1c1*, and *OATP2a1* mRNA transcripts in the brain and liver in patterns that varied with time and dose, possibly as a compensatory response to hypothyroidism. Taken together, data generated in adult and juvenile fathead minnows provide strong evidence that low doses of BDE-

209 disrupted thyroid hormone regulation and signaling at both the central HPT axis and in peripheral tissues of fish. Moreover, brains of adult male fathead minnows exposed to BDE-209 appeared to be resistant to apparent compensatory mechanisms measured in the liver. Thyroid perturbations at the low dose tested (~95 ng/g food-3 % bw/day) generally were equal to those measured at the higher dose tested (~10 µg/g food-3%bw/day), suggesting non-linear relationships between PBDE exposures and thyroid dysfunction.

5.4 Possible Mechanisms of BDE-209 Thyroid Toxicity

Based on evidence gathered here in juvenile and adult fathead minnows with other published studies, a mechanism for BDE-209 disruption of thyroid hormone regulation can be proposed. As illustrated in Figure 28, upon uptake, BDE-209 may enter circulation (1) where it can be transported into the liver and other tissues. In circulation, BDE-209 metabolites may competitively bind with plasma transport proteins, possibly transthyretin, leading to increased elimination of unbound hormone (2) (Meerts et al. 2000; Morgado et al. 2007). In the liver, dio enzymes may catalyze the reductive debromination of BDE-209 to lower congeners (3) (Noyes et al. 2010; Roberts et al. 2011), which may interfere with T4 activation and cause declines in thyroid hormones (5). In

addition, CAR and PXR may be activated, similar to that measured in rodents, stimulating upregulation of xenobiotic metabolizing enzymes (3) (Fery et al. 2009; Pacyniak et al. 2007; Richardson et al. 2008; Sanders et al. 2005; Staskal et al. 2006; Szabo et al. 2009). The activation of CAR or PXR may enhance the conjugation, metabolism, and biliary elimination of thyroid hormones (4), which may cause declines in circulating thyroid hormone levels (5). As described in the next section, additional investigation of the role of BDE-209 in inducing the CAR and PXR of fishes is merited, although previous studies have raised questions about whether the piscivore CAR is functionally homologous to mammalian forms (Iwata et al. 2002).

The hypothyroidism in treated adult fish appeared to induce a rapid compensatory response of the liver that involved an increase in *dio 2* expression and T4-ORD activity possibly to maintain T3 homeostasis (6a). However, although *dio 2* transcripts also increased in the brain, T4-ORD activity declined, suggesting that brain compensatory responses to hypothyroidism may be limited (6b). A limitation of the study in adult fish is that changes in protein levels were not examined but could have been altered in the brain. Compensatory upregulation of high affinity thyroid hormone transporters (*MCT8*, *OATP1c1*) may be another response to hypothyroidism to increase

the delivery of thyroid hormones to target tissues (6c-d). A secondary mechanism may also occur that involves the upregulation of *TRs* in response to enhanced transport of T3 (e.g., via MCT8, OATP1c1) into cells to maintain cellular homeostasis (7a-b).

By day 28 of the exposure, T4-ORD activity was severely reduced in the adult fathead minnow brain and liver possibly because the enhanced thyroid-related transcription and activity in response to BDE-209 are transient (8a-b). Reduced levels of circulating thyroid hormones continue as the thyroid system is unable to compensate for the BDE-209 exposures (9). It is notable that depressed levels of thyroid hormone continued over the 14-day depuration (Figure 22), and relative *dio 2* mRNA transcript levels and T4-ORD activity are again enhanced in the liver perhaps as an ongoing compensatory response to the sustained hypothyroid status (10a). The increased relative *dio 1* mRNA levels measured in the liver and brains of BDE-209 dosed fish at days 28 and 42 may represent an additional mechanism of peripheral tissue responses to extended, chronic deficits in circulating thyroid hormones (10b).

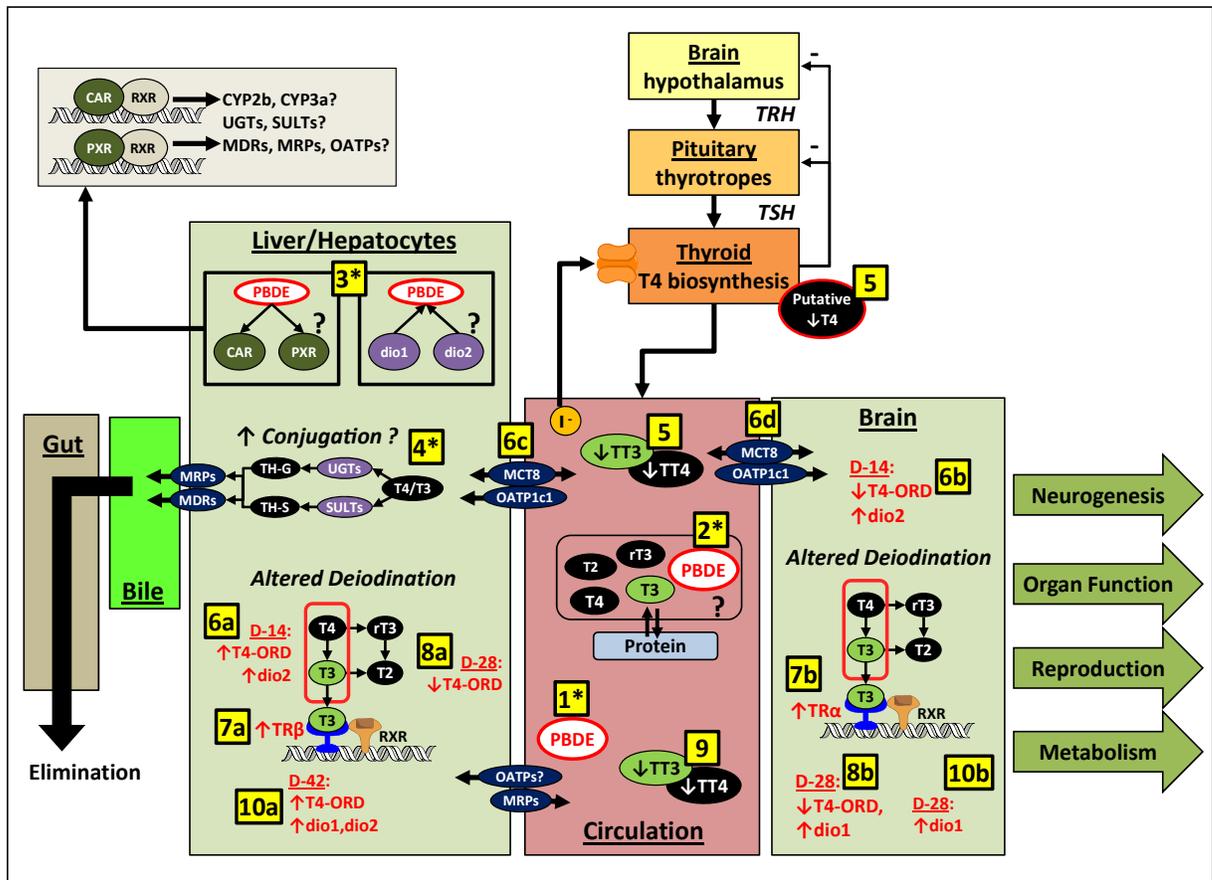


Figure 28: Possible mechanism of thyroid hormone dysfunction in fathead minnow adult males exposed to BDE-209. Asterisks denote pathways shown in other studies. (1) BDE-209 enters circulation; transported into liver, other tissues. (2) Possible competitive interference with plasma transporters; increased elimination of unbound hormone. (3) Possible debromination catalyzed by dios and activation of CAR/PXR. (4) Conjugation and elimination of thyroid hormones by metabolizing enzymes. (5) Declines in plasma thyroid hormones. (6a-b) Compensatory response of liver, brain to hypothyroidism. (6c-d) Compensatory upregulation of thyroid hormone transporters (MCT8, OATP1c1) in response to reduced thyroid hormones. (7a-b) Increased expression of TRs; possible secondary mechanism. (8a, 8b) T4-ORD declines suggest compensatory responses under (6) are transient. (9) Declines in plasma thyroid hormone as compensation transient. (10a-b) Ongoing thyroid effects.

5.5 Data Limitations and Research Needs

While the mechanism described in Figure 28 is well-supported, there exist areas of uncertainty that were beyond the scope of work here that would help to more fully describe the biological pathway of thyroid perturbations in fish exposed to BDE-209. Data generated here suggest that the relative abundance of transcripts encoding *dio 1* and *dio 2* were increased in livers of BDE-209 exposed adult fish, which could be increasing the capacity of the liver to reductively debrominate PBDEs. However, the BDE-209 study in adult minnows did not measure whether dio protein levels were being altered in BDE-209 dosed fish compared to controls. PBDEs appear to operate through non-dioxin, AhR-independent toxicity mechanisms in rodents (Sanders et al. 2005). However, the extent to which these pathways are operational in fish exposed to PBDEs like BDE-209 is less clear. Thus, it would be instructive to examine the role of BDE-209 and other PBDEs in activating CAR/PXR toxic pathways in fishes leading to expression of CYP2B and CYP3A, oxidative metabolism of PBDEs, and Phase II transferase activity. There is mRNA available from livers of BDE-209 treated and control fathead minnows that could be used for investigating these toxicity pathways. CYP3A is the major constitutive CYP form expressed in livers of most species, including fishes, and has been

shown to have broad substrate affinities, including hormones, xenobiotics, and pharmaceuticals (Celander et al. 1996). A number of CYP3A genes have been characterized in teleosts that could serve as a starting point for work in fathead minnows (or other species) (Celander and Stegeman 1997; Kullman and Hinton 2001; McArthur et al. 2003).

It is also important that we increase our understanding of whether PBDEs upregulate the expression and activity of transferase enzymes in fishes as this may also affect thyroid hormone regulation by increasing their biliary elimination. In addition to mRNA being available for use from the adult fathead minnow/BDE-209 study, several UGTs and SULT gene isoforms have been characterized in zebrafish that could facilitate this work (Clarke et al. 1991; Leaver et al. 2007; T Liu et al. 2010; Mortensen and Arukwe 2007). In addition, it would be informative to pair this work with measurements of UGT-T4 activity (perhaps by developing methods that allow for the direct measurement of TH-glucuronidated metabolites by LC/MS/MS). Current methods use radioactive isotopes (Zhou et al. 2002). A method has been developed in our laboratory to measure SULT activity by the formation of T2-sulfate in T2-incubated cytosolic fractions. It would be interesting to further evaluate SULT activity in cytosol from livers and brains of BDE-

209 treated fish. These cytosolic fractions have been preserved and some preliminary testing undertaken suggests the possibility of altered activity of these enzymes in BDE-209 dosed fish.

Another avenue that would be useful to explore is the role of Phase III hepatic efflux transporters, such as the Mrps and p-glycoproteins (Mdr1), in PBDE and thyroid hormone metabolism. For instance, studies have measured increased relative mRNA transcript levels encoding Mdr1, Mrp2, and Mrp3 in rat pups exposed to PentaBDE that was accompanied by decreased levels of T4. The increased transcription of these transporters was postulated to be a detoxification response to the PBDE exposures (Szabo et al. 2009). Studies to localize and characterize these transport proteins support that they operate in the fish liver (Bard et al. 2002; Kleinow et al. 2008; Klobucar et al. 2010; Loncar et al. 2010; Zaja et al. 2007; Zaja et al. 2008; Zaja et al. 2011), and testing their role in PBDE metabolism and disrupted thyroid homeostasis of fishes would be informative.

The mechanistic work conducted under this research dissertation was largely focused on adult male fathead minnows with less examination of mechanisms of toxicity in young fish. Given the substantial thyroid perturbations measured in juvenile fish

exposed to BDE-209 and the importance of thyroid hormones in development, it would be useful to understand patterns of altered thyroidal gene and protein expression in young fish exposed to BDE-209. Moreover, several reductive metabolites were measured in adult and juvenile fish and additional work is needed to better describe the relative contribution of these metabolites to thyroid perturbations observed in fish in comparison to parent BDE-209. There also is an important need to characterize whether and how thyroid perturbations associated with BDE-209 are mediating effects on other apical endpoints of concern in fish, including neurodevelopment and reproduction. For instance, there are many thyroid hormone responsive genes in vertebrates, and a finite number of these have been examined and shown to be altered by PBDEs. For instance, in fish, relative mRNA transcript abundance of the transcription factor BTEB, which regulates neuron differentiation, was reduced in brains of adult male fathead minnows exposed orally to BDE-47 (Lema et al. 2008). BDE-209 effects on BTEB transcription could be tested with relative ease using mRNA available here from the BDE-209/adult fathead minnow study and may provide additional insights into downstream effects of BDE-209 on neurogenesis. Declines in GSI measured in adult male fish suggest potential impacts on reproductive fitness, and the histological analysis planned will help to

elucidate these impacts. However, additional research is needed to better understand the potential for PBDEs to impair reproductive functioning in fish and whether there is an interactive role of PBDE-related thyroid toxicity in mediating these effects.

5.6 Conclusions

Results of this dissertation research suggest that the still widely used flame retardant BDE-209 is causing severe disruption of normal thyroid hormone regulation and signaling in young and adult fathead minnows at low, environmentally relevant concentrations. Generally, doses of BDE-209 tested in these studies were lower than what has been tested in laboratory rodents and other ecotoxicity studies. Moreover, the thyroid perturbations measured in adult male fathead minnows at the lower dose tested were similar to those observed at the higher dose tested, suggesting non-linear relationships between PBDE exposures and effects on thyroid signaling. Based on the evidence gathered, future biological effects studies with BDE-209 should examine endocrine system toxicity at lower exposures and over chronic durations to better assess these potential non-linear dose-response relationships as typical high dose extrapolation may be overlooking important sensitivities. These types of non-linear dose-response relationships have been detected with other endocrine disrupting compounds

(Vandenberg et al. 2012) and may extend into reproduction based on the roughly equivalent GSI reductions measured at both doses tested.

The thyroid system is highly conserved and is crucial to the normal development and biological functioning of all vertebrates. The current body of toxicological evidence, including data generated here, in combination with other studies showing continued and in some cases rising PBDE body burdens, raise concerns about BDE-209 effects on human and wildlife health. This body of evidence compels taking more concerted regulatory and policy actions to reduce future exposures. The pattern of extended delays in removing PBDEs from use despite strong evidence of exposure and toxicity is a repeat of the response taken with other persistent organic pollutants, such as DDT and PCBs. Long delays in removing PBDEs from the market, their ongoing presence in many products still in use, and their active use outside the U.S. and European Union will leave a lasting legacy of contamination, similar to what has occurred for other legacy pollutants. An overhaul of laws and regulations must be a priority to prevent this pattern of chemical contamination and delayed response from repeating itself.

Appendix A: Summary of major studies in piscivores that have measured PBDE metabolism and associated biological effects

Species	Sex, Stage	Exposure Route	Treatment	Dose	Duration	Biological Material	Effects Observed	Reference
PBDE Metabolism and Biotransformation								
Common carp (<i>C. carpio</i>)	Juvenile	In vitro	BDE-99	12-29 pmol/mg protein	60 m incub	GI, liver microsome	Reductive debromination; BDE-47 formation BDE-99 metabolism, liver > intestine Debromination not NADPH-driven No debromination by GI microflora ↓debromination in GI w/rT3	Benedict et al. 2007
Chinook salmon (<i>O. tshawytscha</i>)	Adult	In vitro	BDE-99	0.03 – 1.8 μM	16 h incub	Liver microsome cytosol	Reductive debromination BDE-49 formation DTT-driven (not NADPH) GSTs not involved, cytosolic debrom (salmon, carp)	Browne et al. 2009
Northern Pike (<i>E. Lucius</i>)	Unspec	Dietary	Cocktail of PCBs, PCNs, BDE-47, -99, -153,	90 ng/μl lipid (10 μl injected into rainbow trout)	9 d	Carcass with GI tract removed	Uptake efficiencies: BDE-47≈~90%; BDE-99≈~60%; BDE-153≈~40% Uptake not correlated with Kow, MW, or effective cross section (ECS), suggesting mediated transport; negative correlation with bromination	Burreau et al. 1997
Northern Pike (<i>E. Lucius</i>)	Unspec	Dietary	¹⁴ C-BDE-47	16.2 μg/μl (10 μl injected into rainbow trout)	9, 18, 36, 65 d	Whole fish radiographs	>90% ¹⁴ C-BDE-47 absorbed BDE-47 disposition in most lipophilic tissues Highest levels in liver, adipose tissue, spinal cord-surrounding tissue, eyes, gall bladder Intermediate levels in brain, spinal cord, heart, kidneys; Lowest in muscle, spleen, gills	Burreau et al. 2000
Crucian carp (<i>C. auratus</i>)	Unspec	Aqueous	BDE-15,	0, 10, 100 μg/l	50 d	Liver microsome, S9 fraction	Bioaccumulation BDE-15, gill, liver 2 mono-brominated 3 hydroxy metabolites	Cheng et al. 2012

Species	Sex, Stage	Exposure Route	Treatment	Dose	Duration	Biological Material	Effects Observed	Reference
Rainbow trout (<i>O. mykiss</i>)	Juvenile	Dietary	decaBDE mixture (Dow FR-300BA; purified)	7.5 - 10 mg/kg bw/day	16, 49, 120 d; 71 d dep	Liver, muscle	Reductive debromination ↑ hexa- to nonaBDE formation (liver, muscle); BDE-154 dominant metabolite; BDE-209 uptake (0.02-0.13%); BDE-209 accumulation (liver 870 ± 219 ng/g ww; muscle 38 ± 14 ng/g ww) Rapid BDE-209 elimination; retention hexaBDEs	Kierkegaard et al. 1999
Zebrafish (<i>D. rerio</i>)	Juvenile	Aqueous	DE-71 (w/w/o PBDD/Fs)	0, 0.1, 1 mg/l	4 wk; static renewal	Whole fish	AhR-mediated effects linked to PBDD/F impurities; Weak induction CYP1a, no DR-CALUX response (purified DE-71)	Kuiper et al. 2006
Lake whitefish (<i>C. clupeaformis</i>)	Juvenile	Dietary	BDE-209	0, 0.1, 1, 2 µg/g	30 d	Liver, carcass	BDE-209 and nonaBDE bioaccumulation (BDE-206, -207, -208)	Kuo et al. 2010
Zebrafish (<i>D. rerio</i>)	Adult	Dietary	Mix of BDE-28, -183, -209	1 and 100 nmol/g ww food at 2% bw/day	42 d, 14 d dep	Whole fish	Reductive debromination (high dose); 12 nmol of BDE-154/g ww fish; 3 nmol of BDE-149/g ww fish; <2 nmol of BDE-153 g ww fish; Uptake: BDE-28 (100%)>BDE-183 (10%)>BDE-209 (<1%)	Nyholm et al. 2009
Common sole (<i>S. solea</i> L.)	Juvenile	Dietary	Mix of BDE-28, -47, -99, -100, -153, -209	~82 ng/g ww food to ~184 ng/g ww food at 0.8% bw/day	84 d, 149 d dep	Liver, muscle, blood	Assimilation efficiency (%): 16 (BDE-28); 15 (BDE-47); 13 (BDE-99); 14 (BDE-100); 10 (BDE-153); 1.4 (BDE-209). Inverse relationship to log Kow, MW, #Br. Debromination major biotransformation route: BDE-49; BDE-154; BDE-183; BDE-202; unk tetra-, penta-, hexaBDEs GC/MS Injection: Splitless	Munsch et al. 2011
Common sole (<i>S. solea</i> L.)	Juvenile	Dietary	Mix of BDE-28, -47, -99, -100, -153, -209	~82 ng/g ww food to ~184 ng/g ww food at 0.8% bw/day	84 d, 149 d dep	Liver, muscle, blood	OH-BDE metabolites detected: 6-OH-BDE-47; 4'-OH-BDE-49; 4'-OH-BDE-101; 4'-OH-BDE-103 No MeO metabolites detected GC/MS Injection: Splitless	Munsch et al. 2010
Atlantic cod (<i>G. morhua</i>)	Juvenile	Aqueous	BDE-47	5 µg/L	21 d	Liver	↓mRNA transcripts for CYP1a, CYP2C33-like, CYP3C1-like, UGT No effects on antioxidant genes (GSH-Px, GR)	Olsevik et al. 2009

