PBDE Metabolism and Effects on Thyroid Hormone Regulation in Human Astrocytes

by

Simon Clay Roberts

Environment
Duke University

Date:____________________

Approved:

____________________
Heather M. Stapleton, Supervisor

____________________
Richard T. Di Giulio

____________________
Theodore A. Slotkin

____________________
Joel N. Meyer

____________________
Patrick Lee Ferguson

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

2014
ABSTRACT

PBDE Metabolism and Effects on Thyroid Hormone Regulation in Human Astrocytes

by

Simon Clay Roberts

Environment
Duke University

Date: ____________________________
Approved:

_______________________________
Heather M. Stapleton, Supervisor

_______________________________
Richard T. Di Giulio

_______________________________
Theodore A. Slotkin

_______________________________
Joel N. Meyer

_______________________________
Patrick Lee Ferguson

An abstract of a dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Environment in the
Graduate School of Duke University

2014
Abstract

Polybrominated diphenyl ether (PBDE) flame retardants are ubiquitous contaminants in the environment due to their heavy usage in plastics, foam, and textiles to comply with flammability standards from the 1970s through the late 2000s. Due to their toxicity and persistence in the environment, two of the three PBDE commercial mixtures (PentaBDE and OctaBDE) were banned by the Stockholm Convention on Persistent Organic Pollutants in 2009. The DecaBDE commercial mixture, which consists primarily of the fully brominated congener BDE-209, has been banned or phased out in the United States and Europe but is still in use in other parts of the world. Human exposure to PBDEs persists via environmental reservoirs of PBDEs and products produced before the bans/phase-outs. PBDEs disrupt thyroid hormone levels and neurodevelopment in fish and rodents and are associated with altered thyroid hormone levels and neurodevelopmental impairments in humans. However, the mechanism by which PBDEs alter neurodevelopment remains unclear. Knowledge of the mechanisms and molecular targets of PBDEs is necessary for a causal link to be established between PBDEs and neurodevelopmental impairments. The hypothesis of this thesis research is that PBDEs alter thyroid hormone levels in the brain by interfering with the activity of PBDE-metabolizing deiodinase enzymes in brain cells, which may result in decreased levels of thyroid hormones in the brain and impaired neurodevelopment.
In the first aim of this thesis research, the biotransformation of PBDEs was examined to determine whether hydroxylated PBDEs (OH-BDEs) are formed in the human brain. In biotransformation assays performed with human astrocytes, which are cells located at the blood brain barrier, no debrominated or OH-BDE metabolites were identified. The results indicate that the enzyme responsible for PBDE hydroxylation (CYP2B6) was not expressed in sufficient quantities to metabolize PBDEs in the astrocyte cells used in this study, but future studies should analyze the potential for PBDE hydroxylation in other brain cells.

In the second aim of this thesis research, the effects of PBDEs on the thyroid-activating enzyme Type 2 deiodinase (DIO2) were determined in human astrocyte cells. DIO2 converts thyroxine (T4) into triiodothyronine (T3), which is the primary ligand that binds to the thyroid nuclear receptors, and is a very important signaling molecule during neurodevelopment. Cultured primary astrocytes and a human glioma cell line (H4 cells) were exposed to PBDEs and OH-BDEs, and changes in DIO2 activity were measured using liquid chromatography with tandem mass spectrometry (LC/MS/MS). Exposure to BDE-99, -153, and -209, 3-OH-BDE-47, and 5′-OH-BDE-99 all resulted in significant decreases in DIO2 activity in the H4 cells by up to 80% at doses of 500-1,000 nM. Further experiments deduced that the primary mechanism responsible for this decrease in activity was attributed to decreased DIO2 mRNA expression, increased post-
translational degradation of DIO2, and competitive inhibition of DIO2. The reduction in DIO2 activity by PBDE and OH-BDE exposures could potentially reduce the concentration of T3 in the brain, which may be responsible for the neurodevelopmental impairments produced by exposure to this class of compounds and needs to be further explored.

In the third aim of this thesis research, the effects of PBDEs and OH-BDEs were examined in the H4 cells and in a mixed culture containing a human neuroblastoma cell line (SK-N-AS cells). The SK-N-AS cells express the thyroid hormone-inactivating enzyme Type 3 deiodinase (DIO3), which works in concert with DIO2 to buffer the concentration of T3 in the brain. Exposure to BDE-99 decreased the concentration of T3 and the inactive thyroid hormone rT3 in the cell culture medium of co-cultured cells by 59-76%. 3-OH-BDE-47 competitively inhibited DIO3 with an IC50 of 19 μM. 5′-OH-BDE-99 increased the rT3 concentrations in cell culture medium by 400%, increased DIO3 activity in exposed cells by 50%, and increased DIO3 catalytic activity in cellular homogenates by over 500%. Further effects on the mRNA expression of several thyroid-regulated genes (DIO3, TR-α, TR-β, MCT8, and ENPP2) and oxidative respiration were also assessed in the SK-N-AS cells. DIO3 mRNA expression increased by 9 fold in cells exposed to 400 nM BDE-99, and ENPP2 mRNA expression increased by 2 fold in cells exposed to 500 nM BDE-99 and a mixture of the three congeners, but no other significant
effects on mRNA expression were observed. The basal respiration rates and other parameters of oxidative respiration were also not significantly altered by exposure to PBDEs or OH-BDEs, but proton leak was increased by over 400% in cells exposed to 2 µM 5’-OH-BDE-99.

This was the first study to examine the effects of an environmental contaminant on human DIO2 and DIO3 in cultured cells. The results indicated that BDE-99 and OH-BDEs decreased the activity of DIO2 and 5’-OH-BDE-99 increased the activity of DIO3, which combined would lead to decreased levels of T3 exported from the cells into the extracellular environment. These results provide more evidence that disruption of DIO2 and DIO3 by PBDEs during development may mediate the neurodevelopment effects associated with PBDEs.
## Contents

Abstract ................................................................................................................................. iv

List of Tables ........................................................................................................................... xiii

List of Figures .......................................................................................................................... xiv

List of Abbreviations ............................................................................................................ xxi

1. Introduction ......................................................................................................................... 1
   1.1 Flame Retardant Overview .............................................................................................. 1
   1.2 Polybrominated Diphenyl Ethers .................................................................................. 6
   1.3 Alternate Flame Retardants .......................................................................................... 10
   1.4 Sources and Continued Exposure to PBDEs ............................................................... 12
   1.5 Toxicity of PBDEs ........................................................................................................ 15
      1.5.1 Thyroid Hormone Effects ...................................................................................... 16
      1.5.2 Neurodevelopmental Effects ................................................................................ 27
   1.6 Thesis Research Aims .................................................................................................... 34

2. Biotransformation of Flame Retardants ............................................................................. 36
   2.1 Biotransformation of Xenobiotics .................................................................................. 36
   2.2 Biotransformation of PBDEs ......................................................................................... 37
      2.2.1 Oxidative Biotransformation ............................................................................... 38
      2.2.2 Reductive Debromination ................................................................................... 41
   2.3 Biotransformation Experiments .................................................................................... 44
      2.3.1 In Vitro Debromination in Fish ............................................................................ 44
2.3.2 PBDE Metabolism in Cultured Astrocyte Cells ........................................... 48
2.3.3 Biotransformation of TBB and TBPH .......................................................... 51

3. Disruption of Type 2 Thyroid Deiodinase Activity in Cultured Human Astrocytes by Polybrominated Diphenyl Ethers .................................................. 55

  3.1 Introduction ........................................................................................................ 56
  3.2 Materials and Methods .................................................................................... 60
    3.2.1 Reagents and Materials .............................................................................. 60
    3.2.2 Cell Culture ............................................................................................... 61
    3.2.3 Dosing ........................................................................................................ 62
    3.2.4 Cytotoxicity Assays .................................................................................. 63
    3.2.5 Deiodinase Assays .................................................................................... 63
    3.2.6 Enzyme Kinetics and Inhibition Assays ..................................................... 64
    3.2.7 DIO2 mRNA Expression .......................................................................... 65
    3.2.8 Statistics ..................................................................................................... 66
  3.3 Results ............................................................................................................... 67
    3.3.1 Basal Type 2 Deiodinase Activity ............................................................... 67
    3.3.2 Cytotoxicity .............................................................................................. 68
    3.3.3 Effects of PBDEs and OH-BDEs on DIO2 Activity .................................... 69
    3.3.4 Effects of PBDEs and OH-BDEs on DIO2 mRNA Expression ...................... 72
    3.3.5 In Vitro Inhibition of DIO2 ........................................................................ 73
    3.3.6 Effects of BDE-99 and 5'-OH-BDE-99 on Proteasomal Degradation ............ 74
  3.4 Discussion ......................................................................................................... 76
4. Disruption of Type 2 and Type 3 Deiodinase Activity in Co-cultured Human Glial and Neuronal Cells by Polybrominated Diphenyl Ethers .........................................................84

4.1 Introduction ..................................................................................................................85

4.2 Materials and Methods ...............................................................................................89
  4.2.1 Reagents and Materials .......................................................................................89
  4.2.2 Cell Culture .........................................................................................................90
  4.2.3 Dosing ................................................................................................................91
  4.2.4 Cytotoxicity Assays ..........................................................................................92
  4.2.5 Deiodinase Assays ............................................................................................92
  4.2.6 Enzyme Kinetics and Inhibition Assays ............................................................94
  4.2.7 mRNA expression analysis ..............................................................95
  4.2.8 Metabolic Analysis ...........................................................................................95
  4.2.9 Statistics ..........................................................................................................97

4.3 Results ........................................................................................................................98
  4.3.1 Transwell Co-culture of H4 and SK-N-AS cells ............................................98
  4.3.2 In Vitro Inhibition and Activation of DIO3 in SK-N-AS Cells .....................105
  4.3.3 Effects of BDE-99 and OH-BDEs on DIO3, ENPP2, THR, and MCT8 mRNA Expression .................................................................107
  4.3.4 Effects of BDE-99 and OH-BDEs on Oxidative Respiration ......................111

4.4 Discussion ..................................................................................................................116

4.5 Supplementary Figures ............................................................................................130

4.6 Funding Information .................................................................................................134
5. Discussion ................................................................................................................. 135

5.1 Potential Mechanism of PBDE Neurodevelopmental Effects ................................. 135

5.2 Novel Findings of DIO2 and DIO3 Biochemistry ............................................... 139

5.3 Methods for Testing Thyroid Disruption Potential ............................................. 140

5.4 Limitations ............................................................................................................. 142

5.5 Data Gaps and Future Research Areas ................................................................. 143

5.6 Conclusions ........................................................................................................... 146

Appendix A. Species-Specific Differences and Structure-Activity Relationships in the
Debromination of PBDE Congeners in Three Fish Species ........................................... 148

A.1 Introduction ........................................................................................................... 149

A.2 Methods and Materials ......................................................................................... 152

A.3 Results and Discussion ......................................................................................... 157

A.4 Conclusions .......................................................................................................... 169

A.5 Funding Information ............................................................................................. 170

Appendix B. In Vitro Metabolism of the Brominated Flame Retardants 2-Ethylhexyl-
2,3,4,5-Tetrabromobenzoate (TBB) and Bis(2-Ethylhexyl) 2,3,4,5-Tetrabromophthalate
(TBPH) in Human and Rat Tissues ............................................................................. 171

B.1 Introduction ........................................................................................................... 173

B.2 Experimental Procedures ....................................................................................... 177

B.2.1 Materials .......................................................................................................... 177

B.2.2 Metabolic Incubations ....................................................................................... 178

B.2.3 Sample Preparation ........................................................................................... 179

B.2.4 GC/MS Identification of Brominated Metabolites ............................................. 179
B.2.5 LC/MS/MS Quantification.................................................................180
B.2.6 Quality Assurance.............................................................................182
B.3 Results and Discussion ........................................................................182
  B.3.1 In Vitro Metabolism of TBB ..............................................................182
  B.3.2 Carboxylesterase-Mediated Metabolism ...........................................184
  B.3.3 Enzyme Kinetics ..............................................................................185
  B.3.4 Multiple Species and Tissue Comparison .........................................188
  B.3.5 TBPH Metabolism ...........................................................................191
  B.3.6 Phase II Metabolism ........................................................................191
Appendix C. Supplemental Statistical Results...............................................194
References ..................................................................................................198
Biography....................................................................................................220
List of Tables

Table 1: Commercial PBDE mixtures with the percent composition of the dominant congeners in each mixture (La Guardia et al., 2006).................................................................8

Table 2: Properties of the three DI isoforms obtained summarized from the following reviews (Bianco and Kim, 2006; Bianco and Larsen, 2005; Köhrle, 2002).............................21

Table 3: Levels of polybrominated diphenyl ethers (PBDEs) and hydroxylated polybrominated diphenyl ethers (OH-BDEs) in human serum in previous studies compared with the lowest dose that significantly decreased Type 2 deiodinase (DIO2) activity in this study..............................................................................................................79

Table 4: Average concentrations of BDE-99 and OH-BDEs during the 48 h exposures measured every 12 h. .........................................................................................................................92

Table 5: Expected cellular responses to altered thyroid hormone concentrations including deiodinase activity, mRNA expression of several thyroid hormone-regulated genes, and cellular energy expenditure. .................................................................................................................................121

Table 6: Percent control values for each parameter of oxidative respiration for A) cocultured cells and B) SK-N-AS cells cultured alone. .................................................................134

Table 7: Hepatic microsomal formation rates of debrominated metabolites in incubations containing 1 nmol of the parent congener expressed as fmol hr⁻¹ mg protein⁻¹ with standard error. ......................................................................................................................161

Table 8: Summary of previous studies detecting TBB and TBPH in the environment...175

Table 9: LC/MS/MS conditions, recovery, and detection limits for negative electrospray ionization for the metabolites, TBBA and TBMHP, and the internal standards used for their quantification, 2,3,5-triiodobenzoic acid (TIBA) and monohexyl-2,3,4,5-tetrachlorophthalate (TCMHP), respectively. ....................................................................................181
List of Figures

Figure 1: Chemical structures of the commonly used flame retardants, polybrominated diphenyl ethers (PBDEs), PBDE metabolites, the components of Firemaster® 550 (various isopropylated analogs of triphenyl phosphate (TPP) are also present in the mixture but are not shown), tetrabromobisphenol-A (TBBPA), decabromodiphenyl ethane (DBDPE), and tris(1,3-dichloro-2-propyl) phosphate (TDCPP), and the thyroid hormones thyroxine (T₄) and triiodothyronine (T₃)..................................................5

Figure 2: Deiodinase-mediated thyroid hormone metabolism. The catalytic roles of each DIO isoform are indicated beside the arrows for each reaction. .........................................................23

Figure 3: Major oxidative metabolites of BDE-99 in human liver tissues and hepatocytes (Erratico et al., 2012; Stapleton et al., 2008c). ..........................................................................................40

Figure 4: Hepatic microsomal biotransformation pathway for (a) common carp, (b) rainbow trout, and (c) Chinook salmon. Boxes indicate congeners used as substrates in incubations. (*) Steps in which two bromine atoms were removed in one incubation were hypothesized based on the structure of the metabolite assuming no rearrangement of bromine atoms. (**) BDE 203 coelutes with BDE 200, which is also a potential metabolite not shown here. ..................................................................................46

Figure 5: Chromatograms of LC/MS/MS analysis of a sample of cell culture medium exposed to BDE-99 for 48 h showing no detectable peaks for OH-BDEs or tribromophenol (top) and an analytical standard containing a mixture of OH-BDEs at a concentration of 10 ng mL⁻¹ (bottom)..................................................................................50

Figure 6: In vitro biotransformation product of TBB in human liver microsomes, human intestinal microsomes, rat liver microsomes, rat intestinal microsomes, and porcine carboxylesterase, and in vitro biotransformation product of TBMEHP in porcine carboxylesterase........................................................................................................53

Figure 7: Structures of PBDE and OH-BDE congeners and thyroid hormones used in this study. .................................................................................................................................58

Figure 8: Mechanisms of intracellular DIO2 regulation in astrocyte cells adapted from (Bianco and Larsen, 2005) with BDE-99 and 5’-OH-BDE-99 added into potential mechanisms based on the results of this study. ........................................................................60
Figure 9: A) Kinetics of DIO2 in microsomal preparations of H4 cells (black line: $K_m = 3.49 \pm 0.94$ nM, $V_{max} = 47.5 \pm 2.9$ fmol min$^{-1}$ mg protein$^{-1}$) and DIO2 kinetics with the addition of 20 $\mu$M BDE-99 (orange line: $K_m = 8.74 \pm 3.2$ nM, $V_{max} = 50.6 \pm 5.6$ fmol min$^{-1}$ mg protein$^{-1}$) or 15 $\mu$M 5’-OH-BDE-99 (green line: $K_m = 19.9 \pm 12.0$ nM, $V_{max} = 47.2 \pm 8.0$ fmol min$^{-1}$ mg protein$^{-1}$). B-D) Inhibition of DIO2 activity in microsomal preparations of H4 cells in assays performed with 1-2 nM T4 and increasing concentrations of B) BDE-99, C) 3-OH-BDE-47, and D) 5’-OH-BDE-99). Data are shown as mean ± SEM. …………………………………68

Figure 10: Time course of effects on DIO2 activity in H4 cells exposed to 500 nM BDE-47, BDE-99, BDE-153, and BDE-209 or 1,000 nM 3-OH-BDE-47, 6-OH-BDE-47, and 5’-OH-BDE-99 for 0, 1, 6, and 12 h. Results are shown as mean ± SEM. Two-factor ANOVA indicated a significant interaction of treatment × exposure duration (p<0.001). * Indicates significant difference from vehicle control cells at the corresponding exposure time of the sample (p<0.05; n=8 from 2 experiments). Dose-response relationships and full statistical results are shown in Figure 42. ………………………………………………………………………………………………………70

Figure 11: DIO2 activity after 6 h exposures to PBDEs and OH-BDEs at the doses identified in the dose legend. One-factor ANOVA indicated a significant effect of treatment (p<0.001). Data are reported as percent relative to the vehicle control. * Indicates significant difference from vehicle control cells (p<0.05; n=8 from 2 experiments). Dose-response relationships and full statistical results are shown in Figure 43. …………………………………………………………………………………………………………………………………………………………………………………………………………………71

Figure 12: Decreased DIO2 activity in primary human astrocyte cells exposed to 500 nM BDE-99 and 100 nM rT3. One-way ANOVA indicated an effect of treatment (p<0.001). * Indicates significant difference from control cells (p<0.05; n=8 from 2 experiments). ………71

Figure 13: DIO2 mRNA expression ratio relative to control cells and normalized to RPL13a as an internal reference gene calculated using the $2^{\Delta\Delta Ct}$ method after 6 h exposures to PBDEs and OH-BDEs at the doses identified in the dose legend. One-factor ANOVA indicated a significant effect of treatment (p<0.01). * Indicates significant difference from vehicle control cells (p<0.05; n=8 from 2 experiments; statistics were performed on $\Delta\Delta Ct$ values before linearization). Dose-response relationships and full statistical results are shown in Figure 44. …………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………...
control cells exposed to MG132 (orange), or control cells exposed to both MG132 and cycloheximide (green). Two-factor ANOVA indicated a significant interaction of treatment × inhibitor coexposure (p<0.001). Bars not sharing letters are significantly different from each other (p<0.05; n=8 from 2 experiments).

Figure 15: Effects of BDE-99, 5’-OH-BDE-99, and rT3 on control cells and cells exposed to MG132 or MG132 and cycloheximide together normalized to the vehicle control cells without MG132 or cycloheximide for comparison with Figure 14. In Figure 14, each treatment group is normalized to the control for the corresponding group (i.e., cells coexposed to BDE-99 and MG132 are normalized to control cells normalized to MG132). Two-factor ANOVA indicated a significant interaction of treatment × inhibitor (p<0.001). Bars not sharing letters are significantly different from each other (p<0.05; n=8 from 2 experiments).

Figure 16: Thyroid hormone signaling and deiodination in astrocytes and neurons.

Figure 17. Co-culture experimental design. Modified from an image created by Sean Mutchnick obtained from http://www.hhhoney.com/transwell-schematic/.

Figure 18: Assay design for the XFe24 Seahorse Bioanalyzer and the Seahorse Mitochondrial Stress Test Kit to measure multiple aspects of cellular energy expenditure. Three measurements were performed between the injections of oligomycin, FCCP, and antimycin A/rotenone.

Figure 19: T3 concentrations in the culture medium of cells exposed to the PBDE and OH-BDE compounds for 48 h. A) H4 and SK-N-AS cells cultured together in a 6-well plate with 500 pM T3 or B) SK-N-AS cells cultured alone with 500 pM T4 and 140 pM T3. Two-factor ANOVA indicated a significant interaction of treatment × culture condition (p<0.001) * Indicates statistically significant difference (p<0.05) from control cells in the corresponding culture condition group using Tukey’s post-hoc test (p<0.05; n=7-8, 2 experiments). Dose-response relationships and full statistical results are shown in Figure 45.

Figure 20: rT3 concentrations in the culture medium of cells exposed to the PBDE and OH-BDE compounds for 48 h. A) H4 and SK-N-AS cells cultured together in a 6-well plate with 500 pM T3 or B) SK-N-AS cells cultured alone with 500 pM T4 and 140 pM T3. Two-factor ANOVA indicated main effects of treatment and culture condition (p<0.05) but no interaction of treatment × culture condition. Therefore, statistics were only performed on the combined data from A and B without separating by treatment
Figure 21: Alteration of the DIO2 activity in H4 cells co-cultured with SK-N-AS cells in a 6-well plate and exposed to the PBDE and OH-BDE compounds for 48 h with 500 pM T4. One-way ANOVA indicated a significant effect of treatment (p<0.001). * Indicates statistically significant difference (p<0.05) from control cells using Tukey’s post-hoc test (p<0.05; n=7-8, 2 experiments). ................................................................. 102

Figure 22: DIO3 activity in the homogenate of SK-N-AS cells exposed to the PBDE and OH-BDE compounds for 48 h. A) H4 and SK-N-AS cells cultured together in a 6-well plate with 500 pM T4 or B) SK-N-AS cells cultured alone with 500 pM T4 and 140 pM T3. Two-factor ANOVA indicated a significant interaction of treatment × culture condition (p<0.001). *Indicates significant differences from the controls in the corresponding culture condition using Tukey’s post-hoc test (p<0.05; n=7-8, 2 experiments). ....................... 103

Figure 23: Kinetics of DIO3 activity in cellular homogenates prepared from SK-N-AS cells with a range of T4 concentrations (black line) and with a range of T4 concentrations and 12 μM 3-OH-BDE-47 added to the assay buffer. B) Inhibition of DIO3 activity in cellular homogenates prepared from SK-N-AS cells in assays with 6 nM T4 and a range of 3-OH-BDE-47 concentrations. (2 experiments; n=8) ................................................................. 104

Figure 24: A) No significant inhibition of DIO3 activity in cellular homogenates prepared from SK-N-AS cells in assays with 6 nM T4 and a range of BDE-99 concentrations. B) Altered DIO3 activity in cellular homogenates prepared from SK-N-AS cells in assays with 6 nM T4 and a range of 5’-OH-BDE-99 concentrations. (2 experiments; n=8) ................................................................. 106

Figure 25: Expression ratios (2^ΔΔCt) of DIO3 mRNA relative to control in SK-N-AS cells. A) H4 and SK-N-AS cells cultured together in a 6-well plate with 500 pM T4 or B) SK-N-AS cells cultured alone with 500 pM T4 and 140 pM T3. Two-factor ANOVA indicated a main effect of treatment but no effect of culture condition (p<0.05) or interaction of treatment × culture condition. Therefore, statistics were only performed on the combined data from A and B without separating by treatment condition. * Indicates statistically significant difference (p<0.05) from the control cells using Tukey’s post-hoc test (p<0.05; n=7-8, 2 experiments). .................................................................................. 108

Figure 26: Expression ratios (2^ΔΔCt) of ENPP2 mRNA relative to control in SK-N-AS cells. A) H4 and SK-N-AS cells cultured together in a 6-well plate with 500 pM T4 or B) SK-N-
AS cells cultured alone with 500 pM T₄ and 140 pM T₃. Two-factor ANOVA indicated main effects of treatment and culture condition (p<0.05) but no interaction of treatment × culture condition. Therefore, statistics were only performed on the combined data from A and B without separating by treatment condition. * Indicates significant difference from control using Tukey’s post-hoc test (p<0.05; n=7-8; 2 experiments). ........................................ 110

Figure 27: Oxygen consumption rate in SK-N-AS cells exposed to T₃ for 2 h. Linear regression demonstrated a significant relationship between the T₃ concentration and the oxygen consumption rate (n=3; 1 experiment). ........................................................................ 111

Figure 28: Oxygen consumption rate (OCR) of SK-N-AS cells exposed to vehicle control (A; blue), rT3 (A; orange), or BDE-99 (B) for 48-h. The following treatments were added to the cells during the analysis: 0-10 min, DMSO control; 15-25 min, oligomycin; 30-40 min, FCCP; 45-55 min, antimycin A and rotenone. The results represent the mean ± SEM of 4 samples analyzed during 2 experiments. ................................................................. 113

Figure 29: Basal oxygen consumption rate of SK-N-AS cells after a 48 h exposure to the PBDE and OH-BDE compounds for 48 h in cells A) co-cultured with H4 cells in a 6-well plate with 500 pM T₄ or B) cultured alone with 500 pM T₄ and 140 pM T₃. Two-factor ANOVA indicated main effects of treatment and culture condition (p<0.05) but no interaction of treatment × culture condition. Therefore, statistics were only performed on the combined data from A and B without separating by treatment condition. There were no significant differences from control using Tukey’s post-hoc test (p<0.05; n=7-8; 2 experiments). ......................................................................................... 114

Figure 30: Increased proton leak in SK-N-AS cells after a 48 h exposure to the PBDE and OH-BDE compounds in cells A) co-cultured with H4 cells in a 6-well plate with 500 pM T₄ or B) cultured alone with 500 pM T₄ and 140 pM T₃. Two-factor ANOVA indicated an interaction between treatment × culture condition (p<0.05). * Indicates significant difference from control in the corresponding culture condition group using Tukey’s post-hoc test (p<0.05; n=7-8; 2 experiments). ........................................................................................................ 115

Figure 31: TR-α mRNA expression in SK-N-AS cells that were cultured A) in the presence of 500 pM T₄ and H4 cells for 48 h or B) alone with the addition of 140 pM T₃ and 500 pM T₄ to the cell culture medium. Two-factor ANOVA indicated main effects of treatment and culture condition (p<0.05) but no interaction of treatment × culture condition. Therefore, statistics were only performed on the combined data from A and B without separating by treatment condition. There were no significant differences (p<0.05) from the control cells using Tukey’s post-hoc test (p<0.05; n=7-8, 2 experiments).
The power of the assay would have allowed significant results to be determined for mRNA expression ratios less than ~0.5 and greater than ~2. .................................................. 130

Figure 32: TR-β mRNA expression in SK-N-AS cells that were cultured A) in the presence of 500 pM T₁ and H4 cells for 48 h or B) alone with the addition of 140 pM T₃ and 500 pM T₄ to the cell culture medium. A global ANOVA analysis showed a main effect of treatment (p<0.001), but no interactions between treatment and dose for A or B (n=8). The power of the assay would have allowed significant results to be determined for mRNA expression ratios less than ~0.6 and greater than ~2. .................................................. 131

Figure 33: MCT8 mRNA expression in SK-N-AS cells that were cultured A) in the presence of 500 pM T₁ and H4 cells for 48 h or B) alone with the addition of 140 pM T₃ and 500 pM T₄ to the cell culture medium. Two-factor ANOVA indicated main effects of treatment and culture condition (p<0.05) but no interaction of treatment x culture condition. Therefore, statistics were only performed on the combined data from A and B without separating by treatment condition. There were no significant differences (p<0.05) from the control cells using Tukey’s post-hoc test (p<0.05; n=7-8, 2 experiments). The power of the assay would have allowed significant results to be determined for mRNA expression ratios less than ~0.5 and greater than ~2. .................................................. 132

Figure 34: Percent control values for A) nonmitochondrial respiration, B) ATP production, C) extracellular acidification rate, D) maximal respiration, and E) cytotoxicity measured as membrane damage. No effect of treatment on any of the parameters in A-E was observed using ANOVA (n=8). .................................................. 133

Figure 35: Hepatic microsomal biotransformation pathway for (a) common carp, (b) rainbow trout, and (c) Chinook salmon. Boxes indicate congeners used as substrates in incubations. (*) Steps in which two bromine atoms were removed in one incubation were hypothesized based on the structure of the metabolite assuming no rearrangement of bromine atoms. (**) BDE 203 coelutes with BDE 200, which is also a potential metabolite not shown here. .................................................. 158

Figure 36: Summed formation rates (fmol hr⁻¹ mg protein⁻¹) for all metabolites formed from BDEs 99, 153, 183, 203, 208, and 209 based on the position of debromination (top). Formation rates for 3,3′ T₂, T₃ and rT₃ in hepatic microsomes incubated with 500 pmol T₁ and the thyroid hormone deiodination pathway (bottom). .................................................. 165

Figure 37: Chemical structures of TBB, TBPH, TBBA, and TBMEHP .................................................. 174
Figure 38: Mass spectra of the TBBA methyl-derivative obtained using GC/MS operated in EI (top) and ECNI (bottom) ionization modes with labeled fragments........................................184

Figure 39: Formation of TBBA over time in (a) human liver microsomes (40 μg protein mL⁻¹) and (b) porcine hepatic carboxylesterase (2 μg protein mL⁻¹) at a TBB concentration of 27.1 ± 1.3 μM showing approximately linear formation for 60 min. The values represent the mean of 4 experiments.................................................................186

Figure 40: Initial velocity of TBBA formation at various substrate concentrations fit to a Michaelis-Menten model using 10-min incubations with human liver microsomes and porcine hepatic carboxylesterase with R² values of 0.782 and 0.862, respectively. The values represent the mean of 4 experiments.................................................................188

Figure 41: TBBA formation rates for incubations performed with human and rat tissues normalized to protein content. In pooled samples, n equals the number of individual experiments performed with the pooled sample. In the rat tissues, n equals the number of animals from which individual microsomal or serum samples were prepared. The asterisk indicates significant difference from samples without an asterisk using Tukey’s post-hoc test (p<0.01) after ANOVA indicated a main effect of tissue on formation rate (p<0.001). ..........................................................................................................................190

Figure 42: Two-way ANOVA and Tukey’s post-hoc results for the time-course effects on DIO2 activity shown in Figure 10. ..................................................................................................................194

Figure 43: One-way ANOVA and Tukey’s post-hoc results for the dose-response effects on DIO2 activity shown in Figure 11........................................................................................................195

Figure 44: One-way ANOVA and Tukey’s post-hoc results for the dose-response effects on DIO2 mRNA expression shown in Figure 13........................................................................................................196

Figure 45: Two-way ANOVA and Tukey’s post-hoc results for the T₃ concentration in the cell culture medium of co-cultured cells and SK-N-AS cells cultured alone as shown in Figure 19........................................................................................................197
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid assay</td>
</tr>
<tr>
<td>BDE-47</td>
<td>2,2′,4,4′-tetrabromodiphenyl ether</td>
</tr>
<tr>
<td>BDE-49</td>
<td>2,2′,4,5′-tetrabromodiphenyl ether</td>
</tr>
<tr>
<td>BDE-99</td>
<td>2,2′,4,4′,5-pentabromodiphenyl ether</td>
</tr>
<tr>
<td>BDE-100</td>
<td>2,2′,4,4′,6-pentabromodiphenyl ether</td>
</tr>
<tr>
<td>BDE-101</td>
<td>2,2′,4,5,5′-pentabromodiphenyl ether</td>
</tr>
<tr>
<td>BDE-153</td>
<td>2,2′,4,4′,5,5′-hexabromodiphenyl ether</td>
</tr>
<tr>
<td>BDE-154</td>
<td>2,2′,4,4′,5,6′-hexabromodiphenyl ether</td>
</tr>
<tr>
<td>BDE-183</td>
<td>2,2′,3,4,4′,5,6-heptabromodiphenyl ether</td>
</tr>
<tr>
<td>BDE-206</td>
<td>2,2′,3,3′,4,4′,5,5,6-nonabromodiphenyl ether</td>
</tr>
<tr>
<td>BDE-207</td>
<td>2,2′,3,3′,4,4′,5,6,6′-nonabromodiphenyl ether</td>
</tr>
<tr>
<td>BDE-208</td>
<td>2,2′,3,3′,4,5,5,6′-nonabromodiphenyl ether</td>
</tr>
<tr>
<td>BDE-209</td>
<td>Decabromodiphenyl ether</td>
</tr>
<tr>
<td>Br</td>
<td>Bromine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>PentaBDE</td>
<td>Commercial PentaBDE mixture</td>
</tr>
<tr>
<td>OctaBDE</td>
<td>Commercial OctaBDE mixture</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>DecaBDE</td>
<td>Commercial DecaBDE mixture</td>
</tr>
<tr>
<td>CAR</td>
<td>Constitutive androstane receptor</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CDNB</td>
<td>Chlorinated dinitrobenzene</td>
</tr>
<tr>
<td>CI</td>
<td>Chlorine</td>
</tr>
<tr>
<td>CI</td>
<td>Chemical ionization</td>
</tr>
<tr>
<td>C&lt;sub&gt;t&lt;/sub&gt;</td>
<td>Threshold Cycle</td>
</tr>
<tr>
<td>CYP450</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DEHP</td>
<td>Di-ethylhexyl phthalate</td>
</tr>
<tr>
<td>DIO1</td>
<td>Type 1 Iodothyronine deiodinase</td>
</tr>
<tr>
<td>DIO2</td>
<td>Type 2 Iodothyronine deiodinase</td>
</tr>
<tr>
<td>DIO3</td>
<td>Type 3 Iodothyronine deiodinase</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECNI</td>
<td>Electron capture negative ionization</td>
</tr>
<tr>
<td>EI</td>
<td>Electron impact</td>
</tr>
<tr>
<td>ENPP2</td>
<td>Ectonucleotide pyrophosphatase/phosphodiesterase 2</td>
</tr>
<tr>
<td>EPA</td>
<td>Environmental Protection Agency</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
</tbody>
</table>

xxii
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>Fluorine</td>
</tr>
<tr>
<td>FCCP</td>
<td>Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone</td>
</tr>
<tr>
<td>GC/MS</td>
<td>Gas chromatography/mass spectrometry</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>I</td>
<td>Iodine</td>
</tr>
<tr>
<td>Kₘₙ</td>
<td>Michaelis constant (1/2 Vₘₐₓ)</td>
</tr>
<tr>
<td>Kₐₜₜₜ</td>
<td>Octanol-water partition coefficient</td>
</tr>
<tr>
<td>LC/MS/MS</td>
<td>Liquid chromatography tandem mass spectrometry</td>
</tr>
<tr>
<td>MCT</td>
<td>Monocarboxylate transporter</td>
</tr>
<tr>
<td>MDL</td>
<td>Method detection limit</td>
</tr>
<tr>
<td>MEHP</td>
<td>Mono-ethylhexyl phthalate</td>
</tr>
<tr>
<td>MeO-PBDE</td>
<td>Methoxylated PBDE</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple reaction monitoring</td>
</tr>
<tr>
<td>NADPH</td>
<td>Reduced nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>OATP</td>
<td>Organic anion transport protein</td>
</tr>
<tr>
<td>OH-PBDE</td>
<td>Hydroxylated PBDE</td>
</tr>
<tr>
<td>PBDE</td>
<td>Polybrominated diphenyl ether</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>PBDD</td>
<td>Polybrominated dibenzo-p-dioxin</td>
</tr>
<tr>
<td>PBDF</td>
<td>Polybrominated dibenzofuran</td>
</tr>
<tr>
<td>PCB</td>
<td>Polychlorinated biphenyl ether</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PND</td>
<td>Postnatal day</td>
</tr>
<tr>
<td>PTU</td>
<td>6-propyl-2-thiouricil</td>
</tr>
<tr>
<td>PTV</td>
<td>Pressure temperature variation</td>
</tr>
<tr>
<td>RPL13</td>
<td>Ribosomal protein 13</td>
</tr>
<tr>
<td>rT3</td>
<td>3,3’,5’-Triiodothyronine T4 Thyroxine</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Quantitative real-time reverse transcription PCR</td>
</tr>
<tr>
<td>SULT</td>
<td>Sulfotransferase</td>
</tr>
<tr>
<td>T2</td>
<td>3,3’-Diiodothyronine</td>
</tr>
<tr>
<td>T3</td>
<td>3,3’,5-Triiodothyronine</td>
</tr>
<tr>
<td>T4</td>
<td>Thyroxine</td>
</tr>
<tr>
<td>TBB</td>
<td>2-Ethylhexyl-2,3,4,5-tetrabromobenzoate</td>
</tr>
<tr>
<td>TBBA</td>
<td>2,3,4,5-Tetrabromobenzoic acid</td>
</tr>
<tr>
<td>TBBPA</td>
<td>Tetrabromobisphenol A</td>
</tr>
<tr>
<td>TBP</td>
<td>Tribromophenol</td>
</tr>
<tr>
<td>TBPH</td>
<td>Bis(2-ethylhexyl) 2,3,4,5-tetrabromophthalate</td>
</tr>
</tbody>
</table>
TR  Thyroid receptor
TRE  Thyroid response element
TSH  Thyroid stimulating hormone
TTR  Transthyretin
UGT  Uridine diphosphate glucuronosyl transferase
V_max  Maximum enzyme velocity
1. Introduction

1.1 Flame Retardant Overview

Many household products such as upholstered furniture, mattresses, electronics, and building materials are composed of highly flammable materials, such as polyurethane foam, synthetic fabrics, and polymers that have historically increased the risk of fire to residents. Fire statistics from the 1970s indicate that 60-70% of house fires originating from furniture were initially started by misplaced cigarettes, and most of the remaining fires were started by children playing with matches and lighters (Damant and Nurbakhsh, 1995). In 1974, approximately 2,500 fire incidents involving upholstered furniture occurred in California, and in response, the state legislature enacted flame retardant standards to decrease the burden of fires in both residential and commercial settings (Damant and Nurbakhsh, 1995). Flame retardant standards for motor vehicles, aviation, and fabrics were currently in place before the 1970s, but the first major flammability standards to address upholstered furniture were passed in 1975 by the California Bureau of Electronic and Appliance Repair, Home Furnishings, and Thermal Insulation. Technical Bulletin 133 (TB133) established guidelines for upholstered furniture used in public areas without sprinkler systems that required flammability testing of the final assembled furniture products (Shaw et al., 2010). TB116 and TB117 established guidelines and standards for furniture manufacturers requiring them to
perform flammability tests on household upholstered products before they were sold in California. TB116 was a voluntary test that was performed by placing smoldering cigarettes directly on a fully assembled piece of furniture, but was not widely adopted as a standard test (Shaw et al., 2010). TB117 was a mandatory standard that required upholstered furniture sold in California to pass open-flame and smoldering-cigarette tests on the cushioning components of the furniture, particularly polyurethane foam cushioning (Damant and Nurbakhsh, 1995). Although TB117 only applied to furniture sold in California, most furniture produced in the US has been manufactured to be compliant with TB117 (Weil and Levchik, 2004). While TB117 does not specifically require the addition of chemical flame retardants, manufacturers have historically added liquid flame retardant formulations to polyurethane foam during production to meet the requirements of TB117.

When TB117 was first passed in 1975, the use of halogenated flame retardants was one of the most economical ways to meet the component-based flammability requirements, even though the flame retardants needed to be added at levels greater than 5% of the weight of the polyurethane foam to impart significant flame retarding effects. The combustion of polymers typically occurs in the gas phase and is driven by the cycling of radicals and chain carriers as shown below in the combustion of methane gas (Camino and Costa, 1988):

2
\[ CH_4 + O_2 \rightarrow CH_3 + H \cdot + O_2 \]
\[ H \cdot + O_2 \rightarrow HO + O \]
\[ CH_4 + HO \rightarrow \cdot CH_3 + H_2 O \]
\[ \cdot CH_3 + O \rightarrow H_2 CO + H \cdot \]
\[ H_2 CO + HO \rightarrow CHO + H_2 O \]
\[ CHO + O_2 \rightarrow H \cdot + CO + O_2 \]
\[ CO + HO \rightarrow CO_2 + H \cdot \]

During the early stages of combustion, halogenated flame retardants added to polyurethane foam release low energy halogen radicals, such as Br\( \cdot \) and Cl\( \cdot \), which compete with the high energy radicals, such as HO\( \cdot \) and H\( \cdot \), in the exothermic fire cycle, thus slowing the propagation of the fire (Rahman et al., 2001):

\[ H \cdot + HBr \rightarrow H_2 + Br \cdot \]
\[ HO \cdot + HBr \rightarrow H_2 O + Br \cdot \]

As a result of TB 117 and other flame retardant standards, approximately 150,000 metric tons of brominated flame retardants were produced annually between 1990 and 2000 worldwide (Darnerud et al., 2001). In 2013, the State of California modified the TB 117 standard and proposed a new superseding standard, TB 117-2013. The tests in TB 117-2013 no longer require open-flame testing of the bare cushioning material and may be performed with a barrier material between the foam and the outer fabric that is present on the finished product (BEARHFTI, 2013). Although the effects of the updated
requirements of TB 117-2013 are not yet known, the updated tests may result in fewer flame retardant chemical applications in polyurethane foam and other cushioning fillers in household products. However, many other flame retardant standards exist and chemical flame retardants will continue to be used in many different products.

Halogenated flame retardants can be synthesized based on a variety of chemical structures and typically contain either bromine or chlorine as shown in Figure 1. The effectiveness of organohalogen flame retardants follows the trend I>Br>Cl>F. However, organoiodines are typically too unstable during polymer processing (temperatures >200°C) to be used as flame retardants, while organofluorines are typically not effective when applied as flame retardants in polymers (Camino and Costa, 1988). Halogenated organophosphate flame retardants (OPFRs) contribute an additional mechanism of flame retardant action in addition to the previously described radical mechanism. When phosphate flame retardants are heated during combustion, they form a layer of intumescent char on the surface of the fuel, which prevents further combustion of the material (van der Veen and de Boer, 2012). Halogenated OPFRs act by both mechanisms additively to help slow combustion.
Figure 1: Chemical structures of the commonly used flame retardants, polybrominated diphenyl ethers (PBDEs), PBDE metabolites, the components of Firemaster® 550 (various isopropylated analogs of triphenyl phosphate (TPP) are also present in the mixture but are not shown), tetrabromobisphenol-A (TBBPA), decabromodiphenyl ethane (DBDPE), and tris(1,3-dichloro-2-propyl) phosphate (TDCPP), and the thyroid hormones thyroxine (T4) and triiodothyronine (T3).
Many of the flame retardants added to consumer products are not chemically bound to the polymer and are referred to as additive flame retardants. In contrast, reactive flame retardants are introduced by chemical modification of the polymer, and are typically more stable and do not migrate from the products as readily as additive flame retardants. Tetrabromobisphenol-A (TBBPA) is commonly used as a reactive flame retardant (although it has been used as an additive flame retardant in some applications) and is actually the highest production flame retardant worldwide (Shaw et al., 2010). Despite its large-volume use and concerns about its potential toxicity, human exposure to TBBPA is generally lower than the additive flame retardants due to its slower release from household products. However, additive flame retardants are generally preferred by polymer manufacturers because of their flexibility of use and fewer effects on the performance and properties of the polymer (Camino and Costa, 1988).

1.2 Polybrominated Diphenyl Ethers

Polybrominated diphenyl ethers (PBDEs) are a class of additive, brominated flame retardants that were used heavily from the 1970s through the early 2000s, and some are currently still in use. Similar to PCBs, PBDEs exhibit a diverse range of properties based on the degree of bromination (209 possible structural configurations of 1-10 bromine atoms) of the diphenyl ether backbone. PBDEs are typically synthesized as mixtures of congeners by direct bromination of diphenyl ether using Freidel Crafts
reactions with excess bromine (Hanari et al., 2006). Unlike PCB commercial mixtures, which typically contain 50-100 unique congeners (Albro and Parker, 1979), the number of PBDE congeners in each mixture is usually limited to 10-20 congeners due to the increased steric hindrance of adding bromine compared with chlorine (Hanari et al., 2006; La Guardia et al., 2006). Three main types of commercial PBDE-mixtures have been produced and distributed and can be categorized into three classes, PentaBDE, OctaBDE, and DecaBDE, as shown in Table 1. The major PBDE congeners in the PentaBDE mixture were BDE-99 and BDE-47, which together comprised over 50% of the congener profile of most PentaBDE commercial mixtures (La Guardia et al., 2006). PentaBDE is thought to be the major flame retardant mixture used in polyurethane foam to meet the requirements for TB 117 starting in the 1970s and was used in other applications as well. OctaBDE was applied to acrylonitrile butadiene styrene (ABS) in plastic office products and electronics at levels of up to 12% of the total weight of the polymer (Shaw et al., 2010). DecaBDE was the main commercial PBDE used in electronics housing and textile backcoatings and primarily contains the fully brominated PBDE congener BDE-209 with small amounts of the three nona-brominated congeners BDE-206, BDE-207, and BDE-208. In 2001, 83.3% of the global PBDE market demand consisted of the DecaBDE formulation, followed by the PentaBDE (11.1%) and OctaBDE (5.6%) formulations (La Guardia et al., 2006).
Table 1: Commercial PBDE mixtures with the percent composition of the dominant congeners in each mixture (La Guardia et al., 2006)

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Congener</th>
<th>Percent of Mixture</th>
<th>Applications in Consumer Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>PentaBDE (DE71)</td>
<td>BDE-99</td>
<td>48.6</td>
<td>Polyurethane foam cushioning in upholstered furniture and automobiles</td>
</tr>
<tr>
<td></td>
<td>BDE-47</td>
<td>38.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-100</td>
<td>13.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-153</td>
<td>5.44</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-154</td>
<td>4.54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-85</td>
<td>2.96</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-49</td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td>OctaBDE (DE79)</td>
<td>BDE-183</td>
<td>42.0</td>
<td>Polymers such as acrylonitrile butadiene styrene used in electronics</td>
</tr>
<tr>
<td></td>
<td>BDE-197</td>
<td>22.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-207</td>
<td>11.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-196</td>
<td>10.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-153</td>
<td>8.66</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-203</td>
<td>4.40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-171</td>
<td>1.81</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-180</td>
<td>1.70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-206</td>
<td>1.38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-209</td>
<td>1.31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-154</td>
<td>1.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-138</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>DecaBDE (Saytex102E)</td>
<td>BDE-209</td>
<td>96.8</td>
<td>Polymers such as polypropylene and high impact polystyrene</td>
</tr>
<tr>
<td></td>
<td>BDE-206</td>
<td>2.19</td>
<td>used in electronics, automobiles and construction materials</td>
</tr>
<tr>
<td></td>
<td>BDE-207</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-208</td>
<td>0.06</td>
<td></td>
</tr>
</tbody>
</table>

Due to their brominated organic structure, PBDEs exhibit Log K_{ow} values ranging from approximately 5 for mono-BDEs to 9 for the fully brominated congener BDE-209 and are very stable in many environmental matrices with half-lives ranging from 0.5 to 2 years in sediments (Wania and Dugani, 2003). Because of these physicochemical properties, PBDEs are considered to be persistent organic pollutants.
(POPs) by the United Nations Environmental Program. The PentaBDE and OctaBDE commercial mixtures were added to Annex A of the Stockholm Convention in May, 2009, which effectively banned the use of these PBDE formulations worldwide (EU, 2009a). The regulatory status of the DecaBDE mixture is currently region-specific and is more complicated than the other PBDE mixtures. In the United States, the DecaBDE mixture was scheduled for voluntary phase out of production by the end of 2013, but the DecaBDE mixture continues to be used in some applications without effective replacements. Several states in the United States have imposed stricter regulations on the use of DecaBDE, including Maine, Oregon, Washington, and Vermont (Shaw et al., 2010).

Despite the various bans and restrictions on the use of PBDEs in commercial products over the past decade, PBDEs are nearly ubiquitous in the environment due to their heavy use starting in the 1970s. One of the first studies to detect PBDEs in environmental samples recorded concentrations up to 110 μg g lipid⁻¹ in pike (Esox lucius) liver tissue in Sweden in 1980 (Andersson and Blomkvist, 1981). Over the next 30 years, a large number of studies observed increasing concentrations of PBDEs in many different environmental matrices including sediment, air, sewage sludge, wastewater, birds, marine mammals, and turtles, with concentration doubling times ranging from 1-5 years in some cases (Law et al., 2014). In general, the concentrations of components of the PentaBDE and OctaBDE mixtures in environmental samples reached a maximum
between 2000 and 2010, while the concentrations of BDE 209, the primary component of DecaBDE, may still be increasing in the environment (Law et al., 2014).

Because the DecaBDE commercial product is still in use in some areas of the world, there is continuing concern that environmental abiotic or biotic debromination of BDE-209 could function as a continuing source of lower brominated PBDEs in the environment. Debromination of BDE-209 to lower brominated PBDE congeners has been observed in lake sediments (Bogdal et al., 2010; Rodenburg et al., 2014; Tokarz et al., 2008) and as a result of photolytic debromination (Davis and Stapleton, 2009; Söderstrom et al., 2004; Wei et al., 2013). Biotransformation of PBDEs generally follows two pathways: reductive debromination, which leads to increased levels of lower brominated PBDE congeners, or hydroxylation, which forms hydroxylated PBDEs (OH-BDEs) that exhibit some unique toxicological effects compared with the parent compounds. Due to the significance of both of these pathways to the toxicology and environmental persistence of PBDEs, Chapter 2 reviews PBDE biotransformation in further detail.

1.3 Alternate Flame Retardants

In the late 1980s, concerns with PBDEs started to emerge as data became available regarding persistence in the environment and the effects of PBDEs on fire toxicity. PBDEs were shown to cause increased smoke formation, which is a major threat to human health during fires, and combustion of PBDEs was shown to form brominated
dioxins and furans (Camino and Costa, 1988; Dumler et al., 1989; Thoma and Hutzinger, 1987). Although PentaBDE was the primary flame retardant used in polyurethane foam, it appears that manufacturers were developing and using alternative flame retardants decades before the bans that occurred in the late 2000s (Shaw et al., 2010).

Identifying suitable alternatives to PBDEs has been complicated by financial considerations and the similar physicochemical properties among compounds that can effectively be used as flame retardants. Although many different types of flame retardants have been applied to products over the past 30 years, the two primary replacements of the PentaBDE mixture in polyurethane foam appear to be tris(1,3-dichloro-2-propyl) phosphate (TDCPP) and Firemaster® 550 (FM 550) (Stapleton et al., 2008a). TDCPP is a chlorinated phosphate flame retardant that was initially used as a replacement for the brominated analogue tris(2,3-dibromopropyl) phosphate (brominated Tris), which was banned from use in children’s sleepwear after it was found to be mutagenic (Blum and Ames, 1977). Shortly after brominated Tris was removed from children’s sleepwear, TDCPP was also voluntarily removed from use in children’s sleepwear due to similar concerns regarding potential mutagenicity (Gold et al., 1978). In samples taken in 2003-2009 in two different studies, TDCPP was the most commonly detected flame retardant in polyurethane foam (58% of samples contained TDCPP in one study, and 36% of samples contained TDCPP in the another study) (Stapleton et al., 2009, 2011b).
In 2008, two previously unidentified brominated flame retardants were detected in house dust samples: 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (TBB) and bis(2-ethylhexyl) 2,3,4,5-tetrabromophthalate (TBPH) (Stapleton et al., 2008a). TBB and TBPH were identified as the brominated components of the commercial flame retardants BZ-54 and FM 550, which were marketed as safer and less persistent replacements to PentaBDE for use in polyurethane foam (Stapleton et al., 2008a). FM 550 contains TBB and TBPH in a ratio of approximately 4:1 along with triphenyl phosphate (TPP) and a mixture of TPP analogs with isopropyl groups on 1-3 of the phenyl rings (commonly referred to as iTPPs) (Stapleton et al., 2008a). A novel metabolite of TBB known as 2,3,4,5-tetrabromobenzoic acid (TBBA) was detected as the major in vitro metabolite of TBB in human liver and intestinal tissues (Roberts et al., 2012) and was successfully used as a biomarker for human TBB exposure in 2 recent studies (Butt et al., 2014; Hoffman et al., 2014). Further details of the in vitro metabolism of TBB are included in Appendix B (Roberts et al., 2012).

1.4 Sources and Continued Exposure to PBDEs

Although the intentional application of Penta- and OctaBDE has effectively stopped in new products, furniture containing foam treated with PentaBDE will continue to be present in homes for years because the lifespan of furniture is approximately 30 years according to the Upholstered Furniture Action Council (Waxman et al., 2008). PBDEs are released from treated products over time by
volatilization or partitioning to small dust particles, where they accumulate in the indoor environment in house dust or are eventually released into the outdoor environment. Human exposure to PBDEs occurs via several mechanisms, including inhalation, dietary exposure, dermal absorption, and unintentional ingestion of contaminated dust particles (Lorber, 2008; Stapleton et al., 2012). The most commonly detected congeners in human serum and other tissues are BDE-47, BDE-99, BDE-153, BDE-154, and BDE-209. The most relevant exposure pathway varies regionally based on the relative usage of PBDEs. For example, ingestion of contaminated dust appears to be the most important exposure pathway for humans in the US, and especially toddlers due to increased hand-to-mouth transfer compared with adults (Sharp and Lunder, 2004; Stapleton et al., 2012). However, in Europe, dietary exposure through food and exposure in the air appear to be more relevant exposure pathways than dust ingestion due to the lower usage of PBDEs in consumer products compared with the US (Voorspoels et al., 2007). PBDEs are also maternally transferred during gestation and lactation and have been detected in human fetuses in the first trimester (Zhao et al., 2013).

Many newly manufactured products include recycled materials, including polyurethane foam and electronics casing in recycled foams and plastics (Ionas et al., 2014). Flame retardants, including PBDEs and especially BDE-209, have been detected in children’s toys that were likely produced from recycled foam and plastics (Chen et al.,
2009; Ionas et al., 2014). The concentrations of flame retardants in these toys were typically lower than the levels required to impart properties of flame retardancy (5-10%), but the maximum concentrations of BDE-209 detected in two studies were 140 μg g⁻¹ (Ionas et al., 2014) and 4.2 mg g⁻¹ (Chen et al., 2009). Because children are in close contact with toys for many hours a day, exposure to PBDEs through toys is an ongoing mechanisms of PBDE exposure, especially due to the use of recycled ingredients in toy production.

Currently, an important pathway for the release of high levels of PBDEs into the environment and high levels of human exposure is the disposal of discarded electronic products (e-waste). The issue of appropriate e-waste disposal is especially relevant in China, where both legitimate and black-market recyclers purchase e-waste from entities around the world and often employ crude techniques to extract precious metals from e-waste (Chi et al., 2011). Many of the discarded electronics contain PBDEs (including OctaBDE congeners if the products were produced before the ban) or other brominated flame retardants in addition to heavy metals and other toxic chemicals. The median level of BDE-209 in the serum of e-waste recyclers in a Chinese study was 256 ng g lipid⁻¹, which is approximately 20-30 times higher than concentrations in the general US population. In the same study, a 36-year-old female who had worked as an e-waste recycler for 6 years exhibited a serum BDE-209 concentration of 2,716 ng g lipid⁻¹ and a total serum PBDE concentration of 4,099 ng g lipid⁻¹ (Zheng et al., 2014), which represent
some of the highest recorded levels of PBDEs in human serum. The pyrolytic techniques used to extract metals from e-waste, which sometimes are as simple as placing circuit boards over a charcoal grill (Chi et al., 2011), may represent a source of PBDEs to the air via volatilization, induce debromination of BDE-209, and expose workers to very high concentrations of PBDEs. Other particularly highly exposed workers include carpet installers and foam recyclers (Stapleton et al., 2008d). Exposure to high levels of PBDEs and other brominated flame retardants to e-waste recyclers and other highly exposed workers should be considered when estimating the doses in risk assessments for decisions regarding the regulation of PBDEs.

1.5 Toxicity of PBDEs

The initial concerns regarding PBDEs related mainly to their persistence in the environmental and their detection in human tissues and breast milk in the late 1990s, although little was known regarding the toxicity of PBDEs besides their structural similarity to other organohalogens, such as PCBs, dibenzo-p-dioxins, and dibenzofurans (Darnerud et al., 2001). Early information regarding PBDE toxicity was complicated by the presence of toxic synthetic byproducts, including 2,3,7,8-tetrabromodibenzo dioxin (2,3,7,8-TBDD) at concentrations of approximately 0.1 ng g⁻¹ and several tetra-, penta-, and hexabromodibenzo furans (BDFs) at concentrations ranging from 3.7-43 ng g⁻¹ in the commercial PentaBDE mixture DE-71 (Sanders et al., 2005). Therefore, studies performed using the DE-71 commercial mixture must be interpreted with caution as the
effects may be mediated by impurities. By the early 2000s, toxicological studies had determined that although specific PBDE congeners caused very little acute toxicity, they impaired neurodevelopment in mice, decreased thyroid hormone levels in mice, and displaced thyroid hormones from the serum thyroid hormone transport protein transthyretin (Eriksson et al., 2001; Hallgren et al., 2001; Meerts et al., 2000). These studies also determined that lower brominated PBDE congeners (<7 bromines) were generally more toxic for most endpoints compared with higher brominated congeners and BDE 209 (Darnerud et al., 2001). Since 2000, more detailed toxicological information regarding specific PBDE congeners and especially OH-BDE metabolites in rodents, fish, and human cohort studies has emerged. Several authors have reviewed the full toxicological profile of PBDEs including effects on reproduction, oxidative stress, and other endpoints (Birnbaum and Staskal, 2004; Costa and Giordano, 2011; Shaw et al., 2010). The following sections will focus detailed toxicological discussion to the current knowledge of the effects of PBDEs on the thyroid system and neurodevelopment because these effects have been repeated in a large number of studies and appear to exhibit the most important risk to human health.

1.5.1 Thyroid Hormone Effects

Thyroid hormones are an important component of the human endocrine system and are vital in regulating development and metabolism (Williams, 2008). Thyroxine (T₄) is considered to be a prohormone because it is converted to triiodothyronine (T₃) in the
tissues of the body to elicit effects. Although T₄ exerts some effects via nongenomic pathways, T₃ is considered the active thyroid hormone and complexes with nuclear thyroid hormone receptors (TRs) to act as a ligand-bound transcription factor. The ligand-bound TRs then bind to thyroid response elements (TREs) located in the promoter region of many important genes regulating various physiological processes including metabolism, proliferation, differentiation, and other processes vital for cell development and homeostasis (Williams, 2008). Disruptions in circulating thyroid hormone levels caused by exposure to toxicants represent a serious concern when assessing the risk of the toxicants to human health. PBDEs and especially hydroxylated PBDE metabolites (OH-BDEs) share a similar polyhalogenated diphenyl ether chemical structure with thyroid hormones, although thyroid hormones are substituted with iodine instead of bromine and contain an amino acid group from the initial tyrosine structure used in thyroid hormone synthesis in the thyroid gland. Due to this structurally similarity to thyroid hormones, many different studies have assessed the effects of PBDEs on several aspects of the thyroid system including serum thyroid hormone concentrations, transmembrane transport proteins, serum transport proteins, thyroid nuclear receptors, thyroid conjugating enzymes, and thyroid selenodeiodinases (DIs).
1.5.1.1 Effects of PBDEs on Thyroid Hormone Clearance and Transport

In rodent studies, the most commonly reported effect of PBDEs on thyroid hormone regulation is altered serum T4 concentrations. Developmental exposure to PBDEs either by maternal exposure during gestation or by dosing 7-week old rats caused decreased total and free (unbound to serum proteins) T4 and T3 concentrations in rats from directly after birth to postnatal day (PND) 22 (Darnerud et al., 2007; Kuriyama et al., 2007). However, adult rats administered PBDEs do not demonstrate the repeatable decrease in thyroid hormone levels observed in developmentally exposed rats and have shown no effect or increased T4 levels (Alonso et al., 2010; Hakk et al., 2002). In humans, increased serum PBDE concentrations have been associated with increased serum T4, T3, and TSH concentrations (Dallaire et al., 2009; Turyk et al., 2008; Yuan et al., 2008) and decreased serum T4 concentrations (Herbstman et al., 2008). The alterations in thyroid hormone levels in rodents and humans do not typically exceed the limits of the normal range of thyroid hormones. However, the altered levels of thyroid hormones, whether increased or decreased, may still disturb the homeostasis of the thyroid system and may indicate that the thyroid system is attempting to compensate the effects to maintain the thyroid hormone levels within the normal range.

Two commonly proposed mechanisms of the observed alterations in serum thyroid hormone levels are changes in the activity of the thyroid hormone-conjugating enzyme uridine 5’-diphospho-glucuronosyltransferase (UGT) and changes in the
binding of thyroid hormones to serum transport proteins (Richardson et al., 2008; Szabo et al., 2009). Increased hepatic UGT activity and subsequent hepatic biliary clearance of conjugated thyroid hormones has been shown to result from constitutive androstane receptor (CAR) induction by PBDEs (Richardson et al., 2008). Thyroid hormones are also conjugated by sulfotransferase enzymes expressed throughout the body, and the sulfated thyroid hormones are rapidly deiodinated and cleared from circulation. OH-BDE metabolites and similar compounds were shown to inhibit the sulfation of T₃ in human liver microsomes with IC₅₀ values in the low nM range in human liver microsomes (Butt and Stapleton, 2013). Disruption of circulating thyroid hormone levels could be mediated by these alterations in thyroid hormone clearance.

Because of their low water solubility, thyroid hormones are bound to transport proteins in the blood as they are distributed to peripheral tissues. The free fraction of T₄ in human serum is only 0.03%, and thyroid-binding globulin (TBG) transports approximately 75%, TTR transports 20%, and human serum albumin transports the remaining 5% of bound T₄ in human serum (Marchesini et al., 2008; Schreiber, 2002). Competitive inhibition of the binding of T₄ to TBG and TTR has been reported at concentrations as low as 22-100 nM by 6-OH-BDE-47 and other OH-BDEs (Marchesini et al., 2008). Significant effects on serum thyroid hormone transport have only been identified for OH-BDEs and 2,4,6-tribromophenol, and only very weak inhibition of serum T₄ binding has been reported for PBDE congeners (Marchesini et al., 2008). These
mechanisms of thyroid disruption may cause changes in the serum concentrations of thyroid hormones, which are easily detected and often reported in toxicology and epidemiology studies. However, the concentrations of thyroid hormones in the peripheral tissues may be more relevant to physiologically significant alterations in thyroid signaling.

1.5.1.2 Effects of PBDEs on Deiodinases

The iodothyronine selenodeiodinases (DIOs) are a class of membrane-bound enzymes that catalyze the reductive deiodination of thyroid hormones. DIs belong to the thioredoxin superfamily of enzymes due to the presence of a disulfide-forming thioredoxin fold in the active center (Bianco and Larsen, 2005). DIs also contain the rare amino acid selenocysteine in the active center, which is critical to the catalytic activity of DIs. Selenocysteine is encoded by the UGA codon, which is normally read as a STOP codon. A sequence known as the selenocysteine insertion sequence located directly downstream from the UGA codon allows the selenocysteine amino acid to be incorporated (Bianco and Larsen, 2005). Three DI isoforms are known to exist and share 50% sequence identity, similar molecular weight ranges (29-32 kd), and high sequence homology in the active center (Bianco and Larsen, 2005). However, the catalytic properties, physiological roles and regions of expression vary among the three DI isoforms, as shown in Table 2.
Table 2: Properties of the three DI isoforms obtained summarized from the following
reviews (Bianco and Kim, 2006; Bianco and Larsen, 2005; Köhrle, 2002)

<table>
<thead>
<tr>
<th>Biochemical Properties</th>
<th>Type 1 Deiodinase (DIO1)</th>
<th>Type 2 Deiodinase (DIO2)</th>
<th>Type 3 Deiodinase (DIO3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalyzed Reactions</td>
<td>T₄→T₃</td>
<td>T₄→T₃</td>
<td>T₄→rT₃</td>
</tr>
<tr>
<td></td>
<td>T₄→rT₃</td>
<td>rT₃→T₂</td>
<td>T₃→T₂</td>
</tr>
<tr>
<td></td>
<td>rT₃→T₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substrates</td>
<td>T₄, T₃, rT₃</td>
<td>T₄, rT₃</td>
<td>T₄, T₃</td>
</tr>
<tr>
<td>Km</td>
<td>T₄: 2 µM</td>
<td>T₄: 1-2 µM</td>
<td>T₄: 5-10 nM</td>
</tr>
<tr>
<td></td>
<td>T₃: 8-10 µM</td>
<td>rT₃: 1-2 µM</td>
<td>T₃: 1-2 nM</td>
</tr>
<tr>
<td></td>
<td>rT₃: 0.2-1 µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tissue Expression</td>
<td>Liver, Kidneys</td>
<td>Brain, Brown Adipose</td>
<td>Brain, Placenta</td>
</tr>
<tr>
<td>Approximate in vivo half-life</td>
<td>5-10 h</td>
<td>15 min- 1 h</td>
<td>5-10 h</td>
</tr>
<tr>
<td>Cellular Location</td>
<td>Plasma Membrane</td>
<td>Endoplasmic Reticulum</td>
<td>Plasma Membrane</td>
</tr>
<tr>
<td>Molecular Weight (kd)</td>
<td>29</td>
<td>30.5</td>
<td>31.5</td>
</tr>
</tbody>
</table>

Type 1 deiodinase (DIO1) is mainly expressed in the liver and kidney and
catalyzes the deiodination of T₄, T₃, and rT₃, although in vivo, the primary roles of DIO1
are production of small amounts of circulating T₃ and clearance of rT₃ (Figure 2) (Bianco
and Larsen, 2005). DIO1 catalyzes deiodination reactions following a two-substrate
ping-pong mechanism in which an intermediate enzyme complex (enzyme-selenium-iodide) is formed after the deiodination reaction with T₄ or rT₃. The intermediate is then reduced by dithiothreitol (DTT) or other reducing thiols in vitro, or by a yet unidentified reducing compound in vivo (Köhrle, 2002). The ping-pong reaction mechanism is not observed in other DIs and allows DIO1 to be distinguished by its susceptibility to inactivation by 6-\textit{n}-propyl-2-thiouracil (PTU) or other thiouracil compounds (Köhrle, 2002). This susceptibility to PTU inhibition allows moderate cases of hyperthyroidism to be controlled by administration of PTU, which decreases serum T₃ concentrations (Bianco and Kim, 2006). DIO1 is also unique among the DIs because it catalyzes the deiodination of T₄ to either T₃ or rT₃, while the other DIs appear to catalyze reactions producing one product per substrate. In humans, the balance between T₃ formation and rT3 formation by DIO1 is approximately equimolar, and because of this balance between T₄ activation and inactivation, the physiological significance of DIO1 is generally thought to be lower than the other DI isoforms (Bianco and Kim, 2006).
Figure 2: Deiodinase-mediated thyroid hormone metabolism. The catalytic roles of each DIO isoform are indicated beside the arrows for each reaction.