Species	Sex, Stage	Exposure Route	Treatment	Dose	Duration	Biological Material	Effects Observed	Reference
Rainbow trout (<i>O. mykiss</i>) Common carp (<i>C. carpio</i>) Chinook salmon (<i>O. tshawatcha</i>)	Juvenile	In vitro	BDE-28, -47, -49, -99, -100, -153, -154, -183, -203, -208, -209	1 µM	24 h (hepta to BDE-209) 1 h (tri- to hexaBDEs)	Liver microsome	Reductive debromination of BDE-99, -153, -183, -203, -208, -209; Carp – <i>meta</i> -position debrom dominated; Salmonids – <i>meta</i> - and <i>para</i> -position debrom; No metabolism w/congeners w/no <i>meta</i> -substituted Br (BDE-28, -47, -100) Metabolism rates 10-100X higher in carp than trout, salmon; BDE-47 only detected in carp	Roberts et al. 2011
Rainbow trout (<i>O. mykiss</i>) Common carp (<i>C. carpio</i>)	Juvenile	Dietary (trout), <i>in vitro</i> (trout, carp)	BDE-209	940 ng/g ww food, 1% bw/day (trout) 15 pmol/mg protein (trout, carp)	5 month (trout) 1, 24 h (in vitro)	Carcass, GI liver, serum, microsome (<i>in vitro</i>)	Reductive debromination (trout) Formation of BDE-207, -208,-188, -201, -202, unk. octaBDEs, unk. heptaBDEs BDE-209 accumulation (trout est. uptake 3.2%); liver>serum>intestine>carcass (lipid-normalized) <i>In vitro</i> : Formation of octa – nonaBDEs (trout), hexa – octaBDEs (carp)	Stapleton et al. 2006
Common carp (<i>C. carpio</i>)	Juvenile	Dietary	BDE-209	940 ng/day-fish	60 d w/40 d dep	Carcass	Reductive debromination Formation of BDE-154, BDE-155, unknown hexa- to octaBDEs; Net formation rate ranged from 0.28 – 1.03 ng/day; No BDE-209 bioaccumulation	Stapleton et al. 2004a
Common carp (<i>C. carpio</i>)	Juvenile	Dietary	BDE-99, BDE-183	400 ng/day-fish (BDE-99) 100 ng/day-fish (BDE-183)	62 d w/37 d dep	GI tract	Reductive debromination. BDE-47 dominant metabolite formed w/BDE-99; BDE-154 and unknown hexaBDE dominant metabolites w/BDE-183 9.5% assimilation efficiency of BDE-99; 17% assimilation efficiency of BDE-183	Stapleton et al. 2004b
Common carp (<i>C. carpio</i>)	Juvenile	Dietary	Mix: BDE-28, -47, -99, -153	470 ng/day-fish	60 d w/40 d dep	Carcass, liver, serum	BDE-47 accumulation, high assimilation No BDE-99 bioaccumulation No hydroxy metabolites detected in serum	Stapleton et al. 2004c

Species	Sex, Stage	Exposure Route	Treatment	Dose	Duration	Biological Material	Effects Observed	Reference
Common carp (<i>C. carpio</i>)	12 male, 8 female	Wild Caught	River Po, downstream of PBDE contaminated tributary, River Lambro			Liver	BDE-209 not detected (although high levels in sediments, macroinverts) Congeners detected: BDE-202, -188, 179, -154, -153, -100, -99, -47; Carp age had no effect on bioaccumulation; Higher accumulation in males	Vigano et al. 2011
Japanese medaka (<i>O. latipes</i>)	Adults; embryos	Maternal, in vitro	6-OH-BDE-47, 6-MeO-BDE-47, BDE-47	Maternal dietary: 21; 8; 0.9 µg/g dw food (2% bw/day)	14 d	Embryos, liver microsomes	No OH-, MeO-BDEs detected in BDE-47 treated fish; In vivo and in vitro conversion of 6-OH-BDE-47 to 6-MeO-BDE-47 (and vice-versa) MATERNAL TRANSFER: All treatments in adult females transferred to eggs	Wan et al. 2010
Common carp (<i>C. carpio</i>)	Juvenile	Dietary	Penta, Octa, DecaBDE mixtures	100, 120, 150 µg/day/fish	20 d	Carcass, serum,	Reductive debromination facilitated by at least one meta- or para- doubly flanked Br 11 OH-BDEs measured in serum of pentaBDE exposed fish; No OH-BDEs in decaBDE exposed fish; No MeO-BDEs detected w/either mixture	Zeng et al. 2012
Maternal Transfer of PBDEs								
Zebrafish (<i>D. rerio</i>)	Embryos	Dietary, maternal	BDE-28, -183, -209	10 and 100 nmol/g food · 2% bw/day	42 d	Embryos, maternal transfer	All three PBDEs transferred to eggs (BDE-28 > BDE-183 > BDE-209) Egg/maternal fish concentration ratios significant > 1.0 for BDE-183, BDE-209	Nyholm et al. 2008
Marine medaka (<i>O. melastigma</i>)	Embryos	Maternal	BDE-47 (bioencap artemia)	Breeding pairs: 1.3±0.2 µg/day	18 d	Embryos, maternal transfer	Maternal transfer of BDE-47 ↑ BDE 47 to 25 ng/egg (day 18) Maternal concentrations BDE-47 < males	Van der Merwe et al. 2011
Neurodevelopmental and Developmental Malformations/Effects								
Zebrafish (<i>D. rerio</i>)	Embryo larvae	Aqueous	BDE-47	0, 1.25, 5, 20 µM	6 – 96 hpf	Whole fish	Impaired motor behavior ↓ touch-response, swimming speed Inhibited axon growth	XJ Chen et al. 2012
Zebrafish (<i>D. rerio</i>)	Larvae	Aqueous	DE-71	0, 31, 68.7, 227.6 µg/l	2 – 120 hpf	Whole fish	Altered behavior (light-dark stimulation) ↑AChE activity, ↑ACh; ↓ mRNA transcripts for MBP, α1-tubulin, sonic hedgehog	Chen et al. 2012b

Species	Sex, Stage	Exposure Route	Treatment	Dose	Duration	Biological Material	Effects Observed	Reference
Zebrafish (<i>D. rerio</i>)	Adult, F1	Aqueous parental	BDE-209	0.001 – 1 µM	150 dph (adults) Bred at 120 dph	Whole fish, gonad, offspring	Parent: ↑ mortality (~44% high dose); Neg ctrl mortality ~38%; PBDE bioaccumulation (congeners not specified); ↓male/female GSI; ↓sperm count, motility Offspring: Delayed hatching, motor neuron development, loose muscle fibers, slow locomotion; hyperactivity (light-dark test)	He et al. 2011
Zebrafish (<i>D. rerio</i>)	Larvae	Aqueous	BDE-47	100 – 5000 µg/l	3 – 168 hpf	Whole fish	Delayed hatching, reduced growth, dorsal curvature, impaired CSF flow Cardiac toxicity at 96 hpf (tachycardia, arrhythmias)	Lema et al. 2007
Zebrafish (<i>D. rerio</i>)	Embryo larvae	Aqueous	BDE-49	4 – 32 µM	5 hpf, 24 hpf	Whole fish	Dorsal curvatures, cardiac toxicity (reduced heart rate); neurobehavioral effects (impaired touch-escape responses)	McClain et al. 2012
Mummichog (<i>Fundulus heteroclitus</i>)	Embryo larvae juvenile	Aqueous	DE-71	0.001 – 100 µg/l	0-7 hpf	Whole fish, embryos, behavior	Delayed hatching; No major deformities but tail curve asymmetry; ↓ activity; impaired fright response (larvae) Impaired learning (juveniles) METABOLISM: <i>No in ovo EROD activity (embryos)</i>	Timme-Laragy et al. 2006
Zebrafish (<i>D. rerio</i>)	Embryo larvae	Aqueous	BDE-28, -47, -99, -100, -153, -183	0.635 – 10 mg/l	Up to 168 hpf	Whole fish, behavior	Swimming rate ↑ (96-120 hpf), ↓ (168 hpf) ↓ swimming rates (BDE-47; 168 hpf) Developmental deformities (dorsal curvature at 120 hpf) w/mortality (BDE-28, -47, -99, -100) No effects w/BDE-153, -183 (uncertainty re: dosing stocks, solubility)	Usenko et al. 2011
Thyroid System Alterations								
Zebrafish (<i>D. rerio</i>)	Larvae	Aqueous	BDE-209	0, 0.08, 0.38, 1.92 mg/l	14 dpf	Whole fish total RNA, TH levels	↑ mRNA transcripts for CRH, TSHβ, Pax8, Nkx2.1, NIS, Tg, Dio1, Dio2, TRα, TRβ ↓ TTR mRNA transcripts; ↑ T3 (0.38, 1.92 mg/l), ↓T4 (1.92 mg/l) (whole fish) METABOLISM: <i>↓mRNA transcripts for UDPGT1ab</i>	Q Chen et al. 2012
Zebrafish (<i>D. rerio</i>)	Larvae	Dietary	BDE-47	100 ng/g; 0.5-1 mg food/fish-d	20-60 dph, sampled 38, 60 dph	Whole fish, total RNA	No change in TTR, Dio1, TSHβ mRNA transcripts METABOLISM: <i>↑CYP1A1 mRNA transcripts</i> REPRODUCTION: <i>No change in Vtg mRNA</i>	Chen et al. 2010

Species	Sex, Stage	Exposure Route	Treatment	Dose	Duration	Biological Material	Effects Observed	Reference
Rainbow trout (<i>O. mykiss</i>)	Juvenile	IP injection	BDE-209	50 – 1000 ng/g bw/day	21 d	Liver, kidney, plasma	↑ TT4 (1000 ng/g bw); ↓ FT3 (all doses); ↓FT4 (100, 200, 500 ng/g bw) <i>METABOLISM: Significant OH- and MeO-BDE metabolites (uncertainty about GC/MS analysis)</i>	Feng et al. 2012
European flounder (<i>P. flesus</i>); Zebrafish (<i>D. rerio</i>)	Adult (flounder) larvae (zebrafish)	Spiked sediment diet (flounder) Aqueous (zebrafish)	DE-71(purified of PBDD/Fs)	0+0.014 – 700 + 14000 [µg/g TOC+µg/g lipid]; 0-500 µg/l (zebrafish)	101 days (flounder) 30 days (zebrafish)	Blood, gonad, liver	↓ plasma TT4 (flounder) ↑ plasma TH, ↓ larval survival; (zebrafish) <i>METABOLISM: Weak ↓ EROD</i> <i>REPRODUCTION: ↓ovarian aromatase (CYP19) activity (flounder); ↓ fecundity (zebrafish)</i>	Kuiper et al. 2008
Fathead minnow (<i>P. promelas</i>)	Adult, breeding pairs	Dietary	BDE-47	2.4 µg/pair/day; 12.3 µg/pair/day	21 d	Liver, Brain, Pituitary	↓ TT4 (no change in TT3); ↑ mRNA transcripts for TSHβ (low dose), TRα (female brains); ↓ TRβ mRNA transcripts (both sexes, brains) <i>NEUROTOXICITY: ↓ BTEB mRNA transcripts (males)</i> <i>REPRODUCTION: ↓ mature spermatozoa</i>	Lema et al. 2008
Chinese rare minnow (<i>G. rarus</i>)	Adult, larvae	Aqueous	BDE-209	0 – 10 µg/L	21 d	Liver, gonad, brain, whole-fish	↑ dio 2, NIS (larvae) ↓TRα, dio2, NIS (adults) <i>REPRODUCTION: ↓spermatogenesis</i>	Li et al. 2011
Gilthead sea bream (<i>S. aurata</i>)	Adults	In vitro, recombinant TTR	PBDEs, 6-OH-BDE47	0-10 µM	2 h	Plasma	BDE-28, 49, -47, -99 potent inhibitors of ¹²⁵ I-T3 binding to TTR; IC50s << T3, T4 6-OH-BDE-47 moderate inhibitor; IC50s > T3,T4	Morgado et al. 2007
Lake trout (<i>S. namaycush</i>)	Juveniles	Dietary	13 PBDE congener mix	~0, 2.5, 25 ng/g dw food · 1.5% bw/day	56 d; 112 d depuration	Plasma, liver, kidney, GI tract, carcass	Bioaccumulation, biotransformation ↓ FT4 (low, high dose); ↓FT3 (low dose only) <i>METABOLISM: Reductive debromination; unknown pentaBDE, BDE-140, unknown hexaBDEs not present in food or control fish</i>	Tomy et al. 2004
Zebrafish (<i>D. rerio</i>)	larvae	Aqueous	DE-71	1, 3, 10 µg/l	14 d	Whole fish	↓ whole fish T4 (T3 not measured) ↑ mRNA transcripts for CRH, TSHβ, NIS, TG, Pax8, Nkx2.1, dio1, dio2; ↓TTR mRNA transcripts <i>METABOLISM: ↑ mRNA transcripts for UGT1ab</i>	Yu et al. 2010

Species	Sex, Stage	Exposure Route	Treatment	Dose	Duration	Biological Material	Effects Observed	Reference
Zebrafish (<i>D. rerio</i>)	Adults, offspring	Parental	DE-71	1, 3, 10 µg/l (parents and offspring) + no DE-71(offspring)	5 m to sexual maturation (parents) 5 and 10 dph (F1 larvae)	Plasma (adults), larvae	<p>↑ plasma TT4 (no Δ TT3) (parents, ELISA); ↓ mRNA transcripts for CRH, TSHβ (parent brain) ↑ whole fish T4, T3; altered HPT axis mRNA transcripts (offspring w/w/o DE-71 exposure)</p> <p>REPRODUCTION/DEVELOPMENT: ↓ hatching rate, ↑ malformation rates (maternally exposed F1); DE-71 to F1 worsened</p> <p>MATERNAL TRANSFER: Maternal transfer of PBDEs, THs to offspring</p>	Yu et al. 2011
Reproductive Alterations								
Atlantic salmon (<i>S. salar</i>)	Juvenile	Oral	PentaBDE, OctaBDE mixtures	10 mg/kg bw (d 1), 50 mg/kg bw (d 4)	7 d, 14 d	Plasma, liver, brain, carcass	No effects on protein expression/activity of Vtg, zona radiata, CYP1A	Boon et al. 2002
Fathead minnow (<i>P. promelas</i>)	Adult, breeding pairs	Dietary	BDE-47	28.7±1.6 µg/pair (bioencapsulated artemia)	25 d	Liver, Gonad	<p>Spawning ceased by 2-wks of exposure</p> <p>Reduced fecundity</p> <p>>50% reduction in sperm counts</p> <p>No change in GSI, LSI; reduced condition, males</p>	Muirhead et al. 2006
Atlantic salmon (<i>S. salar</i>)	Juveniles, males	In vitro	BDE-47, -153, -154 (alone and mixed)	0.01 – 100 µM	48 hrs	Hepatocytes, transcriptomic, proteomic approaches	<p>Disturbed glucose homeostasis (PBDE mix, BDE-153)</p> <p>Altered cell proliferation processes (PBDE mix)</p> <p>Estrogenic responses (↑ Vtg; ZP3 mRNA) in males (BDE-47, PBDE mix)</p> <p>METABOLISM: CYP1A induction (BDE-47, mix)</p>	Softeland et al. 2011
Oxidative Stress								
Zebrafish (<i>D. rerio</i>)	Embryos, 1.5 yr old adults, Embryonic fibroblasts	Aqueous, microarray	6-OH-BDE-47, 6-MeO-BDE-47, BDE-47	6.25-100 nM (embryos); 100 nM-1 µM (adults) 1 µM (PAC2 cultures)	3 - 72 hpf (embryos) 96 hrs (adults) 24 hrs (PAC2 cultures)	Mitochondria (pooled adult GI tract, heart, liver, kidneys)	<p>6-OH-BDE-47 only; no effects for 6-MeO-BDE-47, BDE-47</p> <p>Inhibition of electron transport complex II (OXPHOS)</p> <p>EC₅₀ = 25 nM (embryos); LC₅₀ ~350 nM (adults)</p> <p>Altered proton transport, carbohydrate metabolism;</p>	Van Boxtel et al. 2008
Rainbow trout (<i>O. mykiss</i>)	Adults	In vitro	BDE-47	0.2 – 50 µM	24 h	Gill cells (RT-gill-W1)	PBDE-induced apoptosis, oxidative stress	Shao et al. 2010

Species	Sex, Stage	Exposure Route	Treatment	Dose	Duration	Biological Material	Effects Observed	Reference
Crucian carp (<i>C. auratus</i>)	Adults	Aqueous	BDE-209	0 – 4 µM	1 – 13 d	Liver	No Δ GSH ↑ GR, GSH-Px; ↓ GST	Zhao et al. 2011
Immune System Effects								
Chinook salmon (<i>O. tshawytscha</i>)	Juveniles	Dietary	PBDE mix (BDE-47, -99, -100, -153, -154)	1X - 190 ng/g food 10X – 1.9 µg/g food (2% bw/day)	40 d	Not applicable	1X mix - ↑ lethality to <i>L. anguillarum</i> infection 10X mix – no significant effects	Arkoosh et al. 2010
Lake trout (<i>S. namaycush</i>)	4-yr old adults	In vitro	BDE-47, BDE-99	10 µg/l - 100 mg/L	6 h, 20 h	Plasma	↓ thymocyte viability ↑ necrosis/apoptosis at >10 mg/l	Birchmeier et al. 2005
Acute Toxicity and Other Biological Effects								
Zebrafish (<i>D. rerio</i>)	Adult, females	Aqueous	DE-71	0.45 µg/l, 9.6 µg/l	60 d	GI, liver, eyes, ovaries, eggs	↓ retinyl ester protein, ↓ CRBP mRNA transcripts (GI) ↑ retinoids (eyes, ovaries, eggs) ↑ CRBP mRNA transcripts (liver, eyes) ↓ retinal dehydrogenase, ↑ CYP26A (eyes)	Chen et al. 2012a
Mummichog (<i>Fundulus heteroclitus</i>)	Adults	Aqueous	BDE-47	0.0125 – 0.1 mg/l	96 h static renewal	Liver, brain	↑ AChE (0.0125 mg/l) LC ₅₀ = 0.1 mg/l (HDT)	Key et al. 2009
Turbot (<i>P. maxima</i>)	Embryos, larvae	Aqueous	BDE-47, BDE-99	2.8 – 24 µg/l (BDE-47); 4.3 – 39.5 µg/l (BDE-99)	6 d	Not applicable	Embryonic LC ₅₀ = 27.4 (BDE-47), 38.3 µg/l (BDE-99) Larval LC ₅₀ = 14.1 (BDE-47), 29.6 µg/l (BDE-99)	Mhadhbi et al. 2012

a1-tubulin = neuron microtubulin protein; ACh = acetylcholine; AChE = acetylcholinesterase; BTEB = basic transcription element-binding protein; CRBP = cellular retinal binding protein; CRH = corticotrophin releasing hormone; CSF = cerebral spinal fluid; dep = depuration; EROD = ethoxyresorufin-O-deethylase; dpf = days post fertilization; dph = days post hatch; dio = iodothyronine deiodinase; DR-CALUX = chemical-activated luciferase gene expression mediated by Ah-receptor activation; FT3 = free triiodothyronine; FT4 = free thyroxine; GR = glutathione reductase; GSH-Px = glutathione peroxidase; HDT = highest dose tested; hpf = hours post fertilization; HPT = hypothalamic-pituitary-thyroid; MBP = myelin basic protein; NIS = Sodium/Iodide Symporter; OXPHOS = oxidative phosphorylation; Tg = thyroglobulin; TH = thyroid hormone; TR = thyroid receptor; TSH = thyroid stimulating hormone; TT4 = Total thyroxine; TTR = transthyretin; UDPGT = uridine diphosphate glucuronosyl phosphate; Vitg = vitellogenin; ZP3 = zona pellucida 3 (eggshell protein)

Appendix B: Adult fathead minnow/BDE-209 study supplemental materials

B.1 PBDE Quantitative Analysis of BDE-209 Treated Food

Table 8: BDE-209 concentrations measured in food used in adult fathead minnow study. (% recov ¹³C-BDE-209 = 99%; F-BDE-69 = 83%; MDLs < 0.2 ng/g ww).