Type 2 deiodinase (DIO2) converts T₄ to T₃ in the brain and in some other tissues with high metabolic demand such as muscle and brown adipose tissue. In rats, DIO2 produces approximately 75% of the T₃ located in the cerebral cortex (Bianco et al., 2002a). Unlike DIO1, the deiodination reaction catalyzed by DIO2 proceeds via a two-substrate sequential mechanism that is not susceptible to inactivation by PTU, but still requires a reducing thiol compound (DTT in vitro, unknown in vivo) as a cosubstrate to perform the reaction (Köhrle, 2002). The half-life of DIO2 is significantly shorter than the other two DI isoforms (15 min-1 h compared with 10-15 h), which allows its levels in tissues to be rapidly adjusted to tightly control the peripheral concentrations of T₃. The
short half-life of DIO2 is due to rapid ubiquitination of DIO2 after binding of a substrate to the active site followed by subsequent removal from the endoplasmic reticulum membrane. Ubiquitinated DIO2 is then either degraded by proteasomes or deubiquitinated and reactivated once it is reinserted into the endoplasmic reticulum membrane (Bianco et al., 2002a). The posttranslational regulation of proteasomal DIO2 degradation appears to be the most important mechanism of DIO2 regulation, and therefore, T3 regulation in the brain. DIO2 synthesis is also regulated via transcriptional mechanisms involving either induction by cyclic adenosine monophosphate (cAMP) or repression by T3. The cAMP-mediated induction of DIO2 is related to adaptive thermogenesis and is important in generating heat in cold environments, especially in brown adipose tissue. The 5′ flanking region of the DIO2 gene contains a cAMP response element, but no studies have been able to prove the presence of a negative thyroid response element for DIO2 although T3 downregulates DIO2 mRNA transcription (Bianco et al., 2002a). DIO2 is expressed in astrocyte cells in the brain, which are specialized glial cells in the brain that function as a metabolic barrier to neurons. Decreased DIO2 activity in response to both T3 and T4 in astrocyte cells ensure that the appropriate level of T3 reaches neuronal cells.

Type 3 deiodinase (DIO3) is known as the inactivating deiodinase because it catalyzes the deiodination of T4 to form rT3 and the deiodination of T3 to form T2. The sequential two-substrate reaction mechanism is similar to DIO2. Regulation of DIO3
activity occurs via transcriptional and post-translational mechanisms. T₃ increases DIO3 activity by activating DIO3 mRNA transcription, and DIO3 activity can be decreased post-translationally by internalization of DIO3 from the plasma membrane to endosomes (Baqui et al., 2003). The primary function of DIO3 is to protect tissues from the proliferative and differentiating effects of excess T₃ concentrations. This physiological role is very significant during the development of highly organized tissues, such as neuronal networks in the brain, in which exposure to adult levels of T₃ would cause premature differentiation of cells and subsequently impaired structural and functional development (Bianco et al., 2002a). DIO3 is primarily expressed in the placenta and in neuronal cells, where controlling the effects of T₃ during early development are most important. The free fractions of T₄ and T₃ in the fetal serum are also higher compared with the maternal serum, so the total concentrations of T₄ and T₃ must be lowered in the placenta by DIO3 to maintain normal free thyroid hormone levels in the fetus (Williams, 2008). In the brain, DIO2 in astrocyte cells and DIO3 in neuronal cells work together to maintain a T₃ concentration that is neither too high nor too low for proper neurodevelopment.

Due to the previously described effects of PBDEs on multiple aspects of the thyroid system, it is reasonable that PBDEs could affect DI activity either as a compensatory mechanism or by competitive inhibition; however, few studies have directly evaluated the effects of PBDEs on DIs. Decreased expression of DIO1 by up to
70% was observed in rats developmentally exposed to the DE-71 commercial PBDE mixture (Szabo et al., 2009). OH-BDEs, including the major metabolite of BDE-99, 5′-OH-BDE-99, inhibited DIO1 activity with an IC₅₀ of approximately 400 nM in human liver microsomal assays, and no inhibition was detected with BDE-99 (Butt et al., 2011). While these studies have suggested that alterations in DI activity might be an important mechanism of toxicity for PBDEs and OH-BDEs, no other studies have measured the activity of DIs in mammals or in mammalian tissues exposed to PBDEs. Several studies, however, have investigated the effects of PBDEs and OH-BDEs on DI activity and mRNA expression in fish such as fathead minnows and zebrafish. Dietary exposure of fathead minnows to BDE-209 caused the DIO2 activity in liver and brain tissue to decrease by 50-70% with corresponding decreases in the serum T₄ and T₃ concentrations in two different studies (Noyes et al., 2011, 2013). In zebrafish embryos, 6-OH-BDE-47 caused increased DIO1 and DIO3 mRNA expression in specific areas of the brain, which were detected using whole mount in situ hybridization (Dong et al., 2013). Several other studies have observed effects on the mRNA expression of deiodinases in tissues exposed to PBDEs, but to date, no other studies have determined the effects of PBDEs and OH-BDEs on DI activity.

1.5.1.3 Effects of PBDEs on Thyroid Receptor Binding

In addition to effects on circulating and peripheral thyroid hormone levels, PBDEs and OH-BDEs appear to alter thyroid hormone-related endpoints via interactions
with thyroid receptors (TRs). Because of the potential consequences of binding to TRs or altering the binding of T₃ to TRs by competitive or noncompetitive interactions, several studies have attempted to determine whether PBDEs and OH-BDEs interact with TRs. The OH-BDEs 4-OH-BDE-90 and 3-OH-BDE 47 competitively inhibited T₃ binding to human TR, and 4-OH-BDE-90 was also found to be a weak TR agonist in a follow-up study, and parent PBDE congeners showed no effects (Kitamura et al., 2008; Kojima et al., 2009). Another study reported that the PentaBDE commercial mixture and BDE-100, -153, -154, and -209 suppressed TR-mediated transcription, but observed no interactions of several OH-BDEs with TR-mediated gene transcription (Ibhazehiebo et al., 2011). In contrast to these results, BDE-127, BDE-185, 4-OH-BDE-69, and 4-OH-BDE-121 were found to be TR agonists (Freitas et al., 2011; Hamers et al., 2008). These results taken together indicate that PBDEs and OH-BDEs have the potential to interact with TRs based on their structural similarity to thyroid hormones, but more studies are necessary to fully characterize these interactions.

1.5.2 Neurodevelopmental Effects

Impaired neurodevelopment is a major concern to public health and may be a sensitive endpoint to toxicant exposure because disruption of many different physiological processes can impair neurodevelopment. Over 2,000 years ago, lead was determined to impair neurodevelopment by the Greek physician Discerides (Szpir, 2006). In the early 1900s, iodine deficiency was found to cause goiter and delayed
development in school children (Zimmermann, 2011). Public health measures to limit lead exposure and iodine deficiency were both designed to combat neurodevelopmental impairment, but other metals, xenobiotics, and infectious pathogens have also been linked with impaired neurodevelopment (Szpir, 2006). PBDEs are often included in lists of suspected neurodevelopmental toxicants due to associations of PBDEs and OH-BDEs with impaired neurodevelopment in human epidemiological studies, rodent exposure studies, and in vitro mechanistic neurotoxicity studies.

1.5.2.1 Rodent Exposure Studies

A series of studies performed between 2001 and 2008 in neonatal mice exposed to a single oral dose of PBDEs on PND 10 have provided a large amount of data regarding the effects of PBDEs on neurodevelopment (Eriksson et al., 2001; Viberg et al., 2003, 2004, 2006). These studies describe significant effects of BDE-47, BDE-99, BDE-153, BDE-203, BDE-206, and BDE-209 on endpoints including decreased spatial learning, locomotion, rearing, and habituation capability. There has been some debate about the relevance of the single PND10 dosing regime and statistical design of the studies performed by Viberg et al., but studies by other groups using different dosing and exposure regimes confirmed many of these effects using neonatal exposures (Kuriyama et al., 2005) and gestational exposures to BDE-99 through maternal transfer and lactation (Branchi et al., 2002, 2003). PBDEs also altered the levels of several important proteins in the brain after neonatal exposures including brain-derived neurotrophic factor,
calcium/calmodulin kinase II, and synaptophysin (Viberg et al., 2008; Viberg, 2009). The results of these studies indicate that PBDEs alter the physiology of the developing brain in mice, especially when exposure occurs during the most sensitive window between birth and PND14. However, these studies were unable to determine the mechanism by which PBDEs alter neurodevelopment in mice.

1.5.2.2 Human Epidemiological Studies

Effective measurements of impaired neurodevelopment in human studies can be difficult to obtain and interpret, especially in humans, and are often confounded by many factors. Nevertheless, numerous studies have reported associations between PBDEs and altered neurodevelopmental endpoints including standardized developmental tests assessing for ages 1-9 and standard IQ tests. To summarize these results, maternal PBDE levels during pregnancy have been significantly associated with impaired attention (Eskenazi and Chevrier, 2012; Gascon et al., 2011; Roze et al., 2009), impaired fine motor coordination (Eskenazi and Chevrier, 2012; Gascon et al., 2011), decreased full-scale IQ (Eskenazi and Chevrier, 2012; Herbstman et al., 2010), impaired psychomotor development (Herbstman et al., 2010), and decreased adaptive behavior (Shy et al., 2011). However, PBDEs have also been significantly associated with better cognition scores (Shy et al., 2011), better coordination, better behavior, and better visual perception (Roze et al., 2009). While a relatively small number of studies have contributed data relating PBDE exposure with neurodevelopmental endpoints, it is clear
that these results should be further examined to determine the mechanisms and toxicological relevance of these associations.

1.5.2.3 In Vitro Studies

In cell-culture based experiments, BDE-47 and BDE-99 cause apoptotic cell death at concentrations greater than 10-50 µM in various cell types including human astrocytoma cells (Madia et al., 2004), mouse neurons (Giordano et al., 2008), and rat cerebellar granule cells (Reistad et al., 2006), among others. PBDEs have also caused more specific effects in cultured neuronal cells including translocation of protein kinase C (Kodavanti et al., 2005; Madia et al., 2004), inhibition of calcium uptake (Coburn et al., 2008; Kodavanti and Ward, 2005), differentiation (Zhang et al., 2010), and migration (Schreiber et al., 2010).

A series of studies analyzing the effects of PBDEs and OH-BDEs on calcium signaling in PC12 cells determined that BDE-47 and, to a much greater extent, its metabolite 6-OH-BDE-47 caused increased exocytosis and intracellular calcium levels (Dingemans et al., 2011). Similar effects were not observed when the cells were exposed to other PBDEs including BDE-49, BDE-99, BDE-100, and BDE-153, which indicated the potential increased toxicity of BDE-47 compared with other congeners. Similar to the previously described thyroid hormone studies, hydroxylation of BDE-47 also greatly increased the toxic effects, indicating the significance of PBDE bioactivation in vivo (Dingemans et al., 2011). All the studies showing effects of PBDEs and especially OH-
BDEs on intracellular signaling and neurotransmitter release indicate a potential mechanism of disrupted neurodevelopment resulting from PBDE exposure. However, these studies were unable to determine the specific molecular target of these effects because all of these processes are interrelated and can be altered by numerous mechanisms. Furthermore, the doses of PBDEs or OH-BDEs required for statistically significant alterations in most of these endpoints were typically greater than 1 μM, which is below the threshold for overt toxicity, but higher than the expected concentrations in exposed humans. Further studies to determine the specific molecular target of altered neuronal physiology may provide better insight into the relevance of these mechanisms for causing impaired neurodevelopment.

1.5.2.4 Thyroid Hormones and Neurodevelopment

Maternal thyroid hormone status plays an important role in neurodevelopment. Until 10-12 weeks gestation, all fetal T₃ is maternally derived, and the fetal thyroid gland starts significantly contributing to T₃ levels at 18-22 weeks (Zimmermann, 2011). Therefore, many different maternal factors can affect the levels of thyroid hormones reaching the developing fetus (Porterfield, 2000). Endemic cretinism caused by iodine deficiency is by far the most preventable cause of neurologic impairment and results from severely decreased thyroid hormone levels during development (Zimmermann, 2011). A very small amount of fetal T₃ is maternally derived; most fetal T₃ formed by DIO2 expressed in fetal tissues. Therefore, decreased maternal T₃ levels are generally
less predictive of impaired neurodevelopment, while decreased maternal T₄ levels can have more significant effects on neurodevelopment (Zimmermann, 2011). Therefore, hypothyroidism, especially decreased T₄ levels during pregnancy, may contribute to decreases in fetal T₄ and T₃ levels.

The role of T₃ in the developing brain is complex, and the T₃ concentration in the brain must be tightly regulated to ensure proper neurodevelopment. In the first trimester, T₃ regulates microtubule formation and organization in radial glial cells and neurons as the cells proliferate and organize into neural networks via TR-mediated transcription (Nunez et al., 1991). Unliganded TRs are also thought to act as repressors of important genes (e.g., Sox2 and neurogranin) during early development to maintain proliferation and prevent differentiation, which may further compound the effects of decreased T₃ concentrations in the brain (Bernal and Morte, 2013). At 10 weeks gestation, 25% of TRs are occupied by T₃ in the normal human fetal brain, and significantly increased occupancy of TRs during the first trimester may also disrupt neurodevelopment by promoting premature differentiation and migration of immature neural structures (Bernal and Morte, 2013; Préau et al., 2014). During the second trimester, the levels of T₃ in the fetal brain slowly increase as ligand-bound TRs signal differentiation, axon and dendrite migration, synapse formation, and myelination (Williams, 2008).
The enzymatic activities of DIO2 and DIO3 during development are the most important regulators of T3 concentrations in the developing fetal brain. During the first trimester, the activity of DIO2 slowly increases while the activity of DIO3 slowly decreases as the levels of T3 increase in the fetal brain (Williams, 2008). An example of the importance of the timing of DIO2 and DIO3 alterations during development is the altered cochlear structure in DIO2 knockout mice resulting in deafness (Ng et al., 2004). In the developing cochlea, DIO2 expression increases rapidly at certain times in certain regions of the cochlea to locally stimulate proliferation and differentiation in the organization of the complex cochlear structure. In DIO2 knockout mice, the cochlear structure does not develop properly due to the lack of cellular-specific DIO2 expression during cochlear development (Ng et al., 2004). Other tissues in the developing brain rapidly alter the expression of DIO2 and DIO3 at critical times during development to ensure successful T3 signaling (Bianco and Kim, 2006). Astrocyte cells located at the blood brain barrier activate T3 by expressing DIO2, and neurons deactivate T3 by expressing DIO3. Because astrocytes and neurons work together to organize neural structures in the developing brain, perturbations in the functioning of these enzymes may impair neurodevelopment. While PBDEs have been shown to inhibit hepatic DIO1 activity, the effects of PBDEs on the regulation of DIO2 and DIO3 activity have not been evaluated and may represent a potential mechanism of the neurodevelopmental impairments associated with PBDE exposure.
1.6 Thesis Research Aims

The objective of this thesis research was to assess the effects of PBDEs and OH-BDEs on thyroid hormone regulation within specialized cells in the human brain. The main hypothesis was that PBDEs and OH-BDE metabolites disrupt thyroid hormone metabolism in human astrocytes and neuronal cells by decreasing the activity of DIO2. The following research aims were conducted to test this hypothesis:

Aim 1: Determine whether PBDEs are hydroxylated in human astrocyte cells [Chapter 2]. In vitro experiments were performed to examine PBDEs metabolism in human astrocyte cells based on the potential of astrocytes to express the well-characterized PBDE-hydroxylating enzyme CYP2B6.

Aim 2: Determine the effects of PBDEs and OH-BDEs on Type 2 deiodinase activity (DIO2) in human astrocyte cells to assess their potential impacts on T₃ concentrations in the developing brain [Chapter 3]. Cultured human astrocyte cells and an astrocyte cell line were exposed to BDE-47, -99, -153, and -209, 3-OH-BDE-47, 6-OH-BDE-47, and 5’-OH-BDE-99 to determine the effects on DIO2 activity, which is the primary source of T₃ to the developing brain. Further experiments were performed to determine the mechanisms of altered DIO2 activity.

Aim 3: Determine the effects of PBDEs and OH-BDEs on thyroid regulation in co-cultured human astrocyte and neuroblastoma cells and evaluate downstream indicators of thyroid hormone disruption in these cells [Chapter 4]. A co-culture
experimental design using a glioma cell line and a neuroblastoma cell line designed to represent thyroid regulation in the human brain was exposed to BDE-99, 3-OH-BDE-47, and 5’-OH-BDE-99 for 48 h, and the effects on DIO2 activity, DIO3 activity, mRNA expression of 5 thyroid-regulated genes, and 6 different parameters of metabolic energy expenditure were evaluated.

Chapter 5 summarizes the major findings of this thesis research and integrates the discussion of these findings with the current state of knowledge regarding the effects of PBDEs on neurodevelopmental toxicity.
2. Biotransformation of Flame Retardants

This chapter provides an overview and summary of the state of the knowledge on PBDE biotransformation reactions in vertebrates. Also presented are summaries of my published studies on metabolism of flame retardants. As part of my dissertation research I conducted two different metabolism experiments that resulted in peer-reviewed publications. The first was a paper investigating PBDE metabolism among several fish species (Roberts et al. 2011). The second study investigated the in vitro metabolism of two new brominated flame retardant chemicals in human and rat hepatic tissues (Roberts et al. 2012). PBDE metabolism was also investigated in the cell culture experiments described in Chapters 3 and 4, and a summary of those experiments are provided below. However, those cells were not capable of PBDE biotransformation, and a more complete study investigating PBDE metabolism in astrocytes was not conducted.

2.1 Biotransformation of Xenobiotics

Biotransformation of xenobiotics is an important area of research and is required to fully understand the ultimate fate and toxicity of a contaminant. The major biotransformation pathways are conserved across many species and consist of enzymatic oxidation, reduction, hydrolysis, and other reactions that add or remove one or two atoms (Phase I metabolism) and conjugation reactions that add more complex and often polar moieties to the xenobiotic structure, such as glucuronic acid, sulfate, amino acids, and glutathione (Phase II metabolism). Most biotransformation reactions
form products that are more water soluble and more easily excreted than the parent compounds. In some cases, and especially in the case of very nonpolar halogenated contaminants, increasing the water solubility by addition of a polar functional group, such as hydroxyl, also increases the reactivity of the compound, and may increase the potential for interactions with macromolecules and thus, increase toxicity (Guengerich, 2008).

### 2.2 Biotransformation of PBDEs

The biotransformation of the flame retardants PBDEs has been studied in many different organisms including fish, rats, human cells, polar bears, seals, birds, and marine organisms (Letcher et al., 2010; Routti et al., 2008; Stapleton et al., 2008c, 2004c). In general, PBDE biotransformation follows two basic pathways: either reductive debromination to produce lower brominated PBDE congeners or aryl hydroxylation to form OH-BDEs. In addition, methoxylated PBDEs, brominated phenols, and PBDE conjugates (e.g., glutathione and glucuronidated conjugates) have also been detected as biotransformation products (Chen et al., 2006; Stapleton et al., 2008c; Wiseman et al., 2011). Biotransformation is generally considered to increase the toxicity and bioaccumulation potential of PBDEs. Reductive debromination of PBDEs in organisms may act as a source of more toxic, persistent, and bioaccumulative lower brominated PBDE congeners. This has been observed for BDE-209 (Noyes et al., 2011; Stapleton et al., 2006). In contrast, OH-BDEs are more polar and have lower half-lives in the body,
but several toxicology studies have found that they are more potent compared with the parent PBDE congeners (Dingemans et al., 2008; Kojima et al., 2009). Therefore, the formation of OH-BDEs is considered to be an important bioactivation pathway for PBDEs.

### 2.2.1 Oxidative Biotransformation

Oxidative PBDE metabolism occurs via aryl hydroxylation mediated by cytochrome P450 (CYP) enzymes forming OH-BDEs. Cleavage of the diphenyl bond can also occur and form brominated phenols (Stapleton et al., 2008c). OH-BDEs have been found to bioaccumulate in wild fish, sometimes at higher concentrations than PBDEs (Valters et al., 2005; Wan et al., 2009), although there is debate about the source of the OH-BDEs. Methoxylated PBDEs are formed naturally in the marine environment by algae and bacteria, and there is some evidence that both naturally formed methoxylated PBDEs and xenobiotic PBDEs may be metabolized to OH-BDEs in fish (Wan et al., 2009; Zeng et al., 2013; Zhang et al., 2014). In mammals, PBDE hydroxylation has been characterized in vivo, in cell cultures, and in vitro. Because of the potential for increased toxicity of OH-BDEs compared with PBDEs, knowledge of the rates, mechanisms, and tissue-specific regions of PBDE hydroxylation is important for the assessment of PBDE toxicity.

In several studies, rats exposed to BDE-209 excreted various debrominated and hydroxylated metabolites with 5-9 bromine atoms (Huwe and Smith, 2007; Morck et al.,
2003; Riu et al., 2008). Rats exposed to BDE-99 excreted OH-BDEs, debrominated OH-BDEs, 2,4,5-tribromophenol, and various glucuronide- and glutathione-conjugated OH-BDEs and 2,4,5-tribromophenol metabolites (Chen et al., 2006). Although multiple in vivo exposures of rats to PBDEs resulted in the formation of debrominated OH-BDE metabolites, in vitro studies using human liver microsomes and human hepatocytes have provided little or no evidence for PBDE debromination. In human liver microsomes, BDE-47 was hydroxylated to form tetra-OH-BDE metabolites (3-OH-BDE-47, 5-OH-BDE-47, 6-OH-BDE-47, 4-OH-BDE-42, 4’-OH-BDE-49), 2,4-dibromophenol, and minor amounts of triOHBDE metabolites (4’-OH-BDE-17 and 2’-OH-BDE-28) (Erratico et al., 2013; Feo et al., 2013). BDE-99 was hydroxylated by human liver microsomes to form 2,4,5-tribromophenol (2,4,5-TBP), 4-OH-BDE-90, 5’-OH-BDE-99, 6’-OH-BDE-99, 4’-OH-BDE-101, and other minor OH-BDE metabolites (Erratico et al., 2012). In these studies, the major metabolites formed from BDE-99 were 2,4,5-tribromophenol and 5’-OH-BDE-99, and the major metabolites formed from BDE-47 were 5-OH-BDE-47 and 6-OH-BDE-47. Similar metabolites of BDE-99 were identified in human hepatocytes, and the most common metabolites of BDE-99 are shown in Figure 3 (Stapleton et al., 2008c).
Figure 3: Major oxidative metabolites of BDE-99 in human liver tissues and hepatocytes (Erratico et al., 2012; Stapleton et al., 2008c).

In humans, PBDE hydroxylation is mediated by CYP2B6, which is one of the major drug metabolizing enzymes (Wang and Tompkins, 2008). Studies assessing PBDE biotransformation using dozens of recombinant CYPs have indicated that PBDE hydroxylation reactions are solely catalyzed by CYP2B6 in human tissues (Erratico et al., 2012, 2013). The addition of CYP2B6 antibodies to human liver microsomes eliminated the formation of hydroxylated metabolites of BDE-99, which provides evidence for the role of CYP2B6 in mediating this metabolism (Erratico et al., 2012).

In humans, CYP2B6 contributes 2-10% of the total hepatic CYP450 content (Wang and Tompkins, 2008), and is also expressed in astrocyte cells located at the blood brain
barrier (Meyer et al., 2007). Because PBDE metabolism appears to be mediated only by CYP2B6, changes in the expression levels and localization of CYP2B6 and polymorphisms in the gene may significantly alter the clearance of PBDEs from human tissues and affect the formation of OH-BDEs. For example, the expression of CYP2B6 was 2-4 times higher in various regions of the brains of alcoholic smokers compared with nonalcoholic nonsmokers (Miksys, 2003). Furthermore, at least five nonsynonymous single nucleotide polymorphisms of CYP2B6 have each been characterized in 1-32% of the population, and the catalytic activity of CYP2B6 in some of these subjects was decreased by up to 95% (Lang et al., 2001). These special cases could greatly impact the biotransformation of PBDEs by decreasing elimination of PBDEs (e.g., polymorphisms causing reduced CYP2B6 activity) or by increasing the amount of OH-BDEs formed in the brain (e.g., through nicotine and alcohol induction of CYP2B6).

2.2.2 Reductive Debromination

The detection of debrominated PBDEs and OH-BDE metabolites in rat tissues following exposure to PBDEs indicates that debromination of PBDEs can occur in mammalian tissues, but the mechanism of this reaction is unclear. In vitro biotransformation assays using mammalian hepatic tissues were not capable of forming debrominated metabolites. It is possible that debromination occurs in the gut microflora or in extrahepatic tissues. Although the relevance of reductive debromination in
mammals remains unclear, reductive debromination of PBDEs in fish tissues has been observed at high rates in multiple studies.

In the 1990s, researchers started to notice that the PBDE congener profiles in wild fish varied significantly among species, suggesting either drastic differences in sources, or differences in metabolism. For example, wild Chinook salmon (*Oncorhynchus tshawytscha*) typically accumulate higher percentages of BDE-47, and -99 compared with other congeners (Montory et al., 2010; Sloan et al., 2010), while wild common carp (*Cyprinus carpio*) typically accumulate higher proportions of BDE-47 and -100, but little or no BDE-99 (Pérez-Fuentetaja et al., 2010; Xia et al., 2008). This was of interest because BDE-99 was the most abundant congener in the common PBDE commercial mixture (PentaBDE).

It is now known that several species of fish can rapidly debrominate some PBDE congeners. This has been confirmed both *in vivo* and *in vitro*, and variability in the rates of PBDE debromination have been observed in several studies (Browne et al., 2009; Noyes et al., 2011, 2010). For example, carp metabolically debrominate BDE-99 at a faster rate than Chinook salmon, and the metabolic products differ between the two species (BDE-47 was formed in carp and BDE-49 was formed in salmon) (Browne et al., 2009; Noyes et al., 2010). Previous studies in carp have also shown that the metabolic debromination of BDE-99, -183, and -209 favored the removal of *meta*-substituted bromine atoms (Stapleton et al., 2006, 2004b), while in Chinook salmon, the
debromination of BDE-99 favored the removal of a \textit{para}-substituted bromine atom (Browne et al., 2009). These differences in PBDE metabolism may influence PBDE accumulation patterns observed in wild fish. Little is known about species-specific differences in PBDE metabolism, and previous studies have not investigated species-specific differences in the metabolism of individual congeners other than BDE-99 and -209 (Browne et al., 2009; Stapleton et al., 2006).

It has been hypothesized that deiodinase enzymes (DIs) may play a role in the debromination of PBDEs in fish due to the structural similarity of PBDEs to thyroid hormones (Stapleton et al., 2004b). DIs are responsible for activating and deactivating thyroid hormones by cleaving iodine atoms from thyroxine (T\textsubscript{4}), 3,3',5-triiodothyronine (T\textsubscript{3}), or 3,3',5'-triiodothyronine (rT\textsubscript{3}). These dehalogenation reactions occur via the removal of an \textit{ortho}- or \textit{meta}-iodine. Definitive evidence establishing the role of DIs in PBDE debromination in fish is still needed (i.e., assessing PBDE debromination with purified DIs), and it is possible that other biotransformation enzyme systems may also be involved, such as glutathione-S-transferases (GSTs). Previous studies have ruled out the likely involvement of cytochrome P450s (Benedict et al., 2007).
2.3 Biotransformation Experiments

2.3.1 In Vitro Debromination in Fish


While it was quite clear that significant differences in PBDE biotransformation could occur among different fish species, no studies to date had assessed the differences in a systematic way. To help fill this data gap, the biotransformation potential of PBDEs was assessed in fish to examine the step-wise transformation of PBDEs via reductive debromination. The metabolism of eleven individual PBDE congeners was compared among carp, rainbow trout (O. mykiss), and Chinook salmon hepatic tissues. We used environmentally relevant PBDE congeners from seven homologue groups (tri- through deca-) to investigate the structural characteristics and bromine substitution patterns that influence debromination. We also compared the activities of two classes of enzymes, GSTs and DIs, among the three fish species to determine if their relative activities were reflective of the differences in PBDE metabolism.
Extracts from microsomal incubations were analyzed using GC/MS to identify and quantify the debrominated metabolites of 11 environmentally relevant PBDE congeners (BDE-28, -47, -49, -99, -100, -153, -154, -183, -203, -208, and -209) (La Guardia et al., 2006). Metabolic debromination was observed in incubations with six of eleven congeners tested (BDE-99, -153, -183, -203, -208, and -209), while no metabolism was observed in incubations with the remaining five (BDE-28, -47, -49, -100, and -154). The most rapidly metabolized congeners were BDE-99 and -183, and the slowest metabolized congeners were BDE-208 and -209. Up to six metabolites were observed for each metabolized congener. Some PBDE congeners detected as metabolites were used as substrates in our experiments. For example, BDE-203 was metabolically debrominated to form BDE-183, and in a separate incubation, BDE-183 was debrominated to form several hexa-BDEs. The results from incubations with individual congeners were combined to create a metabolic pathway that characterized the potential debromination of BDE-209 to tetra- and penta-BDEs (Figure 4).
Figure 4: Hepatic microsomal biotransformation pathway for (a) common carp, (b) rainbow trout, and (c) Chinook salmon. Boxes indicate congeners used as substrates in incubations. (*) Steps in which two bromine atoms were removed in one incubation were hypothesized based on the structure of the metabolite assuming no rearrangement of bromine atoms. (**) BDE 203 coelutes with BDE 200, which is also a potential metabolite not shown here.

Samples were also screened for oxidative metabolites using LC/MS/MS. OH-BDEs and 2,4,5-tribromophenol have been observed as BDE-99 metabolites in
mammalian studies (Chen et al., 2006; Stapleton et al., 2008c). However, OH-BDEs (i.e., tri- through nona-OH-BDEs) or brominated phenols were not detected in any samples in this study. Reductive debromination appears to be the primary pathway of PBDE metabolism in fish, but further studies are necessary to fully characterize the potential for oxidative PBDE metabolism in fish.

Metabolism likely plays an important role in influencing congener distributions of PBDEs in wild fish. Differences in metabolite formation rates observed in this study corresponded with PBDE congener distributions in wild fish. In wild salmon, PBDE congener distributions included higher proportions of BDE-47 and -99 than other congeners (Hites et al., 2004). In wild common carp, BDE-47, -100, and -154 dominated the congener distribution, with little or no detectable BDE-99 (Pérez-Fuentetaja et al., 2010). According to the results of our study, the enrichment of BDE-47 in wild carp may attributable in part to the rapid metabolism of BDE-153 and -99 to BDE-47. Salmon demonstrated much slower debromination rates of BDE-99 than carp, which may allow BDE-99 to accumulate in salmon to a greater extent.

While understanding the characteristics of PBDE biotransformation is important in understanding the environmental fate of PBDEs, it may also be important from a toxicological perspective. Because lower brominated congeners are often regarded as more toxic and have a higher biomagnification potential than higher brominated congeners, debromination may enhance the toxicity of PBDEs in fish and may increase
the effects on higher trophic level organisms. Furthermore, the potential involvement of DIs in PBDE metabolism may result in altered DI activity, thereby disrupting thyroid regulation. In lake trout and fathead minnows, PBDEs caused decreases in circulating $T_4$ and $T_3$, which may be indicative of altered thyroid hormone homeostasis (Lema et al., 2008; Noyes et al., 2013; Tomy et al., 2004).

In conclusion, we observed debromination in six of eleven PBDE congeners analyzed in this study, including penta- through deca-BDEs, in carp and rainbow trout. Species-specific differences in metabolic rates and products were observed for each substrate. Meta-debromination was predominant in carp, while there was no preference between meta- or para-debromination in rainbow trout and salmon. The results of these experiments provide strong evidence that stepwise debromination of BDE-209 in aquatic organisms will result in the accumulation of lower brominated PBDE congeners in the environment.