Blank Samples							
PBDE Congener	Blank - 1		Blank - 2		Blank - 3		Amount
	Retention Time	Amount	Retention Time	Amount	Retention Time	Amount	Mean
CDE 141	10.10	50.00	10.1	50	10.1	50	
F-BDE 69	10.04	45.00	10.04	42.84	10.04	39.77	
13CBDE209	29.30	49.84	29.3	48.29	29.28	45.67	
FBDE 69	10.04	50.00	10.04	50	10.04	50	
BDE 47	10.51	1.93	10.51	2.34	10.51	1.78	2.02
BDE 99	11.89	1.27	11.89	1.85	11.89	1.48	1.53
BDE 154	13.11	0.11	13.1	0.13	13.08	0.16	0.13
BDE 153	13.99	0.09	13.99	0.16	14.01	0.11	0.12
BDE 208	22.57	0.16	22.59	0.22	22.57	0.21	0.20
BDE 207	22.91	0.25	22.93	0.34	22.93	0.31	0.30
BDE 206	23.84	0.49	23.88	0.84	23.9	0.46	0.60
13C BDE 209	29.30	50.00	29.3	50	29.28	50	
BDE 209	29.30	13.63	29.3	14.06	29.32	13.24	13.64

Negative Control Food											
PBDE Congener	Negative Control - 1			Negative Control - 2			Negative Control - 3			Concentration (ng/g ww)	
	Retention Time	Amount	Blank-Corrected Amount	Retention Time	Amount	Blank-Corrected Amount	Retention Time	Amount	Blank-Corrected Amount	Mean	SEM
CDE 141	10.1	50		10.1	50		10.1	50			
F-BDE 69	10.04	41.66		10.04	38.67		10.04	42.63			
13CBDE209	29.3	51.9		29.3	51.15		29.3	52.18			
FBDE 69	10.04	50		10.04	50		10.04	50			
BDE 47	10.51	3.89	1.87	10.51	4.35	2.33	10.51	3.16	1.14	1.78	0.35
BDE 99	11.89	2.78	1.25	11.89	2.42	0.89	11.89	2.55	1.02	1.05	0.11
BDE 154	13.1	0.21	0.08	13.1	2.3	2.17	13.1	0.22	0.09	0.78	0.70
BDE 153	13.99	0.11	nd	14.01	0.12	nd	14.01	0.1	nd	nd	
BDE 208	22.59	0.15	nd	22.59	0.19	nd	22.59	0.16	nd	nd	
BDE 207	22.93	0.22	nd	22.93	0.26	nd	22.93	0.27	nd	nd	
BDE 206	23.92	0.57	nd	23.88	0.45	nd	23.88	0.48	nd	nd	
13C BDE 209	29.34	50		29.3	50		29.34	50			
BDE 209	29.32	13.61	nd	29.34	13.08	nd	29.34	13.52	0.00	nd	

BDE-209 Low Dose Treatment											
PBDE Congener	BDE-209 Low Dose - 1			BDE-209 Low Dose - 2			BDE-209 Low Dose - 3			Concentration (ng/g ww)	
	Retention Time	Amount	Blank-Corrected Amount	Retention Time	Amount	Blank-Corrected Amount	Retention Time	Amount	Blank-Corrected Amount	Mean	SEM
CDE 141	10.1	50		10.1	50		10.1	50			
F-BDE 69	10.04	42.4		10.04	37.89		10.04	43.03			
13CBDE209	29.85	49.01		29.96	44.89		29.34	51.11			
FBDE 69	10.04	50		10.04	50		10.04	50			
BDE 47	10.51	3.31	1.29	10.51	3.08	1.06	10.51	3.19	1.17	1.18	0.07
BDE 99	11.89	1.8	0.27	11.89	2.39	0.86	11.89	3.49	1.96	1.03	0.50
BDE 154	13.1	0.21	0.08	13.1	0.27	0.14	13.1	0.38	0.25	0.15	0.05
BDE 153	13.99	0.14	0.02	14.01	0.24	0.12	14.01	0.36	0.24	0.13	0.06
BDE 208	22.59	0.69	0.49	22.59	0.55	0.35	22.59	0.62	0.42	0.42	0.04
BDE 207	22.95	1.27	0.97	22.95	1.11	0.81	22.93	1.22	0.92	0.90	0.05
BDE 206	23.9	1.92	1.32	23.9	1.31	0.71	23.9	2.6	2.00	1.35	0.37
13C BDE 209	29.32	50		29.32	50		29.34	50			
BDE 209	29.32	108.76	95.12	29.34	109.75	96.11	29.34	108.38	94.74	95.32	0.41

BDE-209 High Dose Treatment											
PBDE Congener	BDE-209 High Dose - 1			BDE-209 High Dose - 2			BDE-209 High Dose - 3			Concentration (ng/g ww)	
	Retention Time	Amount	Blank-Corrected Amount	Retention Time	Amount	Blank-Corrected Amount	Retention Time	Amount	Blank-Corrected Amount	Mean	SEM
CDE 141	10.1	50		10.1	50		10.1	50			
F-BDE 69	10.04	44.91		10.04	42.99		10.04	39.83			
13CBDE209	29.3	84.33		29.3	88.73		29.28	82.8			
FBDE 69	10.04	50		10.04	50		10.04	50			
BDE 47	10.53	6.7	4.68	10.54	5.28	3.26	10.54	4.63	4.63	4.19	0.46
BDE 99	11.91	5.12	3.59	11.89	5.18	3.65	11.89	3.86	3.86	3.70	0.08
BDE 154	13.1	1.08	0.95	13.1	0.61	0.48	13.1	0.64	0.64	0.69	0.14
BDE 153	13.95	0.27	0.15	13.99	1.48	1.36	13.97	0.91	0.91	0.81	0.35
BDE 208	22.57	50.96	50.76	22.57	38.17	37.97	22.57	40.69	40.69	43.14	3.89
BDE 207	22.93	102.78	102.48	22.93	83.92	83.62	22.93	82.59	82.59	89.56	6.47
BDE 206	23.88	157.57	156.97	23.88	138.19	137.59	23.86	126.18	126.18	140.25	8.99
13C BDE 209	29.3			29.3	50	50.00	29.28	50			
BDE 209	29.32	10441.6	10428.00	29.3	10098.2	10084.56	29.3	10209.8	10196.17	10134.89	101.15

B.2 Isolation and Sequencing of Partial cDNA for Dios

Primers were designed with degenerate PCR from consensus regions of sequences for *dio1*, *dio2*, and *dio3* cDNAs isolated from other teleost fish (Tables 4 and 5). The deduced amino acid sequences for identified fathead minnow *dio* cDNAs were aligned with *dio* sequences from other teleosts using Clustal X software (Table 6) (Larkin et al. 2007). A phylogenetic analysis was conducted using MEGA v.5 and the Neighbor-Joining method and the a p-distance model for tree construction (See Appendix B, Figure 29) (Saitou and Nei 1987; Tamura et al. 2007). Confidence values for clusters of associated taxa were obtained by bootstrap tests (1000 replicates). Degenerate PCR was also performed previously by the Lema Laboratory to deduce partial sequences for the three reference genes evaluated (*beta actin (B-actin)*, *elongation factor-1 α (EF1 α)*, *ribosomal protein-l8 (rpl8)*), monocarboxylate transporters (*MCT8*, *MCT10*), and several organic anion transporter proteins (*OATP1c1*, *OATP1f1*, *OATP1f2*, *OATP2a1*, *OATP2b1*, *OATP3a1*, *OATP4a1*, and *OATP5a1*).

B.3 Reverse Transcription of Total RNA

Total RNA was isolated using TRI-Reagent (Molecular Research Center, Inc., Cincinnati, OH) with bromochloropropane and further treated with DNase I (Turbo DNA-free kit, Ambion, Grand Island, NY). The resulting total RNA was quantified by Nanodrop spectrophotometry (260:280 \geq 1.99) and diluted to 20 ng/ μ l. Total RNA was

reverse transcribed to cDNA in 40 μ l reactions by incubating 8.0 μ l of template with 11.4 μ l nuclease-free water (Sigma-Aldrich), 8.0 μ l of 5X buffer, 6.0 μ l of 0.1 M MgCl₂, 2.0 μ l of random hexamers (500 ng/ml; Promega, Madison, WI), 2.0 μ l of deoxynucleoside triphosphates (dNTPs; stock of 10 mM each of dCTP, dGTP, dTTP, dATP; Promega), 1.0 μ l of RNasin inhibitor (40 U/ μ l), and 1.6 μ l of Superscript III Reverse Transcriptase (200 U/ml; Invitrogen). All RT reactions were performed in 96 well plates with the following thermal profile: 25°C for 5 min., 42°C for 60 min, and 70°C for 5 min (T-100 Thermal Cycler, BIO-RAD, Hercules, CA).

B.4 Taqman Real-Time RT-PCR

Quantitative real-time PCR (RT-qPCR) was conducted in 20 μ L reactions containing 10 μ l of Taqman Universal Master Mix II Reagent (Applied Biosystems, Carlsbad, CA), 6.5 μ l of nuclease free water (Sigma-Aldrich), 0.5 μ l of Taqman probe (10 mM), 0.5 μ l of forward and reverse primers (45 μ M), and 2.0 μ l of reverse transcribed cDNA template (4 ng/ μ l). The thermal profile for the PCR reactions was 50°C for 2 min., 95°C for 10 min., 45 cycles of 95°C for 15 sec., and 60°C for 1 min. All assays were performed on a 7300 Real-Time PCR System (Applied Biosystems).

B.5 Primer and Taqman probe sequences

Table 9: Primer and Taqman probe sequences of fathead minnow deiodinases, thyroid receptors, and reference genes for quantitative real-time RT-PCR.

Transcript	Primer or probe	Nucleotide Sequence (5' to 3')	Amplicon Size (bp)	Effic. (Avg.)
<i>dio1</i>	Forward primer	TGCCCTGTGGTCGTGGAT	66 bp	97.85
	Probe	AGATGACCAACATCACTGCCAGCAAA		
	Reverse primer	CCTCTCAGGAAGGGCTCCAT		
<i>dio2</i>	Forward primer	CGCCCGCTAGTGGTCAAC	62 bp	101.03
	Probe	CGGCTCAGCCACTTGACCCCC		
	Reverse primer	ACCGGCAGCTGGCTTATAAA		
<i>dio3</i>	Forward primer	TTTAATGAAGATGCGGGAAAGC	68 bp	99.06
	Probe	ACATCGCGAGCCCTGACGACC		
	Reverse primer	TGGAGTCGGACACGCACAT		
<i>TRα</i>	Forward primer	TGCAGGCTGTACTCCTCATGA	83 bp	98.88
	Probe	AGATCGTTCTGGACTGACATGTGTGGAAAAGAT		
	Reverse primer	CAGGTACGTCTCCTGACACTTCTC		
<i>TRβ</i>	Forward primer	TTGCTCCAAGCCGTGATTCT	74 bp	98.66
	Probe	CTTTCCTCTGATCGTCCAGGTTTAACGAGC		
	Reverse primer	TGACAACGCTCTATCCGCTCTA		
<i>EF1α</i>	Forward primer	GTTTGAGAAGGAAGCTGCCGAGAT	100 bp	98.06
	Probe	AGGGCTCCTCAAATATGCCTGGGTGCT		
	Reverse primer	ATCAATGGTGATACCACGCTCAC		
<i>rpl8</i>	Forward primer	CTGTTGTTGGTGTGTTGCTGGTG	95 bp	100.72
	Probe	ACCCATCCTGAAGGCAGGACGTGCATACCA		
	Reverse primer	GCAGTTCCTCTTGGCCTTGTACTT		
<i>β-actin-1</i>	Forward primer	GGCATTGCTGACCGTATGCAGAAA	163 bp	98.44
	Probe	AAATCACCTCCCTTGCTCCTTCCACCAT		
	Reverse primer	ACTCCTGCTTGCTGATCCACATCT		

Table 10: Primer and Taqman probe sequences of fathead minnow monocarboxylate transporters (MCTs) and organic anion transport proteins (OATPs) for quantitative real-time RT-PCR.

Transcript	Primer or Probe	Nucleotide Sequence (5' to 3')	Amplicon Size (bp)	Effic. (Avg.)
<i>MCT8</i>	Forward primer	ACCAGGCGTCTCAGTTTAAAGTG	72	102.18
	Probe	CATGGGTCGGCGCTCTGGC		
	Reverse primer	CCGGCGAGCAGAAGAAGAT		
<i>MCT10</i>	Forward primer	GCTCTCCGATCGTCAGTGTGT	61	99.98
	Probe	CACGGATCTGCTGGGATGCCG		
	Reverse primer	TCCTCCGACAGCCGTGAT		
<i>OATP1c1</i>	Forward primer	GAGAGACGGTCACCGGAGAA	67	97.85
	Probe	TCCAAGGCCCTGTTGCTCAAGCC		
	Reverse primer	AGGCCACGAGGAACATCTTG		
<i>OATP1f1</i>	Forward primer	TGACGCCCAGAATACTGTTCAA	64	94.36
	Probe	AGCTTGTGGCCGGATGCCA		
	Reverse primer	CGAGCTTCTCTTCCCTTT		
<i>OATP1f2</i>	Forward primer	TGCAGAATGTATGACACTGAGACTT	71	93.48
	Probe	AGGTTCTGTTTCTGGGACTGG		
	Reverse primer	CCTAACACCCGCAGAGAGTTG		
<i>OATP2a1</i>	Forward primer	TGGCCTTGACAGCCTCTCTT	65	96.08
	Probe	AGGAACCGTCTCGTCCCTCCAT		
	Reverse primer	TCAGCACTGTGTCCCAACCT		
<i>OATP2b1</i>	Forward primer	GAAAAATGGTGACGCCAGATG	65	97.54
	Probe	TTGGAGCTTGGTGGTTGGGA		
	Reverse primer	GGAGGGTGGCAGCTATCAAG		
<i>OATP3a1</i>	Forward primer	CAACCTGGCCCTCATTCTCTTTGT	168	Not detected
	Probe	ATGGCTTTGGGAGCGCTGCTCTCCGCATTA		
	Reverse primer	CCCAATTCCTTGCTCCATGTGTCT		
<i>OATP4a1</i>	Forward primer	TCGCTCATTGCAGGCATCATCTAC	160	Not detected
	Probe	AGCTGTGAGAGCACAGATGTGGGAGGCCAT		
	Reverse primer	TGATTGGCAGGTCTCTGTTCTTCC		
<i>OATP5a1</i>	Forward primer	TTGACCAGAGTGACCCACGATTTG	169	94.44 (Brain)
	Probe	TGGAGTGGGTTTCTCCTGTGTGCTGTGGCA		
	Reverse primer	CTGGACACATCACCTGCACTCTTT		

B.6 GenBank Accession Numbers/Phylogenetic Analysis of Dios

Table 11: GenBank Accession numbers for taxa used in phylogenetic analysis of deiodinase (dio) partial cDNAs isolated from fathead minnow.

Transcript	Common Name	Scientific Name	GenBank Accession No.
dio1	fathead minnow	<i>Pimephales promelas</i>	
dio1	human	<i>Homo sapiens</i>	NM_000792
dio1	mouse	<i>Mus musculus</i>	NM_007860
dio1	chicken	<i>Gallus gallus</i>	NM_001097614
dio1	clawed frog	<i>Xenopus laevis</i>	NM_001095667
dio1	zebrafish	<i>Danio rerio</i>	BC155742
dio1	goldfish	<i>Carassius auratus</i>	EU313785
dio1	striped parrotfish	<i>Scarus iseri</i>	HM120252
dio1	seabream	<i>Sparus aurata</i>	DQ888894
dio1	pufferfish	<i>Takifugu rubripes</i>	AB360767
dio1	walleye	<i>Sander vitreus</i>	EF405953
dio1	sapphire devil	<i>Chrysiptera cyanea</i>	GU583740
dio1	tilapia	<i>Oreochromis niloticus</i>	Y11109
dio1	bastard halibut	<i>Paralichthys olivaceus</i>	AB362421
dio2	fathead minnow	<i>Pimephales promelas</i>	
dio2	walleye	<i>Sander vitreus</i>	EF405954
dio2	striped parrotfish	<i>Scarus iseri</i>	HM120253
dio2	pufferfish	<i>Takifugu rubripes</i>	AB360768
dio2	seabream	<i>Sparus aurata</i>	DQ888895
dio2	bastard halibut	<i>Paralichthys olivaceus</i>	AB362422
dio2	Senegalese sole	<i>Solea senegalensis</i>	AM902723
dio2	turbot	<i>Scophthalmus maximus</i>	AF467779
dio2	mummichog	<i>Fundulus heteroclitus</i>	FHU70869
dio2	medaka	<i>Oryzias latipes</i>	AB383147
dio2	zebrafish	<i>Danio rerio</i>	NM_212789
dio2	goldfish	<i>Carassius auratus</i>	EU313786
dio2	Japanese eel	<i>Anguilla japonica</i>	AB199797
dio2	rainbow trout	<i>Oncorhynchus mykiss</i>	AF207900
dio2	clawed frog	<i>Xenopus laevis</i>	AF354707
dio2	chicken	<i>Gallus gallus</i>	NM_204114
dio2	human	<i>Homo sapiens</i>	NM_013989
dio2	mouse	<i>Mus musculus</i>	BC125383
dio3	fathead minnow	<i>Pimephales promelas</i>	
dio3	mouse	<i>Mus musculus</i>	NM_172119
dio3	human	<i>Homo sapiens</i>	NM_001362
dio3	snake	<i>Pituophis deppei</i>	GQ862344
dio3	chicken	<i>Gallus gallus</i>	NM_001122648
dio3	clawed frog	<i>Xenopus laevis</i>	NM_001087863
dio3	pufferfish	<i>Takifugu rubripes</i>	AB360769
dio3	goldfish	<i>Carassius auratus</i>	EF190704
dio3	bastard halibut	<i>Paralichthys olivaceus</i>	AB362423
dio3	spinefoot	<i>Siganus guttatus</i>	GU385469
dio3	Senegalese sole	<i>Solea senegalensis</i>	AM902722

dio3	tilapia	<i>Oreochromis niloticus</i>	Y11111
dio3	pufferfish	<i>Takifugu rubripes</i>	NM_001136147
dio3	striped parrotfish	<i>Scarus iseri</i>	HM120254
dio3	rare Chinese minnow	<i>Gobiocypris rarus</i>	GU290040
dio3	walleye	<i>Sander vitreus</i>	EF405955
dio3	seabream	<i>Sparus aurata</i>	DQ888896

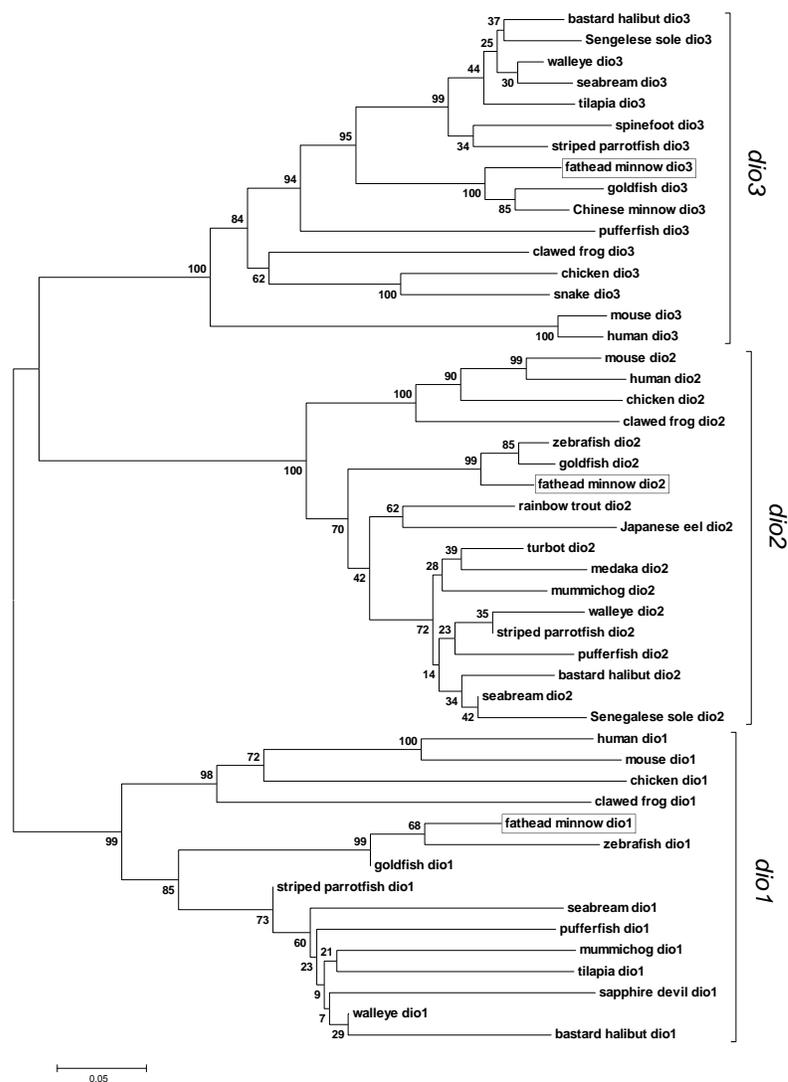


Figure 29: Phylogenetic tree of deiodinase (dio) enzymes based on alignment of deduced amino acid sequences from fish with clades for *dio1*, *dio2*, and *dio3* (marked with brackets). Trees were assembled with the Neighbor-Joining method and pairwise deletion of gaps. Bootstrap values are indicated at each node. GenBank accession numbers for taxa are provided in Tables 8.