### 2.3.2 PBDE Metabolism in Cultured Astrocyte Cells

CYP2B6 catalyzes the formation of OH-BDEs and brominated phenols in human liver tissues (Erratico et al., 2012). As mentioned previously, CYP2B6 is also expressed in astrocyte cells in the human brain, which could result in peripheral hydroxylation of PBDEs to OH-BDEs. This could represent a local source of OH-BDEs in the brain at a very important site for toxicity. Therefore it is essential that cell culture experiments
performed with human astrocytes consider potential CYP2B6 expression and OH-BDE formation during these experiments.

Experiments designed to assess the metabolism of PBDEs in the human brain were performed using H4 glioma cells from the American Type Culture Collection (ATCC® catalog number HTB-148™), primary human astrocytes from Lonza (Basel, Switzerland), and mixed-gender human brain microsomes (Chicago, IL). The cells were plated in 100 mm dishes and exposed to culture medium containing 1 μM BDE-99, and human brain microsomes were exposed to 1 and 10 μM BDE-99 for 2 h using published assay conditions (Erratico et al., 2010). After 48 h, the cell culture medium and the cells were collected and extracted following a previously published method for analysis of OH-BDEs using LC/MS/MS with electrospray ionization (Erratico et al., 2010) and 13C-6-OH-BDE-47 and 13C-3-OH-BDE-100 as surrogate standards for tetra- and penta-OH-BDEs, respectively.

OH-BDEs or brominated phenols were not detected in the cells, cell culture medium, or brain microsomal assays, and the amount of BDE-99 did not decrease significantly compared with controls (Figure 5). The detection limit of the LC/MS/MS method ranged from was 0.1-0.3 ng mL⁻¹ for 5’-OH-BDE-99, 6-OH-BDE-47 and 2,3,5-tribromophenol. While this result was not expected, further analysis with RT-qPCR demonstrated that CYP2B6 (the primary CYP isozyme responsible for PBDE
hydroxylation) did not appear to be endogenously expressed in quantifiable amounts in the primary human astrocytes or the H4 glioma cell line (C\textsubscript{50} > 40; 20 ng assay\textsuperscript{-1}).

![Chromatograms of LC/MS/MS analysis](image)

**Figure 5:** Chromatograms of LC/MS/MS analysis of a sample of cell culture medium exposed to BDE-99 for 48 h showing no detectable peaks for OH-BDEs or tribromophenol (top) and an analytical standard containing a mixture of OH-BDEs at a concentration of 10 ng mL\textsuperscript{-1} (bottom).

Although no oxidative PBDE metabolism was detected, this was the first study to assess the potential PBDE biotransformation in cultured human astrocyte cells or human brain microsomes. Further studies in the brain should however consider potential OH-
BDE formation due to CYP2B6 activity. Other astrocyte cell lines or primary astrocyte preparations may express relevant quantities of CYP2B6 because the expression of CYP2B6 in astrocytes in the brain differs by region (Wang and Tompkins, 2008). Furthermore, CYP2B6 is induced by nicotine, ethanol, rifampin, and phenobarbital, and exposure to any of these compounds could increase the potential for OH-BDE formation in astrocytes (Faucette et al., 2004; Miksys, 2003). The lack of observable OH-BDE formation in the astrocytes indicates that the effects of PBDEs using the H4 glioma cell line and this preparation of primary human astrocytes can be attributed to PBDE congeners dosed into the culture medium and not in vitro biotransformation products.

2.3.3 Biotransformation of TBB and TBPH

The following section includes selections of the results of original research published in Chemical Research in Toxicology, and the full text is included in Appendix B: Simon C. Roberts, Laura J. Macaulay, and Heather M. Stapleton (2012). In vitro metabolism of the brominated flame retardants 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (TBB) and bis(2-ethylhexyl) 2,3,4,5-tetrabromophthalate (TBPH) in human and rat tissues. Chemical Research in Toxicology, 25, 1435–41.

While I was performing the previously described experiments assessing PBDE metabolism as part of this thesis work, I continued to explore flame retardant biotransformation in similar brominated compounds. Unlike PBDEs, much less is known regarding the biotransformation pathways of new and alternative flame
retardants. The brominated components of the major PentaBDE replacement in polyurethane foam, TBB and TBPH, exhibit similar structures to di-ethylhexyl phthalate (DEHP). Because metabolism may lead to bioactivation of these compounds similar to the bioactivation of DEHP to mono-ethylhexyl phthalate (MEHP) (Tickner et al., 2001), and because knowledge of the metabolic stability will allow for predictions of the half-life of these compounds in human tissues, it is important to understand the metabolic potential of TBB and TBPH in humans. The objectives of this study were to identify the metabolites of TBB and TBPH in human liver microsomes (HLM), calculate the enzyme kinetics, and compare the metabolic rates among liver and intestinal subcellular fractions from both humans and rats.

The results from in vitro metabolic assays in human and rat liver and intestinal microsomes indicated that TBB was rapidly metabolized to 2,3,4,5-tetrabromobenzoic acid (TBBA) (Figure 6). TBBA was initially identified as the major metabolite of TBB based on mass spectra of the methyl-derivative of TBBA, and no other potential metabolites were identified. A peak with an m/z of 452.0 was present in both ECNI and EI mass spectra that corresponded to the molecular ion of the TBBA-methyl derivative along with other fragment ions. To further confirm the identification, an analytical standard for TBBA was synthesized, and a more robust LC/MS/MS method using negative ESI was developed using 2,3,5-triiodobenzoic acid (TIBA) as an internal
standard. Comparison of the metabolite with the synthesized TBBA confirmed that
TBBA was the primary metabolite of TBB.

![Chemical structures of TBB, TBBA, TBPH, and TBMEHP]

**Figure 6:** *In vitro* biotransformation product of TBB in human liver microsomes, human intestinal microsomes, rat liver microsomes, rat intestinal microsomes, and porcine carboxylesterase, and *in vitro* biotransformation product of TBMEHP in porcine carboxylesterase.

Unlike TBB, in experiments with human liver microsomes, significant loss of TBPH was not observed, and no metabolites were detected by GC/MS analysis of the sample extracts. An LC/MS/MS method was developed to monitor tetrabromo-MEHP (TBMEHP), a potential hydrolysis metabolite of TBPH (Figure 6). In assays with purified porcine carboxylesterase, TBPH was slowly metabolized to form TBMEHP at an approximate rate of 1.08 pmol min⁻¹ mg esterase⁻¹. In a previous study, DEHP (50 µM) was metabolized to form MEHP in purified carboxylesterase at a rate of 127 pmol min⁻¹ mg protein⁻¹ (Niino et al., 2003), which was approximately 100 times faster than the hydrolysis of TBPH observed in this study. The prominent difference between the
metabolic hydrolysis of DEHP and TBPH may be a result of steric hindrance by the fully brominated phenyl ring of TBPH.

The metabolism of TBB to form TBBA has several toxicological implications for TBB. While metabolism apparently reduces the potential for TBB bioaccumulation, it introduces a metabolite, TBBA, with unknown toxicity and fate. After the publication of these results in 2012, TBBA was used a biomarker for human exposure to TBB, in which TBBA was detected in 72% of human urine samples and correlated with the concentrations of TBB in handwipe samples (Butt et al., 2014; Hoffman et al., 2014). The metabolism of TBPH to form TBMEHP may have implications on the toxicity of TBPH, but may not be rapid enough to affect the bioaccumulation of TBPH. However, its persistence in tissues and the ubiquity of carboxylesterases in other organs and tissues may facilitate TBPH metabolism in mammalian tissues. In vivo toxicological studies should assess the in vivo accumulation and metabolism of both TBB and TBPH in mammals and evaluate the toxicity of both TBBA and TBMEHP.
3. Disruption of Type 2 Thyroid Deiodinase Activity in Cultured Human Astrocytes by Polybrominated Diphenyl Ethers

The following chapter was submitted for review to Toxicological Sciences on July 31, 2014

Polybrominated diphenyl ether (PBDE) flame retardants are endocrine disruptors and suspected neurodevelopmental toxicants. While the mechanisms of neurodevelopmental toxicity have not been fully elucidated, it is conceivable that alterations in thyroid hormone levels in the developing brain may contribute to these effects. The brain activates thyroid hormone by locally converting thyroxine (T4) to the biologically active triiodothyronine (T3) through the action of the selenodeiodinase Type 2 deiodinase (DIO2). Previous studies have demonstrated that PBDEs can alter hepatic deiodinase activity both in vitro and in vivo; however, the effects of PBDEs on the deiodinase isoforms expressed in the brain are not well understood. Here we studied the effects of individual PBDEs and their hydroxylated metabolites (OH-BDEs) on DIO2 activity in astrocytes, a specialized glial cell in which >50% of the T3 in the brain is produced. Primary human astrocytes and H4 glioma cells were exposed to individual PBDEs or OH-BDEs at concentrations up to 5 μM. BDE-99 decreased DIO2 activity by 50% in primary astrocyte cells and by up to 80% in the H4 cells at doses of 500 nM or greater. BDE-153, BDE-209, 3-OH-BDE-47, 6-OH-BDE-47, and 5′-OH-BDE-99 all
decreased DIO2 activity in cultured cells by 45-80%. Multiple mechanisms appear to contribute to the decreased DIO2 activity, including decreased expression of DIO2 mRNA, competitive inhibition of DIO2, and increased posttranslational degradation of DIO2. We conclude that reductions in DIO2 activity caused by exposure to PBDEs may be playing a role in the neurodevelopmental deficits caused by these toxicants.

3.1 Introduction

Polybrominated diphenyl ether (PBDE) flame retardants were added to commercial products to slow the propagation of flame until production was banned (Penta- and OctaBDE) or phased out and restricted to certain applications (DecaBDE) over the past decade due to their persistence and toxicity (de Wit et al., 2010). However, PBDEs are still a concern because they are persistent in the environment, both in commercial products produced before the phase-out and in products made from recycled materials (Ionas et al., 2014). Human exposure to PBDEs occurs via multiple pathways, including inhalation, dermal absorption, ingestion of contaminated food, and most importantly, ingestion of contaminated housedust (Johnson et al., 2010). Toddlers exhibit a higher risk of exposure to PBDEs compared with adults due to increased hand-mouth transfer of contaminated dust during important stages of neurodevelopment (Stapleton et al., 2012, 2014).

Toxic effects of PBDEs have been observed in a variety of organisms including fish, birds, and mammals (Fernie et al., 2005; Noyes et al., 2013; Vonderheide et al.,...
In rodent laboratory exposures, PBDEs were associated with neurodevelopmental toxicity (Viberg and Eriksson, 2011), and human birth cohort studies have observed significant associations between serum PBDEs and decreased performance on neurodevelopmental cognitive and behavioral tests (Gascon et al., 2011; Herbstman et al., 2010). PBDEs and their hydroxylated metabolites (OH-BDEs) are structurally similar to thyroid hormones (Figure 7) and significantly disrupt circulating thyroid hormone levels in fish, rats, mice, and birds. The proposed mechanisms include disruption of proteins involved in thyroid hormone transport (TTR, TBG) and metabolism (deiodinase, sulfotransferase, and glucuronyl transferase) (Butt et al., 2011; Fernie et al., 2005; Kudo and Yamauchi, 2005; Meerts et al., 2000; Szabo et al., 2009).
Figure 7: Structures of PBDE and OH-BDE congeners and thyroid hormones used in this study.

Thyroid hormones are important regulators of neurodevelopment; the biologically active thyroid hormone triiodothyronine (T₃) signals the growth, organization, and differentiation of neurons, and T₃ deficiency in the developing brain impairs neurodevelopment (Bernal et al., 2003; Préau et al., 2014). Therefore, a possible mechanism for the observed effects of PBDEs on neurodevelopment may involve alterations in the levels of thyroid hormones in the brain during critical windows of development. Previous studies have determined that PBDE exposure decreases serum thyroxine (T₄) and occasionally T₃ concentrations, but little is known regarding potential changes in the levels of thyroid hormones in the affected tissues, such as the brain.
Type 2 deiodinase (DIO2) is a major regulator of $T_3$ levels in the brain because it locally converts $T_4$ into $T_3$ via deiodination (Guadaño-Ferraz et al., 1999). DIO2 is primarily regulated by thyroid hormone levels via two homeostatic mechanisms to tightly control the level of $T_3$ reaching the neuronal cells: $T_3$ negatively regulates DIO2 transcription and $T_4$ negatively regulates DIO2 activity by accelerating DIO2 posttranslational ubiquitination and its subsequent proteasomal degradation (Figure 8) (Mohácsik et al., 2011). Similar to many other enzymes, some molecules can affect DIO2 activity by interfering with the catalytic reaction (Bianco et al., 2002b; Rosene et al., 2010). Because DIO2 regulation is affected by $T_4$ and $T_3$, which are structurally similar to PBDEs and especially OH-BDEs, it is conceivable that exposure to PBDEs and OH-BDEs disrupts DIO2 regulation and alters $T_3$ levels in the brain. While previous studies have suggested that the association between PBDEs and neurodevelopment may be driven by disruption of thyroid hormone levels, the effects of PBDEs on human DIO2 are unknown. The purpose of this study is to investigate whether PBDEs and OH-BDEs affect DIO2 expression/activity, and if so, to determine the mechanism(s) responsible.
3.2 Materials and Methods

3.2.1 Reagents and Materials

Individual PBDE congeners (BDE-47, -99, -153, and -209) and OH-BDEs (3-OH-BDE-47, 6-OH-BDE-47, and 5′-OH-BDE-99) were purchased as neat standards (purity >97%) from Accustandard (New Haven, CT). Stable isotopically labelled surrogate standards \(^{13}\)C-6-OH-BDE-47 and \(^{13}\)C-6′-OH-BDE-100 were purchased from Wellington...
Laboratories (Ontario, Canada). Dithiothreitol (DTT), T₄, T₃, rT₃, and T₂ were purchased from Sigma Aldrich (St. Louis, MO). Advanced Dulbecco's Modified Eagle Medium (A-DMEM) cell culture medium and other cell culture reagents were purchased from Life Technologies (Carlsbad, CA). Cell culture plastics were purchased from Genesee Scientific (San Diego, CA). H4 glioma cells and primary human astrocytes that were originally purchased from the American Type Culture Collection (ATCC® catalog number HTB-148™) and Lonza (Basel, Switzerland), respectively, were obtained from the Duke University Cell Culture Facility (Durham, NC). All solvents and other reagents were purchased from VWR (Radnor, PA).

3.2.2 Cell Culture

H4 glioma cells were grown in DMEM supplemented with 10% fetal bovine serum, 30 nM selenium (as sodium selenite), 100 units mL⁻¹ penicillin, and 100 µg mL⁻¹ streptomycin at 37°C and 5% CO₂. Normal primary human astrocyte cells were grown on tissue culture dishes coated with gelatin in A-DMEM medium supplemented with 3% FBS, 30 nM selenium, 2 mM l-glutamine, 100 units mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin, and 2 ng mL⁻¹ human epidermal growth factor. All experiments were performed with cells thawed from the same passage number. For both cell types, the culture medium was changed to A-DMEM supplemented with 30 nM selenium, 2 mM l-glutamine, 100 units mL⁻¹ penicillin, and 100 µg mL⁻¹ streptomycin without serum 24 h before each experiment. A-DMEM medium contains nonessential amino acids, insulin,
transferrin, and 0.4 mg L⁻¹ albumin, and is designed to be used as reduced-serum or serum-free culture medium. Cells were thawed and plated at a density of 2 × 10⁴ cells cm⁻² in T-75 flasks and transferred to either 70 mm dishes or 96-well plates for experiments.

3.2.3 Dosing

PBDEs, OH-BDEs, and other test compounds were dissolved in pure DMSO in 1,000× the desired final concentration to achieve a final DMSO concentration of 0.1% in the cell culture medium for each tested concentration and control. Because PBDEs exhibit low aqueous solubility, the concentrations of all the test compounds in the dosed culture medium were determined after dosing in all the experiments using a published GC/MS method (Stapleton et al., 2008b) for PBDEs and a published LC/MS/MS method (Erratico et al., 2010) for OH-BDEs. In preliminary experiments, the concentrations of the PBDEs decreased by approximately 50% after a 12 h exposure. Therefore, to maintain a constant concentration of BDE-47, -99, -153, and -209, an additional dose (50% of the initial dose) was added to the cell culture medium at 6 h for 12-h continuous exposures. The exposure concentrations reported represent the average concentration of the compounds measured over the entire experiment. The measured concentrations of the PBDEs (50 nM, 500 nM, and 2,500 nM) were lower than the nominal concentrations due to low solubility in the DMSO stock, while the measured concentrations of the OH-BDEs (100 nM, 1,000 nM, 5,000 nM) were similar to the nominal concentrations.
3.2.4 Cytotoxicity Assays

Cell viability was assessed in 96-well plates by measuring resazurin reduction to resarufin in the cultured cells after 24 h exposures to the PBDEs and OH-BDEs (O’Brien et al., 2000). The culture medium was removed and replaced with 100 μL of A-DMEM containing 44 μM resazurin. The formation of the fluorescent resarufin product was measured after 1 h, and the results were normalized to the DNA content in each well and the results from the controls cells dosed with the DMSO vehicle. Damage to the cell membrane was assessed by measuring the activity of glucose-6-phosphate dehydrogenase in cell culture medium exposed to the cells for 24 h (Batchelor and Zhou, 2004). The reduction of resazurin (30 μM) to the fluorescent resarufin product was measured in 100 μL of cell culture medium with the addition of 2 mM glucose-6-phosphate, 1 U/mL diaphorase, and 0.5 mM NADP+. The formation of resarufin over 45 minutes was measured to calculate the amount of G6PDH released from the cells into the culture medium, and the results were normalized to the DNA content in each well and the controls cells dosed with the DMSO vehicle.

3.2.5 Deiodinase Assays

Cells were scraped into KPO₄ buffer containing 0.25 M sucrose, 30 mM DTT, and 1 mM EDTA and sonicated for 15 s on ice. Microsomal subcellular fractions were prepared from the cellular homogenate by ultracentrifugation at 100,000×g for 60 min. DIO2 assays were performed using KPO₄ buffer containing 30 mM DTT, 1 mM EDTA,
10% glycerol, and 100-200 µg of microsomal protein. After 75 min incubations at 37°C, 1 mL of 1 M HCl and 0.5 ng of each 13C-labelled surrogate standard for T4, T3, rT3, and T2 were added to stop the reaction. The reaction mixtures were extracted using Agilent OPT Solid Phase Extraction tubes and analyzed using LC/MS/MS following our previously published analytical method (Roberts et al., 2011). The total mass of T3 quantified in each reaction mixture was corrected for the low background levels of T3 in the cultured cells or T4 dosing stock to calculate the net T3 formed via deiodination of T4 in the reactions. The protein content of the cell homogenates was determined using the Bradford assay, and the DNA content was determined using the PicoGreen® double-stranded DNA assay (Bradford, 1976; Otto, 2005). The DIO2 activity measurements were calculated as fmol T3 formed min⁻¹ mg protein⁻¹ and normalized to the values of controls containing equivalent amounts of the dosing vehicle, DMSO, for each experiment and are reported as percent control with the standard error of the mean (SEM).

### 3.2.6 Enzyme Kinetics and Inhibition Assays

The kinetic parameters of DIO2 were determined in microsomal preparations of H4 cells using a range of T4 concentrations from 0.1 nM to 50 nM for 75 min incubations with 100 µg protein. Kinetics experiments were also performed using the same conditions but with the addition of 20 µM BDE-99 or 15 µM 5’-OH-BDE-99. The kinetic parameters were calculated and compared with JMP Pro 11 (Cary, NC) using the Hill
equation, which is a modified form of the Michaelis Menten model with the addition of coefficients to account for substrate cooperativity or multiple binding sites:

\[ v = \frac{V_{\text{max}} \times S^n}{K_m^n + S^n} \]

where \( V_{\text{max}} \) is the maximum reaction rate, \( S \) is the substrate concentration, \( n \) is the Hill coefficient, and \( K_m \) is the substrate concentration at 50% of the maximum reaction rate, similar to the Michaelis Menten equation (Porter and Miller, 2012). In vitro DIO2 inhibition assays were performed with 100 \( \mu \)g protein of microsomal preparations of H4 cells, 2 nM T4, and 9 concentrations of BDE-99, 5'-OH-BDE-99, and 3-OH-BDE-47 ranging from 0.05 \( \mu \)M to 100 \( \mu \)M. The values are reported as the average DIO2 activity normalized to the control assays. The IC50 values were calculated using a 3-parameter nonlinear model in JMP Pro 11, and the Ki values were calculated using the following equation, which relates the IC50 to the concentration of T4 (S) and the Km of T3 formation:

\[ K_i = \frac{IC_{50}}{S} \frac{K_m}{K_m + 1} \]

### 3.2.7 DIO2 mRNA Expression

Total RNA was extracted from a 300 \( \mu \)L aliquot of the scraped cells (approximately 30% of the total cells scraped from the dish) using the Quick-RNA™ MicroPrep kit from Zymo Research (Irvine, CA) and quantified using the Nanodrop 1000 (Thermo Scientific). Total RNA was converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit from Life Technologies (Carlsbad, CA). Approximately
10 ng of cDNA was analyzed in 20 μL quantitative PCR (qPCR) reactions using TaqMan® Gene Expression Assays (Life Technologies) for DIO2 (Hs00988260_m1), RPL13A (Hs04194366_g1), GAPDH (Hs02758991_g1), SDHA (Hs00417200_m1), and CYP2B6 (Hs04183483_g1) with an Applied Biosystems (Foster City, CA) 7300 Real-Time PCR System. The threshold cycles (Ct) of RPL13A, GAPDH, and SDHA were compared in an initial experiment to determine the best reference standard, and RPL13A was chosen due to its stable expression between control cells and cells treated with BDE-99 using DataAssist 3.01 (Applied Biosystems). Expression values for DIO2 mRNA are reported as the expression ratio relative to control samples normalized to RPL13A using the 2^{-\Delta Ct} method.

3.2.8 Statistics

ANOVA was performed using JMP Pro 11 to test for significant effects of the treatment, experiment number, or exposure time on the DIO2 activity or DIO2 mRNA expression. Significant effects and interactions were further tested using Tukey's post-hoc test with a significance level of α=0.05. DIO2 activity data were log transformed before statistical analysis, and statistical analysis of mRNA expression was performed using the (ΔΔCt) values before conversion to the linear expression ratio for graphical presentation. All experiments were performed with 3-4 samples and repeated on a separate day, and when the results were not significantly different between experimental days, the results were combined for a total of 6-8 samples per treatment
group. In the experiments reported in this study, there was no significant effect of the
treatments on the ratio of membrane to total protein, total protein to DNA, or membrane
protein to DNA. The results for each experiment were normalized to the control values
of that experiment, combined with the results from a repeated experiment, and are
presented as mean ± SEM.

3.3 Results

3.3.1 Basal Type 2 Deiodinase Activity

Primary human astrocyte cells expressed DIO2 activity (4.10 ± 0.24 fmol T₃ min⁻¹
mg protein⁻¹) in microsomal subcellular fractions prepared from homogenates of cells
grown in serum-free A-DMEM supplemented with 10 μM forskolin, a well-known
inducer of DIO2 expression (Pallud et al., 1997). In contrast, H4 cells expressed DIO2
mRNA and DIO2 activity under serum-free culture conditions without the addition of
forskolin to culture medium. T₂ and rT₃ were not formed as deiodination products in
assays with T₄ as a substrate, indicating the absence of detectable DIO3 activity in the H4
cells. The calculated Kₘ and Vₘₐₓ values of T₃ formation in the H4 microsomal fraction
were 3.49 ± 0.94 nM and 47.5 ± 2.9 fmol min⁻¹ mg protein⁻¹, respectively (Figure 9).
Figure 9: A) Kinetics of DIO2 in microsomal preparations of H4 cells (black line: $K_m = 3.49 \pm 0.94$ nM, $V_{max} = 47.5 \pm 2.9$ fmol min$^{-1}$ mg protein$^{-1}$) and DIO2 kinetics with the addition of 20 μM BDE-99 (orange line: $K_m = 8.74 \pm 3.2$ nM, $V_{max} = 50.6 \pm 5.6$ fmol min$^{-1}$ mg protein$^{-1}$) or 15 μM 5′-OH-BDE-99 (green line: $K_m = 19.9 \pm 12.0$ nM, $V_{max} = 47.2 \pm 8.0$ fmol min$^{-1}$ mg protein$^{-1}$). B-D) Inhibition of DIO2 activity in microsomal preparations of H4 cells in assays performed with 1-2 nM T4 and increasing concentrations of B) BDE-99, C) 3-OH-BDE-47, and D) 5′-OH-BDE-99). Data are shown as mean ± SEM.

3.3.2 Cytotoxicity

Significant effects on membrane damage and viability were not detected at any doses less than 7.5 μM for BDE-47, -99, -153, -209, or 5′-OH-BDE-99. Viability decreased significantly by 31.7 ± 7.8% and 32.0 ± 8.2% at doses of 5 μM 3-OH-BDE-47 and 6-OH-BDE-47, respectively, but membrane damage was not significantly different from control at doses less than 50 μM. Potentially confounding effects on DIO2 activity could only be
considered for the 5 μM doses of 3-OH-BDE-47 and 6-OH-BDE-47, although the effects of reduced cell viability on DIO2 activity are unknown.

### 3.3.3 Effects of PBDEs and OH-BDEs on DIO2 Activity

The effects of PBDEs and OH-BDEs on DIO2 activity over time were determined in 1, 6, and 12 h exposures (Figure 10). BDE-209 (500 nM) and 3-OH-BDE-47 (1,000 nM) significantly decreased DIO2 activity by 66.0 ± 9.9% and 71.1 ± 13.7%, respectively, after 1 h exposures; no other PBDEs or OH-BDEs significantly altered DIO2 activity after 1 h exposures. Treatment of H4 cells with BDE-99, -153, and -209 (500 nM) for 6 h significantly decreased DIO2 activity by 82.9 ± 6.7%, 79.6 ± 4.8%, and 54.2 ± 11.8%, respectively (Figure 10). DIO2 activity decreased significantly after a 6 h exposure to 3-OH-BDE-47 and 5′-OH-BDE-99 (1,000 nM) by 52.1 ± 8.5% and 57.4 ± 1.2% (Figure 10). After a 12 h exposure, BDE-99, BDE-153, and 5′-OH-BDE-99 significantly decreased DIO2 activity by 64.6 ± 2.8%, 50.9 ± 8.3%, and 55.7 ± 5.4%, respectively.
Figure 10: Time course of effects on DIO2 activity in H4 cells exposed to 500 nM BDE-47, BDE-99, BDE-153, and BDE-209 or 1,000 nM 3-OH-BDE-47, 6-OH-BDE-47, and 5’-OH-BDE-99 for 0, 1, 6, and 12 h. Results are shown as mean ± SEM. Two-factor ANOVA indicated a significant interaction of treatment × exposure duration (p<0.001). * Indicates significant difference from vehicle control cells at the corresponding exposure time of the sample (p<0.05; n=8 from 2 experiments). Dose-response relationships and full statistical results are shown in Figure 42.

Effects on DIO2 activity were also investigated at 3 different doses for a 6 h exposure (Figure 11). BDE-47 did not significantly alter DIO2 activity at any of the exposure concentrations. None of the PBDEs or OH-BDEs significantly altered DIO2 activity at the lowest dose tested (50 nM for PBDEs and 100 nM for OH-BDEs). However, treatment of H4 cells with 2,500 nM BDE-99 for 6 h significantly decreased DIO2 activity by 80.8 ± 4.7%, and treatment with 5,000 nM 3-OH-BDE-47, 6-OH-BDE-47, and 5’-OH-BDE-99 for 6 h significantly decreased DIO2 activity by 68.2 ± 13.9%, 57.1 ± 11.4%, and 63.6 ± 2.0%, respectively. Due to the limitations of using primary human astrocytes, experiments were conducted primarily with H4 cells and confirmed with primary astrocytes for a 6 h exposure with BDE-99 using rT3 as a positive control.
Treatment of primary human astrocytes with BDE-99 (500 nM) and rT₃ (100 nM) for 6 h decreased DIO2 activity by 51.8 ± 8.6% and 49.4 ± 4.1%, respectively (Figure 12).

Figure 11: DIO2 activity after 6 h exposures to PBDEs and OH-BDEs at the doses identified in the dose legend. One-factor ANOVA indicated a significant effect of treatment (p<0.001). Data are reported as percent relative to the vehicle control. * Indicates significant difference from vehicle control cells (p<0.05; n=8 from 2 experiments). Dose-response relationships and full statistical results are shown in Figure 43.

Figure 12: Decreased DIO2 activity in primary human astrocyte cells exposed to 500 nM BDE-99 and 100 nM rT₃. One-way ANOVA indicated an effect of treatment (p<0.001). * Indicates significant difference from control cells (p<0.05; n=8 from 2 experiments).
3.3.4 Effects of PBDEs and OH-BDEs on DIO2 mRNA Expression

*DIO2* mRNA expression was evaluated in H4 cells exposed to 3 doses of PBDEs and OH-BDEs for 6 h using RT-qPCR (Figure 13). Exposure to 500 nM and 2,500 nM BDE-99 for 6 h significantly decreased the *DIO2* mRNA expression levels to 0.63 ± 0.06 and 0.59 ± 0.10 (expression ratio relative to the control value of 1.0), respectively. Exposure to 5,000 nM 6-OH-BDE-47 and 5′-OH-BDE-99 significantly decreased *DIO2* gene expression levels to 0.59 ± 0.08 and 0.64 ± 0.12 (expression ratio relative to the control value of 1.0), respectively. The expression levels of *DIO2* mRNA in cells treated with BDE-47, -153, and -209 and 3-OH-BDE-47 were not significantly different from control.

![Graph showing DIO2 mRNA expression ratio relative to control](image)

Figure 13: *DIO2* mRNA expression ratio relative to control cells and normalized to *RPL13a* as an internal reference gene calculated using the 2^ΔΔCt^ method after 6 h exposures to PBDEs and OH-BDEs at the doses identified in the dose legend. One-factor ANOVA indicated a significant effect of treatment (p<0.01). * Indicates significant difference from vehicle control cells (p<0.05; n=8 from 2 experiments;
3.3.5 In Vitro Inhibition of DIO2

To evaluate whether BDE-99 and 5’-OH-BDE-99 competitively or noncompetitively inhibited DIO2 activity, 20 μM BDE-99 or 15 μM 5’-OH-BDE-99 were added to microsomal fractions over a range of T4 concentrations from approximately 0.05 nM to 50 nM. The kinetics were modeled using the Hill equation, which provided a slightly better fit than the Michaelis Menten equation (r²=0.96 vs. 0.94) due to the addition of the Hill coefficient to the equation (n=0.6 in all three models indicating negative cooperativity of T4 binding to DIO2). The presence of BDE-99 caused the K_m of DIO2 activity (T3 formation) to increase significantly from 3.49 ± 0.94 nM to 8.74 ± 3.2 nM T4 (Figure 9A). The calculated V_max of 50.6 ± 5.6 fmol min⁻¹ mg protein⁻¹ with the addition of BDE-99 was not significantly different from the control V_max of 47.5 ± 2.9 fmol min⁻¹ mg protein⁻¹. The presence of 5’-OH-BDE-99 caused the K_m to increase significantly from 3.49 ± 0.94 nM to 19.9 ± 12.0 nM, while the V_max of 47.2 ± 8.0 fmol min⁻¹ mg protein⁻¹ was not significantly different from the control V_max of 47.5 ± 2.9 fmol min⁻¹ mg protein⁻¹.

In assays with 2 nM T4, BDE-99, 5’-OH-BDE-99, and 3-OH-BDE-47 inhibited DIO2 activity at concentrations above ~1 μM (Figure 9C-D). The calculated IC₅₀ values for BDE-99, 5’-OH-BDE-99, and 3-OH-BDE-47 were 77.6 ± 2.9 μM, 16.6 ± 1.1 μM, and 3.74 ± 1.20 μM, respectively. The IC₅₀ values at the measured T4 concentrations for each
experiment were used to calculate $K_i$ values of 33.3 μM, 7.11 μM, and 1.60 μM for BDE-99, 5’-OH-BDE-99, and 3-OH-BDE-47, respectively.