B.7 PBDE Quantitative Analyses of Fathead Minnow Carcasses

Date of PBDE Extractions: 1/26/12 to 3/12/12

Date of GC/ECNI-MS Analysis: 3/16/12

Surrogate and Internal Standards:

F-BDE-69 (500 mg/ml; 2/9/9 HMS)

¹³C-BDE-209 (500 mg/ml; 4/29/11 PDN)

¹³C-CDE-141 (500 mg/ml; 4/6/11 PDN)

nd = non detect; MDL = method detection limit; IDL = instrument detection limit

Adult Fathead Minnow/BDE-209 Study: Fish mass and percent lipid measurements

Sample ID	Mass (g)	% Lipid	Sample ID	Mass (g)	% Lipid	Sample ID	Mass (g)	% Lipid
FHD28H1	1.05	0.33	FHD28L1	1.68	1.87	FHD28NC1	1.66	3.79
FHD28H2	1.13	0.30	FHD28L2	1.8	1.06	FHD28NC2	2.05	1.11
FHD28H3	1.36	0.41	FHD28L3	1.79	0.68	FHD28NC3	1.72	0.43
FHD14H1	1.58	0.39	FHD14L1	1.73	0.64	FHD14NC1	1.68	2.30
FHD14H2	2.13	0.89	FHD14L2	1.4	0.62	FHD14NC2	1.93	2.54
FHD14H3	1.49	0.50	FHD14L3	2.46	0.38	FHD14NC3	1.61	0.46
FHD0H1	2.36	0.74	FHD0L1	2.47	2.75	FHD0NC1	2.12	3.85
FHD0H2	2.37	1.68	FHD0L2	2.14	0.29	FHD0NC2	1.49	2.27
FHD0H3	1.21	0.79	FHD0L3	2.24	1.74	FHD0NC3	1.96	2.19
FHD42H1	2.37	3.62	FHD42L1	1.78	0.25	FHD42NC1	2.14	0.78
FHD42H2	1.55	0.66	FHD42L2	2.84	3.51	FHD42NC2	1.56	0.51
FHD42H3	1.31	0.45	FHD42L3	2.09	0.86	FHD42NC3	1.94	2.16

Laboratory Blank Samples (n=3)

Sample	FHBLKST1			FHBLKST2			FHBLKST 3			TOTAL		MDL CALCULATIONS		
Name	Ret Time	Amt	Target Resp	Ret Time	Amt	Target Resp	Ret Time	Amt	Target Resp	AVG	STD DEV	(3*SD)	Avg tissue (g)	MDL (IDL) (3*SD)/Avg tissue; ng/g)
CDE 141	10.4	50.00	184637	10.4	50.00	238334	10.4	50.00	264269				1.96	
F-BDE 69	10.4	35.21	29516	10	38.83	42032	10.4	25.24	30258					
13CBDE209	32.6	32.84	8923	32.6	46.15	17475	32.6	31.83	12303					
FBDE 69	10.4	50.00	29516	10.4	50.00	42032	10.4	50.00	30232					
BDE 30	9.1	0.02	17.0	9.1	0.03	33	9.1	0.03	32	0.03	0.01	0.02		0.01
BDE 17	9.6	0.03	20.0	9.6	0.03	22	9.6	0.01	10	0.02	0.01	0.03		0.02
BDE 25	9.6	0.02	14.0	9.6	0.02	17	9.6	0.03	22	0.02	0.01	0.02		0.01
BDE 28,33	9.8	0.15	96.0	9.8	0.20	189	9.8	0.16	131	0.17	0.03	0.08		0.04
BDE 75	10.6	0.01	3.0	10.6	0.03	24	10.6	0.06	44	0.03	0.03	0.08		0.04
BDE 49	10.7	0.01	20.0	10.7	0.02	44	10.7	0.01	13	0.01	0.01	0.02		0.01
BDE 71	10.6	0.02	169.0	10.6	0.04	14	10.6	0.05	16	0.04	0.02	0.05		0.02
BDE 47	10.9	4.91	3584	10.9	3.89	3732	10.9	4.29	4927	4.36	0.51			
BDE 66	11.1	0.06	37.0	11.1	0.08	63	11.1	0.09	67	0.08	0.02	0.05		0.02
BDE 101	12.0	0.05	30.0	12.0	0.03	24	12.0	0.05	37	0.04	0.01	0.03		0.02
BDE 99	12.7	6.44	4959	12.7	5.34	4796	12.7	5.56	6028	5.78	0.58			
BDE 116	13.4	0.02	9.0	13.4	0.03	20	13.4	0.04	26	0.03	0.01	0.03		0.02
BDE 155	14.0	0.03	25.0	14.0	0.01	13	14.0	0.03	32	0.02	0.01	0.03		0.02
BDE 154	15.0	0.03	17.0	15.0	0.03	29	15.0	0.04	32	0.03	0.01	0.02		0.01
BDE 153	16.5	0.03	22.0	16.5	0.02	23	16.5	0.05	44	0.03	0.02	0.05		0.02
HexaBDE1	17.2	0.02	9.0	17.2	0.01	9	17.2	0.02	13	0.02	0.01	0.02		0.01
HexaBDE2	17.6	0.01	6.0	17.6	0.01	7	17.6	0.01	8	0.01	0.00	0.00		0.00
BDE 188	17.7	0.02	7.0	17.7	0.02	10	17.7	0.05	20	0.03	0.02	0.05		0.03
BDE 179	18.1	0.03	9.0	18.1	0.02	10	18.1	0.04	17	0.03	0.01	0.03		0.02
BDE 176	18.2	0.02	7.0	18.2	0.02	9	18.2	0.04	19	0.03	0.01	0.03		0.02
BDE 183	18.9	0.02	11.0	18.9	0.03	17	18.9	0.04	28	0.03	0.01	0.03		0.02
BDE 191	19.1	0.01	7.0	19.1	0.01	9	19.1	0.02	10	0.01	0.01	0.02		0.01
BDE 181	19.2	0.01	5.0	19.2	0.01	5	19.2	0.04	23	0.02	0.02	0.05		0.03
BDE 171	19.4	0.01	3.0	19.4	0.01	9	19.4	0.01	6	0.01	0.00	0.00		0.00
BDE 190	19.9	0.03	10.0	19.9	0.02	9	19.9	0.05	17	0.03	0.02	0.05		0.02
BDE 202	20.0	0.03	8.0	20.0	0.02	8	20.0	0.02	5	0.02	0.01	0.02		0.01
BDE 201	20.4	0.04	10.0	20.4	0.05	18	20.4	0.06	20	0.05	0.01	0.03		0.02
BDE 197	21.5	0.04	14.0	21.5	0.03	15	21.5	0.02	9	0.03	0.01	0.03		0.02
BDE 203,200	21.0	0.02	8.0	21.0	0.01	11	21.0	0.02	11	0.02	0.01	0.02		0.01
BDE 196	21.5	0.03	10.0	21.5	0.04	20	21.5	0.04	17	0.04	0.01	0.02		0.01
BDE 205	24.1	0.01	3.0	24.1	0.01	7	24.1	0.04	20	0.02	0.02	0.05		0.03
BDE 208	24.6	0.26	153.0	24.6	0.22	184	24.6	0.14	104	0.21	0.06	0.18		0.09
BDE 207	25.8	0.43	241.0	25.8	0.31	249	25.8	0.19	136	0.31	0.12	0.36		0.18
BDE 206	32.6	0.18	16.0	32.6	0.22	28	32.6	0.21	24	0.20	0.02	0.06		0.03
BDE 209	32.7	12.32	2365	32.7	13.02	6051	32.7	10.71	3700	12.02	1.18			

Negative Control - Sampling Day 0

Name	FHD0NC1						FHD0NC2						FHD0NC3						TOTALS (BW NORM.)				TOTALS (LW NORM.)				
	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	MEAN (ng/g ww)	STD DEV (ng/g ww)	SEM (ng/g ww)	%CV	MEAN (ng/g lw)	STD DEV (ng/g lw)	SEM (ng/g lw)	%CV	
CDE 141	10.4	50.0	157454				10.4	50.0	143794				10.4	50.0	186701												
F-BDE 69	10.4	39.4	28157				10.4	39.6	25832				10.4	30.6	25888												
13CBDE209	32.6	54.0	13438				32.6	50.3	11767				32.6	36.7	10316												
FBDE 69	10.4	50.0	28157				10.4	50.0	25832				10.4	50.0	25888												
BDE 30	9.1	nd	nd				9.1	nd	nd				9.1	nd	nd												
BDE 17	9.6	nd	nd				9.6	nd	nd				9.6	nd	nd												
BDE 25	9.6	nd	nd				9.6	nd	nd				9.6	nd	nd												
BDE 28,33	9.8	nd	nd				9.8	nd	nd				9.8	nd	nd												
BDE 75	10.6	nd	nd				10.6	nd	nd				10.6	nd	nd												
BDE 49	10.7	nd	nd				10.7	nd	nd				10.7	nd	nd												
BDE 71	10.6	nd	nd				10.6	nd	nd				10.6	nd	nd												
BDE 47	10.9	5.8	3049	1.4	0.7	17	10.9	2.4	1197	-2.0			10.9	3.3	1688	-1.0											
BDE 66	11.1	nd	nd				11.1	nd	nd				11.1	nd	nd												
BDE 101	12.0	nd	nd				12.0	nd	nd				12.0	nd	nd												
BDE 99	13.4	7.1	3964	1.3	0.6	16	13.4	4.3	2658	-1.5	-1.0	-44	13.4	6.1	3496	0.4	0.2	8									
BDE 116	14.0	nd	nd				14.0	nd	nd				14.0	nd	nd												
BDE 155	15.0	0.1	55	0.1	0.0	1	15.0	nd	nd	nd	nd	nd	15.0	0.1	90	0.1	0.1	3	0.0	0.0	0.0	47	2	1	1	78	
BDE 154	16.5	0.3	168	0.3	0.1	3	16.5	0.1	80	0.1	0.1	4	16.5	0.5	290	0.5	0.3	12	0.2	0.1	0.1	55	7	5	3	73	
BDE 153	17.2	0.0	27	0.0	0.0	0	17.2	0.1	49	0.1	0.1	2	17.2	0.3	193	0.3	0.2	7	0.1	0.1	0.0	96	3	4	2	105	
HexaBDE1	17.6	nd	nd				17.6	nd	nd				17.6	nd	nd												
HexaBDE2	17.7	nd	nd				17.7	nd	nd				17.7	nd	nd												
BDE 188	17.9	nd	nd				17.9	nd	nd				17.9	nd	nd												
BDE 179	18.2	nd	nd				18.2	nd	nd				18.2	nd	nd												
BDE 176	18.9	nd	nd				18.9	nd	nd				18.9	nd	nd												
BDE 183	19.1	nd	nd				19.1	nd	nd				19.1	nd	nd												
BDE 191	19.2	nd	nd				19.2	nd	nd				19.2	nd	nd												
BDE 181	19.4	nd	nd				19.4	nd	nd				19.4	nd	nd												
BDE 171	19.9	nd	nd				19.9	nd	nd				19.9	nd	nd												
BDE 190	20.0	nd	nd				20.0	nd	nd				20.0	nd	nd												
BDE 202	20.4	nd	nd				20.4	nd	nd				20.4	nd	nd												
BDE 201	21.5	nd	nd				21.5	nd	nd				21.5	nd	nd												
BDE 197	21.0	nd	nd				21.0	nd	nd				21.0	nd	nd												
BDE 203,200	21.5	nd	nd				21.5	nd	nd				21.5	nd	nd												
BDE 196	24.1	nd	nd				24.1	nd	nd				24.1	nd	nd												
BDE 205	24.6	nd	nd				24.6	nd	nd				24.6	nd	nd												
BDE 208	25.8	0.2	103	0.0	0.0	0	25.8	0.2	127	-0.1	0.0	0	25.8	0.3	140	0.1	0.0	1	0.0	0.0	0.0	-584	0	1	1	251	
BDE 207	32.6	0.3	153	0.0	0.0	0	32.6	0.2	151	-0.1	-0.1	0	32.6	0.4	212	0.1	0.1	3	0.0	0.1	0.0	-965	1	2	1	214	
BDE 206	32.6	0.1	7	-0.1	0.0	0	32.6	0.2	33	0.0	0.0	0	32.6	0.4	28	0.2	0.1	4	0.0	0.0	0.0	143	1	2	1	143	
BDE 209	32.7	12.1	3738	0.0	0.0	0	32.7	12.0	3229	0.0	0.0	0	32.7	12.7	3270	0.7	0.4	17	0.1	0.2	0.1	166	6	10	6	170	

Negative Control - Sampling Day 14

Name	FHD14NC1						FHD14NC2						FHD14NC3						TOTALS (BW NORM.)				TOTALS (LW NORM.)				
	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	MEAN (ng/g ww)	STD DEV (ng/g ww)	SEM (ng/g ww)	%CV	MEAN (ng/g lw)	STD DEV (ng/g lw)	SEM (ng/g lw)	%CV	
CDE 141	10.4	50.0	194312				10.4	50.0	181309				10.4	50.0	201691												
F-BDE 69	10.4	45.8	40475				10.4	28.9	23757				10.4	30.6	28050												
13CBDE209	32.6	42.0	12872				32.6	43.2	12378				32.6	59.6	19033												
FBDE 69	10.4	50.0	40475				10.4	50.0	23757				10.4	50.0	28050												
BDE 30	9.1	nd	nd				9.1	nd	nd				9.1	nd	nd												
BDE 17	9.6	nd	nd				9.6	nd	nd				9.6	nd	nd												
BDE 25	9.6	nd	nd				9.6	nd	nd				9.6	nd	nd												
BDE 28,33	9.8	nd	nd				9.8	nd	nd				9.8	nd	nd												
BDE 75	10.6	nd	nd				10.6	nd	nd				10.6	nd	nd												
BDE 49	10.7	nd	nd				10.7	nd	nd				10.7	nd	nd												
BDE 71	10.6	nd	nd				10.6	nd	nd				10.6	nd	nd												
BDE 47	10.9	2.9	3621	-1.5			10.9	5.1	5420	0.7	0.4	15	10.9	3.8	2071	-0.6	-0.4										
BDE 66	11.1	nd	nd				11.1	nd	nd				11.1	nd	nd												
BDE 101	12.0	nd	nd				12.0	nd	nd				12.0	nd	nd												
BDE 99	12.7	5.5	5565	-0.3			12.7	3.7	4381	-2.1			12.7	7.1	6441	1.3	0.8	177									
BDE 116	13.4	nd	nd				13.4	nd	nd				13.4	nd	nd												
BDE 155	14.0	nd	nd	nd	0.0	0	14.0	0.3	195	0.3	0.1	6	14.0	0.1	105	0.1	0.1	16	0.1	0.1	0.0	98	7	8	5	114	
BDE 154	15.0	0.4	385	0.4	0.3	11	15.0	1.1	655	1.1	0.5	22	15.0	0.2	136	0.2	0.1	30	0.3	0.2	0.1	67	21	9	5	44	
BDE 153	16.5	0.6	553	0.6	0.4	15	16.5	0.2	85	0.2	0.1	3	16.5	0.2	94	0.2	0.1	20	0.2	0.2	0.1	88	13	9	5	69	
HexaBDE1	17.2	nd	nd				17.2	nd	nd				17.2	nd	nd												
HexaBDE2	17.6	nd	nd				17.6	nd	nd				17.6	nd	nd												
BDE 188	17.7	nd	nd				17.7	nd	nd				17.7	nd	nd												
BDE 179	18.1	nd	nd				18.1	nd	nd				18.1	nd	nd												
BDE 176	18.2	nd	nd				18.2	nd	nd				18.2	nd	nd												
BDE 183	18.9	nd	nd				18.9	nd	nd				18.9	nd	nd												
BDE 191	19.1	nd	nd				19.1	nd	nd				19.1	nd	nd												
BDE 181	19.2	nd	nd				19.2	nd	nd				19.2	nd	nd												
BDE 171	19.4	nd	nd				19.4	nd	nd				19.4	nd	nd												
BDE 190	19.9	nd	nd				19.9	nd	nd				19.9	nd	nd												
BDE 202	20.0	nd	nd				20.0	nd	nd				20.0	nd	nd												
BDE 201	20.4	nd	nd				20.4	nd	nd				20.4	nd	nd												
BDE 197	21.5	nd	nd				21.5	nd	nd				21.5	nd	nd												
BDE 203,200	21.0	nd	nd				21.0	nd	nd				21.0	nd	nd												
BDE 196	21.5	nd	nd				21.5	nd	nd				21.5	nd	nd												
BDE 205	24.1	nd	nd				24.1	nd	nd				24.1	nd	nd												
BDE 208	24.6	0.2	175	0.0	0.0	0	24.6	0.2	105	0.0	0.0	0	24.6	0.2	134	0.0	0.0	5	0.0	0.0	0.0	65	2	2	1	142	
BDE 207	25.8	0.2	171	-0.1	0.0	0	25.8	0.3	142	0.0	0.0	0	25.8	0.3	169	0.0	0.0	0	0.0	0.1	0.0	173	2	3	2	173	
BDE 206	32.6	0.1	6	-0.2	0.0	0	32.6	0.5	32	0.2	0.1	5	32.6	0.1	9	-0.1	0.0	0	0.0	0.1	0.0	221	29	52	30	178	
BDE 209	32.9	12.2	3349	0.1	0.1	4	32.7	11.8	3277	-0.2	-0.1	-5	32.7	12.7	5419	0.7	0.4	90	0.1	0.3	0.2	221	29	52	30	178	

Negative Control - Sampling Day 28

Name	FHD28NC1						FHD28NC2						FHD28NC3						TOTALS (BW NORM.)				TOTALS (LW NORM.)				
	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	MEAN (ng/g ww)	STD DEV (ng/g ww)	SEM (ng/g ww)	%CV	MEAN (ng/g lw)	STD DEV (ng/g lw)	SEM (ng/g lw)	%CV	
CDE 141	10.4	50.0	229200				10.4	50.0	207372				10.4	50.0	199333												
F-BDE 69	10.4	23.0	23893				10.4	32.0	30120				10.4	39.6	35855												
13CBDE209	32.6	47.2	17107				32.6	49.0	16071				32.6	53.8	16945												
FBDE 69	10.4	50.0	24315				10.4	50.0	30000				10.4	50.0	35928												
BDE 30	9.1	nd	nd				9.1	nd	nd				9.1	nd	nd												
BDE 17	9.6	nd	nd				9.6	nd	nd				9.6	nd	nd												
BDE 25	9.6	nd	nd				9.6	nd	nd				9.6	nd	nd												
BDE 28,33	9.8	nd	nd				9.8	nd	nd				9.8	nd	nd												
BDE 75	10.6	nd	nd				10.6	nd	nd				10.6	nd	nd												
BDE 49	10.7	nd	nd				10.7	nd	nd				10.7	nd	nd												
BDE 71	10.6	nd	nd				10.6	nd	nd				10.6	nd	nd												
BDE 47	10.9	5.8	3171	1.4	0.9	23	10.9	3.7	2197	-0.6			10.9	1.0	687	-3.4											
BDE 66	11.1	nd	nd				11.1	nd	nd				11.1	nd	nd	nd											
BDE 101	12.0	nd	nd				12.0	nd	nd				12.0	nd	nd	nd											
BDE 99	12.5	7.0	4377	1.2	0.7	19	12.5	5.4	3760	-0.4			12.5	2.1	1165	-3.7											
BDE 116	12.7	nd	nd	nd	nd	nd	12.7	nd	nd	nd	nd	nd	12.7	nd	nd	nd											
BDE 155	13.4	0.1	83	0.1	0.1	2	13.4	0.1	108	0.1	0.1	5	13.4	0.0	45	0.0	0.0	5	0.0	0.0	0.0	46	4	2	1	50	
BDE 154	14.0	0.5	265	0.5	0.3	8	14.0	0.3	273	0.4	0.2	19	14.0	0.1	98	0.1	0.1	17	0.2	0.1	0.1	57	15	6	3	41	
BDE 153	15.0	0.2	114	0.2	0.1	3	15.0	0.2	127	0.2	0.1	8	15.0	0.2	162	0.2	0.1	27	0.1	0.0	0.0	16	13	13	7	99	
HexaBDE1	16.5	nd	nd				16.5	nd	nd				16.5	nd	nd												
HexaBDE2	17.2	nd	nd				17.2	nd	nd				17.2	nd	nd												
BDE 188	17.6	nd	nd				17.6	nd	nd				17.6	nd	nd												
BDE 179	17.9	nd	nd				17.9	nd	nd				17.9	nd	nd												
BDE 176	18.1	nd	nd				18.1	nd	nd				18.1	nd	nd												
BDE 183	18.2	nd	nd				18.2	nd	nd				18.2	nd	nd												
BDE 191	18.9	nd	nd				18.9	nd	nd				18.9	nd	nd												
BDE 181	19.1	nd	nd				19.1	nd	nd				19.1	nd	nd												
BDE 171	19.2	nd	nd				19.2	nd	nd				19.2	nd	nd												
BDE 190	19.4	nd	nd				19.4	nd	nd				19.4	nd	nd												
BDE 202	19.9	nd	nd				19.9	nd	nd				19.9	nd	nd												
BDE 201	20.0	nd	nd				20.0	nd	nd				20.0	nd	nd												
BDE 197	20.4	nd	nd				20.4	nd	nd				20.4	nd	nd												
BDE 203,200	21.5	nd	nd				21.5	nd	nd				21.5	nd	nd												
BDE 196	21.0	nd	nd				21.0	nd	nd				21.0	nd	nd												
BDE 205	21.5	nd	nd				21.5	nd	nd				21.5	nd	nd												
BDE 208	24.1	0.3	119	0.0	0.0	1	24.1	0.2	93	0.0	0.0	0	24.1	0.1	97	-0.1	0.0	0	0.0	0.0	0.0	-286	0	0	0	173	
BDE 207	24.6	0.3	151	0.0	0.0	0	24.6	0.2	119	-0.1	0.0	0	24.6	0.2	121	-0.1	0.0	0	0.0	0.0	0.0	-211	0	0	0	173	
BDE 206	25.8	0.3	22	0.1	0.1	2	25.8	0.3	23	0.0	0.0	2	25.8	0.1	7	-0.1	0.0	0	0.0	0.0	0.0	109	1	1	1	89	
BDE 209	32.6	12.2	4812	0.1	0.1	2	32.6	12.1	4363	0.0	0.0	1	32.6	12.8	4687	0.7	0.4	98	0.2	0.2	0.1	124	34	56	32	164	