3.3.6 Effects of BDE-99 and 5’-OH-BDE-99 on Proteasomal Degradation

To determine the mechanisms responsible for the decrease in DIO2 activity, further experiments were conducted to examine the role of PBDEs on DIO2 protein degradation and synthesis. In H4 cells treated with 500 nM BDE-99 and 5’-OH-BDE-99 and 100 nM rT3 for 6 h, DIO2 activity decreased significantly by 78.6 ± 3.9%, 58.9 ± 1.6%, and 55.9 ± 3.6%, respectively, compared with control (Figure 14). Treatment of H4 cells with 10 μM MG132, a proteasomal inhibitor, increased the basal DIO2 activity by 73.5 ± 17.9% (Figure 15). The addition of 500 nM BDE-99 and 5’-OH-BDE-99 and 100 nM rT3 to cells treated with MG132 caused the DIO2 activity to decrease significantly by 45.5 ± 3.8%, 37.0 ± 2.6%, and 25.8 ± 2.3%, respectively, compared with the cells treated with MG132 alone. The addition of cycloheximide (a protein synthesis inhibitor) and MG132 together caused the DIO2 activity to decrease by 31.6 ± 8.0% compared with control cells (Figure 15). The addition of 500 nM BDE-99 and 5’-OH-BDE-99 and 100 nM rT3 to cells treated with cycloheximide and MG132 together caused decreases in activity (~15%) that were not significantly different from the control cells treated with cycloheximide and MG132 (Figure 14). The DIO2 activity of the cells treated with BDE-99 was significantly different among all 3 treatment groups (basal vs. MG132 vs. MG132+cycloheximide).
Figure 14: DIO2 activity in H4 cells treated with 500 nM BDE-99, 500 nM 5’-OH-BDE-99, or 100 nM rT3 and in H4 cells coexposed with BDE-99, 5’-OH-BDE-99, and rT3 and either 10 μM MG132 or a combination of 10 μM MG132 and 100 μM cycloheximide for 6 h. Data are reported mean ± SEM of the percent relative to vehicle control cells (blue), control cells exposed to MG132 (orange), or control cells exposed to both MG132 and cycloheximide (green). Two-factor ANOVA indicated a significant interaction of treatment × inhibitor coexposure (p<0.001). Bars not sharing letters are significantly different from each other (p<0.05; n=8 from 2 experiments).
3.4 Discussion

The present study indicates that exposure of primary human astrocytes and a glioma cell line to PBDE flame retardants and their OH-BDE metabolites disrupts DIO2 expression and activity, potentially compromising the main source of T3 to the brain and dampening thyroid hormone signaling in neurons.

To our knowledge, this is the first study to characterize DIO2 activity in cultured human astrocytes, which required induction with 10 μM forskolin to become readily
detectable; this is similar to DIO2 expression in primary rat astrocytes (Leonard et al., 1990; Leonard, 1988; Pallud et al., 1997). Due to limited availability of human primary astrocytes, we also used human H4 glioma cells that were previously validated as a suitable cell model to study the DIO2 pathway in glial cells (Freitas et al., 2010). Exposure of both cell types to PBDEs or OH-BDEs for a few hours decreased DIO2 activity by approximately 50% through transcriptional, post-translational, and catalytic mechanisms, which all cause decreased T3 production. This is particularly relevant because most T3 in the brain is generated by DIO2-expressing glial cells. Via a paracrine mechanism, T3 leaves the glial cells and enters the neighboring neurons where it modifies expression of T3-responsive genes cells (Freitas et al., 2010).

In humans, PBDEs are hydroxylated by the cytochrome P450 enzyme, CYP2B6, which is expressed in the liver and in astrocytes (Erratico et al., 2012; Meyer et al., 2007). In fact, the expression of CYP2B6 is highly variable in the brain (Wang and Tompkins, 2008), and local biotransformation of PBDEs is considered to be a potential source of OH-BDEs. However, no OH-BDEs or brominated phenol metabolites were detected in cells or cell culture medium. The detection limit for 5′-OH-BDE-99, the major metabolite of BDE-99, was 0.244 nM, well below the concentration of 1,000 nM at which OH-BDEs caused significant effects. Therefore, the present results appear to be directly caused by the compounds dosed into the cell culture medium.
Robust decreases in DIO2 activity occurred at concentrations of approximately 500 nM of BDE-99, BDE-153, BDE-209 and 1,000 nM 3-OH-BDE-47 and 6-OH-BDE-47, with no significant acute effects observed at a lower, more environmentally relevant concentration of 50-100 nM of any of the tested compounds. The average PBDE and OH-BDE concentrations used in the present studies are higher than the concentrations measured in human serum (Table 3). However, the maximum concentrations of BDE-47, -99, and -153 in human serum are in the low nM range, and the maximum combined concentrations of PBDEs have been detected as high as 46-78 nM (Stapleton et al., 2011a; Zheng et al., 2014). PBDEs are expected to readily cross the blood brain barrier and could potentially accumulate in the brain. For example, in mice, the brain:blood BDE-47 and -99 ratios are approximately 1:1, and for BDE-153, the ratio is approximately 4:1 (Staskal et al., 2006). Therefore, the actual concentration of PBDEs reaching human astrocytes in vivo could differ from the levels measured in the serum due to differential partitioning and/or transport in the human brain. Furthermore, the present studies were based on acute short-term exposures to PBDE and OH-BDE. More studies are needed to analyze the effects of long-term exposure to lower concentrations of PBDEs on the DIO2 pathway.
Table 3: Levels of polybrominated diphenyl ethers (PBDEs) and hydroxylated polybrominated diphenyl ethers (OH-BDEs) in human serum in previous studies compared with the lowest dose that significantly decreased Type 2 deiodinase (DIO2) activity in this study.

<table>
<thead>
<tr>
<th></th>
<th>Average Serum Concentrationa (ng/g lipid)</th>
<th>Maximum Serum Concentration (ng/g lipid)</th>
<th>Sourcea</th>
<th>Significant DIO2 Effects in H4 Cells (%Ctrl; Dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nM)b</td>
<td>(nM)c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BDE-47</td>
<td>77.8 1.02</td>
<td>4.640 60.7</td>
<td>1</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>23.3 0.305</td>
<td>350 4.58</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>77 1.01</td>
<td>148 1.94</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>BDE-99</td>
<td>26.1 0.294</td>
<td>1.200 13.5</td>
<td>1</td>
<td>82.9%; 500 nM</td>
</tr>
<tr>
<td></td>
<td>6.39 0.072</td>
<td>225 2.53</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.0 0.034</td>
<td>33 0.371</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>BDE-153</td>
<td>33.1 0.327</td>
<td>200 1.98</td>
<td>1</td>
<td>79.6%; 500 nM</td>
</tr>
<tr>
<td></td>
<td>5.34 0.053</td>
<td>83.1 0.821</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>29 0.286</td>
<td>82 0.810</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>BDE-209</td>
<td>256 1.70</td>
<td>2.716 18.0</td>
<td>3</td>
<td>66.0%; 500 nM</td>
</tr>
<tr>
<td></td>
<td>10.5 0.0697</td>
<td>60.9 0.404</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>ΣPBDEs4</td>
<td>160 1.80</td>
<td>7.003 78.8</td>
<td>1</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>42.9 0.483</td>
<td>668 7.52</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>753 8.48</td>
<td>4.010 46.1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>3-OH-BDE-47</td>
<td>1.6 0.020</td>
<td>- -</td>
<td>5</td>
<td>71.1%; 1,000 nM</td>
</tr>
<tr>
<td>6-OH-BDE-47</td>
<td>- 0.0131</td>
<td>- 0.336</td>
<td>4</td>
<td>57.1%; 5,000 nM</td>
</tr>
<tr>
<td></td>
<td>9.9 0.13</td>
<td>- -</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.17 0.0022</td>
<td>10.8 0.137</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>5′-OH-BDE-99</td>
<td>- 0.0268</td>
<td>- 0.389</td>
<td>4</td>
<td>57.4%; 1,000 nM</td>
</tr>
<tr>
<td></td>
<td>22 0.24</td>
<td>- -</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

a Values represent geometric mean or median as reported by the authors of the corresponding study
b Calculated assuming a serum lipid concentration of 6.36 mg mL⁻¹ (Bernert et al., 2007)

c References numbered as follows: 1 (Stapleton et al., 2008d) (median values from foam workers and maximum values from control group); 2 (Stapleton et al., 2012); 3 (Zheng et al., 2014); 4 (Chen et al., 2013); 5 (Qiu et al., 2009); 6 (Stapleton et al., 2011a)
d Combined PBDE concentration of all PBDEs quantified as reported by the authors. Values for nM calculated using molecular weight for pentaBDE of 565 g mol⁻¹

The DIO2 pathway exhibits multilevel control, including transcriptional repression by T₃ (Gereben and Salvatore, 2005), reduced translational efficiency by endoplasmic reticulum stress (Arrojo et al., 2013a), and post-translational ubiquitination
followed by proteasomal degradation (Arrojo et al., 2013b). Most of these mechanisms are homeostatic and mediate DIO2 repression triggered by exposure to thyroid hormone. Thus, given the structural similarities between PBDEs and thyroid hormones we tested whether PBDEs alter DIO2 activity following similar mechanisms.

The relative expression level of DIO2 mRNA decreased significantly in cells exposed to BDE-99, 5’-OH-BDE-99, and 6-OH-BDE-47 by approximately 45%, which explains in part the accompanying decrease in DIO2 activity. However, the degree to which a 45% decrease in mRNA expression may affect DIO2 activity is unclear. Previous studies have determined that PBDEs and OH-BDEs bind to and trigger thyroid receptor-mediated transcriptional repression in the nM concentration range (Ibhazehiebo et al., 2011); thus, the reduction in DIO2 mRNA levels observed here may be mediated by transcriptional repression.

In previous studies, 5’-OH-BDE-99 inhibited DIO1 activity in human liver microsomes with an IC₅₀ of 400 nM (Butt and Stapleton, 2013; Butt et al., 2011). In the present study, the Kₘ values increased significantly with the addition of BDE-99 and 5’-OH-BDE-99 to the DIO2 assay, while the Vₘₐₓ was not significantly altered, which is the classic indicator of competitive inhibition because increasing substrate concentrations negated the competitive inhibition of DIO2 activity. The most potent inhibitor was 3-OH-BDE-47, followed by 5’-OH-BDE-99 and BDE-99, which inhibited DIO2 by a maximum of 45% at the highest concentration tested (90 μM). It appears that the
addition of the hydroxyl group to the BDEs greatly increases the affinity of the BDE structure for DIO2, which may be expected based on the presence of an aromatic hydroxyl group in thyroid hormones. The $K_i$ values of 3-OH-BDE-47 and 5'-OH-BDE-99 (1.60 μM and 7.11 μM, respectively) were in the same range as the high dose of 5 μM in the cell exposures; therefore, competitive inhibition of DIO2 is a likely additional mechanism explaining the observed decreases in DIO2 activity for 3-OH-BDE-47 and 5'-OH-BDE-99, but not BDE-99. These are remarkable findings given that to our knowledge, these are the first non-thyroid hormone-related compounds shown to competitively inhibit DIO2 (Bianco et al., 2014).

Ubiquitination of DIO2 followed by removal from the endoplasmic reticulum membrane and proteasomal degradation is believed to be the most important mechanism of DIO2 regulation in vivo (Arrojo et al., 2013a). The rate of ubiquitination is driven by the binding of substrate (T₄ and rT₃) to DIO2, and could therefore be affected by other compounds that bind to the active site in DIO2 (Sagar et al., 2007). In fact, the loss in DIO2 activity caused by BDE-99 was significantly minimized by exposure to the proteasome inhibitor MG132 (Figure 14), but DIO2 activity was still significantly lower than control cells. These data confirm that BDE-99 binds to the DIO2 active site, accelerating loss of DIO2 catalytic activity similarly to its natural substrate T₄. In addition, because loss of DIO2 activity was only partially inhibited by MG132, these data also indicate the involvement of transcriptional mechanisms. The latter was
confirmed in experiments in which H4 cells were treated with cycloheximide to prevent
the reduction in DIO2 activity mediated by transcriptional pathways.

Overall, the present results indicate that both increased proteasomal degradation
and reduced DIO2 expression result from exposure to BDE-99 and 5′-OH-BDE-99. Given
that exposure of MG132 and cycloheximide rescued the effects of the BDE-99 and rT3 on
DIO2 activity, competitive inhibition does not appear to be a major contributor to the
reduced DIO2 activity observed in whole cell experiments. However, these observations
resolve an important aspect of T3-induced acceleration of DIO2 ubiquitination of
whether substrate binding or substrate catalysis triggers DIO2 ubiquitination (Sagar et
al., 2007). Because PBDEs or OH-BDEs are competitive inhibitors that are not
deiodinated by DIO2 but trigger loss of DIO2 activity that is preventable by exposure to
MG132, it is logical to conclude that binding to the catalytic active center of DIO2 and
not enzymatic catalysis is the key molecular mechanism that initiates the conformational
changes in DIO2 that trigger its ubiquitination.

Reductions in DIO2 activity from PBDE and OH-BDE exposure may result in
decreased T3 levels in the brain, which has not been addressed in previous studies. The
importance of local T3 actions in the development of tissues is supported by the hearing
impairments due to altered differentiation and organization of cochlear cells in DIO2
knockout mice. Other effects of decreased T3 during development may involve impaired
differentiation and organization of neuronal networks in the brain (Préau et al., 2014).
Therefore, subtle changes in local T₃ concentrations in the brain mediated by alterations in DIO2 activity by PBDEs and OH-BDEs could similarly impair neurodevelopment.

In addition to DIO2, other mechanisms also control the level of T₃ in the brain. T₃ is transported across the blood-brain barrier into astrocytes, and T₃ is transported in and out of astrocytes and neurons by multiple transport proteins including MCT8 and OATP1C1 (Suzuki and Abe, 2008). In addition to fluctuations in DIO2 activity, changes in the expression of these transporters could regulate the amount of T₃ reaching the neurons. Neurons also express DIO3, which deactivates T₃ and T₄, and thus limit the impact of T₃ in these cells (Freitas et al., 2010). However, DIO3 is an inner ring deiodinase that has lower affinity for T₄ and is regulated differently than DIO2. Thus, further studies are needed to assess the effects of PBDEs on DIO3 activity and evaluate the effects of PBDEs and OH-BDEs on thyroid hormone transporters in the brain.
4. Disruption of Type 2 and Type 3 Deiodinase Activity in Co-cultured Human Glial and Neuronal Cells by Polybrominated Diphenyl Ethers

Polybrominated diphenyl ether (PBDE) flame retardants are persistent organic pollutants and suspected neurodevelopmental toxicants. Humans are exposed to PBDEs via multiple mechanisms including inhalation, dermal absorption, and ingestion of contaminated housedust. PBDEs and their hydroxylated metabolites (OH-BDEs) alter serum thyroid hormone levels, which are vital for signaling proper growth and development. However, little is known regarding the effects of PBDEs on thyroid hormone signaling in the brain. Our previous study showed that PBDEs and OH-BDEs decreased the activity of the thyroid-activating enzyme Type 2 deiodinase (DIO2) in human astrocyte cells. We hypothesized that decreased DIO2 activity would impair the functioning of neurons, which express the thyroid hormone inactivating enzyme Type 3 deiodinase (DIO3). The goal of the present study was to determine the effects of PBDEs and OH-BDEs on the export of T3 from glial cells (expressing DIO2) and rT3 in neuronal cells (expressing DIO3) cultured together in a transwell plate. A secondary goal was to determine whether alterations in thyroid hormone levels would alter the physiology of the co-cultured neurons. BDE-99 decreased the concentration of T3 and the inactive thyroid hormone rT3 in the cell culture medium of co-cultured cells by 50-80%. 3-OH-BDE-47 competitively inhibited DIO3 with an IC50 of 19 μM. 5′-OH-BDE-99 increased the rT3 concentration in cell culture medium by 400%, increased DIO3 activity in exposed
cells by 50%, and increased DIO3 catalytic activity in cellular homogenates by over 500%. Further effects on the mRNA expression of several thyroid-regulated genes (DIO3, TR-α, TR-β, MCT8, and ENPP2) and oxidative respiration were also assessed in the SK-N-AS cells. DIO3 mRNA expression increased by 9 fold in cells exposed to 400 nM BDE-99, and ENPP2 mRNA expression increased by 2 fold in cells exposed to 500 nM BDE-99 and a mixture of the three congeners, but no other significant effects on mRNA expression were noted. The basal respiration rates and other parameters of oxidative respiration were not significantly altered by the PBDEs or OH-BDEs, but proton leak was increased by over 400% in cells exposed to 2 μM 5’-OH-BDE-99. The results of this study indicate that PBDEs and their OH-BDE metabolites alter thyroid hormone signaling in these specialized brain cells.

4.1 Introduction

Polybrominated diphenyl ether (PBDE) flame retardants are commonly detected in many environmental matrices, including air, arctic organisms, food, and housedust, due to their heavy use in furniture foam and electronics casing (de Wit, 2002). The use of PBDEs has declined over the past decade due to the addition of the PentaBDE and OctaBDE commercial PBDE mixtures to the Stockholm Convention on Persistent Organic Pollutants and phase-outs and restricted usage of the DecaBDE commercial mixture in the United States and Europe (EU, 2009a). Due to the persistence of PBDEs in the environment and especially the indoor environment, where household items
containing PBDEs are still in use, human exposure to PBDEs will likely continue for decades. Further research regarding the potential toxicity of PBDEs is necessary to make appropriate decisions regarding these persistent contaminants, including the regulation of recycled products and e-waste containing PBDEs (Chi et al., 2011). PBDEs and their hydroxylated metabolites (OH-BDEs) have been associated with impaired neurodevelopment in human birth cohorts and animal exposure models (Gascon et al., 2011; Herbstman et al., 2010). However, the specific mechanisms underlying the negative effects on neurodevelopment have not been fully elucidated. Previous studies have suggested that alterations in neuronal function, glial cell migration, and thyroid hormone levels could negatively affect neurodevelopment (Viberg and Eriksson, 2011). Due to their structural similarity to thyroid hormones, PBDEs and OH-BDEs have been shown to alter the transport, metabolism, and receptor binding of thyroid hormones (Butt et al., 2011; Kudo and Yamauchi, 2005; Meerts et al., 2000; Szabo et al., 2009). Alterations in the levels of thyroid hormones reaching the brain during neurodevelopment could impact the growth, differentiation, and migration of neurons and negatively affect neurodevelopment (Porterfield, 2000).

In our previous study, we determined that PBDEs and OH-BDEs decrease the activity of the thyroid hormone activating enzyme Type 2 deiodinase (DIO2) in cultured human astrocyte cells. Because DIO2 converts thyroxine (T4) transported from the thyroid gland to triiodothyronine (T3), which is the most potent ligand of thyroid
receptors, decreased DIO2 activity could cause the concentration of T3 in the brain to decrease and may negatively affect the development and functioning of neurons. To compensate for potential alterations in the concentrations of T3 in the brain, neurons express Type 3 deiodinase (DIO3), which inactivates T4 and T3. We hypothesized that changes in thyroid hormone levels in astrocyte cells by PBDEs would alter the functioning of neuronal cells cultured together with the astrocytes.

Figure 16: Thyroid hormone signaling and deiodination in astrocytes and neurons.

A previous study introduced a co-culture experimental design using a glial cell line and a neuronal cell line separated by a membrane in a 6-well plate and suggested that the experimental design could be used for in vitro investigations of thyroid
hormone function in the brain (Freitas et al., 2010). In that study and in another study, the authors determined that the mRNA expression of ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2) in SK-N-AS cells exhibited a dose-dependent response to the concentration of T₃ in the cell culture medium, and could be used as a marker for disrupted thyroid hormone levels (Freitas et al., 2010; Jo et al., 2012). Other thyroid hormone-related genes include, DIO3, thyroid receptor alpha (TR-α), thyroid receptor beta (THR-β), and monocarboxylate transporter 8 (MCT8). Another study found that the relative rates of oxidative respiration and glycolysis in SK-N-AS cells exhibited a dose-dependent response to the T₃ concentration in the cell culture medium (Simonides et al., 2008). The combined use of ENPP2 mRNA expression and measurements of oxidative metabolism rates may therefore be an effective method for determining alterations in thyroid hormone metabolism in the SK-N-AS cells. Combining this approach with a co-culture experimental design using H4 glioma cells could allow complex alterations in DIO2 and DIO3 activity, as well as the functioning of thyroid hormone transporters and receptors to be investigated in response to drugs or toxicants, such as PBDEs.

In the current study, we used a co-culture experimental design to determine the effects of the commonly detected PBDE congener, BDE-99, and two OH-BDE metabolites, 3-OH-BDE-47 and 5’-OH-BDE-99, in H4 glioma cells and SK-N-AS neuroblastoma cells. The goal of the study was to determine if PBDEs alter the: 1)
production and transport of T₃ and rT₃ in the cell cultures, 2) DIO2 or DIO3 activity, 3) mRNA expression of several thyroid-related genes, and 4) cellular respiration and mitochondrial function in SK-N-AS cells.

4.2 Materials and Methods

4.2.1 Reagents and Materials

BDE-99, 3-OH-BDE-47, and 5′-OH-BDE-99 were purchased as neat standards (purity >97%) from Accustandard (New Haven, CT). Dithiothreitol (DTT), T₄, T₃, rT₃, and T₂ were purchased from Sigma Aldrich (St. Louis, MO). Advanced Dulbecco’s Modified Eagle Medium (A-DMEM) cell culture medium and other cell culture reagents were purchased from Life Technologies (Carlsbad, CA). All solvents and other reagents were purchased from VWR (Radnor, PA). H4 glioma cells (ATCC® catalog number HTB-148™) and SK-N-AS cells (ATCC® catalog number CRL-2137™), which were originally purchased from the American Type Culture Collection, were obtained from the Duke University Cell Culture Facility (Durham, NC). Two types of Corning (Corning, NY) transwell inserts were used for either deiodinase activity experiments (6 well, 0.4 μM pore size, 24 mm diameter polyester membranes) or respiration experiments (24 well, 0.4 μM pore size, 6.5 mm diameter polyester membrane). For mitochondrial respiration experiments, the Corning Transwell inserts were placed in Seahorse Bioscience (North Billerica, MA) 24-well cell culture plates for analysis using the Seahorse Bioscience XFe24 Bioanalyzer and the Seahorse Bioscience Mitochondrial Stress Test Kit containing
oligomycin, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), antimycin A, and rotenone.

### 4.2.2 Cell Culture

H4 glioma cells and SK-N-AS neuroblastoma cells were grown in DMEM supplemented with 10% FBS, 30 nM selenium (as sodium selenite), 100 units mL$^{-1}$ penicillin, and 100 μg mL$^{-1}$ streptomycin at 37°C and 5% CO$_2$. All experiments were performed with cells that were thawed from the same passage number. The culture medium was changed to A-DMEM supplemented with 30 nM selenium, 2 mM L-glutamine, penicillin/streptomycin, 0.1% bovine serum albumin (BSA), and 500 pM T$_4$ 24 h before each experiment. Assuming a 3.5% free fraction of T$_4$, which has been reported in a previous study with 0.1% BSA (Bianco et al., 2014), the free T$_4$ concentration was ~18 pM, which is within the normal range of free T$_4$ concentrations in human serum (16-36 pM) (Soldin et al., 2009). Cells were thawed and plated at a density of 2 x 10$^4$ cells cm$^{-2}$ in T-75 flasks and transferred to either 6-well plates or 24-well Seahorse plates for experiments.

Transwell inserts in 6-well plates were used to simultaneously expose both cell types to the same culture medium to determine changes in the metabolism of T$_4$ over a 48 h exposure as shown in Figure 17. H4 cells were plated in 24 mm inserts in the 6-well plates at a density of 5 x 10$^4$ cells cm$^{-2}$, and SK-N-AS cells were plated in the 6-well plates at a density of 5 x 10$^4$ cells cm$^{-2}$. Cells were maintained in separate 6-well plates for the
first 48 h after plating to prevent cross-contamination between unattached cells in the
culture medium. All experiments were performed both in plates with co-cultured cells
and in plates with SK-N-AS cells grown alone with 140 pM total T₃ added to the culture
medium.

![Co-culture experimental design. Modified from an image created by Sean Mutchnick obtained from http://www.hhhoney.com/transwell-schematic/.

4.2.3 Dosing

BDE-99, 3-OH-BDE-47, and 5’-OH-BDE-99 and other test compounds were
dissolved in DMSO at 1,000× the desired final concentration in the cell culture medium.
The concentrations of the compounds in the dosed culture medium were determined
after dosing in all the experiments using GC/MS for BDE-99 (Stapleton et al., 2008b) and
LC/MS/MS (Erratico et al., 2010) for 3-OH-BDE-47 and 5’-OH-BDE-99. In 48 h
experiments with BDE-99, an additional dose of 50% of the initial dose was added to the
cell culture medium every 12 h. The exposure concentrations are shown in Table 4 and
represent the average concentrations of the compounds over the entire experiment, which were measured at 0, 11.5, 12.5, 23.5, 24.5, 35.5, 36.5 and 48 h.

**Table 4: Average concentrations of BDE-99 and OH-BDEs during the 48 h exposures measured every 12 h.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Average Concentration in Dosing Group (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
</tr>
<tr>
<td>BDE-99</td>
<td>0.2</td>
</tr>
<tr>
<td>3-OH-BDE-47</td>
<td>0.4</td>
</tr>
<tr>
<td>5'-OH-BDE-99</td>
<td>0.4</td>
</tr>
</tbody>
</table>

1 Single refers to treatment groups containing each congener separately  
2 Mixture refers to the concentration of each congener in a mixture of all 3 congeners

### 4.2.4 Cytotoxicity Assays

Damage to the cell membrane was assessed by measuring the activity of glucose-6-phosphate dehydrogenase in cell culture medium exposed to the cells for 48 hours (Batchelor and Zhou, 2004). The reduction of resazurin (30 µM) to resarufin over 45 min was analyzed to measure the amount of G6PDH released from the cells into the culture medium, and the results were normalized to the DNA content in each well and the controls cells dosed with the DMSO vehicle.

### 4.2.5 Deiodinase Assays

Cells were scraped into KPO₃ buffer containing 0.25 M sucrose, 30 mM DTT, and 1 mM EDTA and sonicated for 15 s on ice. DIO2 assays were performed using microsomal subcellular fractions after centrifugation at 100,000 x g. DIO3 assays were
performed using whole cellular homogenate from SK-N-AS cells. Assays were performed for 75 min, and 1 mL of 1 M HCl and 0.5 ng of each stable isotope-labelled surrogate standard were added to stop the reaction mixture. The reactions mixtures were extracted using Agilent OPT Solid Phase Extraction tubes and analyzed using LC/MS/MS following our previously published analytical method (Roberts et al., 2011). The total mass of T₃ or rT₃ formed in each assay was adjusted to the background levels of T₃ or rT₃ in the T₄ dosing stock and to background levels of T₃ or rT₃ in the cultured cells. The protein content of the cell homogenates was determined using the Bradford assay, and the DNA content was determined using the PicoGreen® double-stranded DNA assay (Bradford, 1976; Otto, 2005). The DIO2 activity measurements were calculated as fmol T₃ formed min⁻¹ mg protein⁻¹, and the DIO3 activity measurements were calculated as fmol rT₃ formed min⁻¹ mg protein⁻¹. All the results are normalized to the values of controls containing equivalent amounts of the dosing vehicle, DMSO, for each experiment.

The concentrations of T₄, T₃, rT₃, and T₂ were measured in 3 mL of cell culture medium from each well of the 6-well plates. The cell culture medium was acidified by adding 1 mL of 1 M HCl, and the samples were processed following the same method used for the DIO2 and DIO3 assays after the addition of 0.5 ng of each stable isotope-labelled surrogate standard. The concentrations reported in this analysis represent total concentrations of each thyroid hormone, including both protein-bound and unbound
hormone. The free concentration of thyroid hormones can be estimated using the following free fraction percentages determined in previous studies using serum-free cell culture medium and 0.1 BSA: 3.5% free fraction of T₄ (Bianco et al., 2014), 8% free fraction of T₃, and 3% free fraction of rT₃ (van der Putten et al., 2003).

4.2.6 Enzyme Kinetics and Inhibition Assays

The DIO3 kinetic parameters in cellular homogenates were evaluated using a range of T₄ concentrations from 1.0-250 nM for 75 min incubations with 300 μg protein. The kinetic parameters were calculated and compared with JMP Pro 11 (Cary, NC) using the Hill equation, which is a modified form of the Michaelis Menten equation with the addition of coefficients to account for substrate cooperativity or multiple binding sites:

$$\nu = \frac{V_{max} \times S^n}{K_m^n + S^n}$$

where $V_{max}$ is the maximum reaction rate, $S$ is the substrate concentration, $n$ is the Hill coefficient, and $K_m$ is the substrate concentration at 50% of the maximum reaction rate, similar to the Michaelis Menten constant (Porter and Miller, 2012). In vitro DIO3 inhibition assays were performed with homogenates of SK-N-AS cells, 300 μg protein, 6 nM T₄, and 9 concentrations of BDE-99, 5′-OH-BDE-99, and 3-OH-BDE-47 ranging from 0.05 μM to 100 μM. The IC₅₀ was calculated using a 3-parameter nonlinear model in JMP Pro 11, and the $K_i$ was calculated using the following equation (Lazareno and Birdsall, 1993):
4.2.7 mRNA expression analysis

Total RNA was extracted from a 300 μL aliquot of the scraped cells (approximately 30% of the total cells scraped from the dish) using the Quick-RNA™ MicroPrep kit from Zymo Research (Irvine, CA) and quantified using the Nanodrop 1000 (Thermo Scientific). Total RNA was converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit from Life Technologies (Carlsbad, CA). Approximately 10 ng of cDNA was analyzed in 20 μL quantitative PCR (qPCR) reactions using TaqMan® Gene Expression Assays (Life Technologies) for DIO3 (Hs00956431_s1), RPL13A (Hs04194366_g1), MCT8 (Hs00185140_m1), TR-α (Hs00268470_m1), TR-β (Hs00230861_m1), and ENPP2 (Hs00905117_m1) with an Applied Biosystems (Foster City, CA) 7300 Real-Time PCR System. We chose RPL13A as the internal reference gene in our previous study due to its stable expression between control cells and cells treated with BDE-99. Expression values are reported as the expression ratio relative to control samples normalized to RPL13A using the 2^{-ΔΔCt} method.

4.2.8 Metabolic Analysis

An XFe24 Seahorse Bioanalyzer was used to measure the extracellular acidification rate (ECAR; mP H min$^{-1}$) and the oxygen consumption rate (OCR; pmol min$^{-1}$

\[
K_i = \frac{IC_{50}}{S} + \frac{1}{K_m}
\]
in SK-N-AS cells grown in 24-well, Seahorse Biosciences cell culture plates. After a 48 h exposure to BDE-99 and the OH-BDEs, the cell culture medium was exchanged with Seahorse Biosciences XF Assay medium, which is a buffer-free and serum-free cell culture medium required for the Seahorse Bioanalyzer. The basal metabolic rate was measured 3 times per well for 5 min cycles before injection of oligomycin (ATP synthase inhibitor), FCCP (electron transport chain uncoupler), and a mixture of antimycin A and rotenone (complex III and I chain inhibitors) in that order. The concentrations of each injected compound were optimized in preliminary experiments following the manufacturer’s instructions (1 μM oligomycin, 1 μM FCCP, and 0.75 μM each of antimycin A and rotenone). The background-corrected OCR was then measured 3 times after each injection of the stress test compounds for 5 min cycles. For further data analysis, the OCR measurements were normalized to the DNA content in each well, and the 3 measurements performed for each injection were used to calculate the basal respiration (basal-antimycin A/rotenone), ATP production (basal-oligomycin), proton leak (oligomycin-antimycin A/rotenone), maximal respiration (FCCP-antimycin A/rotenone), spare respiratory capacity (FCCP-basal), and non-mitochondrial respiration (antimycin A/rotenone-background), as shown in Figure 18.
Figure 18: Assay design for the XFe24 Seahorse Bioanalyzer and the Seahorse Mitochondrial Stress Test Kit to measure multiple aspects of cellular energy expenditure. Three measurements were performed between the injections of oligomycin, FCCP, and antimycin A/rotenone.

4.2.9 Statistics

ANOVA tests were performed using JMP Pro 11 to test for significant effects of the treatment, culture condition (i.e., co-cultured cells or SK-N-AS cells cultured alone), and experimental day on the measured values. Significant effects and interactions were further tested using Tukey’s post-hoc test. In cases where significant main effects of treatment were determined without interactions of treatment × culture condition, the statistical results are reported for the combined samples in both culture condition groups without separating by culture condition. The mRNA expression results were analyzed using the ΔΔCt values and parametric statistics (data were log2 normal) and
are reported graphically as the $2^{-\Delta\Delta Ct}$ expression ratios. The minimum significant
difference from control was estimated for nonsignificant data using the control
difference limits reported by Tukey’s post-hoc test for data tested using parametric
statistics. The results for thyroid hormone levels in the culture medium and DIO2 and
DIO3 activity were log transformed before analysis because the percent control data are
expressed as ratios. For all analyses and reported data, statistical significance is defined
as p<0.05. All experiments were performed with 3-4 samples and repeated on a separate
day. When there was no significant effect of the experimental day, the results from each
experiment were combined for a total of 7-8 samples. In the experiments reported in this
study, there was no significant effect of the treatments on the ratio of total protein to
DNA. The results for each experiment were normalized to the control values in that
experiment and combined with the results from a repeated experiment.