Negative Control - Sampling Day 42

Name	FHD42NC1						FHD42NC2						FHD42NC3						TOTALS (BW NORM.)				TOTALS (LW NORM.)				
	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	MEAN (ng/g ww)	STD DEV (ng/g ww)	SEM (ng/g ww)	%CV	MEAN (ng/g lw)	STD DEV (ng/g lw)	SEM (ng/g lw)	%CV	
CDE 141	10.4	50.0	188672				10.4	50.0	188177				10.4	50.0	216601												
F-BDE 69	10.4	42.1	36094				10.4	25.2	21486				10.4	33.1	32588												
13CBDE209	32.6	49.5	15108				32.6	46.3	13844				32.6	44.1	15006												
FBDE 69	10.4	50.0	36196				10.4	50.0	21297				10.4	50.0	32709												
BDE 30	9.1	nd	nd				9.1	nd	nd				9.1	nd	nd												
BDE 17	9.6	nd	nd				9.6	nd	nd				9.6	nd	nd												
BDE 25	9.6	nd	nd				9.6	nd	nd				9.6	nd	nd												
BDE 28,33	9.8	nd	nd				9.8	nd	nd				9.8	nd	nd												
BDE 75	10.6	nd	nd				10.6	nd	nd				10.6	nd	nd												
BDE 49	10.7	nd	nd				10.7	nd	nd				10.7	nd	nd												
BDE 71	10.6	nd	nd				10.6	nd	nd				10.6	nd	nd												
BDE 47	10.9	5.1	4875	0.7	0.3	44	10.9	3.1	1277	-1.3			10.9	4.9	4569	0.5	0.3	12	0.3	0.1	0.0	18	28	22	13	80	
BDE 66	11.1	nd	nd				11.1	nd	nd				11.1	nd	nd												
BDE 101	12.0	nd	nd				12.0	nd	nd				12.0	nd	nd												
BDE 99	12.7	6.0	4376	0.2	0.1	13	12.7	6.0	3470	0.2	0.1	24	12.7	7.0	5625	1.2	0.6	29	0.3	0.3	0.2	106	22	8	5	38	
BDE 116	13.4	nd	nd				13.4	nd	nd				13.4	nd	nd												
BDE 155	14.0	0.4	402	0.4	0.2	22	14.0	0.0	0	0.0	0.0	0	14.0	0.3	287	0.3	0.1	7	0.1	0.1	0.1	87	10	11	6	116	
BDE 154	15.0	1.2	926	1.2	0.6	71	15.0	0.2	75	0.2	0.1	0	15.0	1.7	1224	1.7	0.9	41	0.5	0.4	0.2	77	37	36	21	95	
BDE 153	16.5	0.8	678	0.8	0.4	49	16.5	1.0	465	1.0	0.6	119	16.5	1.0	786	1.0	0.5	25	0.5	0.1	0.1	23	64	49	28	76	
HexaBDE1	17.2	0.1	65	0.1	0.0	6	17.2	nd	nd				17.2	nd	nd												
HexaBDE2	17.6	nd	nd				17.6	nd	nd				17.6	nd	nd												
BDE 188	17.7	nd	nd				17.7	nd	nd				17.7	nd	nd												
BDE 179	18.1	nd	nd				18.1	nd	nd				18.1	nd	nd												
BDE 176	18.2	nd	nd				18.2	nd	nd				18.2	nd	nd												
BDE 183	18.9	nd	nd				18.9	nd	nd				18.9	nd	nd												
BDE 191	19.1	nd	nd				19.1	nd	nd				19.1	nd	nd												
BDE 181	19.2	nd	nd				19.2	nd	nd				19.2	nd	nd												
BDE 171	19.4	nd	nd				19.4	nd	nd				19.4	nd	nd												
BDE 190	19.9	nd	nd				19.9	nd	nd				19.9	nd	nd												
BDE 202	20.0	nd	nd				20.0	nd	nd				20.0	nd	nd												
BDE 201	20.4	nd	nd				20.4	nd	nd				20.4	nd	nd												
BDE 197	21.5	nd	nd				21.5	nd	nd				21.5	nd	nd												
BDE 203,200	21.0	nd	nd				21.0	nd	nd				21.0	nd	nd												
BDE 196	21.5	nd	nd				21.5	nd	nd				21.5	nd	nd												
BDE 205	24.1	nd	nd				24.1	nd	nd				24.1	nd	nd												
BDE 208	24.6	0.2	119	0.0	0.0	-2	24.6	0.2	101	0.0	0.0	4	24.6	0.2	121	0.0	0.0	0	0.0	0.0	0.0	-1392	1	3	2	628	
BDE 207	25.8	0.2	148	-0.1	0.0	-6	25.8	0.3	120	0.0	0.0	-3	25.8	0.2	147	-0.1	0.0	-2	0.0	0.0	0.0	-54	-3	2	1	-64	
BDE 206	32.6	0.3	43	0.1	0.0	5	32.6	0.2	34	0.0	0.0	3	32.6	0.4	54	0.1	0.1	4	0.0	0.0	0.0	66	4	1	1	26	
BDE 209	32.7	12.0	4207	0.0	0.0	0	32.6	11.7	3857	-0.3	-0.2	-40	32.6	12.6	4201	0.6	0.3	14	0.0	0.3	0.1	771	-9	28	16	-322	

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BDE-209 Low Dose - Sampling Day 0

Name	FHD0L1						FHD0L2						FHD0L3						TOTALS (BW NORM.)				TOTALS (LW NORM.)				
	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	MEAN (ng/g ww)	STD DEV (ng/g ww)	SEM (ng/g ww)	%CV	MEAN (ng/g lw)	STD DEV (ng/g lw)	SEM (ng/g lw)	%CV	
CDE 141	10.4	50.0	163525				10.4	50.0	200336				10.4	50.0	201462												
F-BDE 69	10.4	45.0	33421				10.4	45.8	41737				10.4	29.0	26492												
13CBDE209	32.6	52.0	13939				32.6	49.6	15695				32.6	46.0	14712												
FBDE 69	10.4	50.0	33421				10.4	50.0	41737				10.4	50.0	26492												
BDE 30	9.1	nd	nd				9.1	nd	nd				9.1	nd	nd												
BDE 17	9.6	nd	nd				9.6	nd	nd				9.6	nd	nd												
BDE 25	9.6	nd	nd				9.6	nd	nd				9.6	nd	nd												
BDE 28,33	9.8	nd	nd				9.8	nd	nd				9.8	nd	nd												
BDE 75	10.6	nd	nd				10.6	nd	nd				10.6	nd	nd												
BDE 49	10.7	nd	nd				10.7	nd	nd				10.7	nd	nd												
BDE 71	10.6	nd	nd				10.6	nd	nd				10.6	nd	nd												
BDE 47	10.9	5.4	5831	1.1	0.4	16	10.9	2.9	2372	-1.5			10.9	2.2	11578	-2.2											
BDE 66	11.1	nd	nd				11.1	nd	nd				11.1	nd	nd												
BDE 101	12.0	nd	nd				12.0	nd	nd				12.0	nd	nd												
BDE 99	12.5	6.4	6518	0.6	0.2	9	12.5	4.4	3197	-1.4			12.5	4.9	32878	-0.9											
BDE 116	12.7	nd	nd				12.7	nd	nd				12.7	nd	nd												
BDE 85,155	13.4	nd	nd				13.4	nd	nd				13.4	nd	nd												
BDE 154	14.0	nd	nd				14.0	nd	nd				14.0	nd	nd												
BDE 153	15.0	nd	nd				15.0	nd	nd				15.0	nd	nd												
HexaBDE1	16.5	nd	nd				16.5	nd	nd				16.5	nd	nd												
HexaBDE2	17.2	nd	nd				17.2	nd	nd				17.2	nd	nd												
BDE 188	17.6	nd	nd				17.6	nd	nd				17.6	nd	nd												
BDE 179	17.9	nd	nd				17.9	nd	nd				17.9	nd	nd												
BDE 176	18.1	nd	nd				18.1	nd	nd				18.1	nd	nd												
BDE 183	18.2	nd	nd				18.2	nd	nd				18.2	nd	nd												
BDE 191	18.9	nd	nd				18.9	nd	nd				18.9	nd	nd												
BDE 181	19.1	nd	nd				19.1	nd	nd				19.1	nd	nd												
BDE 171	19.2	nd	nd				19.2	nd	nd				19.2	nd	nd												
BDE 190	19.4	nd	nd				19.4	nd	nd				19.4	nd	nd												
BDE 202	19.9	nd	nd				19.9	nd	nd				19.9	nd	nd												
BDE 201	20.0	nd	nd				20.0	nd	nd				20.0	nd	nd												
BDE 197	20.4	nd	nd				20.4	nd	nd				20.4	nd	nd												
BDE 203,200	21.5	nd	nd				21.5	nd	nd				21.5	nd	nd												
BDE 196	21.0	nd	nd				21.0	nd	nd				21.0	nd	nd												
BDE 205	21.5	nd	nd				21.5	nd	nd				21.5	nd	nd												
BDE 208	24.1	0.2	143	0.0	0.0	0	24.1	0.1	176	-0.1	0.0	-16	24.1	0.4	231	0.2	0.1	6	0.0	0.1	0.0	380	-3	11	7	-345	
BDE 207	24.6	0.3	215	0.0	0.0	0	24.6	0.2	196	-0.1	-0.1	0	24.6	0.3	246	0.0	0.0	0	0.0	0.0	0.0	-299	0	0	0	95	
BDE 206	25.8	0.3	34	0.1	0.0	2	25.8	0.2	40	0.0	0.0	3	25.8	0.1	5	-0.1	0.0	-4	0.0	0.0	0.0	135	0	3	2	1646	
BDE 209	32.6	12.1	4277	0.1	0.0	1	32.6	12.1	4374	0.0	0.0	7	32.6	12.2	4511	0.2	0.1	4	0.04	0.0	0.02	78	4	3	2	79	

BDE-209 Low Dose - Sampling Day 14

Name	FHD14L1						FHD14L2						FHD14L3						TOTALS (BW NORM.)				TOTALS (LW NORM.)				
	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	MEAN (ng/g ww)	STD DEV (ng/g ww)	SEM (ng/g ww)	%CV	MEAN (ng/g lw)	STD DEV (ng/g lw)	SEM (ng/g lw)	%CV	
CDE 141	10.4	50.0	222708				10.4	50.0	203650				10.4	50.0	318228												
F-BDE 69	10.4	32.0	32347				10.4	34.3	31726				10.4	25.1	36298												
13CBDE209	32.6	59.3	22504				32.6	53.9	17359				32.6	46.5	23361												
FBDE 69	10.4	50.0	32372				10.4	50.0	31726				10.4	50.0	36298												
BDE 30	9.1	nd	nd				9.1	nd	nd				9.1	nd	nd												
BDE 17	9.6	nd	nd				9.6	nd	nd				9.6	nd	nd												
BDE 25	9.6	nd	nd				9.6	nd	nd				9.6	nd	nd												
BDE 28,33	9.8	nd	nd				9.8	nd	nd				9.8	nd	nd												
BDE 75	10.6	nd	nd				10.6	nd	nd				10.6	nd	nd												
BDE 49	10.7	nd	nd				10.7	nd	nd				10.7	nd	nd												
BDE 71	10.6	nd	nd				10.6	nd	nd				10.6	nd	nd												
BDE 47	10.9	4.6	2946	0.3	0.2	24	10.9	4.3	3689	-0.1			10.9	4.0	2961	-0.3											
BDE 66	11.1	nd	nd				11.1	nd	nd				11.1	nd	nd												
BDE 101	12.0	0.1	89	0.1	0.1	9	12.0	4.4	2748	4.3	3.1	500	12.0	3.3	2266	3.2	1.3	346	1.5	1.5	0.9	102	285	251	145	88	
BDE 99	12.5	5.2	3379	-0.6			12.5	6.1	4754	0.3	0.2	38	12.5	6.2	4901	0.4	0.2	47									
BDE 116	12.7	nd	nd				12.7	nd	nd				12.7	nd	nd												
BDE 155	13.4	0.2	232	0.2	0.1	21	13.4	0.7	665	0.7	0.5	79	13.4	1.0	1071	1.0	0.4	102	0.3	0.2	0.1	54	67	42	24	62	
BDE 154	14.0	10.7	10495	10.7	6.2	973	14.0	2.2	1505	2.2	1.6	254	14.0	29.3	23109	29.3	11.9	3119	6.6	5.2	3.0	79	1449	1491	861	103	
BDE 153	15.0	0.2	127	0.2	0.1	15	15.0	1.4	1026	1.4	1.0	162	15.0	0.2	196	0.2	0.1	25	0.4	0.5	0.3	131	67	82	48	122	
HexaBDE1	16.5	0.2	93	0.2	0.1	14	16.5	0.0	0	0.0	0.0	0	16.5	0.0	0	0.0	0.0	0	0.0	0.1	0.0	173	5	8	5	173	
HexaBDE2	17.2	0.2	112	0.2	0.1	17	17.2	1.6	927	1.6	1.1	182	17.2	4.6	3079	4.6	1.9	486	1.0	0.9	0.5	85	228	238	137	104	
BDE 188	17.6	0.4	151	0.4	0.2	35	17.6	0.4	139	0.4	0.3	43	17.6	0.4	175	0.4	0.2	44	0.2	0.0	0.0	22	41	5	3	11	
BDE 179	17.9	0.4	128	0.4	0.2	35	17.9	0.2	80	0.2	0.2	28	17.9	0.3	129	0.3	0.1	36	0.2	0.0	0.0	25	33	5	3	14	
BDE 176	18.1	nd	nd				18.1	nd	nd				18.1	nd	nd												
BDE 183	18.2	0.0	22	0.0	0.0	4	18.2	0.1	53	0.1	0.1	10	18.2	0.1	48	0.1	0.0	7	0.0	0.0	0.0	58	7	3	2	48	
BDE 191	18.9	nd	nd				18.9	nd	nd				18.9	nd	nd												
BDE 181	19.1	nd	nd				19.1	nd	nd				19.1	nd	nd												
BDE 171	19.2	nd	nd				19.2	nd	nd				19.2	nd	nd												
BDE 190	19.4	nd	nd				19.4	nd	nd				19.4	nd	nd												
BDE 202	19.9	0.1	32	0.1	0.1	11	19.9	0.2	52	0.2	0.1	23	19.9	0.3	99	0.3	0.1	35	0.1	0.0	0.0	35	23	12	7	53	
BDE 201	20.0	0.2	65	0.2	0.1	20	20.0	0.1	39	0.1	0.1	15	20.0	0.1	25	0.1	0.0	7	0.1	0.1	0.0	61	14	6	4	45	
BDE 197	20.4	0.1	40	0.1	0.1	10	20.4	nd	nd	nd	0.0	0	20.4	0.0	0	0.0	0.0	0	0.0	0.0	0.0	173	3	6	3	173	
BDE 203,200	21.5	nd	nd				21.5	nd	nd				21.5	nd	nd												
BDE 196	21.0	nd	nd				21.0	nd	nd				21.0	nd	nd												
BDE 205	21.5	nd	nd				21.5	nd	nd				21.5	nd	nd												
BDE 208	24.1	0.2	167	0.0	0.0	-2	24.1	0.2	113	0.0	0.0	0	24.1	0.2	107	-0.1	0.0	0	0.0	0.0	0.0	-20	-1	1	1	-173	
BDE 207	24.6	0.4	201	0.1	0.1	9	24.6	0.2	141	0.0	0.0	0	24.6	0.2	141	-0.1	0.0	0	0.0	0.1	0.0	1178	3	5	3	173	
BDE 206	25.8	0.9	221	0.7	0.4	62	25.8	0.2	62	-0.1	0.0	-6	25.8	0.3	27	0.0	0.0	5	0.1	0.2	0.1	188	20	37	21	180	
BDE 209	32.6	14.3	8725	2.2	1.3	204	32.6	15.3	5641	3.3	2.4	385	32.6	13.6	6531	1.6	0.7	171	1.4	0.9	0.5	60	253	116	67	46	