4.3 Results

4.3.1 Transwell Co-culture of H4 and SK-N-AS cells

Human SK-N-AS neuroblastoma and H4 glioma cells were co-cultured and
exposed to either reverse triiodothyronine (rT3) as a positive control, or to increasing
doses of BDE-99, 5’OH-BDE-99 or 3-OH-BDE-47 for 48 h. In each experiment, the
concentration of T3 in the culture medium was measured at the end of the exposure, and
the basal DIO activity (DIO2 and DIO3) was measured in homogenates prepared from
the cells at the end of the experiment. In the negative control samples, the shared cell
culture medium contained 48.4 ± 3.4 pM T₃ and 38.8 ± 5.7 pM rT₃ at the end of the exposure period. When rT₃ (100 nM) was added to the co-cultured SK-N-AS and H4 cells as a positive control, the T₃ concentration decreased significantly by 63.1 ± 3.7% (Figure 19). The concentration of T₃ also decreased significantly in the culture medium of cells exposed to 0.5 μM BDE-99 (59.1 ± 4.5% decrease) and 2 μM BDE-99 (76.4 ± 2.9% decrease), but was not significantly different from control cells exposed to the lowest dose of BDE-99 tested (0.2 μM). The T₃ concentration also decreased significantly (68.6 ± 3.4%) after exposure to 2 μM 5′-OH-BDE-99, but no significant differences were observed for the two lower doses of 5′-OH-BDE 99, or any dose of 3-OH-BDE-47. Similar to BDE-99, exposure to the higher two doses of the BDE/OH-BDE mixture significantly decreased the T₃ concentration by 56.6 ± 3.2% (middle dose) and 67.9 ± 1.9% (high dose). In contrast, in SK-N-AS cells cultured without H4 cells, there was no significant change in the concentration of T₃ after 48 h for any of the tested compounds.
Figure 19: T$_3$ concentrations in the culture medium of cells exposed to the PBDE and OH-BDE compounds for 48 h. A) H4 and SK-N-AS cells cultured together in a 6-well plate with 500 pM T$_4$ or B) SK-N-AS cells cultured alone with 500 pM T$_4$ and 140 pM T$_3$. Two-factor ANOVA indicated a significant interaction of treatment × culture condition (p<0.001) * Indicates statistically significant difference (p<0.05) from control cells in the corresponding culture condition group using Tukey’s post-hoc test (p<0.05; n=7-8, 2 experiments). Dose-response relationships and full statistical results are shown in Figure 45.

The concentration of rT$_3$ in the culture medium of the co-cultured H4 and SK-N-AS cells increased significantly by up to 518 ± 88% after exposure to 0.7 µM and 2 µM 5’-OH-BDE-99 and decreased significantly after exposure to 0.5 µM BDE-99 (55.0 ± 10.5% decrease) and 2 µM BDE-99 (45.7 ± 4.2% decrease) (Figure 20A). No significant effects on
the concentration of rT$_3$ were observed in cells treated with 3-OH-BDE-47 or the mixture. Similar effects were observed in SK-N-AS cells cultured without H4 cells (Figure 20B). Because there was no significant interaction effect of culture condition (i.e., co-cultured cells or SK-N-AS cells cultured alone) on the concentrations of rT$_3$ in the culture medium, statistics were performed on the combined samples from both culture groups. The concentration of rT$_3$ increased significantly by 495 ± 99% in cells exposed to 2 μM 5′-OH-BDE-99 and decreased significantly after exposure to 0.5 μM BDE-99 (52.7 ± 4.5% decrease) and 2 μM BDE-99 (53.7 ± 5.5% decrease). The results of cells exposed to the positive control (100 nM rT$_3$) were excluded from this analysis due to the high background concentration in the culture medium.
Figure 20: rT₃ concentrations in the culture medium of cells exposed to the PBDE and OH-BDE compounds for 48 h. A) H4 and SK-N-AS cells cultured together in a 6-well plate with 500 pM T₄ or B) SK-N-AS cells cultured alone with 500 pM T₄ and 140 pM T₃. Two-factor ANOVA indicated main effects of treatment and culture condition (p<0.05) but no interaction of treatment x culture condition. Therefore, statistics were only performed on the combined data from A and B without separating by treatment condition. * Indicates statistically significant difference (p<0.05) from the control cells using Tukey’s post-hoc test (p<0.05; n=7-8, 2 experiments).

The basal activity of DIO2 in the microsomal fraction of the control H4 cells was 4.35 ± 0.22 fmol min⁻¹ mg protein⁻¹ after the 48 h exposure, which was measured as the formation of T₃ from T₄. The concentrations of T₂ and rT₃ were below the detection limit of 0.2 fmol min⁻¹ mg protein⁻¹. Treatment of the co-cultured H4 cells with 2 μM BDE-99
and 100 nM rT3 for 48 h significantly decreased the DIO2 activity measured in the cell homogenate by 34.9 ± 5.3% and 29.1 ± 6.0%, respectively (Figure 21). DIO2 activity was not detected in SK-N-AS cells.

Figure 21: Alteration of the DIO2 activity in H4 cells co-cultured with SK-N-AS cells in a 6-well plate and exposed to the PBDE and OH-BDE compounds for 48 h with 500 pM T4. One-way ANOVA indicated a significant effect of treatment (p<0.001). * Indicates statistically significant difference (p<0.05) from control cells using Tukey’s post-hoc test (p<0.05; n=7-8, 2 experiments).

After the 48 h co-culture exposures, the basal DIO3 activity in the SK-N-AS cell homogenates was 4.82 ± 0.35 fmol min⁻¹ mg protein⁻¹, which was measured as the formation of rT3 from T4. The concentrations of T2 and T3 were below the detection limit of 0.2 fmol min⁻¹ mg protein⁻¹. The mean DIO3 activity in the cell homogenates increased significantly by 80.6 ± 11.2% after a 48 h exposure to 2 μM 5'-OH-BDE-99 but was not significantly altered after exposure to any of the other compounds or doses (Figure 22A). In SK-N-AS cells cultured without H4 cells, the DIO3 activity was not significantly altered compared with control cells for any of the treatments (Figure 22B). Cells cultured
in the presence of 100 nM rT3 (positive control for DIO2 inhibition) contained high levels of rT3 absorbed from the culture medium, which prevented the accurate measurement of DIO3 activity and these results were excluded from the analysis.

Figure 22: DIO3 activity in the homogenate of SK-N-AS cells exposed to the PBDE and OH-BDE compounds for 48 h. A) H4 and SK-N-AS cells cultured together in a 6-well plate with 500 pM T4 or B) SK-N-AS cells cultured alone with 500 pM T4 and 140 pM T3. Two-factor ANOVA indicated a significant interaction of treatment × culture condition (p<0.001). *Indicates significant differences from the controls in the corresponding culture condition using Tukey’s post-hoc test (p<0.05; n=7-8; 2 experiments).
4.3.2 In Vitro Inhibition and Activation of DIO3 in SK-N-AS Cells

The calculated $K_m$ and $V_{max}$ values for DIO3 activity were $5.03 \pm 0.71$ nM T$_4$ and $15.3 \pm 0.51$ fmol min$^{-1}$ mg protein$^{-1}$, respectively, in the homogenate of SK-N-AS cells cultured in serum-free culture medium in the presence of physiologically relevant T$_4$ and T$_3$ concentrations. Nonlinear modeling indicated a slightly better fit of the Hill equation ($r^2$=0.96) compared with the Michaelis Menten equation ($r^2$=0.93) due to the addition of the Hill coefficient. In the basal kinetic experiments, the Hill coefficient ($n$) was 0.8, which indicated negative cooperativity of T$_4$ binding to DIO3. The addition of 12 µM 3-OH-BDE-47 to the kinetics assays caused the $K_m$ of rT$_3$ formation to increase significantly to $17.2 \pm 5.1$ nM T$_4$, while the $V_{max}$ of $16.6 \pm 1.3$ fmol min$^{-1}$ mg protein$^{-1}$ was not significantly different from control ($15.3 \pm 0.51$ fmol min$^{-1}$ mg protein$^{-1}$) (Figure 23A). The Hill coefficient in cell homogenate exposed to 3-OH-BDE-47 decreased to 0.6, which may indicate increased negative cooperativity of T$_4$ binding to DIO3 in the presence of 3-OH-BDE-47. In inhibition assays with SK-N-AS cell homogenate and approximately 6 nM T$_4$, 3-OH-BDE-47 inhibited DIO3 activity (Figure 23B), which was measured as the formation of rT$_3$, over a range of concentrations from approximately 10-90 µM. The calculated IC$_{50}$ for DIO3 inhibition by 3-OH-BDE-47 was $18.6 \pm 1.1$ µM and the $K_i$ was 7.75 µM.
Figure 23: Kinetics of DIO3 activity in cellular homogenates prepared from SK-N-AS cells with a range of T₄ concentrations (black line) and with a range of T₄ concentrations and 12 μM 3-OH-BDE-47 added to the assay buffer. B) Inhibition of DIO3 activity in cellular homogenates prepared from SK-N-AS cells in assays with 6 nM T₄ and a range of 3-OH-BDE-47 concentrations. (2 experiments; n=8)

BDE-99 did not significantly alter DIO3 activity at concentrations up to 85 μM BDE-99 (Figure 24A). At concentrations ranging from 5 to 21 μM 5’-OH-BDE-99, DIO3 activity increased from approximately 3.90 fmol min⁻¹ mg protein⁻¹ to a maximum of 29.2 ± 1.34 fmol min⁻¹ mg protein⁻¹ at a concentration of 21 μM 5’-OH-BDE-99 as shown in (Figure 24B). At concentrations of 35 μM 5’-OH-BDE-99 and higher, DIO3 activity decreased to a minimum of 1.87 ± 0.41 fmol min⁻¹ mg protein⁻¹. Typical enzymatic inhibition or activation models did not fit the unusual shape of the dose response curve of 5’-OH-BDE-99 for DIO3 activity.
Figure 24: A) No significant inhibition of DIO3 activity in cellular homogenates prepared from SK-N-AS cells in assays with 6 nM T3 and a range of BDE-99 concentrations. B) Altered DIO3 activity in cellular homogenates prepared from SK-N-AS cells in assays with 6 nM T3 and a range of 5'-OH-BDE-99 concentrations. (2 experiments; n=8).

4.3.3 Effects of BDE-99 and OH-BDEs on DIO3, ENPP2, THR, and MCT8 mRNA Expression

To measure the relative mRNA expression of DIO3, ENPP2, TR-α, TR-β, and MCT8, RNA was extracted from SK-N-AS cells after 48 h exposures to BDE-99 and the OH-BDEs. Because there was no significant interaction of treatment × co-culture condition, statistics were performed on the combined samples from both culture conditions (i.e., co-cultured cells or SK-N-AS cells grown without H4 cells). Treatment with 0.5 μM BDE-99 significantly increased the expression of DIO3 mRNA by up to 10 fold (Figure 25), but DIO3 mRNA expression was not altered by any of the other tested compounds including the positive control rT3. When the data from both exposures (co-culture and SK-N-AS alone) are normalized to the co-culture control, the mRNA
expression of the SK-N-AS cells grown alone (T3=140 pM) is ~1.5 fold higher than the co-cultured SK-N-AS cells (T3=48 pM), but the difference was not statistically significant.

Figure 25: Expression ratios ($2^{-\Delta\Delta Ct}$) of DIO3 mRNA relative to control in SK-N-AS cells. A) H4 and SK-N-AS cells cultured together in a 6-well plate with 500 pM T3 or B) SK-N-AS cells cultured alone with 500 pM T3 and 140 pM T3. Two-factor ANOVA indicated a main effect of treatment but no effect of culture condition ($p<0.05$) or interaction of treatment $\times$ culture condition. Therefore, statistics were only performed on the combined data from A and B without separating by treatment condition. * Indicates statistically significant difference ($p<0.05$) from the control cells using Tukey’s post-hoc test ($p<0.05; n=7-8$, 2 experiments).
Similar to the *DIO3* mRNA expression, because there was no significant
interaction of treatment × co-culture condition, statistics were performed on the
combined *ENPP2* mRNA expression ratios from both culture conditions (i.e., co-cultured
cells or SK-N-AS cells grown without H4 cells). The *ENPP2* mRNA expression ratio
decreased to 0.68 in SK-N-AS cells treated with 100 nM rT₃, but the difference was not
statistically significant. BDE-99 at all 3 doses and the low and middle doses of the mixed
compounds significantly increased mRNA expression of *ENPP2* by 2-4 fold (Figure 26).
None of the other compounds significantly altered *ENPP2* mRNA expression. The
relative mRNA expression of the control SK-N-AS cells in Figure 26B was approximately
1.5 times higher than the control SK-N-AS cells cultured with H4 cells shown in Figure
26A, but the difference was not statistically significant.
Figure 26: Expression ratios (2^{-ΔΔCt}) of ENPP2 mRNA relative to control in SK-N-AS cells. A) H4 and SK-N-AS cells cultured together in a 6-well plate with 500 pM T3 or B) SK-N-AS cells cultured alone with 500 pM T3 and 140 pM T3. Two-factor ANOVA indicated main effects of treatment and culture condition (p<0.05) but no interaction of treatment × culture condition. Therefore, statistics were only performed on the combined data from A and B without separating by treatment condition. * Indicates significant difference from control using Tukey’s post-hoc test (p<0.05; n=7-8; 2 experiments).

BDE-99, 5’-OH-BDE-99, and 3-OH-BDE-47 did not significantly alter the mRNA expression of TR-α, TR-β, or MCT8 at any of the 3 tested doses in SK-N-AS cells cultured with or without H4 cells (see 4.5 Supplementary Figures Figure 31, Figure 32, and Figure...
33. Given the variability among the 7-8 samples in each treatment group in the mRNA analyses there was only statistical power to identify decreases greater than ~0.6 or increases greater than ~2 in the mRNA expression ratios at p<0.05.

4.3.4 Effects of BDE-99 and OH-BDEs on Oxidative Respiration

Oxygen consumption rates (OCR) were evaluated in exposed and control SK-N-AS cells. To determine the responsiveness of the basal oxidative respiration rate to T₃, cells were treated with increasing concentrations of T₃ from 30 to 400 pM (Figure 27). As expected, the OCR increased significantly by 150 ± 30% and 180 ± 60% cells treated with 200 pM and 400 pM T₃, respectively, for 2 h compared with control cells with 30 pM T₃.

![figure27.png](attachment:figure27.png)

**Figure 27:** Oxygen consumption rate in SK-N-AS cells exposed to T₃ for 2 h. Linear regression demonstrated a significant relationship between the T₃ concentration and the oxygen consumption rate (n=3; 1 experiment).

Further experiments were performed to determine the bioenergetics profiles of the SK-N-AS cells using an XFe24 Seahorse Bioanalyzer, which measures the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) in living cells.
The OCR measured in the SK-N-AS cells was altered by each of the injected compounds (oligomycin, FCCP, and antimycin A/rotenone) and used to calculate various aspects of the energy consumption profile (Figure 28A). The same bioenergetics profile was analyzed in SK-N-AS cells exposed to BDE-99 and the OH-BDEs for 48 h, and the results were plotted as shown in Figure 28B. These results were used to calculate the various endpoints of oxidative respiration as shown in Figure 18.
Figure 28: Oxygen consumption rate (OCR) of SK-N-AS cells exposed to vehicle control (A; blue), rT3 (A; orange), or BDE-99 (B) for 48-h. The following treatments were added to the cells during the analysis: 0-10 min, DMSO control; 15-25 min, oligomycin; 30-40 min, FCCP; 45-55 min, antimycin A and rotenone. The results represent the mean ± SEM of 4 samples analyzed during 2 experiments.

Figure 29A shows the basal oxygen consumption rate in SK-N-AS cells after a 48 h co-culture. Treatment of the co-cultured cells with 100 nM rT3 caused the basal oxygen consumption rate to decrease by approximately 25%, but the difference was not statistically significant. There was no significant effect on the basal oxygen consumption rate in co-cultured SK-N-AS cells or SK-N-AS cells cultured alone (Figure 29B).
Figure 29: Basal oxygen consumption rate of SK-N-AS cells after a 48 h exposure to the PBDE and OH-BDE compounds for 48 h in cells A) co-cultured with H4 cells in a 6-well plate with 500 pM T<sub>4</sub> or B) cultured alone with 500 pM T<sub>4</sub> and 140 pM T<sub>3</sub>. Two-factor ANOVA indicated main effects of treatment and culture condition (p<0.05) but no interaction of treatment × culture condition. Therefore, statistics were only performed on the combined data from A and B without separating by treatment condition. There were no significant differences from control using Tukey’s post-hoc test (p<0.05; n=7-8; 2 experiments).

Proton leak, which was calculated as the portion of the basal oxygen consumption rate not attributed to ATP production, increased by approximately 200% in SK-N-AS cells treated with the high dose of 5′-OH-BDE-99 (Figure 30). However, no other compounds affected the proton leak parameter. No significant alterations were
observed in any treatments for any of the following parameters: basal oxygen consumption rate, basal extracellular acidification rate, maximal respiration rate, metabolic reserve capacity, or nonmitochondrial respiration (4.5 Supplemental Figure 34).

Figure 30: Increased proton leak in SK-N-AS cells after a 48 h exposure to the PBDE and OH-BDE compounds in cells A) co-cultured with H4 cells in a 6-well plate with 500 pM T4 or B) cultured alone with 500 pM T4 and 140 pM T3. Two-factor ANOVA indicated an interaction between treatment × culture condition (p<0.05). * Indicates significant difference from control in the corresponding culture condition group using Tukey’s post-hoc test (p<0.05; n=7-8; 2 experiments).

Similar to the mRNA analysis, high variability among the 7-8 samples within the treatment groups in the respiration analysis restricted the statistical power to identify
decreases less than 24-40% or increases less than 40-280% for ATP production, basal respiration, maximum respiration, proton leak, nonmitochondrial respiration, cytotoxicity, and the basal extracellular acidification rate with statistical significance at p<0.05.

4.4 Discussion

This is the first study to demonstrate the potential of an environmental contaminant (i.e., BDE-99 and its metabolite 5’-OH-BDE-99) to disrupt the regulation of T₃ concentrations by decreasing DIO2 activity and increasing DIO3 activity in co-cultured human brain cells. PBDEs have been associated with decreased performance on developmental tests and fine motor coordination (among other endpoints) in humans (Herbstman et al., 2010). However, identification of the mechanisms driving these effects is necessary to strengthen the confidence in these associations between PBDEs and impaired neurodevelopment. DIO2 and DIO3 tightly control the level of T₃ in the brain, and the interplay between thyroid-hormone activating DIO2 activity and thyroid hormone-inactivating DIO3 is especially important during neurodevelopment. The goal of this study was to determine the effects of PBDEs and OH-BDEs on DIO2 and DIO3 activity in H4 glioma cells and SK-N-AS neuroblastoma cells cultured together. The T₃ concentration decreased by 60-76% in co-cultured H4 glioma and SK-N-AS neuroblastoma cells exposed to BDE-99, providing a potential mechanism for the previously described associations between PBDEs and impaired neurodevelopment.
The co-cultured cells were exposed for 48 h to 3 doses of BDE-99, 3-OH-BDE-47, and 5′-OH-BDE-99 individually and to a mixture of the 3 compounds designed to represent the approximate in vivo proportions of the congeners in human serum (~12:1:0.5) (Chen et al., 2013; Qiu et al., 2009). In our previous study, BDE-99, 5′-OH-BDE-99, and 3-OH-BDE-47 significantly decreased DIO2 activity in primary human astrocytes and H4 glioma cells. In the current study, we determined the effects of decreased DIO2 activity on the T₃ and rT₃ concentrations in the culture medium of co-cultured H4 and SK-N-AS neuroblastoma cells as a method of examining thyroid hormone signaling between glial and neuronal cells in shared culture medium. In addition to the co-culture experiments, the SK-N-AS cells were also cultured without H4 cells to evaluate the direct effects of BDE-99 and the OH-BDEs on SK-N-AS cells that had a fixed concentration of T₃ in the cell culture medium (140 pM T₃).

At the beginning of the 48 h exposures, the culture medium contained T₃ concentrations less than 0.01 pM. The T₃ concentration in the culture medium of the control cells increased to 48.4 ± 3.4 pM at the end of the 48 h co-culture due to DIO2 activity (outer ring deiodination of T₄ to form T₃) in the H4 glioma cells. In a previous study, the T₃ free fraction was determined to be 8% of the total T₃ concentration in serum-free cell culture medium with 0.1% BSA, which is the same conditions used in this study (van der Putten et al., 2003). Therefore, the free T₃ concentration in the cell
culture medium of the control cells after 48 h was approximately 4 pM, which is within
the normal serum free T3 concentration range of 2.3-9.2 pM free T3 (Soldin et al., 2009).
The 48 h exposure duration was chosen based on a previous study with co-cultured H4
and SK-N-AS cells in which the effects of decreased DIO2 and DIO3 activity were most
clearly observed after a 48 h exposure compared with 12-24 h exposures (Freitas et al.,
2010). Treatment of the co-cultured cells with BDE-99, 5'-OH-BDE-99, and a mixture of
the compounds for 48 h caused T3 concentrations to decrease by 36-76% compared with
control culture medium. Based on the assumed free fraction of 8% (van der Putten et al.,
2003), the free T3 concentrations decreased to 0.96-2.6 pM, which are in the low limits of
or below the normal range in human serum (i.e., 2.3-9.2 pM free T3) (Soldin et al., 2009).
However, PBDEs and OH-BDEs may effect the binding of T4 and T3 to albumin;
therefore, the assumptions of the free fraction may be affected by the exposures. Future
studies should directly measure the free T3 and T4 concentrations in this type of
experiment.

The DIO2 activity decreased significantly in cells exposed to 100 nM rT3 (positive
control) and the highest dose of BDE-99 compared with the negative control; however,
the OH-BDEs did not significantly alter DIO2 basal activity in the H4 cells. In our
previous study, we observed stronger decreases in DIO2 activity over a shorter exposure
time of 1-12 h, which was likely due to the absence of T4 in the cell culture medium in
the previous study. The 48 h exposure in this study was instead performed with a
physiological concentration of T₄. Therefore, the less pronounced decrease in DIO2 activity observed in this study compared with our previous study could be driven by the suppressed DIO2 activity via T₃-mediated increases in proteasomal degradation of DIO2 in cultured cells (Bianco and Larsen, 2005). Although significantly decreased DIO2 activity in H4 cells was not detected after 48 h exposures to most of the tested compounds, the reduced T₃ concentrations in the culture medium could result from subtle decreases in DIO2 activity over the 48 h exposure.

Other mechanisms could also mediate the decreased T₃ concentrations in the culture medium. For example, the binding of both T₄ and T₃ to albumin may be altered by PBDEs and OH-BDEs. A previous study showed that BDE-47 and 6-OH-BDE-47 competitively displaced T₄ and T₃ from gull albumin at IC₅₀ concentrations of ~110 and 1-5 nM, respectively (Ucán-Marin et al., 2010). Competitive displacement of bound T₄ and T₃ would increase the free T₄ and T₃ concentrations in the cell culture medium. This may cause the cells to decrease the DIO2 activity and increase the DIO3 activity in response to the increased free T₄ and T₃ concentrations. Future studies should measure the free thyroid hormone concentrations in the cell culture medium of cells exposed to PBDEs to assess the effects of competitive displacement from serum proteins. Decreased uptake of T₄ into H4 cells, increased sulfation of T₄ and T₃ in H4 and SK-N-AS cells, or increased uptake of T₃ into SK-N-AS and H4 cells, may also effect the bulk concentration of T₃ in the cell culture medium.
Because of the similarity of the substrates and known competitors for DIO1, DIO2, and DIO3 such as T4 and iodoacetate, we expected to observe decreased DIO3 activity in SK-N-AS cells exposed to BDE-99 and the OH-BDEs based on the observed decrease in DIO2 activity in H4 cells. Similar to DIO2, DIO3 activity can be altered via 3 homeostatic mechanisms: increased DIO3 synthesis mediated via transcriptional activation by T3-bound thyroid receptors, post-translational regulation via internalization of DIO3 from the plasma membrane to endosomes (and subsequent recycling back to the plasma membrane or degradation in lysosomes) (Baqui et al., 2003), and catalytic inhibition of DIO3 activity. The concentration of rT3 in the cell culture medium of SK-N-AS cells reflects the DIO3 activity (inactivation of T4 to rT3) over the 48 h exposure to BDE-99 and the OH-BDEs. Two-factor ANOVA indicated that the rT3 concentrations in each treatment were not significantly affected between the co-cultured cells and the SK-N-AS cells cultured alone. Therefore, the effects on the rT3 concentrations are likely driven by direct effects of the PBDEs and OH-BDEs on the SK-N-AS cells, rather than the effects of the decreased T3 levels released from the exposed H4 cells. Exposure to BDE-99 resulted in a decrease in rT3 in the low and high dose groups. However, DIO3 activity was not significantly decreased in the homogenates of cells exposed to BDE-99 for 48 h, and BDE-99 did not significantly inhibit DIO3 activity in vitro. Furthermore, an increase in DIO3 mRNA was observed after treatment with BDE-99. These conflicting results imply that other potential mechanisms may be driving
the reductions in the rT3 concentration in the culture medium of cells treated with BDE-99, such as increased uptake of rT3 into the cells or increased sulfation of rT3. Table 5 presents a summary of previously observed effects, or compensation mechanisms, following changes in TH levels in these types of cells.

Table 5: Expected cellular responses to altered thyroid hormone concentrations including deiodinase activity, mRNA expression of several thyroid hormone-regulated genes, and cellular energy expenditure.

<table>
<thead>
<tr>
<th>TH Condition</th>
<th>DIO Activity</th>
<th>DIO mRNA</th>
<th>ENPP2 mRNA</th>
<th>TR mRNA</th>
<th>MCT8 mRNA</th>
<th>Energy Expenditure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decreased T3</td>
<td>↑ DIO2</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>
| Decreased T3 | ↑ DIO2  ↓ DIO3 | ↑ DIO2  ↓ DIO3 | ↑ ENPP2  ↑ TR-α  ↑ TR-β | ↑ MCT8  ↓ Respiration
| Decreased rT3 | ↑ DIO2      | n/a      | n/a         | n/a     | n/a       | n/a               |

1 These effects are summarized from the results of multiple studies (Arrojo et al., 2013a; Bianco et al., 2002b; Freitas et al., 2010; Simonides et al., 2008)

Surprisingly, the high dose of 5’-OH-BDE-99 caused the concentration of rT3 in the co-cultured medium to increase by over 4 fold compared with the control cells. The increased rT3 concentration in the culture medium corresponded with increased DIO3 activity in the SK-N-AS cell homogenate after the 48 h exposure and increased DIO3 activity in the in vitro DIO3 inhibition experiments. The mRNA expression of DIO3 also doubled in cells treated with all 3 doses of 5’-OH-BDE-99, although the difference was not statistically significant due to the high variability among replicates and treatments.
The results suggest that 5′-OH-BDE-99 activates the DIO3 enzyme and increases the T4 inner ring deiodination rate, but we were not able to determine the mechanism responsible. Direct allosteric or active site activators of DIO1, DIO2, or DIO3 have not been reported previously, and further experiments are required to determine the mechanism.

When added to homogenates of unexposed cells, 3-OH-BDE-47 competitively inhibited both DIO3 ($K_i = 7.75 \mu M$) in this study and DIO2 ($K_i = 1.60 \mu M$) in our previous study; however, the affinity was nearly 5 times lower for DIO3 compared with DIO2. Surprisingly, exposure of living cells to 3-OH-BDE-47 for 48 h did not significantly alter the T3 or rT3 concentrations in the cell culture medium or the DIO2 or DIO3 activity measured in the homogenates of exposed cells. In our previous study, the BSA concentration in the cell culture medium of H4 cells was 0.04%, while in this study, the BSA concentration was 0.1%. The higher BSA concentration may have therefore decreased the free concentration of 3-OH-BDE-47 available to the cells compared with our previous study. Furthermore, OH-BDEs bind to albumin with ~20-100× greater affinity than PBDEs, which would explain why the potency BDE-99 was not also affected by the increased BSA concentration (Ucán-Marin et al., 2010).

The observed decreases in the T3 concentration in the culture medium may alter the functioning of neurons, and we attempted to test whether several thyroid-dependent parameters were altered in the co-cultured SK-N-AS neuronal cells. A previous study
found that the mRNA expression of ENPP2 was increased up to 7 fold in SK-N-AS co-cultured with H4 cells and 20 pM free T4 for 48 h compared with cells not treated with T4 (Freitas et al., 2010; Jo et al., 2012). The mRNA expression of thyroid receptor beta (TR-β) in cultured neurons and astrocytes also increases by 2-4 fold in response to 10x increasing concentrations of T3 (Baas et al., 1998; Blanco et al., 2011; Lebel et al., 1993). Unlike TR-β, thyroid receptor alpha (TR-α) mRNA expression does not typically change in response to T3 concentrations in cell culture medium (Baas et al., 1998; Lebel et al., 1993), and the effects of T3 concentrations on MCT8 mRNA expression in cultured cells are not well-characterized. Significant changes in the expressions of any of these genes would indicate that disruption of the thyroid hormone levels in the culture medium (by changes in DIO2 or DIO3 activity) altered the functioning of the SK-N-AS neuronal cells. We observed increased ENPP2 mRNA expression in cells treated with BDE-99 and the mixture compared with control cells, but the increase occurred both in co-cultured and individually cultured SK-N-AS cells. This implies that BDE-99 altered ENPP2 mRNA expression via a thyroid hormone independent mechanism. Although the levels of T3 were decreased in the culture medium by up to 76% in some treatments including the positive control rT3, the mRNA expression of TR-α, TR-β, and MCT8 were not significantly altered by any of the exposures. Previous studies demonstrating effects on the mRNA transcription of TR-β in cells dosed with 0.03-30 nM T3 reported significant effects between 10-fold differences in T3 concentrations. The control mRNA expression
values of the SK-N-AS cell cultured alone were within 0.5 fold of the co-cultured controls for TR-α, TR-β, MCT8, ENPP2, and DIO3, and were therefore not significantly different, even though the T₃ concentration was nearly tripled in the SK-N-AS cells grown alone compared with the co-cultured cells. A larger decrease or increase in the T₃ concentration may have generated a significant effect on TR-β, ENPP2, and DIO3 mRNA based on the results of previous studies. Unlike the thyroid hormone measurements, the mRNA expression ratio measurements were not adequately powered to determine changes that may be physiologically relevant to the cells. Adequate quality control was performed in all experiments, and the variability among triplicate qPCR reactions was acceptably low. However, high variability occurred between samples within the same treatment group and may be expected based on the adaptable and fluid regulation of these genes. Future experiments incorporating a similar design could use a larger number of samples to overcome the variability in the mRNA expression of these genes or measure the protein concentrations directly.

A previous study showed that increased concentrations of T₃ in the cell culture medium of SK-N-AS cells caused by DIO3 inhibition caused significant increases in basal oxidative metabolism using a Seahorse Bioanalyzer (Simonides et al., 2008). TREs are present on several genes directly responsible for mitochondrial biogenesis including peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and mitochondrial transcription factor A (TFAM) (Song et al., 2011). Therefore, increased T₃
concentrations result in increased numbers of mitochondria per cell and subsequent increases in glucose and fatty acid oxidation. As shown in Figure 27, oxidative metabolism significantly increased in 2 h exposures of SK-N-AS cells to T3 concentrations of 50-400 nM. Figure 28A indicates that SK-N-AS cells cultured alone with T3 added to the culture medium exhibited higher basal and maximal OCR values compared with the co-cultured cells, which may be due to the 3x difference in the T3 concentration between these two culture conditions. However, the addition of rT3 to the co-cultured cells did not decrease the basal OCR even though the concentration of T3 was decreased by greater than 50% in cells treated with rT3. This unexpected result may be due to other confounding factors, including effects on the release of growth factors and other signaling molecules into the cell culture medium and other effects of rT3 on thyroid hormone transport. Similarly, although the thyroid hormone concentration was decreased by up to 76% in cells treated with BDE-99, the metabolic profiles of the cells treated with BDE-99 were very similar to the control cells. As shown in Figure 28B, increasing doses of BDE-99 decreased the basal OCR and altered the metabolic profiles in SK-N-AS cells grown alone with a constant T3 concentration, although the changes in the calculated metabolic parameters were not statistically significant. Similar nonsignificant alterations in the metabolic profile for cells grown in a constant concentration of T3 were observed for 5'-OH-BDE 99 and 3-OH-BDE-47. These results indicate that these compounds may be altering the metabolic profile of the SK-N-AS
cells following other mechanisms (e.g., inhibition of electron transport complexes) in addition to decreased T₃ concentrations in the cell culture medium. This is supported by a previous study, which showed that BDE-49 altered oxidative respiration by inhibiting electron transport complexes at concentrations less than 100 nM (Napoli et al., 2013).

Due to high variability among samples within the same treatment group, our analysis was not adequately powered to significantly determine subtle (50%) increases or decreases in 6 different aspects of oxidative metabolism with the exception of proton leak. Treatment of co-cultured cells with 5′-OH-BDE-99 caused a significant 200% increase in the measured metabolic parameter of proton leak. Proton leak refers to protons that enter the mitochondria independent of ATP synthase through carrier proteins such as adenine nucleotide translocase (ANT) and uncoupling protein 1 (UCP1) (Jastroch et al., 2010). ANT is inducible by T₃ via nongenomic mechanisms, and UCP1 contains a thyroid response element (TRE) in its promoter region (Song et al., 2011; Wrutniak-Cabello et al., 2001). Therefore, it is possible that 5′-OH-BDE-99 may induce the activity of these proton carriers via T₃ mimicry, leading to increased proton leak. However, determining the mechanisms of this effect was beyond the scope of this work. Further studies should further investigate the potential of OH-BDEs to interfere with cellular metabolism by altering proton leak, especially because proton leak is very important in thermogenesis and body temperature regulation, especially during early development (Jastroch et al., 2010).
The co-culture setups in our study allowed 2 types of cells to share culture medium and represent a more physiologically relevant exposure scenario compared with individually cultured cells. The 6-well co-culture setup appears to be a good model for detecting alterations in thyroid metabolism in both cell types. Future studies could utilize this model to test the thyroid hormone disrupting potential of other compounds and mixtures of compounds to determine the potential for thyroid disruption in the brain. The mRNA expression and oxidative metabolism endpoints we measured in the SK-N-AS exhibit significant variation over time and between samples due to the tightly regulated homeostasis by the cell. Unlike the more stable parameters of DIO activity and thyroid hormone levels, the mRNA expression and oxidative metabolism assays were not appropriately powered to detect potentially relevant changes. Future studies could use additional samples in each experiment (8 samples per experiment; 16 total) for each treatment group to improve the ability to measure changes in these endpoints or use different, more stable measurements of protein quantity. A different type of cell could also be exchanged for the SK-N-AS cells to measure endpoints such as neurite organization and outgrowth.

The low doses used in this study represent high but environmentally relevant levels of human exposure to BDE-99 (150 nM) compared with the maximum concentrations of PBDEs detected in previous studies: 79 nM in one occupational exposure study focusing on carpet installers and 46 nM in electronic recyclers (Stapleton
et al., 2008d; Zheng et al., 2014). However, similar to our previous study, significant
effects were not observed at these environmentally relevant doses. The effects seen at
higher doses represent potential mechanisms of thyroid hormone disruption in the
brain, and over a longer exposure or in more sensitive individuals, significant effects
may also be observed at lower doses. The experiments performed in this study used 2
cancer cell lines, which may respond differently to primary cells, although previous
studies have determined that the regulation of DIO2 and DIO3 in these cells closely
represent normal cells (Freitas et al., 2010).

Decreased T3 formation in the brain could be a potential contributor to the
observed effects of PBDEs and OH-BDEs on neurodevelopment. BDE-99 and 5′-OH-
BDE-99 decreased the concentration of T3 in the cell culture medium by over 50% in co-
cultured brain cells. Our previous study showed that PBDEs and OH-BDEs decrease the
activity of DIO2, and it appears that the neuronal cells were unable to compensate for
this effect by decreasing the activity of the T3 inactivating DIO3. Neurons in the placenta
and in the developing brain highly expresses DIO3 to protect developing brain cells
from the proliferative and differentiating effects of T3 while the cells are being organized
into neural structures (Préau et al., 2014). Knockout of the DIO3 gene in mice, which
results in increased T3 concentrations in the brain, profoundly impairs several important
aspects of neurodevelopment, including neural crest migration and development of the
cerebellum and cochlea (Préau et al., 2014). During neurodevelopment, a specific range
of T₃ levels is necessary for proper signaling of differentiation, migration, and proliferation, and disruption of the T₃ equilibrium via effects on DIO2 or DIO3 may impair neurodevelopment. The results of this study indicate that in utero exposure to BDE-99 and 5′-OH-BDE-99 has the potential to alter important regulators of neurodevelopment at by altering DIO2 and DIO3 activity. Further studies are necessary to determine the mechanisms of the decreased rT3 concentrations caused by BDE-99 and the increased DIO3 activity caused by 5′-OH-BDE-99.
4.5 Supplementary Figures

Figure 31: TR-α mRNA expression in SK-N-AS cells that were cultured A) in the presence of 500 pM T₄ and H4 cells for 48 h or B) alone with the addition of 140 pM T₃ and 500 pM T₄ to the cell culture medium. Two-factor ANOVA indicated main effects of treatment and culture condition (p<0.05) but no interaction of treatment x culture condition. Therefore, statistics were only performed on the combined data from A and B without separating by treatment condition. There were no significant differences (p<0.05) from the control cells using Tukey’s post-hoc test (p<0.05; n=7-8, 2 experiments). The power of the assay would have allowed significant results to be determined for mRNA expression ratios less than ~0.5 and greater than ~2.
Figure 32: TR-β mRNA expression in SK-N-AS cells that were cultured A) in the presence of 500 pM T₃ and H4 cells for 48 h or B) alone with the addition of 140 pM T₃ and 500 pM T₄ to the cell culture medium. A global ANOVA analysis showed a main effect of treatment (p<0.001), but no interactions between treatment and dose for A or B (n=8). The power of the assay would have allowed significant results to be determined for mRNA expression ratios less than ~0.6 and greater than ~2.
Figure 33: MCT8 mRNA expression in SK-N-AS cells that were cultured A) in the presence of 500 pM T₄ and H4 cells for 48 h or B) alone with the addition of 140 pM T₃ and 500 pM T₄ to the cell culture medium. Two-factor ANOVA indicated main effects of treatment and culture condition (p<0.05) but no interaction of treatment x culture condition. Therefore, statistics were only performed on the combined data from A and B without separating by treatment condition. There were no significant differences (p<0.05) from the control cells using Tukey’s post-hoc test (p<0.05; n=7-8, 2 experiments). The power of the assay would have allowed significant results to be determined for mRNA expression ratios less than ~0.5 and greater than ~2.
Figure 34: Percent control values for A) nonmitochondrial respiration, B) ATP production, C) extracellular acidification rate, D) maximal respiration, and E) cytotoxicity measured as membrane damage. No effect of treatment on any of the parameters in A-E was observed using ANOVA (n=8).
Table 6: Percent control values for each parameter of oxidative respiration for A) cocultured cells and B) SK-N-AS cells cultured alone.

<table>
<thead>
<tr>
<th></th>
<th>ATP Production</th>
<th>Basal Respiration</th>
<th>Proton Leak</th>
<th>Normochondrial Respiration</th>
<th>Maximal Respiration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
</tr>
<tr>
<td>Ctrl</td>
<td>100%</td>
<td>17%</td>
<td>100%</td>
<td>21%</td>
<td>100%</td>
</tr>
<tr>
<td>rT3</td>
<td>92%</td>
<td>15%</td>
<td>81%</td>
<td>12%</td>
<td>127%</td>
</tr>
<tr>
<td>BDE 99 Low</td>
<td>83%</td>
<td>23%</td>
<td>77%</td>
<td>18%</td>
<td>62%</td>
</tr>
<tr>
<td>BDE 99 Med</td>
<td>7%</td>
<td>28%</td>
<td>68%</td>
<td>14%</td>
<td>63%</td>
</tr>
<tr>
<td>BDE 99 High</td>
<td>91%</td>
<td>12%</td>
<td>96%</td>
<td>20%</td>
<td>98%</td>
</tr>
<tr>
<td>OHBDE 47 Low</td>
<td>94%</td>
<td>12%</td>
<td>108%</td>
<td>10%</td>
<td>90%</td>
</tr>
<tr>
<td>OHBDE 47 Med</td>
<td>89%</td>
<td>14%</td>
<td>104%</td>
<td>12%</td>
<td>98%</td>
</tr>
<tr>
<td>OHBDE 47 High</td>
<td>86%</td>
<td>12%</td>
<td>100%</td>
<td>9%</td>
<td>98%</td>
</tr>
<tr>
<td>OHBDE 99 Low</td>
<td>93%</td>
<td>11%</td>
<td>92%</td>
<td>11%</td>
<td>90%</td>
</tr>
<tr>
<td>OHBDE 99 Med</td>
<td>84%</td>
<td>10%</td>
<td>88%</td>
<td>10%</td>
<td>103%</td>
</tr>
<tr>
<td>OHBDE 99 High</td>
<td>54%</td>
<td>7%</td>
<td>114%</td>
<td>10%</td>
<td>322%</td>
</tr>
<tr>
<td>MIX Low</td>
<td>89%</td>
<td>24%</td>
<td>102%</td>
<td>13%</td>
<td>143%</td>
</tr>
<tr>
<td>MIX Med</td>
<td>68%</td>
<td>24%</td>
<td>94%</td>
<td>19%</td>
<td>133%</td>
</tr>
<tr>
<td>MIX High</td>
<td>86%</td>
<td>17%</td>
<td>82%</td>
<td>10%</td>
<td>108%</td>
</tr>
</tbody>
</table>

4.6 Funding Information
This project was funded by NIEHS R01ES016099 and EPA STAR Fellowship FP-91749601.
5. Discussion

5.1 Potential Mechanism of PBDE Neurodevelopmental Effects

The results of this thesis research support the main hypothesis that PBDEs and OH-BDEs have the potential to disrupt thyroid hormone metabolism in the human brain. While several studies have provided evidence of the toxic effects of PBDEs and OH-BDEs on various aspects of neurophysiology, the specific mechanism or molecular target of these effects in the brain has not been clearly identified. Data generated by this thesis research contributes additional knowledge of the potential mechanisms of PBDE toxicity that should be considered in risk assessments and when designing future studies to assess neurodevelopmental effects of PBDEs.

This research demonstrates for the first time that an environmental toxicant (i.e., PBDEs) can reduce the activity of human DIO2 (Chapter 3) and decrease the extracellular release of T₃ (Chapter 4). Alteration of T₃ concentrations in the developing brain is a widely accepted cause of impaired neurodevelopment. Although nearly all of the T₄ in the developing brain is maternally derived, 75% of the T₃ in the developing brain is produced locally by DIO2 (Bianco and Kim, 2006). The progression of neurodevelopmental events coincides with increasing T₃ concentrations in the brain over time and in very specific locations. For example, deafness results from impaired cochlear development in DIO2 knockout mice (Ng et al., 2004). At high PBDE doses, reductions in DIO2 activity and T₃ concentrations by over 50% could have negative consequences
for the developing brain. The effect of reducing T₃ concentrations by over 50% could be similar to congenital hypothyroidism and severe iodine deficiency, which can result in severe learning disabilities, dwarfism, impaired bone ossification, and hearing loss (Bath and Rayman, 2013; Préau et al., 2014). At the average and maximum serum PBDE concentrations found in humans, subtle effects on DIO2 activity that decrease local T₃ concentrations by 10-30% may cause effects similar to mild iodine deficiency. Mild iodine deficiency and maternal hypothyroidism during pregnancy are associated with decreased IQ by 1-20 points and decreased fine motor coordination (Bath and Rayman, 2013; Dunn and Delange, 2001). These effects are very similar to the decreases in developmental test scores and fine motor coordination associated with PBDEs in human cohort studies (Herbstman et al., 2010). However, while other endpoints of thyroid disruption can be tested *in vivo* by measuring the serum concentrations of T₄, T₃, and TSH in maternal and cord blood, measuring DIO2 activity and T₃ concentrations in the developing brain are not possible. Therefore, it is difficult to identify the threshold level of T₃ or DIO2 disruption at the cellular level that would lead to neurodevelopmental deficits associated with PBDE exposures.

Although the doses at which significant effects were observed in this study were an order of magnitude higher than the highest measured doses in human serum, variability in the sensitivity of the human population to neurodevelopmental toxicants may contribute to increased risk for a portion of the population. The EPA typically
recommends a 100× safety factor compared with the lowest observed effect level when identifying the neurodevelopmental risk of pesticides to a population (Schardein, 1999). However, in endpoints that involve infants and children, this safety factor can be increased to 1,000× by the Food Quality Protection Act (Schardein, 1999). By applying this safety factor, the population adjusted dose (PAD) for neurodevelopmental effects could be as low as 500 pM. As shown in Table 3, the average serum levels of individual congeners in several studies is around 100-500 pM. Therefore, to adequately assess the risk of PBDE exposure, impaired neurodevelopment via inhibition of DIO2 activity should be further explored in other models.

The experiments in this thesis research were not designed to quantitatively assess structure-activity relationships among the PBDE and OH-BDE congeners; however, clear differences were observed. OH-BDEs have been shown to be more potent than their PBDE analogs in other studies (Dingemans et al., 2008). However, in the work reported here, BDE-99 and its major oxidative metabolite, 5′-OH-BDE-99, exhibited similar potency (effects ranging from 500 nM to 1 μM) in decreasing DIO2 activity, although the mechanism of decreased DIO2 activity differed between the compounds. BDE-99 exposure led to enhanced DIO2 degradation, while 5′-OH-BDE-99 was a more potent competitive inhibitor of DIO2. Not surprisingly, these results imply that certain structural characteristics (in this case, the additional hydroxyl group) are required to bind to the DIO2 active site and either increase ubiquitination and proteasomal
degradation (stronger effect of BDE-99) or competitively inhibit T₄ to T₃ conversion
(stronger effect of 5’-OH-BDE-99). Surprisingly, BDE-47 did not significantly alter DIO2
activity, but instead caused variable, nonsignificant changes in activity, while the effects
of 3-OH-BDE-47 on DIO2 were similar to 5’-OH-BDE-99.

One of the most surprising and unexpected observations from this thesis
research was the effect of 5’-OH-BDE-99 on the catalytic activity of DIO3. 5’-OH-BDE-99
profoundly increased DIO3 activity, increasing rT₃ concentrations released in the cell
culture medium by over 400% and increasing the DIO3 activity in cellular homogenates
by over 50%. The mechanism of this effect was extremely dose dependent and occurred
at doses ranging from 5 to 35 μM, although at higher doses (> 35 μM), the expected
inhibitory effects (decreased DIO3) were observed. Similar to decreased DIO2 activity,
increased DIO3 activity would result in lower concentrations of T₃ in the developing
brain due to the inactivating role of DIO3. Therefore, the maintenance of T₃ homeostasis
may be dually impaired by simultaneous disruption of the two major enzymes used to
control the levels of T₃ in the developing brain. However, the mechanism by which DIO3
activity increased is not clear, and further studies are required to determine if the effects
are due to allosteric activation of DIO3 or other mechanisms.

Exposure to both BDE-99 and 5’-OH-BDE-99 decreased mRNA expression of
DIO2 in H4 cells. Although a TRE has not been found on the DIO2 promoter, the
transcription of DIO2 mRNA is negatively controlled by T₃, which indicates the presence
of a negative TRE (Bianco et al., 2002b). The ability of BDE-99 and 5’-OH-BDE-99 to affect DIO2 mRNA expression could therefore be mediated by a TR-based mechanism. Previous studies testing the competitive binding and antagonist/agonist effects of BDE-99 and 5’-OH-BDE-99 have indicated that these compounds have the ability to disrupt human TRs (Freitas et al., 2011; Hamers et al., 2008; Kitamura et al., 2008; Kojima et al., 2009). The results of this work provide indirect evidence that BDE-99 and 5’-OH-BDE-99 may disrupt TRs, although the mechanism will remain unclear until more knowledge regarding transcriptional control of DIO2 becomes available.

5.2 Novel Findings of DIO2 and DIO3 Biochemistry

Although the goal of this thesis research was to determine the effects of PBDEs on DIO2 and DIO3 activity, the results provide some insights into the regulation and catalytic properties of DIO2 and DIO3. Because of the difficulties of expressing the rare selenocysteine amino acid found in DIs, data gaps still exist regarding the mechanisms of DIO2 regulation and the binding characteristics of the active site. For example, little is known regarding the cellular mechanism that causes the DIO2 structure to undergo rapid ubiquitination and subsequent removal from the endoplasmic reticulum membrane. Previous work has suggested that catalytic activity of DIO2 (i.e., deiodination of T4) is required to promote ubiquitination and proteasomal degradation (Arrojo et al., 2013a). However, the results of this work demonstrate that BDE-99 also induced proteasomal degradation of DIO2, but BDE-99 was not debrominated by DIO2.
This result indicates that catalytic activity of DIO2 may not be required to promote ubiquitination, and simply binding to the DIO2 active site (such as BDE-99) may induce a structural conformation in the enzyme, leading to ubiquitination and proteasomal degradation. Of course, further studies are necessary to fully elucidate the mechanism of proteasomal degradation of DIO2 using recombinant enzymes.

Using the H4 and SK-N-AS cells as sources of DIO2 and DIO3, 3-OH-BDE-47, 5’-OH-BDE-99, and to a lesser extent BDE-99, were the first xenobiotic competitive inhibitors of DIO2 and DIO3 to be identified besides thyroid hormone analogs. Based on the data, it appears that competitive displacement of T4 from the active site by the OH-BDEs and BDE-99 inhibited the catalytic activity of DIO2 and DIO3. We also identified 5’-OH-BDE-99 as the first compound to increase the catalytic activity (i.e., enzyme activation) of thyroid hormone deiodination without acting as a co-substrate by reductively regenerating deiodinases after catalysis (i.e., DTT and glutathione). This novel alteration of DIO3 activity requires further investigation and may allow further knowledge of the biochemical properties of the deiodinases to be discovered, such as the presence of an allosteric activation site.

5.3 Methods for Testing Thyroid Disruption Potential

The cell culture models used in this research (H4 and SK-N-AS cells) were validated as models of thyroid hormone metabolism in the human brain in a previous study (Freitas et al., 2010). These cell lines afford several key benefits. The expression of
DIO2 and DIO3 in these cells is stable and responds predictably to known regulators of
DIO2 and DIO3, such as cAMP inducers or sonic hedgehog protein, and changes in cell
culture conditions, such as decreased activity with serum-containing culture medium or
with increased T4 concentrations in the culture medium (Freitas et al., 2010). This allows
the co-cultured cells to be used to screen effects on DIO2 and DIO3 activity, extracellular
release of thyroid hormones into the cell culture medium, and other endpoints. In the
co-culture setup used in the previous study, and in the studies presented in this thesis,
the cells were separated by a porous membrane. This membrane allows the two cell
types to be sampled independently and distinct effects to be determined in the separate
cell types. This is a significant advantage over mixed cell cultures.

H4 and SK-N-AS cells are some of the only cell lines that consistently express
DIO2 and DIO3 in physiologically relevant levels. The important selenocysteine enzyme
in the active center of DIO2 and DIO3 is encoded by a UGA Stop codon, which makes it
difficult to transfec and express DIO2 and DIO3 in large quantities for biochemical
experiments. Therefore, cell lines that express intact human DIO2 and DIO3 in sufficient
quantities, such as H4 and SK-N-AS cells, are useful sources of these enzymes for
experiments assessing inhibition or other catalytic parameters.

The experiments performed here are the first experiments to use a cell culture
model to assess the effects of an environmental toxicant on human DIO2 or DIO3
activity. Similar experiments could be performed to determine if other environmental
toxicants and thyroid disrupting compounds affect DIO2 or DIO3 following similar mechanisms. This assay could be especially useful for testing replacement flame retardants, which may have similar structures to PBDEs.

5.4 Limitations

While this research provided some novel insights into the effects of PBDEs on thyroid hormone regulation in the brain, there are several limitations that should be considered. The use of cancer cell lines as models for primary human cells is a significant limitation of this thesis work. Although the cancer cell lines provided an accurate model of thyroid function and exhibited stable expression of the deiodinase enzymes studied, certain aspects of the physiology may influence the uptake of PBDEs and thyroid hormones into the cells and may not reflect in vivo conditions. While we did confirm that BDE-99 decreased DIO2 activity in primary human astrocyte cells, the availability of these cells is limited to one or two commercial vendors who recommend that the cells not be grown for more than 4-6 passages. Furthermore, the doubling time of the primary human astrocytes was on the order of 1-2 weeks. For these reasons, performing large-scale studies to evaluate multiple compounds and mechanisms would not be possible using primary cells. The cells used in this study may also not reflect the complex physiology of cells present in the developing brain. During development, the activity and regional expression of deiodinases changes rapidly in response to many factors to coordinate neurodevelopmental events (Préau et al., 2014). Therefore, the
sensitivity of developing cells to thyroid disruption and downstream effects of thyroid
disruption may be much greater than the effects observed in the cells used in this study.
Furthermore, cellular models designed to assess neurodevelopmental endpoints such as
synaptogenesis may provide better toxicological endpoints for reduced thyroid hormone
levels. The cells used in this work are validated and useful models for assessing changes
in the regulation of DIO2 and DIO3, although the regulation of these enzymes in these
cells may differ from cells present in the developing brain.

The binding of PBDE congeners to the plastic components of the cell culture
containers was also problematic. Concentrations of PBDEs in the culture medium
decreased over time during the experiments due to binding to plastics. For the OH-BDEs
and BDE-47, binding to the plasticware was not as profound, but for BDE-99, BDE-153,
and BDE-209, treated cell culture medium exposed to the cells for longer than 6 h
required replenishment of the PBDEs as the concentrations decreased by approximately
50% in cell culture dishes with culture medium and no cells. This limitation could also
be overcome by using glass cell culture dishes, but our co-culture setup required the use
of plastic Corning Transwell dishes.

5.5 Data Gaps and Future Research Areas

The results of this study provide evidence that PBDEs and OH-BDEs have the
potential to disrupt DIO2 and DIO3 activity after short exposures (1-12 h). In rodent
studies, developmental exposure to PBDEs either by maternal exposure during gestation
or by dosing 7-week old rats typically decreases the total and free (unbound to serum proteins) T₄ and T₃ concentrations in (Darnerud et al., 2007; Kuriyama et al., 2007). However, increased serum PBDE concentrations in humans have been associated with increased serum T₄, T₃, and TSH concentrations (Dallaire et al., 2009; Turyk et al., 2008; Yuan et al., 2008). It is unclear whether the changes in the serum concentrations of thyroid hormones are a direct effect of PBDEs (i.e., lowered thyroid hormone concentrations by displacement from serum transport proteins or increasing clearance) or if the changes are compensatory mechanisms as an attempt of the organisms to return the thyroid hormone concentrations to normal levels. In general, while measurements of thyroid hormones in the serum may reflect the response of the organism to a toxicant, they may not represent the levels of thyroid hormones in peripheral tissues. Measurements of T₃ concentrations and DIO2 and DIO3 activity in the developing brain of exposed animal models may provide further evidence of the thyroid disrupting potential of PBDEs. Furthermore, it would be interesting to determine whether treatment of exposed rats with a DIO2 inducer such as forskolin may rescue the effects of decreased DIO2 activity. This experimental design could indicate whether the neurodevelopmental impairments caused by PBDEs are mediated via alterations in DIO2 activity.

An increasing number of studies have provided evidence that some flame retardants may be acting as environmental obesogens, which are compounds that can
cause weight gain and increased proliferation of adipocytes via multiple pathways. DIO2 is an important regulator of T₃ signaling and fatty acid oxidation. DIO2-knockout mice experience significant weight gain in response to high-fat diets compared with normal mice (Arrojo et al., 2013b). This weight gain in DIO2-knockout mice is related to the high basal activity of DIO2 in brown adipose tissue and preadipocytes, in which T₃ produced by DIO2 promotes fatty acid oxidation (Harada et al., 2011). A TRE is present in the promoter region of uncoupling protein 1 (UCP1), which is a protein in brown adipose tissue that is responsible for uncoupling substrate oxidation from ATP production by increasing proton leak (Song et al., 2011). This causes increased heat to be released from the cells and is a major mechanism of adaptive thermogenesis. It is possible that BDE-99 also decreases DIO2 activity in these tissues in addition to the brain, and the concentrations of BDE-99 in fat are much higher than the concentrations in other tissues (e.g., 100-500 times higher in rat fat tissues compared with brain or serum) (Staskal et al., 2006). The effects of BDE-99 on DIO2 should therefore also be determined in brown adipose cells.

The SK-N-AS cell line was chosen for this work based on its previous validation as a model for DIO3 regulation (Freitas et al., 2010; Jo et al., 2012; Simonides et al., 2008). Similar co-culture studies could use a different neuronal cell model using a similar setup to determine the downstream effects of DIO2 disruption on thyroid-dependent parameters. This work attempted to evaluate the effects of T₃ disruption on metabolic
energy expenditure, but the results were highly variable, and the measured endpoints
were not significantly altered by the decreases in T3 concentrations mediated by
disrupted DIO2 activity. Other cell lines or primary neurons may exhibit a phenotype of
T3 disruption that is more similar to the expected phenotype. For example, T3-induced
differentiation of a neuronal cell line or embryonic neural stem cells or T3-induced
migration of granular cells could be endpoints of disrupted DIO2 activity in the H4 cells
(Chen et al., 2012). The results of these experiments would provide further evidence that
dereased DIO2 activity caused by PBDEs is a major mediator of downstream toxic
effects on developing neurons.

5.6 Conclusions

The results of this thesis work indicate that PBDEs and OH-BDEs may disrupt
thyroid signaling in the brain by altering the activities of DIO2 and DIO3. Because no
OH-BDE metabolites were detected as biotransformation products, the results of these
experiments appear to be mediated directly by the compounds dosed into the cell
culture medium. BDE-99, BDE-153, BDE-209, 3-OH-BDE-47, 6-OH-BDE-47, and 5′-OH-
BDE-99 all reduced the activity and/or expression of DIO2 in the H4 human glioma cell
line by 35-80%. BDE-99 mediated these effects via reduced mRNA expression and
increased proteasomal degradation of DIO2. In a co-culture experimental design, the
concentration of T3 in cells treated with BDE-99 decreased by up to 76%, which indicates
a potential mechanism of thyroid disruption in the developing brain. Exposure to BDE-
99, 3-OH-BDE-47, and 5′-OH-BDE-99 competitively inhibited DIO2 activity, and 3-OH-
BDE-47 competitively inhibited DIO3 activity. A novel DIO3 activating effect of 5′-OH-
BDE-99 was also observed. These results indicate that PBDEs and OH-BDEs disrupt
thyroid hormone metabolism in human brain cells, likely due to their structural
similarity to thyroid hormones, and the relevance of these mechanisms to PBDE-
impaired neurodevelopment should be evaluated in future studies.
Appendix A. Species-Specific Differences and Structure-Activity Relationships in the Debromination of PBDE Congeners in Three Fish Species


Previous studies have suggested that there may be species-specific differences in the metabolism of polybrominated diphenyl ethers (PBDEs) among different fish species. In this study, we investigated the in vitro hepatic metabolism of eleven individual PBDE congeners (tri- through decaBDEs) in three different fish species: rainbow trout (Oncorhynchus mykiss), common carp (Cyprinus carpio), and Chinook salmon (O. tshawytscha). In addition, we evaluated the influence of PBDE structural characteristics (i.e., bromine substitution patterns) on metabolism. Six of the eleven congeners we evaluated, BDEs 99, 153, 183, 203, 208, and 209, were metabolically debrominated to lower brominated congeners. All of the congeners that were metabolized contained at least one meta-substituted bromine. Metabolites were not detected for congeners without one meta-substituted bromine (e.g., BDEs 28, 47, and 100). Metabolite formation rates were generally 10-100 times faster in carp than in trout.
and salmon. BDEs 47, 49, 101, 154, and 183 were the major metabolites observed in all three species with the exception of BDE 47, which was only detected in carp. Carp demonstrated a preference towards meta-debromination, while trout and salmon debrominated meta- and para- bromine atoms to an equal extent. We compared glutathione-S-transferase (GST) and deiodinase (DI) activity among all three species as these enzyme systems have been hypothesized to play a role in PBDE debromination among teleosts. Carp exhibited a preference for meta-deiodination of the thyroid hormone thyroxine, which was consistent with the preference for meta-debromination of PBDEs observed in carp.

A.1 Introduction

Polybrominated diphenyl ethers (PBDEs) are flame retardants that are incorporated into consumer products such as textiles, polyurethane foam, and casing for electronics. Two of the commercial PBDE flame retardant mixtures, PentaBDE and OctaBDE, were listed as persistent organic pollutants (POPs) according to the Stockholm Convention on POPs due to their persistence and biomagnification potential (EU, 2009b). Currently, DecaBDE, which consists almost entirely of the fully brominated congener BDE 209, is the only PBDE mixture still used today, and it is scheduled for phase out by the end of 2013 for similar reasons (USEPA, n.d.). Due to their persistence and ability to bioaccumulate, lower brominated PBDEs are often detected in aquatic
organisms, which may be attributable in part to biotransformation of higher brominated congeners (Hites, 2004; Letcher et al., 2010). The metabolism of PBDEs in humans and other mammals typically occurs via oxidative pathways, producing hydroxylated PBDEs (OH-BDEs) and brominated phenols (Chen et al., 2006; Stapleton et al., 2008c). Contrary to mammals, fish have not been shown to form oxidative metabolites of PBDEs, but have instead demonstrated the ability to reductively debrominate PBDEs both in vivo and in vitro (Stapleton et al., 2004c).

Studies have shown that PBDE congener profiles in wild fish vary among species. Wild Chinook salmon (Oncorhynchus tshawytscha) typically accumulate higher percentages of BDEs 47, 49, and 99 compared to other congeners (Montory et al., 2010; Sloan et al., 2010), while wild common carp (Cyprinus carpio) typically accumulate higher proportions of BDE 47 and 100 and little or no BDE 99 (Pérez-Fuentetaja et al., 2010). We previously observed that carp could metabolically debrominate BDE 99 to a greater extent than Chinook salmon and that the metabolic products differed between species (BDE 47 was formed in carp and BDE 49 was formed in salmon) (Browne et al., 2009; Noyes et al., 2010). Previous studies in carp have shown that the metabolic debromination of BDEs 99, 183, and 209 favored the removal of meta-substituted bromine atoms (Stapleton et al., 2004b, 2004c), while in Chinook salmon, the debromination of BDE 99 favored the removal of a para-substituted bromine atom (Browne et al., 2009). These differences in PBDE metabolism may influence PBDE
accumulation patterns observed in wild fish. Little is known about species-specific differences in PBDE metabolism, and previous studies have not investigated species-specific differences in the metabolism of individual congeners other than BDEs 99 and 209 (Browne et al., 2009; Stapleton et al., 2006).

It has been hypothesized that deiodinase enzymes (DIs) may play a role in the debromination of PBDEs in fish due to the structural similarity of PBDEs to thyroid hormones (Stapleton et al., 2006). DIs are responsible for activating and deactivating thyroid hormones by cleaving iodine atoms from thyroxine (T₄), 3,3',5-triiodothyronine (T₃), or 3,3',5'-triiodothyronine (rT₃). These dehalogenation reactions occur via the removal of an ortho- or meta-iodine. Definitive evidence establishing the role of DIs in PBDE debromination in fish is still needed (i.e., assessing PBDE debromination with purified DIs), and it is possible that other biotransformation enzyme systems may also be involved, such as glutathione-S-transferases (GSTs). Previous studies have ruled out the likely involvement of cytochrome P450s (Benedict et al., 2007). PBDEs are well known disruptors of thyroid hormone regulation in fish (Tomy et al., 2004), but little is known about the mechanisms of thyroid toxicity. Understanding the metabolism of PBDEs in fish, as well as their potential interactions with DIs, may allow for better understanding of the toxicological mechanisms.

The objective of this study was to compare the metabolism of eleven individual PBDE congeners among carp (Cyprinus carpio), rainbow trout (Oncorhynchus mykiss), and
Chinook salmon (O. tshawytscha). We used environmentally relevant PBDE congeners from seven homologue groups (tri- through deca-) to investigate the structural characteristics and bromine substitution patterns that influence debromination. We also compared the activities of two classes of enzymes, GSTs and DIs, among the three fish species to determine if their relative activities were reflective of the differences in PBDE metabolism.

**A.2 Methods and Materials**

*Materials.* Individual PBDE congeners used for dosing, BDEs 28, 47, 49, 99, 100, 153, 154, 183, 203, 208, and 209, were purchased as neat standards from AccuStandard, Inc. (New Haven, CT, USA). Quantification standards for the previously mentioned congeners and BDEs 101, 144, 149, 180, and 187 were also purchased from AccuStandard, Inc. Internal and surrogate standards $^{13}$C-2,2',3,4,5,5'-hexachlorodiphenylether (CDE 141) and $^{13}$C-decabromodiphenylether ($^{13}$C-BDE 209) were purchased from Wellington Labs (Guelph, Canada), 4'-fluoro-2,3',4,6-tetrabromodiphenylether (FBDE 69) was purchased from Chiron (Trondheim, Norway), and $^{13}$C-3,3' diiodothyronine ($^{13}$C-3,3' T₂), $^{13}$C-T₃, $^{13}$C-rT₃, and $^{13}$C-T₄ were purchased from Isotec (Miamisburg, OH). Unlabeled thyroid hormones (T₄, T₃, rT₃, 3,3' T₂ and 3,5 T₂), reduced glutathione, 1-chloro-2,4-dinitrobenzene (CDNB), and dithiothreitol (DTT) were
purchased from Sigma Aldrich (St. Louis, MO). All solvents and other materials were HPLC grade.

**Animals.** Adult common carp livers were donated by Dr. Carys Mitchelmore from the Chesapeake Biological Laboratory (Solomons, MD) and were originally purchased as juveniles from Hunting Creek Fisheries in Thurmont, MD. Adult rainbow trout livers were donated by the Armstrong State Fish Hatchery in Marion, NC. Juvenile Chinook salmon livers were donated by Dr. Evan Gallagher from the University of Washington in Seattle, WA.

**Preparation of Hepatic Sub-Cellular Fractions.** Five grams of liver tissue pooled from five individual fish (1 g per fish) of each species was homogenized for five minutes using a Bullet Blender with 0.5 mm zirconium oxide beads (Next Advance, Averill Park, NY). Using previously published methods, microsomal suspensions were prepared from homogenized liver tissues with 10 mM dithiothreitol (DTT) in all buffers (McKinney et al., 2004). Protein concentrations were determined using a bicinchoninic acid assay (Pierce, Rockford, IL). Heat-inactivated microsomes (submersed in boiling water for 15 min) spiked with individual PBDEs were used as negative controls.