BDE-209 Low Dose - Sampling Day 28

Name	FHD28L1						FHD28LH2						FHD28LH3						TOTALS (BW NORM.)				TOTALS (LW NORM.)				
	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	MEAN (ng/g ww)	STD DEV (ng/g ww)	SEM (ng/g ww)	%CV	MEAN (ng/g lw)	STD DEV (ng/g lw)	SEM (ng/g lw)	%CV	
CDE 141	10.4	50.0	253076				10.4	50.0	180049				10.4	50.0	216306												
F-BDE 69	10.4	26.1	29967				10.4	50.4	41243				10.4	37.6	36914												
13CBDE209	32.6	51.4	21277				32.6	55.6	16732				32.6	47.7	16529												
FBDE 69	10.4	50.0	29954				10.4	50.0	41243				10.4	50.0	36812												
BDE 30	9.1	nd	nd				9.1	nd	nd				9.1	nd	nd												
BDE 17	9.6	nd	nd				9.6	nd	nd				9.6	nd	nd												
BDE 25	9.6	nd	nd				9.6	nd	nd				9.6	nd	nd												
BDE 28,33	9.8	nd	nd				9.8	nd	nd				9.8	nd	nd												
BDE 75	10.6	nd	nd				10.6	nd	nd				10.6	nd	nd												
BDE 49	10.7	nd	nd				10.7	nd	nd				10.7	nd	nd												
BDE 71	10.6	nd	nd				10.6	nd	nd				10.6	nd	nd												
BDE 47	10.9	4.2	2812	-0.2			10.9	5.3	3319	0.9	0.5	47	10.9	2.6	1846	-1.8											
BDE 66	11.1	nd	nd				11.1	nd	nd				11.1	nd	nd												
BDE 101	12.0	0.1	27	0.0	0.0	0	12.0	1.3	1086	1.3	0.7	67	12.0	0.3	220	0.3	0.1	21	0.3	0.4	0.2	131	29	34	20	116	
BDE 99	12.5	5.7	3918	0.0			12.5	5.3	3601	-0.5			12.5	1.4	1073	-4.3											
BDE 116	12.7	nd	nd				12.7	nd	nd				12.7	nd	nd												
BDE 155	13.4	0.7	647	0.7	0.4	22	13.4	0.3	419	0.3	0.2	17	13.4	0.1	118	0.1	0.1	8	0.2	0.2	0.1	84	16	7	4	45	
BDE 154	14.0	2.5	987	2.5	1.5	80	14.0	3.0	1884	3.0	1.7	157	14.0	2.2	1149	2.2	1.2	180	1.5	0.2	0.1	15	139	52	30	38	
BDE 153	15.0	2.2	1541	2.2	1.3	71	15.0	1.7	1624	1.7	1.0	90	15.0	1.4	1180	1.4	0.8	114	1.0	0.3	0.2	28	91	22	13	24	
HexaBDE1	16.5	0.7	384	0.7	0.4	22	16.5	0.3	230	0.3	0.2	16	16.5	nd	nd	0.0	0.0	0	0.2	0.2	0.1	107	13	11	7	90	
HexaBDE2	17.2	0.3	152	0.3	0.2	9	17.2	0.1	90	0.1	0.1	6	17.2	nd	nd	0.0	0.0	0	0.1	0.1	0.0	107	5	4	3	90	
BDE 188	17.6	1.4	508	1.4	0.9	45	17.6	0.4	170	0.4	0.2	18	17.6	0.1	40	0.1	0.1	7	0.4	0.4	0.2	117	24	20	11	83	
BDE 179	17.9	1.3	388	1.3	0.8	40	17.9	0.4	154	0.4	0.2	19	17.9	nd	nd	nd	0.0	0	0.3	0.4	0.2	123	20	20	12	102	
BDE 176	18.1	nd	nd				18.1	nd	nd				18.1	nd	nd												
BDE 183	18.2	0.1	58	0.1	0.1	3	18.2	nd	nd	nd	0.0	0	18.2	nd	nd	nd	0.0	0	0.0	0.0	0.0	173	1	2	1	173	
BDE 191	18.9	nd	nd				18.9	nd	nd				18.9	nd	nd												
BDE 181	19.1	nd	nd				19.1	nd	nd				19.1	nd	nd												
BDE 171	19.2	nd	nd				19.2	nd	nd				19.2	nd	nd												
BDE 190	19.4	nd	nd				19.4	nd	nd				19.4	nd	nd												
BDE 202	19.9	0.9	217	0.9	0.5	29	19.9	0.2	78	0.2	0.1	12	19.9	nd	nd	nd	0.0	0	0.2	0.3	0.2	127	14	14	8	106	
BDE 201	20.0	0.2	56	0.2	0.1	6	20.0	0.1	37	0.1	0.1	5	20.0	nd	nd	nd	0.0	0	0.1	0.1	0.0	102	4	3	2	88	
BDE 197	20.4	nd	nd				20.4	nd	nd				20.4	nd	nd												
BDE 203,200	21.5	nd	nd				21.5	nd	nd				21.5	nd	nd												
BDE 196	21.0	nd	nd				21.0	nd	nd				21.0	nd	nd												
BDE 205	21.5	nd	nd				21.5	nd	nd				21.5	nd	nd												
BDE 208	24.1	0.3	171	0.1	0.0	3	24.1	0.2	127	-0.1	0.0	0	24.1	0.1	86	-0.1	0.0	0	0.0	0.1	0.0	-519	1	2	1	173	
BDE 207	24.6	0.3	181	0.0	0.0	0	24.6	0.2	145	-0.1	0.0	0	24.6	0.2	124	-0.1	0.0	0	0.0	0.0	0.0	173	0	0	0	173	
BDE 206	25.8	0.6	56	0.4	0.2	13	25.8	0.5	60	0.3	0.2	14	25.8	0.3	32	0.1	0.0	7	0.2	0.1	0.1	67	12	4	2	34	
BDE 209	32.6	14.4	6394	2.4	1.4	75	32.6	14.0	4926	2.0	1.1	103	32.6	13.5	4695	1.5	0.8	122	1.1	0.3	0.2	26	100	23	14	23	

BDE-209 Low Dose - Sampling Day 42

Name	FHD42L1						FHD42L2						FHD42L3						TOTALS (BW NORM.)				TOTALS (LW NORM.)				
	Ret Time	Amt	Resp	Blk Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	Ret Time	Amt	Resp	Blk Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	Ret Time	Amt	Resp	Blk Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	MEAN (ng/g ww)	STD DEV (ng/g ww)	SEM (ng/g ww)	%CV	MEAN (ng/g lw)	STD DEV (ng/g lw)	SEM (ng/g lw)	%CV	
CDE 141	10.4	50.0	313595				10.4	50.0	263381				10.4	50.0	217004												
F-BDE 69	10.4	31.6	44916				10.4	25.7	30722				10.4	33.7	33205												
13CBDE209	32.6	43.2	21162				32.6	43.0	17674				32.6	59.1	21837												
FBDE 69	10.4	50.0	45047				10.4	50.0	30722				10.4	50.0	33205												
BDE 30	9.1	nd	nd				9.1	nd	nd				9.1	nd	nd												
BDE 17	9.6	nd	nd				9.6	nd	nd				9.6	nd	nd												
BDE 25	9.6	nd	nd				9.6	nd	nd				9.6	nd	nd												
BDE 28,33	9.8	nd	nd				9.8	nd	nd				9.8	nd	nd												
BDE 75	10.6	nd	nd				10.6	nd	nd				10.6	nd	nd												
BDE 49	10.7	nd	nd				10.7	nd	nd				10.7	nd	nd												
BDE 71	10.6	nd	nd				10.6	nd	nd				10.6	nd	nd												
BDE 47	10.9	4.2	3700	-0.2			10.9	4.9	5407	0.5	0.2	5	10.9	5.2	5369	0.9	0.4	48	0.3	0.2	0.1	54	nd	nd	nd	nd	
BDE 66	11.1	nd	nd				11.1	nd	nd				11.1	nd	nd												
BDE 101	12.0	0.1	88	0.1	0.0	13	12.0	0.7	442	0.7	0.2	7	12.0	0.1	67	0.1	0.0	3	0.1	0.1	0.1	122	8	5	3	64	
BDE 99	12.5	4.9	4250	-0.8	0.0	0	12.5	6.3	6729	0.5	0.2	5	12.5	6.3	5977	0.5	0.2	28	0.1	0.1	0.1	90	11	15	9	137	
BDE 116	12.7	nd	nd				12.7	nd	nd				12.7	nd	nd												
BDE 155	13.4	0.5	642	0.5	0.3	103	13.4	1.8	1693	1.8	0.6	18	13.4	0.4	453	0.4	0.2	25	0.4	0.2	0.1	63	49	48	27	98	
BDE 154	14.0	1.0	953	1.0	0.6	220	14.0	7.4	4901	7.4	2.6	74	14.0	1.4	1032	1.4	0.7	80	1.3	1.1	0.7	89	125	83	48	67	
BDE 153	15.0	1.3	1322	1.3	0.7	286	15.0	1.4	1003	1.4	0.5	14	15.0	2.5	1882	2.5	1.2	137	0.8	0.3	0.2	43	145	136	79	93	
HexaBDE1	16.5	0.3	283	0.3	0.2	76	16.5	2.0	1162	2.0	0.7	20	16.5	0.2	138	0.2	0.1	12	0.3	0.3	0.2	98	36	35	20	96	
HexaBDE2	17.2	0.3	286	0.3	0.2	76	17.2	0.6	317	0.6	0.2	6	17.2	nd	nd	0.0	0.0	0	0.1	0.1	0.1	87	27	43	25	156	
BDE 188	17.6	0.7	367	0.7	0.4	155	17.6	1.4	513	1.4	0.5	14	17.6	0.3	103	0.3	0.1	14	0.3	0.2	0.1	57	61	81	47	133	
BDE 179	17.9	0.5	217	0.5	0.3	106	17.9	1.2	384	1.2	0.4	12	17.9	nd	nd	0.0	0.0	0	0.2	0.2	0.1	94	39	58	33	147	
BDE 176	18.1	nd	nd				18.1	nd	nd				18.1	nd	nd												
BDE 183	18.2	0.1	41	0.1	0.0	11	18.2	nd	nd	nd	0.0	0	18.2	0.1	43	0.1	0.0	4	0.0	0.0	0.0	88	5	6	3	113	
BDE 191	18.9	nd	nd				18.9	nd	nd				18.9	nd	nd												
BDE 181	19.1	nd	nd				19.1	nd	nd				19.1	nd	nd												
BDE 171	19.2	nd	nd				19.2	nd	nd				19.2	nd	nd												
BDE 190	19.4	nd	nd				19.4	nd	nd				19.4	nd	nd												
BDE 202	19.9	0.3	119	0.3	0.2	74	19.9	0.8	198	0.8	0.3	8	19.9	0.1	14	0.1	0.0	3	0.2	0.1	0.1	80	28	40	23	140	
BDE 201	20.0	nd	nd		0.0	0	20.0	0.1	29	0.1	0.0	1	20.0	nd	nd	0.0	0.0	0	0.0	0.0	0.0	173	0	1	0	173	
BDE 197	20.4	nd	nd		0.0	0	20.4	0.1	45	0.1	0.0	1	20.4	nd	nd	0.0	0.0	0	0.0	0.0	0.0	173	0	1	0	173	
BDE 203,200	21.5	nd	nd				21.5	nd	nd				21.5	nd	nd												
BDE 196	21.0	nd	nd				21.0	nd	nd				21.0	nd	nd												
BDE 205	21.5	nd	nd				21.5	nd	nd				21.5	nd	nd												
BDE 208	24.1	0.2	154	0.0	0.0	-8	24.1	0.2	120	0.0	0.0	0	24.1	0.2	130	0.0	0.0	0	0.0	0.0	0.0	-118	-3	5	3	-160	
BDE 207	24.6	0.3	231	0.0	0.0	-9	24.6	0.3	145	-0.1	0.0	-1	24.6	0.3	190	0.0	0.0	-1	0.0	0.0	0.0	-61	-3	5	3	-144	
BDE 206	25.8	0.5	67	0.3	0.2	64	25.8	0.6	57	0.4	0.1	4	25.8	0.3	26	0.1	0.0	3	0.1	0.1	0.0	66	24	35	20	147	
BDE 209	32.6	13.9	6641	1.9	1.1	421	32.6	13.8	5124	1.8	0.6	18	32.6	14.7	6813	2.6	1.3	147	1.0	0.3	0.2	34	195	206	119	105	

BDE-209 High Dose - Sampling Day 0

Name	FHD0H1						FHD0H2						FHD0H3						TOTALS (BW NORM.)				TOTALS (LW NORM.)				
	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	MEAN (ng/g ww)	STD DEV (ng/g ww)	SEM (ng/g ww)	%CV	MEAN (ng/g lw)	STD DEV (ng/g lw)	SEM (ng/g lw)	%CV	
CDE 141	10.4	50.0	256516				10.4	50.0	162988				10.4	50.0	246799												
F-BDE 69	10.4	26.4	30764				10.4	54.9	40720				10.4	26.5	29701												
13CBDE209	32.6	33.0	12457				32.6	64.9	18570				32.6	49.7	19892												
FBDE 69	10.4	50.0	30232				10.4	50.0	40720				10.4	50.0	29650												
BDE 30	9.1	nd	nd				9.1	nd	nd				9.1	nd	nd												
BDE 17	9.6	nd	nd				9.6	nd	nd				9.6	nd	nd												
BDE 25	9.6	nd	nd				9.6	nd	nd				9.6	nd	nd												
BDE 28,33	9.8	nd	nd				9.8	nd	nd				9.8	nd	nd												
BDE 75	10.6	nd	nd				10.6	nd	nd				10.6	nd	nd												
BDE 49	10.7	nd	nd				10.7	nd	nd				10.7	nd	nd												
BDE 71	10.6	nd	nd				10.6	nd	nd				10.6	nd	nd												
BDE 47	10.9	4.3	2904	-0.1			10.9	4.1	5002	-0.3			10.9	3.7	2136	-0.7											
BDE 66	11.1	nd	nd				11.1	nd	nd				11.1	nd	nd												
BDE 101	12.0	nd	nd				12.0	nd	nd				12.0	nd	nd												
BDE 99	12.5	6.2	4228	0.4	0.2	21	12.5	4.9	5254	-0.8	-0.4	-21	12.5	5.1	4559	-0.7											
BDE 116	12.7	nd	nd				12.7	nd	nd				12.7	nd	nd												
BDE 155	13.4	0.2	148	0.2	0.1	9	13.4	0.2	198	0.2	0.1	4	13.4	0.1	73	0.1	0.1	2	0.1	0.0	0.0	1	5	4	2	73	
BDE 154	14.0	0.7	445	0.7	0.3	39	14.0	1.3	1109	1.3	0.5	32	14.0	0.4	229	0.4	0.3	9	0.4	0.1	0.1	37	27	16	9	59	
BDE 153	15.0	0.1	67	0.1	0.0	6	15.0	0.3	324	0.3	0.1	9	15.0	0.2	149	0.2	0.2	6	0.1	0.1	0.0	59	7	2	1	25	
HexaBDE1	16.5	nd	nd				16.5	nd	nd				16.5	nd	nd												
HexaBDE2	17.2	nd	nd				17.2	nd	nd				17.2	nd	nd												
BDE 188	17.6	nd	nd				17.6	nd	nd				17.6	nd	nd												
BDE 179	17.9	nd	nd				17.9	nd	nd				17.9	nd	nd												
BDE 176	18.1	nd	nd				18.1	nd	nd				18.1	nd	nd												
BDE 183	18.2	nd	nd				18.2	nd	nd				18.2	nd	nd												
BDE 191	18.9	nd	nd				18.9	nd	nd				18.9	nd	nd												
BDE 181	19.1	nd	nd				19.1	nd	nd				19.1	nd	nd												
BDE 171	19.2	nd	nd				19.2	nd	nd				19.2	nd	nd												
BDE 190	19.4	nd	nd				19.4	nd	nd				19.4	nd	nd												
BDE 202	19.9	nd	nd				19.9	nd	nd				19.9	nd	nd												
BDE 201	20.0	nd	nd				20.0	nd	nd				20.0	nd	nd												
BDE 197	20.4	nd	nd				20.4	nd	nd				20.4	nd	nd												
BDE 203,200	21.5	nd	nd				21.5	nd	nd				21.5	nd	nd												
BDE 196	21.0	nd	nd				21.0	nd	nd				21.0	nd	nd												
BDE 205	21.5	nd	nd				21.5	nd	nd				21.5	nd	nd												
BDE 208	24.1	0.1	76	-0.1			24.1	0.1	85	-0.1			24.1	0.2	139	0.0	0.0	1									
BDE 207	24.6	0.2	95	-0.2			24.6	0.2	118	-0.2			24.6	0.2	135	-0.1											
BDE 206	25.8	0.1	9	-0.1			25.8	0.4	54	0.2	0.1	6	25.8	0.1	11	-0.1											
BDE 209	32.6	12.8	3667	0.7	0.3	43	32.6	13.0	5442	1.0	0.4	25	32.6	12.9	5372	0.9	0.0	0	0.2	0.2	0.1	90	23	22	12	95	

BDE-209 High Dose - Sampling Day 14

Name	FHD14H1						FHD14H2						FHD14H3						TOTALS (BW NORM.)				TOTALS (LW NORM.)				
	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	MEAN (ng/g ww)	STD DEV (ng/g ww)	SEM (ng/g ww)	%CV	MEAN (ng/g lw)	STD DEV (ng/g lw)	SEM (ng/g lw)	%CV	
CDE 141	10.4	50.0	226145				10.4	50.0	276299				10.4	50.0	296277												
F-BDE 69	10.4	29.3	30082				10.4	34.8	43601				10.4	37.6	50608												
13CBDE209	32.6	58.6	22511				32.6	53.2	24268				32.6	49.8	23912												
FBDE 69	10.4	50.0	30082				10.4	50.0	42857				10.4	50.0	51168												
BDE 30	9.1	nd	nd				9.1	nd	nd				9.1	nd	nd												
BDE 17	9.6	nd	nd				9.6	nd	nd				9.6	nd	nd												
BDE 25	9.6	nd	nd				9.6	nd	nd				9.6	nd	nd												
BDE 28,33	9.8	nd	nd				9.8	nd	nd				9.8	nd	nd												
BDE 75	10.6	nd	nd				10.6	nd	nd				10.6	nd	nd												
BDE 49	10.7	nd	nd				10.7	nd	nd				10.7	nd	nd												
BDE 71	10.6	nd	nd				10.6	nd	nd				10.6	nd	nd												
BDE 47	10.9	2.2	1283	-2.2			10.9	4.3	5891	0.0			10.9	4.1	6692	-0.3											
BDE 66	11.1	nd	nd				11.1	nd	nd				11.1	nd	nd												
BDE 101	12.0	3.9	2321	3.8	2.4	621	12.0	2.9	2431	2.8	1.3	148	12.0	26.1	26624	26.0	17.5	3495	7.1	9.0	5.2	128	1421	1811	1046	127	
BDE 99	12.5	2.6	2928	-3.2			12.5	5.9	6184	0.2			12.5	5.4	8311	-0.4											
BDE 116	12.7	nd	nd				12.7	nd	nd				12.7	nd	nd												
BDE 155	13.4	25.5	23591	25.5	16.1	4130	13.4	18.5	24392	18.5	8.7	974	13.4	2.2	3399	2.2	1.4	290	8.7	7.3	4.2	84	1798	2048	1183	114	
BDE 154	14.0	42.1	27575	42.1	26.7	6834	14.0	30.8	28685	30.8	14.5	1624	14.0	18.0	20048	18.0	12.1	2421	17.7	7.8	4.5	44	3626	2806	1620	77	
BDE 153	15.0	0.4	245	0.4	0.2	57	15.0	0.4	350	0.4	0.2	18	15.0	1.7	2001	1.7	1.1	227	0.5	0.5	0.3	107	101	111	64	110	
HexaBDE1	16.5	17.5	9811	17.5	11.1	2837	16.5	12.9	10295	12.9	6.0	679	16.5	1.3	1228	1.3	0.9	173	6.0	5.1	2.9	85	1230	1415	817	115	
HexaBDE2	17.2	4.7	2652	4.7	3.0	769	17.2	4.7	3744	4.7	2.2	248	17.2	0.3	303	0.3	0.2	43	1.8	1.4	0.8	79	353	374	216	106	
BDE 188	17.6	48.2	17101	48.2	30.5	7822	17.6	35.4	17944	35.4	16.6	1869	17.6	2.2	1338	2.2	1.5	295	16.2	14.5	8.4	90	3329	3970	2292	119	
BDE 179	17.9	31.5	9719	31.5	19.9	5112	17.9	29.6	12998	29.6	13.9	1560	17.9	1.8	916	1.8	1.2	235	11.7	9.6	5.5	82	2302	2522	1456	110	
BDE 176	18.1	nd	nd				18.1	nd	nd				18.1	nd	nd												
BDE 183	18.2	0.0	0	0.0	0.0	0	18.2	0.5	393	0.5	0.2	26	18.2	0.1	59	0.1	0.0	8	0.1	0.1	0.1	136	11	13	8	117	
BDE 191	18.9	nd	nd				18.9	nd	nd				18.9	nd	nd												
BDE 181	19.1	nd	nd				19.1	nd	nd				19.1	nd	nd												
BDE 171	19.2	nd	nd				19.2	nd	nd				19.2	nd	nd												
BDE 190	19.4	nd	nd				19.4	nd	nd				19.4	nd	nd												
BDE 202	19.9	24.6	6007	24.6	15.5	3986	19.9	24.8	8649	24.8	11.7	1309	19.9	1.4	576	1.4	0.9	188	9.4	7.6	4.4	81	1828	1951	1127	107	
BDE 201	20.0	2.2	605	2.2	1.4	354	20.0	3.9	1544	3.9	1.8	206	20.0	0.2	93	0.2	0.1	27	1.1	0.9	0.5	79	195	164	95	84	
BDE 197	20.4	0.6	185	0.6	0.4	93	20.4	0.9	411	0.9	0.4	47	20.4	0.1	60	0.1	0.1	15	0.3	0.2	0.1	65	51	39	23	76	
BDE 203,200	21.5	nd	nd				21.5	nd	nd				21.5	nd	nd												
BDE 196	21.0	nd	nd				21.0	nd	nd				21.0	nd	nd												
BDE 205	21.5	nd	nd				21.5	nd	nd				21.5	nd	nd												
BDE 208	24.1	1.3	757	1.1	0.7	171	24.1	2.5	2117	2.3	1.1	119	24.1	0.3	351	0.1	0.1	18	0.6	0.5	0.3	81	103	78	45	76	
BDE 207	24.6	0.9	491	0.5	0.3	88	24.6	1.1	912	0.8	0.4	42	24.6	0.4	398	0.1	0.1	13	0.3	0.2	0.1	65	48	37	22	78	
BDE 206	25.8	1.5	137	1.3	0.8	212	25.8	0.9	110	0.6	0.3	34	25.8	0.7	100	0.4	0.3	60	0.5	0.3	0.2	64	102	96	56	94	
BDE 209	32.6	23.1	10982	11.0	7.0	1792	32.6	27.3	14458	15.2	7.2	804	32.6	18.1	9195	6.0	4.0	810	6.1	1.7	1.0	29	1135	569	328	50	

BDE-209 High Dose Group: Sampling Day 28:

Name	FHD28H1						FHD28H2						FHD28H3						TOTALS (BW NORM.)				TOTALS (LW NORM.)				
	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	Ret Time	Amt	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	MEAN (ng/g ww)	STD DEV (ng/g ww)	SEM (ng/g ww)	%CV	MEAN (ng/g lw)	STD DEV (ng/g lw)	SEM (ng/g lw)	%CV	
CDE 141	10.4	50.0	255456				10.4	50.0	323350				10.4	50.0	226161												
F-BDE 69	10.4	28.8	33419				10.4	27.1	39804				10.4	27.8	28551												
13CBDE209	32.6	53.4	22556				32.6	34.1	16348				32.6	59.3	22854												
FBDE 69	10.4	50.0	32517				10.4	50.0	38921				10.4	50.0	27316												
BDE 30	9.1	nd	nd				9.1	nd	nd				9.1	nd	nd												
BDE 17	9.6	nd	nd				9.6	nd	nd				9.6	nd	nd												
BDE 25	9.6	nd	nd				9.6	nd	nd				9.6	nd	nd												
BDE 28,33	9.8	nd	nd				9.8	nd	nd				9.8	nd	nd												
BDE 75	10.6	nd	nd				10.6	nd	nd				10.6	nd	nd												
BDE 49	10.7	nd	nd				10.7	nd	nd				10.7	nd	nd												
BDE 71	10.6	nd	nd				10.6	nd	nd				10.6	nd	nd												
BDE 47	10.9	3.6	2271	-0.8			10.9	3.0	2296	-1.4			10.9	3.4	2176	-0.9											
BDE 66	11.1	nd	nd				11.1	nd	nd	nd			11.1	nd	nd												
BDE 101	12.0	8.4	5424	8.4	8.0	2416	12.0	5.9	3804	5.9	5.2	1728	12.0	7.8	4245	7.8	5.7	1391	6.3	1.5	0.9	24	1845	522	301	28	
BDE 99	12.5	3.8	3088	-2.0			12.5	5.8	7606	0.0			12.5	5.4	6888	-0.4											
BDE 116	12.7	nd	nd				12.7	nd	nd				12.7	nd	nd												
BDE 155	13.4	20.8	20843	20.8	19.8	6003	13.4	11.8	14119	11.8	10.4	3475	13.4	38.3	32240	38.3	28.1	6863	19.5	8.9	5.1	46	5447	1761	1017	32	
BDE 154	14.0	59.6	42225	59.6	56.7	17192	14.0	40.5	35797	40.5	35.8	11941	14.0	80.0	47722	80.0	58.8	14345	50.5	12.7	7.3	25	14493	2629	1518	18	
BDE 153	15.0	1.8	1358	1.8	1.7	522	15.0	1.7	1494	1.7	1.5	490	15.0	3.2	2035	3.2	2.4	579	1.9	0.5	0.3	25	530	45	26	9	
HexaBDE1	16.5	34.8	21147	34.8	33.2	10046	16.5	17.2	12458	17.2	15.2	5062	16.5	41.0	20919	41.0	30.1	7349	26.2	9.6	5.6	37	7486	2495	1440	33	
HexaBDE2	17.2	3.9	2350	3.9	3.7	1120	17.2	4.0	2875	4.0	3.5	1171	17.2	9.0	4585	9.0	6.6	1618	4.6	1.8	1.0	38	1303	274	158	21	
BDE 188	17.6	24.0	9249	24.0	22.9	6935	17.6	22.2	10216	22.2	19.6	6540	17.6	48.9	15757	48.9	36.0	8772	26.2	8.6	5.0	33	7415	1191	688	16	
BDE 179	17.9	16.6	5528	16.6	15.8	4788	17.9	17.2	6839	17.2	15.2	5059	17.9	44.2	12394	44.2	32.5	7925	21.2	9.8	5.7	46	5924	1738	1004	29	
BDE 176	18.1	nd	nd				18.1	nd	nd				18.1	nd	nd												
BDE 183	18.2	0.1	66	0.1	0.1	32	18.2	0.2	154	0.2	0.2	62	18.2	0.3	162	0.3	0.2	57	0.2	0.1	0.0	38	50	16	9	32	
BDE 191	18.9	nd	nd				18.9	nd	nd				18.9	nd	nd												
BDE 181	19.1	nd	nd				19.1	nd	nd				19.1	nd	nd												
BDE 171	19.2	nd	nd				19.2	nd	nd				19.2	nd	nd												
BDE 190	19.4	nd	nd				19.4	nd	nd				19.4	nd	nd												
BDE 202	19.9	10.5	2760	10.5	10.0	3027	19.9	11.9	3736	11.9	10.5	3499	19.9	21.0	4667	21.0	15.5	3773	12.0	3.0	1.7	25	3433	377	218	11	
BDE 201	20.0	0.6	177	0.6	0.6	170	20.0	0.9	328	0.9	0.8	268	20.0	2.5	622	2.5	1.8	443	1.1	0.7	0.4	63	294	138	80	47	
BDE 197	20.4	0.2	66	0.2	0.2	55	20.4	0.2	99	0.2	0.2	71	20.4	0.8	231	0.8	0.6	142	0.3	0.2	0.1	68	89	46	27	52	
BDE 203,200	21.5	nd	nd				21.5	nd	nd				21.5	nd	nd												
BDE 196	21.0	0.0	0	0.0	0.0	0	21.0	0.0	0	0.0	nd	0	21.0	0.4	126	0.4	0.3	77	0.2	0.2	0.1	141	26	45	26	173	
BDE 205	21.5	nd	nd				21.5	nd	nd				21.5	nd	nd												
BDE 208	24.1	0.5	307	0.3	0.3	76	24.1	0.6	433	0.4	0.3	104	24.1	1.4	775	1.2	0.9	218	0.5	0.4	0.2	73	133	75	43	57	
BDE 207	24.6	0.4	270	0.1	0.1	35	24.6	0.4	283	0.1	0.1	21	24.6	1.3	682	1.0	0.7	178	0.3	0.4	0.2	123	78	87	50	112	
BDE 206	25.8	0.7	68	0.5	0.5	140	25.8	0.6	66	0.4	0.3	105	25.8	1.6	133	1.4	1.0	252	0.6	0.4	0.2	63	166	77	44	46	
BDE 209	32.6	17.3	8194	5.3	5.0	1516	32.6	17.1	5822	5.0	4.5	1488	32.6	40.3	19420	28.3	20.8	5076	10.1	9.3	5.4	92	2693	2064	1191	77	

BDE-209 High Dose - Sampling Day 42

Name	FHD42H1						FHD42H2						FHD42H3						TOTALS (BW NORM.)				TOTALS (LW NORM.)				
	Ret Time	Amf	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	Ret Time	Amf	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	Ret Time	Amf	Resp	Blk Corr. Amt (ng)	Conc (ng/g ww)	Conc (ng/g lw)	MEAN (ng/g ww)	STD DEV (ng/g ww)	SEM (ng/g ww)	%CV	MEAN (ng/g lw)	STD DEV (ng/g lw)	SEM (ng/g lw)	%CV	
CDE 141	10.4	50.0	263921				10.4	50.0	253399				10.4	50.0	288896												
F-BDE 69	10.4	34.9	41809				10.4	24.4	28087				10.4	30.7	40305												
13CBDE209	32.6	57.2	25482				32.6	45.4	18178				32.6	53.4	25517												
FBDE 69	10.4	50.0	41782				10.4	50.0	26743				10.4	50.0	40305												
BDE 30	9.1	nd	nd				9.1	nd	nd				9.1	nd	nd												
BDE 17	9.6	nd	nd				9.6	nd	nd				9.6	nd	nd												
BDE 25	9.6	nd	nd				9.6	nd	nd				9.6	nd	nd												
BDE 28,33	9.8	nd	nd				9.8	nd	nd				9.8	nd	nd												
BDE 75	10.6	nd	nd				10.6	nd	nd				10.6	nd	nd												
BDE 49	10.7	nd	nd				10.7	nd	nd				10.7	nd	nd												
BDE 71	10.6	nd	nd				10.6	nd	nd				10.6	nd	nd												
BDE 47	10.9	4.2	8850	-0.2	0	-2	10.9	4.9	4826	0.5	0.4	53	10.9	3.4	3288	-1.0	-0.7	-163									
BDE 66	11.1	nd	nd				11.1	nd	nd				11.1	nd	nd												
BDE 101	12.0	12.7	10609	12.7	5.4	148	12.0	14.8	7869	14.7	9.5	1437	12.0	0.9	679	0.8	0.6	138	5.2	4.4	2.6	86	574	747	431	130	
BDE 99	12.5	6.2	10312	0.4	0.2	4	12.5	5.6	5229	-0.2			12.5	4.6	5213	-1.2											
BDE 116	12.7	nd	nd				12.7	nd	nd				12.7	nd	nd												
BDE 155	13.4	97.7	126211	97.7	41.2	1139	13.4	39.9	32898	39.9	25.7	3895	13.4	6.9	8591	6.9	5.3	1186	24.1	18.0	10.4	75	2074	1578	911	76	
BDE 154	14.0	135.7	124427	135.7	57.2	1583	14.0	93.2	54473	93.2	60.1	9096	14.0	19.2	16780	19.2	14.6	3286	44.0	25.5	14.7	58	4655	3939	2274	85	
BDE 153	15.0	1.7	1619	1.7	0.7	20	15.0	1.7	1066	1.7	1.1	169	15.0	2.4	2245	2.4	1.8	413	1.2	0.6	0.3	47	201	199	115	99	
HexaBDE1	16.5	52.1	40752	52.1	22.0	608	16.5	47.5	23761	47.5	30.7	4640	16.5	6.3	4741	6.3	4.8	1082	19.2	13.2	7.6	69	2110	2204	1272	104	
HexaBDE2	17.2	20.1	15653	20.1	8.5	235	17.2	7.4	3689	7.4	4.8	724	17.2	0.5	367	0.5	0.4	84	4.5	4.1	2.3	89	347	334	193	96	
BDE 188	17.6	200.2	96344	200.2	84.5	2336	17.6	33.0	10440	33.0	21.3	3224	17.6	18.0	5103	18.0	18.0	3080	41.3	37.5	21.6	91	2880	477	275	17	
BDE 179	17.9	117.7	50787	117.7	49.7	1373	17.9	21.0	5743	21.0	13.5	2046	17.9	10.2	2061	10.2	7.7	1740	23.6	22.7	13.1	96	1720	337	194	20	
BDE 176	18.1	nd	nd				18.1	nd	nd				18.1	nd	nd												
BDE 183	18.2	1.8	1374	1.8	0.7	21	18.2	0.0	nd	0.0	0.0	0	18.2	0.0	nd	0.0	0.0	nd	0.2	0.4	0.2	173	10	15	8	141	
BDE 191	18.9	nd	nd				18.9	nd	nd				18.9	nd	nd												
BDE 181	19.1	nd	nd				19.1	nd	nd				19.1	nd	nd												
BDE 171	19.2	nd	nd				19.2	nd	nd				19.2	nd	nd												
BDE 190	19.4	nd	nd				19.4	nd	nd				19.4	nd	nd												
BDE 202	19.9	80.0	27679	80.0	33.8	933	19.9	11.5	2498	11.5	7.4	1127	19.9	11.1	2442	11.1	8.5	1908	16.6	14.9	8.6	90	1323	516	298	39	
BDE 201	20.0	12.3	4747	12.3	5.2	143	20.0	1.1	280	1.1	0.7	111	20.0	nd	nd	nd	nd	nd	3.0	3.1	1.8	106	127	22	13	18	
BDE 197	20.4	4.4	1981	4.4	1.9	51	20.4	0.3	73	0.3	0.2	24	20.4	nd	nd	nd	nd	nd	1.0	1.2	0.7	119	38	19	11	50	
BDE 203,200	21.5	nd	nd				21.5	nd	nd				21.5	nd	nd												
BDE 196	21.0	nd	nd				21.0	nd	nd				21.0	nd	nd												
BDE 205	21.5	nd	nd				21.5	nd	nd				21.5	nd	nd												
BDE 208	24.1	4.6	3814	4.4	1.8	51	24.1	0.7	357	0.5	0.3	45	24.1	0.3	229	0.1	0.1	14	0.7	1.0	0.6	131	37	20	11	53	
BDE 207	24.6	2.7	2128	2.3	1.0	27	24.6	0.6	292	0.3	0.2	25	24.6	0.4	281	0.1	0.0	9	0.4	0.5	0.3	129	20	10	6	50	
BDE 206	25.8	1.1	139	0.9	0.4	10	25.8	1.0	82	0.8	0.5	80	25.8	0.8	94	0.6	0.4	97	0.4	0.1	0.0	17	62	46	26	73	
BDE 209	32.6	23.5	12587	11.4	4.8	133	32.6	15.8	5763	3.8	2.4	366	32.6	14.6	7821	2.5	1.9	436	3.1	1.5	0.9	50	312	158	91	51	

B.8 Rates of T3-IRD and rT3-ORD in fathead minnow adults

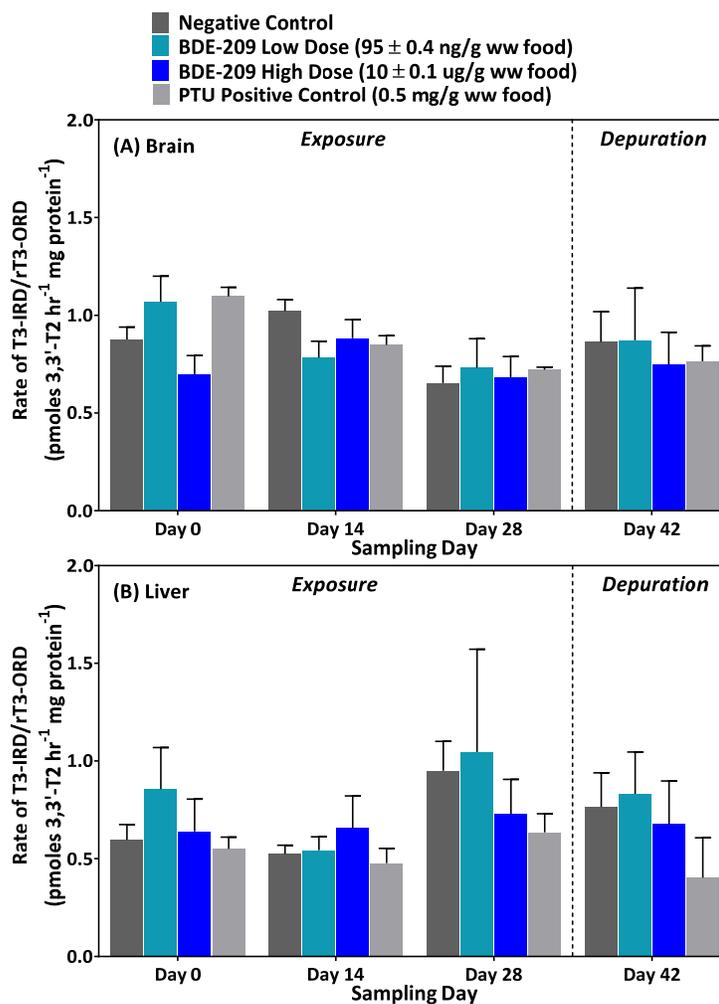


Figure 30: Rates of T3-inner ring deiodination (IRD)/rT3-outer ring deiodination (ORD) in brain (A) and liver (B) microsomes from adult fathead minnows exposed orally to BDE-209 or PTU (n=3 pools; mean \pm SE). Microsomes incubated with $0.64 \mu\text{M}$ of thyroxine (T4) for 90 minutes at 25°C . Statistical significance evaluated within sampling day with one-way ANOVA and Tukey's test ($*p < 0.05$).

B.9 Relative expression of OATP mRNA levels in adult male fathead minnows

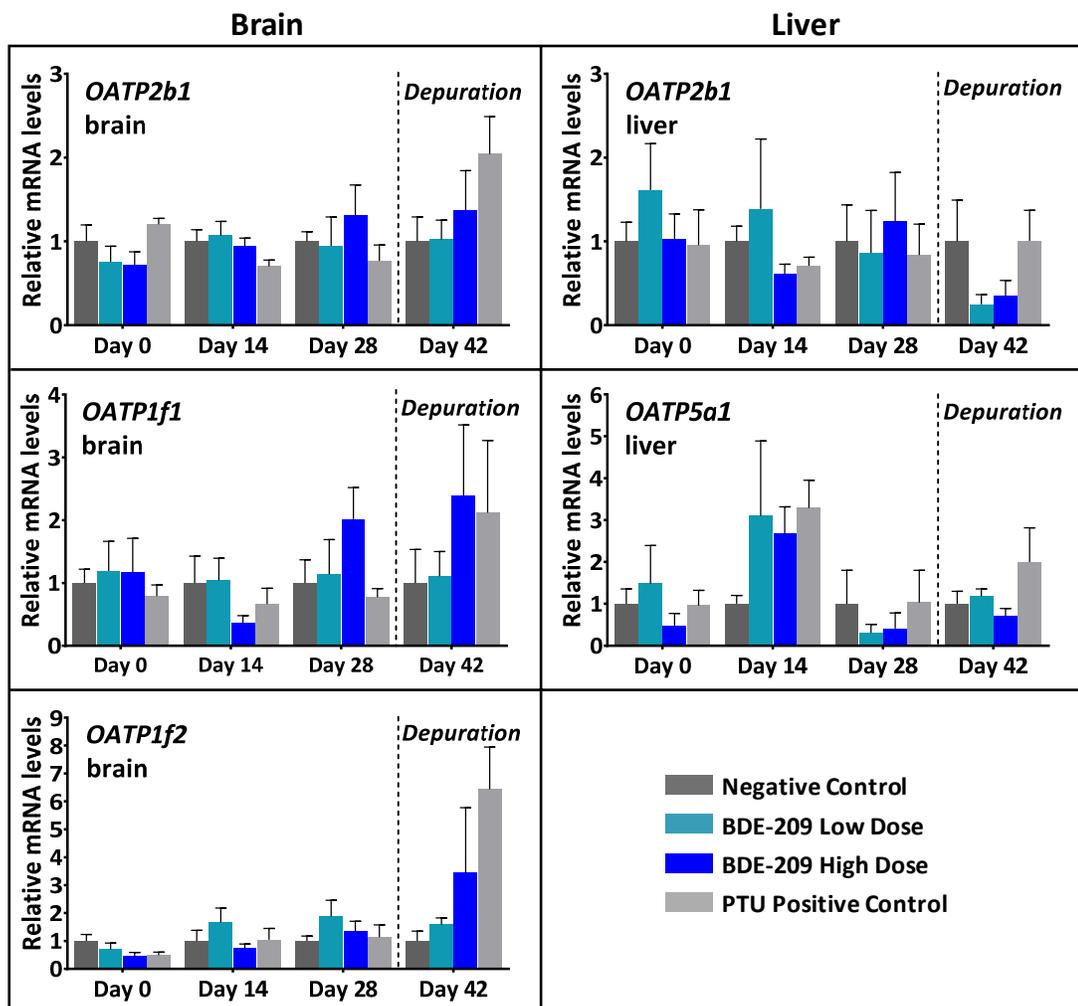


Figure 31: Relative expression of organic anion transport protein (OATP) mRNA levels (n=6; mean \pm SE) in brains and livers of adult male fathead minnows exposed to BDE-209 for 28-days followed by a 14-day depuration. Statistical significance evaluated with a one-way ANOVA and Tukey's test within sampling day ($p < 0.05$).

Appendix C: Rapid method to extract and measure thyroid hormones from fish plasma by LC-ESI/MS/MS

C.1 Introduction

One important biomarker for discerning thyroid system dysfunction associated with contaminant exposures, disease etiology, and other biologic perturbations in vertebrates is through the measurement of circulating levels of thyroid hormones. Thyroid hormone levels are frequently measured by radio-immunoassay (RIA) methods with many clinical laboratories routinely measuring T4 and T3 levels in human sera using automated RIA approaches. RIA methods have also been the preferred approach to measure thyroid hormone levels in fish species (Crane et al. 2004; Dickhoff et al. 1982; Van der Geyten et al. 1998). While thyroid hormones are structurally identical across all vertebrates, immunoassay approaches used to measure these hormones have several weaknesses, including that they can be compromised by a lack of specificity and accuracy due to analytical interferences that may involve, for example, biomolecules that may vary with species and cross-react with antibodies (Ghosh et al. 2008; Steele et al. 2005; Tate and Ward 2004). Thyroid hormone RIAs also require the use of radioactive tracers (^{125}I -TH) that present safety and disposal issues. Moreover, although a number of commercial RIA kits are available to measure thyroid hormones levels in human sera, these kits have not been validated for use in non-human animals and so their use in fish

and other species is problematic. For instance, commercial RIA kits typically rely on the use of T4 anti-serum from a mammalian species (e.g., rabbit) and have been validated over a narrow range of standards that are usually too high for the relatively lower levels of thyroid hormones in fish. Thus, there is a need to develop approaches that allow for more direct and reliable measurements of thyroid hormones in fishes. The use of tandem mass spectrometry (MS/MS) is increasingly the preferred tool of choice for overcoming some of the analytical difficulties raised by immunoassay approaches (Soldin and Soldin 2011). For instance, our laboratory has developed an analytical methodology that allows for the simultaneous measurement of T4, T3, rT3, and T2 using liquid chromatography tandem mass spectrometry (LS/MS/MS) operated in positive electrospray ionization (ESI+) mode with multiple reaction monitoring (MRM) transitions (Butt et al. 2011; Wang and Stapleton 2010). The purpose of this work was to develop a method that allows for the rapid isolation of native thyroid hormones (T4 and T3), both bound to protein and unbound (total thyroid hormones), from the plasma of different species of fishes that takes advantage of our LC/MS/MS analytical methods for thyroid hormones.

C.2 Materials and Methods

C.2.1 Chemicals and Materials

Unlabeled THs (T4, T3), citric acid, ascorbic acid, and dithiothreitol (DTT) were purchased from Sigma-Aldrich (St. Louis, MO). The stable isotope internal standards used were $^{13}\text{C}_{12}$ -T4 and $^{13}\text{C}_6$ -T3 (Cambridge Isotope Laboratories, Andover, MA). The solid phase extractions used SampliQ OPT polymer cartridges (60 mg/3 ml; Agilent). A standard stock solution of T4 and T3 target analytes was prepared in an amber bottle in methanol (MeOH) and water (1:1) at a concentration of 10 ng/ml. This stock solution was used to prepare a six point calibration curve (0.0, 0.05, 0.1, 1.0, 5.0 ng) of T4 and T3 in 0.4 ml volumes for LC/MS/MS analysis. An internal standard stock solution of $^{13}\text{C}_{12}$ -T4 and $^{13}\text{C}_6$ -T3 was also prepared in MeOH at a concentration of 10 ng/ml and was spiked into each calibration standard (50 μl at 10 ng/ml; 0.5 ng). All solvents used were High Performance Liquid Chromatography (HPLC) grade.

C.2.2 Fish Plasma Samples

Blood was collected from adult male fathead minnows (*Pimephales promelas*) purchased by Aquatic BioSystems (Fort Collins, CO) and from adult rainbow trout (*Oncorhynchus mykiss*) donated by the Armstrong Hatchery, Marion, NC. Fish were euthanized using MS-222 and blood samples were taken from the caudal vein using

either heparin-coated 75-mm capillary tubes (fathead minnow) or by cardiac puncture using 10 ml syringes/18 gauge needles rinsed with heparin (rainbow trout). The plasma fraction was isolated by centrifugation at $3,000 \times G$ for five minutes and was stored at -20°C until thyroid hormone extraction and analysis. Three replicate pools ($n=3$) of plasma were measured for each fish species. Each replicate pool consisted of plasma collected from 8-12 fathead minnows/replicate pool or from 3-5 rainbow trout/replicate pool. Plasma concentrations of TT4 and TT3 were measured in triplicate for each species and extraction technique tested.

C.2.3 Thyroid Hormone Extractions

Thyroid hormones were extracted from plasma by first incubating 50 μl of plasma with 50 μl of ^{13}C -T4/-T3 (10 ng/ml in MeOH) in 15-ml sterile polypropylene conical test tubes (Sigma-Aldrich) for one hour covered on ice to allow for equilibration of endogenous and labeled thyroid hormones with plasma proteins. The incubation medium also contained 100 μl of an antioxidant/reducing solution (25 g/L) containing ascorbic acid, citric acid, and DTT to prevent the deiodination of T4 to T3 in the incubation medium. Plasma samples were well-vortexed before and after adding standards and antioxidant solutions. After this equilibration step, one ml of hydrochloric acid (6 M; Sigma-Aldrich) was added to each sample, vortexed, and incubated covered

for 60 min in a 50°C water bath oscillating at 180 rpm to allow for denaturation of plasma proteins and release of hormone. Thyroid hormones were then isolated from extracts using a liquid-liquid solid-phase extraction procedure and SampliQ OPT polymer cartridges (60 mg/3 ml; Agilent). Polymer cartridges were conditioned with 3 ml of MeOH followed by 3 ml of HPLC grade deionized water (dH₂O). Samples were added to filter cartridges using sterile graduated Pasteur pipettes. The cartridges were further washed of protein and lipid matrix using 3 ml of HPLC grade dH₂O followed by 0.5 ml of 30% MeOH (H₂O) and dried gently under vacuum. Thyroid hormones were then eluted from the filter cartridges with 4 ml of MeOH into polypropylene test tubes. The extract was evaporated in a heated manifold block at 35°C under carbon-filtered nitrogen gas to approximately 50 µl and reconstituted with 400 µl (1:1 MeOH/H₂O) and vortexed. The reconstituted extract was transferred to a Mini-Uniprep Syringeless Filter (0.2 µm; Agilent) for final filtration and analysis by LC/MS/MS.

C.2.4 Instrumental Analysis by LC/MS/MS

Levels of TT4 and TT3 in plasma were determined using our published LC/MS/MS method (Butt et al. 2011; Wang and Stapleton 2010) with additional modifications. Mobile phases used were acetonitrile (ACN) and water (buffered with 10 mM of formic acid). Chromatography was performed using a Synergi Polar RP column

(50 x 2.0 mm, 2.5 μ m particle size; Phenomenex, Torrence, CA) and a SecurityGuard Polar-RP (4 x 2.0 mm) guard cartridge. The injection volume was 20 μ l and the flow rate was 400 μ l/min. Initial conditions were 70:30 water:ACN that was held for 3.1 min, ramping to 50:50 of water:ACN for 0.4 min followed by 1:99 water:ACN for 5.0 min, and returning to baseline conditions of 70:30 water:ACN for 3.5 min. An additional column cleaning method was integrated into longer batch runs (>30 samples) that was run between ~6 samples under the following conditions: 1:99 water:ACN for 7.0 min and 0.5 min 70:30 water:ACN for 0.5 min. The MS/MS operating conditions included multiple reaction monitoring (MRM) transitions (Table 12) based on ESI operated in positive mode with the multiplier voltage set at 900 delta EMV.

Table 12: Tandem mass spectrometer (MS/MS) parameters and multiple reaction monitoring (MRM) transitions operated in ESI positive mode.

Targeted Compound	MRM Transitions	Fragmenter (V)	CE (V)
¹³ C ₁₂ -T4	789.7 (precision ion) 743.6 (product ion)	150	24
T4	777.7 (precision ion) 731.7 (product ion)	150	24
¹³ C ₆ -T3	657.8 (precision ion) 611.3 (product ion)	150	24
T3	651.8 (precision ion) 605.8 (product ion)	150	24

Labeled internal standards, $^{13}\text{C}_{12}$ -T4 and $^{13}\text{C}_6$ -T3 (50 μL at 10 ng/ml), added to each sample at the start of the extraction were used to quantify levels of T4 and T3, respectively. These values were normalized to the plasma volume extracted (50 μl). Blank controls that contained 50 μl of ^{13}C -T4/-T3 alone were extracted alongside plasma samples to correct for trace levels (~0.5%) of unlabeled hormones present as commercial impurities in the ^{13}C -labeled internal standards. Method detection limits (MDLs) were calculated as three times the standard deviation of TH detected in blanks containing no plasma. MDLs normalized to the amount of plasma extracted were: T4 = 0.17 ng/ml and T3 = 0.09 ng/ml. The thyroid hormones rT3 and 3,3'-T2 were not targeted because preliminary testing in fish plasma showed that they were not detected at levels above instrument detection limits (IDLs).

C.2.5 Intra-assay/Inter-assay CVs

Two indicators of precision in immunoassay analyses are the repeatability (intra-assay variability) and reproducibility (inter-assay variability). The intra-assay variability is a measure of the closeness of agreement between results of successive measurements carried out under the same conditions. It is evaluated by performing repeat measurements within a single run and calculating the coefficient of variation (CV). For the method development, all samples were extracted and measured in triplicate and the

resulting TT3 and TT4 values were used to derive intra-assay CVs. In fathead minnow plasma, intra-assay coefficients of variation (CVs) within single batch runs were $9.2\pm 2.0\%$ for TT3 and $9.8\pm 2.0\%$ for TT4, and were derived from triplicate extractions of thyroid hormones from plasma collected from adult male fathead minnows under the BDE-209 study (Chapter 4). In rainbow trout plasma, intra-assay CVs were derived from results of optimized extraction methods run in triplicate and were $6\pm 3\%$ for TT3 and $12\pm 0.5\%$ for TT4. The inter-assay variability is the closeness of agreement between results of measurements performed under changed conditions of measurements (e.g., time, extraction media, and LC/MS/MS conditions). This was determined by performing repeat extractions and measurements of the same samples over different days and calculating the CV. In the adult fathead minnow/BDE-209 study, inter-assay CVs were $11\pm 1.9\%$ for TT3 and $12\pm 2.0\%$ for TT4 based on repeated triplicate extractions and measurements of the same plasma pool over different batch runs. For the rainbow trout plasma, it was not possible to derive inter-assay CVs because conditions of the extraction were being altered across different batches by design to optimize the extraction and measurement performance of thyroid hormones.

C.3 Results and Discussion

The deproteination of plasma samples using HCl (1 ml; 6M) and heating to 50 °C for 60 min coupled with a liquid-liquid SPE procedures and filtration (0.2 µm) produced a clean low interference extract that could be analyzed effectively by LC-ESI/MS/MS. In addition, for the rainbow trout optimization, recovery of the ¹³C₁₂-T₄ ranged from 70

C.3.1 Extraction Optimization

As outlined in Table 13, several extraction conditions were evaluated to optimize the performance of the extraction that centered largely on altering conditions to optimize the upfront conditions used to denature the plasma protein matrix. Unless otherwise noted, the liquid-liquid SPE procedures and filtration were identical to procedures described in the methods section (Section C.2.3). For the optimization, acid-base conditions were modified along with incubations times and temperatures. All conditions were evaluated in triplicate. As shown in Table 13, the use of HCl and heating the plasma samples to 50°C appeared to facilitate denaturing and deproteination with %CVs for TT3 and TT4 optimized at 8.3% and 11.3%, respectively. While KOH (0.5 M) and elevated incubation temperatures appeared to perform for the extraction of TT3, this method did not appear to work as well for isolating TT4 with %CVs at over 30%. The optimal temperature and incubation times appear to be at 50°C with 60 min

incubations based on the low variability and overall levels of TT3 and TT4 measured, although the 70°C/60 min incubations also performed well. Finally, the performance of the method was improved further by washing the SPE filter cartridges during the liquid-liquid SPE extraction with 0.5 ml of 30% MeOH. In addition, 0.5 ng of the stable isotope $^{13}\text{C}_6\text{-T4}$ (Isotec) was added to these fathead minnow samples prior to LC/MS/MS analysis to monitor recovery of the $^{13}\text{C}_{12}\text{-T4}$ and evaluate matrix effects leading to ion suppression or enhancement. The recovery of the $^{13}\text{C}_{12}\text{-T4}$ ranged from $67 \pm 4.2\%$ (no 30% MeOH wash) to $96 \pm 3.6\%$ (0.5 ml 30% MeOH wash), further demonstrating the usefulness of the wash step in improving method recovery and reducing matrix effects. These were the only samples that were spiked with the $^{13}\text{C}_6\text{-T4}$ because of the need to minimize background levels of T4 and T3 in the blanks ($^{13}\text{C}_6\text{-T4}$ contained low levels of T4 and T3 impurities). In rainbow trout plasma samples (50 μl) spiked with 2 ng of T4, recoveries of T3 and T4 were 89.7% and 110.8%, respectively (Table 13). These results provide further evidence that the sample extraction and LC-ESI/MS/MS method used in fish plasma were reliably measuring T4 and T3 hormone. These samples were subjected to the same sample extraction and clean-up procedure as described in the methods section (C.2.3).

Table 13: Optimization of thyroid hormone extraction conditions from rainbow trout and fathead minnow plasma and thyroid hormone matrix spikes to evaluate recovery (50 μ l plasma samples in triplicate; mean \pm SE).

Extraction condition tested ¹	TT3 (ng/ml)	%CV	TT4 (ng/ml)	%CV
Acid vs. base and Heat vs. no Heat				
KOH (0.5 M) + 50°C	3.51 \pm 0.28	7.9	3.50 \pm 1.18	34
KOH (0.5 M) + no heat	2.51 \pm 1.06	42	3.12 \pm 1.45	46
HCl (6 M) + 50°C	4.03 \pm 0.42	8.3	3.12 \pm 0.35	11
HCl (6 M) + no heat	3.43 \pm 0.89	26	3.31 \pm 1.55	47
Deproteination Temperature and Incubation Time				
50°C, 20 min	2.40 \pm 0.36	15	2.41 \pm 0.97	41
50°C, 60 min	1.85 \pm 0.06	3.5	4.08 \pm 0.22	5.3
50°C, 90 min	1.50 \pm 0.16	11	1.39 \pm 0.81	58
50°C, 120 min	1.95 \pm 0.70	36	3.11 \pm 1.07	34
50°C, 180 min	2.40 \pm 0.36	15	3.58 \pm 1.60	45
70°C, 20 min	1.29 \pm 0.11	8.7	3.38 \pm 0.56	17
70°C, 60 min	2.08	9.1	4.21 \pm 0.51	12.0
Solid Phase Extraction, Filter Wash with 30% MeOH (H₂O)³				
No 30% MeOH wash	3.23 \pm 0.33	10	2.20 \pm 1.23	56
30% MeOH – 0.5 ml	2.98 \pm 0.12	4.0	3.38 \pm 0.05	1.4
30% MeOH – 1.0 ml	3.33 \pm 0.38	12	2.06 \pm 0.26	13
Plasma Matrix Spikes of T4 and T3²				
Plasma + 2 ng TH	35.9 \pm 0.60	1.7	44.3 \pm 2.16	7.8
Plasma alone	2.54 \pm 0.14	5.4	5.19 \pm 1.01	20

1. Unless otherwise noted, extraction conditions employed HCl (1 ml at 6M)) with 50°C and 20 min incubations.
2. Target T4 and T3 = ~40 ng/ml; 2 ng of T3 and T4 added to 50 μ l plasma samples.
3. Fathead minnow plasma samples (50 μ l) in triplicate. Recovery of ¹³C₁₂-T4: 67 \pm 4.2% (no MeOH wash); 96 \pm 3.6% (0.5 ml 30% MeOH wash); 84 \pm 1.8% (1.0 ml 30% MeOH wash).

C.3.2 Accuracy of Thyroid Hormone Levels

As a validation component of this work and to gain insights into the accuracy of the extraction and LC/MS/MS analysis, thyroid hormone levels were measured in the same rainbow trout and fathead minnow plasma pools using previously published thyroid hormone RIA approaches (Dickhoff et al. 1982). These RIAs were conducted by our co-author, Dr. Sean Lema, California Polytechnic State University, at NOAA's Northwest Fisheries Science Center, Seattle, WA, where the RIA method for TH measurements in fishes by Dickhoff et al. (1982) was developed. Generally, the absolute and relative (T4:T3) magnitude of thyroid hormone concentrations measured with RIAs were consistent with those measured here by LC/MS/MS. However, there were notable differences. In particular, TT3 and TT4 levels in rainbow trout using RIA were measured at 2.42 ± 0.14 ng/ml and 12.0 ± 2.86 ng/ml, respectively (~5:1 T4:T3). The extraction and LC/MS/MS method used here to measure thyroid hormones in the same trout pool measured TT3 at 1.85 ± 0.06 ng/ml and T4 at 4.08 ± 0.22 ng/ml (~2:1 T4:T3). Thus, levels of TT3 measured by LC/MS/MS and RIA were generally consistent with one another, but TT4 levels were higher in trout when measured by RIA. Other studies that have measured thyroid hormones in rainbow trout have reported levels of TT3 and TT4 that are consistent with the levels measured here with LC/MS/MS. For instance, early studies

using RIA approaches reported circulating levels of both TT4 and TT3 levels in rainbow trout to be in the range of approximately 1-4 ng/ml, depending on physiological and environmental factors (e.g., dietary restrictions) (Eales et al. 1981). More recent studies with RIA have also measured thyroid hormone levels in rainbow trout to be in a range of 2-4 ng/ml (Feng et al. 2012).

It is also notable that levels of optimized TT4 and TT3 in fathead minnows were measured with this LC/MS/MS-based approach at 3.38 ± 0.05 ng/ml and 2.98 ± 0.12 ng/ml, respectively (or $\sim 1.1:1$ T4:T3). These values are consistent with the negative control levels measured in the adult male fathead minnow study that used the same method (Figure 22). However, in contrast, RIA analysis of this same fathead minnow plasma pool measured TT4 and TT3 values at 13.2 ± 0.23 ng/ml and 9.97 ± 0.52 ng/ml, respectively ($\sim 1.3:1$). While the absolute levels of thyroid hormones measured by RIA were three to four times greater than those measured by LC/MS/MS, the relative ratio of TT4 to TT3 were approximately equal at about 1:1 of T4:T3. The underlying reasons for the differences in absolute values of thyroid hormones measured by LC/MS/MS versus RIA are not exactly clear. It is possible that the extraction method here did not fully deproteinate the plasma sample or that matrix interferences produced small amounts of ion suppression, resulting in lower levels of T4. However, matrix effects were not

observed and high recovery of $^{13}\text{C}_{12}$ -T4 was measured in fathead minnow samples evaluated (Table 13) with additional SPE clean-up steps. It is also possible that the RIA methodology could be over-estimating TT4 levels in rainbow trout due to analytical interferences with the antibody or other matrix interferences as has been previously reported with immunoassay approaches (Ghosh et al. 2008; Steele et al. 2005; Tate and Ward 2004).

C.4 Conclusions

The thyroid hormone extraction method developed here provides a rapid and effective tool for isolating thyroid hormones from fish plasma using a combination of acid and heating for deproteination coupled with liquid-liquid SPE procedures and filtration. It produced a clean, low interference extract that could be analyzed effectively using our previously published LC-ESI/MS/MS methodologies. Additional steps will be taken to measure its effectiveness in other fish species, including mummichogs (*Fundulus heteroclitus*) and Chinook salmon (*Oncorhynchus tshawytscha*).

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Biography

Pamela Noyes was born in Binghamton, New York and grew up in Manhattan Beach, California, Satellite Beach, Florida, and the Washington, D.C. suburbs. She received her B.S. from the University of Maryland and M.S. in Environmental Science from Johns Hopkins University in 2002. She worked for the U.S. Environmental Protection Agency for several years starting in 1999 where, over the course of her tenure, she worked on EPA science policy issues and conducted scientific analyses in support of wildlife protection, water quality, and pesticide regulations. For her doctoral research, Pam was awarded an EPA STAR fellowship to pursue lines of investigation to elucidate PBDE flame retardant effects in fishes. She received the Otto Hutzinger best student presentation award at the 2010 Dioxin International Symposium where she presented results of some of this work. While pursuing her doctorate, Pam was also invited to participate with leading scientists in a 2011 SETAC Pellston Workshop to examine effects of climate change on chemical toxicity and develop recommendations for assessing these interactions, results of which were published. She has coauthored several published articles describing results of her research and climate change-chemical interactions, including:

Noyes PD, Hinton DE, Stapleton HM. 2011. Accumulation and debromination of decabromodiphenyl ether (BDE-209) in juvenile fathead minnows induces thyroid disruption and liver alterations. *Toxicological Sciences* 122(2): 265-274.

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