**Incubations.** Incubations were performed in glass test tubes at 25°C in a shaking water bath at 140 rpm using our published method (Noyes et al., 2010). The 1-mL incubation mixtures contained 0.1 M potassium phosphate buffer (pH 7.4) with 10 mM DTT, 100 μM NADPH, microsomes at a concentration of 1 mg protein mL⁻¹, and
approximately 1 nmol of a PBDE congener delivered in 5 μL of acetone. Previous studies have shown that higher substrate concentrations (>50 μM) result in a wider variety and higher abundance of metabolites (Noyes et al., 2010). However, for this study, we chose a lower substrate concentration of 1 μM to more closely reflect environmentally relevant concentrations, while still producing detectable levels of metabolites. All rainbow trout and salmon incubations, as well as carp microsomal incubations with higher brominated PBDEs (i.e., heptaBDEs and higher) were conducted for 24 hours. Incubations containing lower brominated PBDE congeners (i.e., hexaBDEs and lower) with carp microsomes were conducted for one hour due to their higher metabolic activity. Previous studies in carp and salmon have shown that debromination can continue for up to 24 hours during in vitro incubations (Browne et al., 2009; Noyes et al., 2010).

**Extraction and Analysis.** Incubations were stopped with 1 mL of ice-cold methanol, extracted with hexane, and cleaned with sulfuric acid using our previously published method (Noyes et al., 2010). FBDE 69 and 13C-BDE 209 were added prior to extraction as surrogate internal standards, and CDE 141 was added prior to GC/MS analysis to measure their recovery. Samples were analyzed using a GC/MS (Agilent Model numbers 6890N and 5975, respectively) operated in electron capture negative ionization (ECNI) mode using published run parameters (Stapleton et al., 2008b). A calibration mixture containing 43 PBDE congeners was used for identification and quantification of metabolites (less brominated congeners) and parent compounds.
Analytes were confirmed by homologue group using GC/MS operated in electron ionization mode (GC/EI-MS). Samples were also analyzed using a liquid chromatography tandem mass spectrometer (LC-MS/MS) (Agilent 1200 SL binary pump and an Agilent 6410 MS/MS) operated in multiple reaction monitoring (MRM) mode (monitoring MRM transitions for tri- through nona-OH-BDEs) with a Hypersil C-18 column following a previously published method (Mas et al., 2007) to determine if OH-BDEs or 2,4,5 tribromophenol were formed as metabolites.

**DI Assay.** To measure the activity of endogenous DI enzymes in microsomal fractions, approximately 500 pmol of T₄ was used as a substrate instead of PBDE congeners in the previously described incubations. Incubations were performed for 90 minutes and were stopped with 1 mL of ice-cold acetone, deproteinated, and extracted using our lab’s published method for thyroid hormones (Wang and Stapleton, 2010). The formation rates of T₃, rT₃, 3,5 T₂ and 3,3’ T₂ were determined using LC/MS/MS in positive-ESI mode by monitoring MRM transitions using ¹³C-3,3’ T₂, ¹³C-T₃, and ¹³C-T₄ as surrogate internal standards. We injected 20 µL of sample onto a Synergi 2.5 µm Polar-RP 100A column (2.5 µm, 50 mm × 2.0 mm) (Phenomenex, Torrance, CA) with water (A) and acetonitrile (B) mobile phases with 0.1 M formic acid. A gradient method at a flow rate of 0.40 mL/min was used with the following parameters: 30% B from 0-2 min, 90% B from 2-5 min, and 30% B from 5-10 min.
Cytochrome C Reductase Assay. The integrity of the microsomal fractions was assessed using an NADPH-cytochrome c reductase assay (Thermo-Pierce, Rockford, IL). NADPH-cytochrome c reductase is an enzyme involved in the hepatic mixed-function monooxygenase system that transfers electrons to cytochrome P-450 and other oxygenases in the endoplasmic reticulum (Shephard et al., 1983). Thus, NADPH-cytochrome c reductase activity is a reliable indicator of microsomal integrity. The values obtained for NADPH-cytochrome c reductase activity were 9 ± 1, 11 ± 1, and 10 ± 1 nmol min⁻¹ mg protein⁻¹ for carp, rainbow trout, and salmon, respectively.

mGST Assay. GSTs are a multigenic family of Phase II metabolizing enzymes that include mitochondrial, cytosolic, and microsomal isoforms with the ability to conjugate glutathione to xenobiotics. The GST activity was assessed in each pool of microsomes using a published method, which measured the conjugation of glutathione to 1-chloro-2,4-dinitrobenzene (CDNB) to form glutathione-2,4-dinitrobenzene (Noyes et al., 2010).

QA. The average recovery of FBDE 69 in the microsomal extracts was 103 ± 12% and the average ¹³C-BDE 209 recovery was 107 ± 7%. The average recoveries of ¹³C-T₂, ¹³C-T₃, ¹³C-rT₃, and ¹³C-T₄ were 103 ± 15%, 100 ± 16%, 102 ± 14%, and 80 ± 10%, respectively. Limits of detection were defined as 3 times the standard deviation of heat-inactivated and buffer controls. Samples with active microsomes were blank corrected for low concentrations of impurities detected in spiking solutions observed in
incubations with heat-inactivated microsomes. Metabolite formation is reported as pmol or fmol hr$^{-1}$ mg protein$^{-1}$. Statistical analyses were performed using a Student’s t-test in Sigma Plot 9.0 with statistical significance was defined as p<0.05.

**A.3 Results and Discussion**

Extracts from the microsomal incubations were analyzed using GC/MS to identify and quantify the debrominated metabolites of eleven environmentally relevant PBDE congeners (BDEs 28, 47, 49, 99, 100, 153, 154, 183, 203, 208, and 209) (La Guardia et al., 2006, 2007). Metabolic debromination was observed in incubations with six of eleven congeners tested (BDEs 99, 153, 183, 203, 208, and 209), while no metabolism was observed in incubations with the remaining five (BDEs 28, 47, 49, 100, and 154). The most rapidly metabolized congeners were BDEs 99 and 183, and the slowest metabolized congeners were BDEs 208 and 209. Up to six metabolites were observed for each metabolized congener. Some PBDE congeners detected as metabolites were used as substrates in our experiments. For example, BDE 203 was metabolically debrominated to form BDE 183, and in a separate incubation, BDE 183 was debrominated to form several hexaBDEs. The results from incubations with individual congeners were combined to create a metabolic pathway that characterized the potential debromination of BDE 209 to tetra- and pentaBDEs (Figure 35).
Figure 35: Hepatic microsomal biotransformation pathway for (a) common carp, (b) rainbow trout, and (c) Chinook salmon. Boxes indicate congeners used as substrates in incubations. (*) Steps in which two bromine atoms were removed in one incubation were hypothesized based on the structure of the metabolite assuming no rearrangement of bromine atoms. (**) BDE 203 coelutes with BDE 200, which is also a potential metabolite not shown here.

Samples were also screened for oxidative metabolites using LC/MS/MS. Hydroxylated BDEs and 2,4,5 tribromophenol have been observed as BDE 99 metabolites in mammalian studies (Chen et al., 2006; Stapleton et al., 2008c). However,
OH-BDEs (i.e., tri- through nona-OH-BDEs) or brominated phenols were not detected in any samples in this study, which is consistent with previous fish studies (Stapleton et al., 2004c). Therefore, reductive debromination appears to be the primary pathway of PBDE metabolism in fish.

*Debromination in Common Carp.* In carp, *in vitro* incubations with BDE 209 generated small masses (<1% of the substrate) of a variety of metabolites from hepta-through nonaBDEs. A previous study detected hexaBDEs as metabolites of BDE 209 in juvenile carp hepatic microsomal incubations and pentaBDEs after *in vivo* exposure to BDE 209 (Stapleton et al., 2004a). The more limited debromination observed in this study may have been due to differences in the expression and activity of the enzyme(s) systems responsible for the metabolism of PBDEs as a result of different aged fish used in each study. The major metabolites of BDE 209 were octaBDE 197 and nonaBDEs 207 and 208. The lowest brominated metabolite of BDE 209 detected in our samples was heptaBDE 184 (40 ± 5 fmol hr⁻¹ mg protein⁻¹). The lowest brominated metabolites of BDE 208 were heptaBDEs 179 and 188 (39 ± 22 and 68 ± 19 fmol hr⁻¹ mg protein⁻¹). The only two metabolites of BDE 209 that were evaluated separately as substrates in this study were BDEs 203 and 208.

BDE 203 was debrominated to form several hepta- and hexaBDEs, including BDEs 183 (1,190 ± 190 fmol hr⁻¹ mg protein⁻¹) and 154 (230 ± 30 fmol hr⁻¹ mg protein⁻¹) and to a lesser extent, BDE 153 (44 ± 12 fmol hr⁻¹ mg protein⁻¹). The formation of BDE 154 as a
metabolite of both BDEs 183 and 203 occurred fairly rapidly in comparison to many of the other reactions observed (Table 7). Furthermore, BDE 154 was resistant to metabolic debromination in our experiments. These results suggest that BDE 154 is likely to accumulate to a greater extent than other higher brominated congeners in vivo as a result of metabolic debromination. Several studies examining PBDEs in marine mammals have observed higher concentrations of BDE 154 relative to BDE 153, even though commercial PBDE formulations contain higher concentrations of BDE 153 (Hites, 2004; La Guardia et al., 2006). This inconsistency may be explained in part by the debromination of higher brominated PBDEs in fish, resulting in accumulation of BDE 154 in higher trophic level marine mammals.
Table 7: Hepatic microsomal formation rates of debrominated metabolites in incubations containing 1 nmol of the parent congener expressed as fmol hr\(^{-1}\) mg protein\(^{-1}\) with standard error.

<table>
<thead>
<tr>
<th>Parent Metabolite [Br] Position</th>
<th>Common Carp</th>
<th>Rainbow Trout</th>
<th>Chinook Salmon</th>
</tr>
</thead>
<tbody>
<tr>
<td>pentaBDE 99</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>47 m</td>
<td>237,980 ± 22,600</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>49 p</td>
<td>1,750 ± 250</td>
<td>140 ± 10</td>
<td>210 ± 10</td>
</tr>
<tr>
<td>66 o</td>
<td>ND</td>
<td>8.2 ± 4</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>hexaBDE 153</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>47 m,m</td>
<td>1,290 ± 300</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>101 p</td>
<td>990 ± 130</td>
<td>6.6 ± 1.3</td>
<td>87 ± 8</td>
</tr>
<tr>
<td>heptaBDE 183</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>144 p</td>
<td>ND</td>
<td>9 ± 1</td>
<td>ND</td>
</tr>
<tr>
<td>149 m</td>
<td>530 ± 60</td>
<td>37 ± 3</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>153 o</td>
<td>320 ± 40</td>
<td>37 ± 1</td>
<td>16 ± 7</td>
</tr>
<tr>
<td>154 m</td>
<td>4,840 ± 490</td>
<td>1,500 ± 70</td>
<td>470 ± 3</td>
</tr>
<tr>
<td>octaBDE 203</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>149 m,p</td>
<td>42 ± 4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>153 m,o</td>
<td>44 ± 12</td>
<td>ND</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>154 m,m</td>
<td>230 ± 30</td>
<td>10 ± 2</td>
<td>61 ± 6</td>
</tr>
<tr>
<td>180 o</td>
<td>28 ± 7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>183 m</td>
<td>1,190 ± 190</td>
<td>150 ± 10</td>
<td>340 ± 60</td>
</tr>
<tr>
<td>187 p</td>
<td>640 ± 90</td>
<td>150 ± 10</td>
<td>440 ± 70</td>
</tr>
<tr>
<td>nonaBDE 208</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>179 m,p</td>
<td>39 ± 22</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>188 m,m</td>
<td>68 ± 19</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>201 m</td>
<td>760 ± 310</td>
<td>110 ± 20</td>
<td>ND</td>
</tr>
<tr>
<td>202 p</td>
<td>690 ± 250</td>
<td>200 ± 50</td>
<td>ND</td>
</tr>
<tr>
<td>decaBDE 209</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>184 m,m,m</td>
<td>40 ± 5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>197 m,m</td>
<td>100 ± 10</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>201 m,p</td>
<td>58 ± 8</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>202 p,p</td>
<td>45 ± 10</td>
<td>16 ± 2</td>
<td>ND</td>
</tr>
<tr>
<td>203/200</td>
<td>45 ± 3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>207 m</td>
<td>250 ± 80</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>208 p</td>
<td>130 ± 40</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

BDE 153 was observed as a metabolite of BDEs 183 and 203. However, unlike BDE 154, BDE 153 was metabolized to form pentaBDE 101 and tetraBDE 47. The
formation of BDE 47 from BDE 153 was the only transformation in which a metabolic product with two fewer bromine atoms than the parent was detected without detecting the intermediate (in this case, BDE 99). This was probably due to the rapid metabolism of BDE 99 to 47 (238 ± 23 pmol hr⁻¹ mg protein⁻¹), which was by far the most rapid transformation observed in this study. Furthermore, similar to BDE 154, BDE 47 was resistant to metabolism, which further increases its potential to bioaccumulate in organisms such as carp.

*Debromination in Rainbow Trout and Chinook Salmon.* In general, the metabolic products and rates were similar for trout and salmon. Unlike carp, which debrominated BDE 209 to form hepta- through nonaBDEs, very limited metabolism of BDE 209 occurred in rainbow trout and salmon. The only significant metabolites of BDEs 209 and 208 in rainbow trout were BDEs 202 and 201. Due to the slow biotransformation of BDEs 208 and 209 in salmon, quantification of metabolites was not possible. However, other congeners that were metabolized in carp (i.e., BDEs 99, 153, 183, and 203) were also metabolized in trout and salmon, albeit at much slower rates. The biotransformation rates for trout and salmon were typically 10-100 times slower than those observed in carp. This indicated a greater ability of carp to metabolically debrominate PBDEs, which was consistent with previous studies (Browne et al., 2009).

The only congener that was debrominated to similar products in all three fish species was BDE 183. BDE 154 was the dominant metabolite and BDEs 153 and 149 were
minor metabolites of BDE 183 in all species, even though the metabolite formation rates were more rapid in carp. For the other congeners, there were differences in both the products and rates of PBDE debromination between carp and the salmonids (trout and salmon). In incubations with BDEs 153 and 99, there were major differences in the metabolic products and formation rates. BDE 47 was not detected as a metabolite in trout or salmon. The absence of this metabolic pathway resulted in much slower metabolism of BDEs 99 and 153 in trout and salmon. Unlike carp, which readily debrominated BDE 153 to form BDE 47, only BDE 101 was observed as a metabolite of BDE 153 in trout and salmon.

*Structure-Activity Relationships.* As mentioned previously, the slowest metabolite formation rates were observed for BDEs 208 and 209 in all three species. In carp, there was a general trend towards higher biotransformation rates in congeners with fewer than six bromine atoms, which was clearly evidenced by the rapid biotransformation of BDE 99 in carp. For trout and salmon, heptaBDE 183 and octaBDE 203 were most rapidly metabolized with slower biotransformation of BDEs 99 and 153.

In all three species, only congeners with at least one *meta*-substituted bromine atom were debrominated. Furthermore, congeners without *meta*-substituted bromines, BDEs 28, 47, and 100, did not undergo metabolism to any extent. Thus, it appeared that the presence of a *meta*-substituted bromine atom increased the potential for metabolic debromination of the congener. Because all of the debrominated metabolites observed in
this study, except BDE 47, possessed at least one meta-substituted bromine, it is possible that many of these metabolites will be further debrominated in vivo based on this structure-activity relationship. However, because BDEs 154 and 49 both contain one meta-substituted bromine and were not metabolized, there are likely other structural factors affecting PBDE metabolism in fish.

Although the substrate preference for PBDE congeners with at least one meta-substituted bromine constituent was conserved across all three species, there were other differences among species in the site of debromination. Figure 36 presents a summation of all of the metabolite formation rates on a logarithmic scale categorized by the position of the bromine atom cleaved during metabolism. Carp demonstrated a clear preference for meta-debromination. However, in trout and salmon, there appeared to be no clear preference between meta- or para-debromination. The metabolism of BDE 183 to 154 was the only case in which meta-debromination was predominant in trout or salmon. In every other incubation, para-debromination was predominant. Figure 36 shows the absence of any preference between para- or meta-debromination in salmon. This was a significant difference between carp and salmonids in their biotransformation of PBDEs.
Figure 36: Summed formation rates (fmol hr⁻¹ mg protein⁻¹) for all metabolites formed from BDEs 99, 153, 183, 203, 208, and 209 based on the position of debromination (top). Formation rates for 3,3’ T₂, T₃ and rT₃ in hepatic microsomes incubated with 500 pmol T₄ and the thyroid hormone deiodination pathway (bottom).

In carp, every debromination involved the removal of a bromine atom that was adjacent to at least one other bromine. For example, out of the three ortho-substituted bromines on BDE 183, only the ortho-bromine adjacent to a meta-substituted bromine underwent debromination to form BDE 153, while the other two ortho-substituted bromines were unmodified, as shown in Figure 35. In trout and salmon, a small amount
of BDE 66 was formed via the removal of an isolated ortho-substituted bromine, but this was only a minor metabolite.

**Biotransformation Enzyme Activity.** Previous work has investigated the involvement of DIs and GSTs in the debromination of PBDEs in fish (Benedict et al., 2007; Browne et al., 2009; Noyes et al., 2010). GSTs are responsible for Phase II conjugation of many xenobiotics with glutathione, and are capable of catalyzing reductive dehalogenation reactions, as evidenced by the reductive dechlorination of DDT by *Drosophila* GST (Tang and Tu, 1994). Although most GST activity is present in the cytosol, about 3% of microsomal protein is GST (mGST) (Morgenstern et al., 1984). We compared mGST activity towards the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione among the three species. The mGST activities for carp, trout, and salmon microsomes were 38 ± 2, 65 ± 7, and 14 ± 3 nmol min⁻¹ mg protein⁻¹, respectively. Trout had higher mGST activity, which was not reflective of the PBDE debromination rates among species but was similar to values observed in a previous study (Laurén et al., 1989). Although this does not definitively rule out the involvement of GSTs in PBDE debromination, it provides further indication, along with a previous study (Browne et al., 2009), that GSTs are not involved in PBDE debromination in fish.

PBDEs are structurally similar to the thyroid hormones T₄, T₃, and rT₃, which are substrates in deiodination reactions mediated by DIs. The earliest evidence for the
involvement of DIs was demonstrated when in vivo debromination of PBDEs in carp generally favored the removal of meta-substituted bromine atoms, which was similar to the removal of a meta-iodine atom from thyroid hormones by DIs (Stapleton et al., 2004c). Later studies showed that debromination of BDE 99 by salmon microsomes decreased when DTT, which is a required co-substrate for in vitro activity of DI enzymes, was omitted from incubation buffers (Benedict et al., 2007; Browne et al., 2009). Other studies showed that a DI inhibitor, iodoacetate, inhibited PBDE debromination, and rT3 and to a lesser extent T4 also inhibited PBDE debromination at various substrate concentrations, which implied that PBDEs and thyroid hormones competed as substrates (Benedict et al., 2007; Noyes et al., 2010).

In this study, we attempted to further investigate the involvement of DIs in PBDE metabolism by assessing DI activity in pooled microsomes from each species. We hypothesized that the species-specific differences we observed in PBDE debromination would correspond with species-specific differences in DI activity. The conversion of T4 to its deiodinated metabolites T3 and rT3 was monitored using LC/MS/MS in microsomal incubations similar to the PBDE incubations, but using T4 instead of PBDEs as the substrate. Distributions of deiodinated metabolites of T4 varied among all three species, as shown in Figure 36. Carp demonstrated faster formation of T3 from T4 than rainbow trout (p<0.05) or salmon (p<0.01) via a meta-deiodination reaction. Trout and salmon demonstrated ortho-deiodination of T4 to form rT3, which was not observed in carp.
These differences in DI activity between carp and salmonids may be responsible for the species differences observed in PBDE debromination (Figure 36), but further studies using purified DIs are needed to confirm the involvement of DIs in PBDE debromination.

*Implications.* Metabolism likely plays an important role in influencing congener distributions of PBDEs in wild fish. Differences in metabolite formation rates observed in this study corresponded with PBDE congener distributions in wild fish. In wild salmon, PBDE congener distributions included higher proportions of BDEs 47 and 99 than other congeners (Hites, 2004). In wild common carp, BDEs 47, 100, and 154 dominated the congener distribution, with little or no detectable BDE 99 (Pérez-Fuentetaja et al., 2010). According to the results of this study, the enrichment of BDE 47 in wild carp may attributable in part to the rapid metabolism of BDEs 153 and 99 to BDE 47. Salmon demonstrated much slower debromination rates of BDE 99 than carp, which may allow BDE 99 to accumulate in salmon to a greater extent.

While understanding characteristics of biotransformation of PBDEs is important in understanding their environmental fate, it may also be important from a toxicological perspective. Because lower brominated congeners are often regarded as more toxic and have a higher biomagnification potential than higher brominated congeners, debromination may enhance the toxicity of PBDEs in fish and may increase the effects on higher trophic level organisms (Tomy et al., 2004). Furthermore, the potential
involvement of DIs in PBDE metabolism may result in altered DI activity, thereby disrupting thyroid regulation. In lake trout and fathead minnows, PBDEs caused decreases in circulating T₄ and T₃, which may be indicative of altered thyroid hormone homeostasis (Lema et al., 2008; Tomy et al., 2004). Furthermore, Szabo et al. (Szabo et al., 2009) observed decreases in DI activity following exposure to PBDEs in rats. Future studies should consider examining the effects of PBDEs on thyroid hormone metabolism to determine whether PBDEs may interfere with basal DI activity.

A.4 Conclusions

In conclusion, we observed debromination in six of eleven PBDE congeners analyzed in this study, including penta- through decaBDEs, in carp and rainbow trout. This provides further evidence that BDE 209 may degrade in the environment to form more toxic, lower brominated congeners. Species-specific differences in metabolic rates and products were observed for each substrate. Meta-debromination was predominant in carp, while there was no preference between meta- or para-debromination in rainbow trout and salmon. These species-specific structure-activity relationships may be important in modeling the distribution of PBDE congeners in the environment. It is likely that carp will accumulate BDEs 47 and 154 as a result of metabolism, while trout and salmon will likely accumulate BDEs 99 and 154 to a greater extent. Species-specific differences were also observed in DI activity; carp microsomes demonstrated a strong preference for meta-deiodination of T₄, while trout and salmon demonstrated higher
rates of ortho-deiodination. These species-specific differences in DI activity corresponded
to the species-specific differences observed in PBDE debromination and provided
further indication of the involvement of DIs in the metabolic debromination of PBDEs.

**A.5 Funding Information**

This study was supported by a grant from the National Institute of
Environmental Health Sciences, R01ES016099. The content is solely the responsibility of
the authors and does not necessarily represent the official views of the National Institute
of Environmental Health Sciences or the National Institutes of Health.
Appendix B. *In Vitro* Metabolism of the Brominated Flame Retardants 2-Ethylhexyl-2,3,4,5-Tetrabromobenzoate (TBB) and Bis(2-Ethylhexyl) 2,3,4,5-Tetrabromophthalate (TBPH) in Human and Rat Tissues

This following section contains research published in 2012 in Chemical Research in Toxicology:

*Simon C. Roberts, Laura J. Macaulay, and Heather M. Stapleton. 2012. In Vitro Metabolism of the Brominated Flame Retardants 2-Ethylhexyl-2,3,4,5-Tetrabromobenzoate (TBB) and Bis(2-Ethylhexyl) 2,3,4,5-Tetrabromophthalate (TBPH) in Human and Rat Tissues. Chemical Research in Toxicology. 25 (7) 1435-1441.*

Due to the phaseout of polybrominated diphenyl ether (PBDE) flame retardants, new chemicals, such as 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (TBB) and bis(2-ethylhexyl) 2,3,4,5-tetrabromophthalate (TBPH), have been used as replacements in some commercial flame retardant mixtures. Both chemicals have been detected in indoor dust at concentrations approaching the concentrations of PBDEs; however, little is known about their fate, metabolism, or toxicity. The goal of this study was to investigate the potential metabolism of these two brominated flame retardants in human and rat tissues by conducting *in vitro* experiments with liver and intestinal subcellular fractions. In all the experiments, TBB was consistently metabolized to 2,3,4,5-tetrabromobenzoic
acid (TBBA) via cleavage of the 2-ethylhexyl chain without requiring any added
cofactors. TBBA was also formed in purified porcine carboxylesterase, but at a much
faster rate of $6.29 \pm 0.58 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$. The estimated $K_m$ and $V_{\text{max}}$ values for
TBB metabolism in human microsomes were $11.1 \pm 3.9 \text{ M}$ and $0.644 \pm 0.144 \text{ nmol min}^{-1}
\text{ mg protein}^{-1}$, respectively. A similar $K_m$ of $9.3 \pm 2.2 \text{ M}$ was calculated for porcine
carboxylesterase, indicating similar enzyme specificity. While the rapid formation of
TBBA may reduce the bioaccumulation potential of TBB in mammals and may be useful
as a biomarker of TBB exposure, the toxicity of this brominated benzoic acid is unknown
and may be a concern based on its structural similarity to other toxic pollutants. In
contrast to TBB, no metabolites of TBPH were detected in human or rat subcellular
fractions. However, a metabolic product of TBPH, mono(2-ethylhexyl)
tetrabromophthalate (TBMEHP), was formed in purified porcine carboxylesterase at an
approximate rate of $1.08 \text{ pmol min}^{-1} \text{ mg protein}^{-1}$. No Phase II metabolites of TBBA or
TBMEHP were observed. More research is needed to understand the $in \ vivo$
toxicokinetics and health effects of these compounds given their current ubiquitous
presence in most US households and the resulting probability of chronic exposure,
particularly to young children.
B.1 Introduction

Brominated compounds are often used as additive flame retardants because of the halogen radicals’ ability to quench gas phase free radicals that propagate the fire cycle (Rahman et al., 2001). The addition of halogens to aromatic and aliphatic backbones increases the flame retardant properties of these compounds, but also increases their potential for bioaccumulation and persistence in the environment. Polybrominated diphenyl ether (PBDEs) flame retardants were used heavily until they were phased out in several regions (EU, 2009a; Tullo, 2003) and were later listed as persistent organic pollutants (POPs) due to their persistence and bioaccumulation potential according to the Stockholm Convention. Currently, DecaBDE is the only commercial PBDE mixture still in use, but it is scheduled for phase out in the United States by 2013 (USEPA, n.d.). Because flame retardants are used in consumer products, such as couches, electronics, and upholstery, they are present in the indoor environment. A study characterizing flame retardants in house dust identified 2-ethylhexyl tetrabromobenzoate (TBB) and 2-ethylhexyl tetrabromophthalate (TBPH) at concentrations often exceeding 1 µg g⁻¹ (Figure 37) (Stapleton et al., 2008a). These compounds are components of several Firemaster® commercial flame retardant mixtures marketed by Chemtura (West Lafayette, IN, USA) for use as replacements for the PentaBDE commercial mixture. However, the physical properties of TBB and TBPH (estimated log $K_{ow}$ of 8.8 and 12, respectively) indicate a potential for biomagnification
similar to other persistent organic pollutants (Barr et al., 2010). These compounds enter wastewater treatment plants, where they have been detected in sewage sludge and eventually enter the environment via effluent discharge or land-applied biosolids (La Guardia et al., 2010). TBB and TBPH were both detected in marine mammals from China, but no other published studies have analyzed sediment, lower trophic level organisms, or marine mammals from other regions (Lam et al., 2009). Therefore, little is known about the environmental fate of TBB and TBPH. A summary of the published studies investigating the levels of TBB and TBPH in the environment is included in Table 8.

![Chemical structures of TBB, TBPH, TBBA, and TBMEHP](image)

**Figure 37**: Chemical structures of TBB, TBPH, TBBA, and TBMEHP
Table 8: Summary of previous studies detecting TBB and TBPH in the environment.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Results</th>
<th>Location</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>House Dust</td>
<td>TBB: 6.6–15,030 ng g(^{-1})</td>
<td>Boston, MA</td>
<td>Stapleton, 2008(^8)</td>
</tr>
<tr>
<td>House Dust</td>
<td>TBPH: 1.5 to 10,630 ng g(^{-1})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dust from E-waste Processing Facility</td>
<td>TBB: 450–75,000 ng g(^{-1})</td>
<td>Boston, MA</td>
<td>Stapleton, 2009(^2)</td>
</tr>
<tr>
<td>Polyurethane Foam From Baby Products</td>
<td>TBPH: 300–47,110 ng g(^{-1})</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TBB: 18 ng g(^{-1}) mean</td>
<td>Thailand</td>
<td>Ali, 2011(^2)</td>
</tr>
<tr>
<td></td>
<td>TBPH: 270 ng g(^{-1}) mean</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Finless Porpoise</td>
<td>TBB: 5.6 ± 17 ng g(^{-1}) lipid</td>
<td>Hong Kong, China</td>
<td>Lam, 2009(^9)</td>
</tr>
<tr>
<td></td>
<td>TBB: 342 ± 883 ng g(^{-1}) lipid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sewage Sludge</td>
<td>TBB: 3,430–89,900 ng g(^{-1}) TOC</td>
<td>Mid-Atlantic Region, USA</td>
<td>La Guardia, 2010(^8)</td>
</tr>
<tr>
<td></td>
<td>TBPH: 64–33,500 ng g(^{-1}) TOC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sewage Sludge</td>
<td>TBB: 128 and 2,490 ng g(^{-1}) dry</td>
<td>North Carolina and California, USA</td>
<td>Davis, 2012(^2)</td>
</tr>
<tr>
<td></td>
<td>TBPH: 220–1340 ng g(^{-1}) dry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atmosphere</td>
<td>TBB/TBPH: 5.85–42.5 mg g(^{-1})</td>
<td>USA</td>
<td>Stapleton, 2011(^2)</td>
</tr>
<tr>
<td></td>
<td>TBB/TBPH: 0.050–290 pg m(^{-3})</td>
<td>Great Lakes Region, USA</td>
<td>Ma, 2011(^2)</td>
</tr>
</tbody>
</table>

Because of their use in consumer products and prevalence in house dust, the probability of human exposure to TBB and TBPH is high, particularly for children. A previous study demonstrated the ability of a commercial mixture containing both TBB and TBPH to bioaccumulate in fathead minnows (Pimephales promelas) after dietary exposure (Bearr et al., 2010). TBB was metabolized to form unidentified brominated metabolites, but TBPH was resistant to metabolic degradation (Bearr et al., 2010). Because TBB and TBPH were only recently discovered in the environment, there is very little toxicological data on these compounds. The nonbrominated analogs of TBB and TBPH, 2-ethylhexyl benzoate (EHB) and bis(2-ethylhexyl) phthalate (DEHP),
respectively, are commonly used industrial chemicals. Only slight toxicity has been observed with EHB, which is approved for use as a food additive (WHO, 2011), but the chronic toxicity of DEHP has been extensively studied (Tickner et al., 2001). In general, the metabolites of DEHP, including the hydrolysis metabolite 2-monoethylhexyl phthalate (MEHP), mediate the toxicity of DEHP (Tickner et al., 2001). Lipases and esterases in various organs mediate the bioactivation of DEHP via hydrolysis of one ethylhexyl group (Niino et al., 2003). For TBB, toxicity data is not available from the manufacturer, and only one previous study that attempted to assess the toxicity of the commercial BZ-54 mixture, which contains both TBPH and TBB, reported bioaccumulation of TBB and TBPH in fathead minnows with evidence of potential genotoxicity (Berr et al., 2010). Some acute toxicological data on TBPH are available from the EPA’s high production volume chemical database, but the potential developmental toxicity and endocrine disrupting effects of TBPH are unknown.

The metabolism of TBB and TBPH in humans has not yet been evaluated, and no studies have investigated the levels of these flame retardants in human tissues. Based on the metabolism of DEHP, it is likely that TBB and TBPH undergo similar cleavage of the ethylhexyl groups during metabolism. Because metabolism may lead to bioactivation of these compounds, and because knowledge of the metabolic stability will allow for predictions of the half-life of these compounds in human tissues, it is important to understand the metabolic potential of TBB and TBPH in humans. Furthermore, because
toxicology studies are usually performed in rats, it is also important to compare species-
specific differences in metabolism, which may mediate differences in toxicity. The
objectives of this study were to identify the metabolites of TBB and TBPH in human liver
microsomes (HLM), calculate the enzyme kinetics, and compare the metabolic rates
among liver and intestinal subcellular fractions from both humans and rats.

**B.2 Experimental Procedures**

**B.2.1 Materials**

HLM (50 donors pooled) and human liver cytosol (50 donors pooled) were
purchased from Invitrogen (Austin, TX, USA). Human intestinal microsomes (20 donors
pooled) were purchased from BD Biosciences (Franklin Lakes, NJ, USA). An additional
preparation of HLM (50 donors pooled) was purchased from Celsis (Chicago, IL, USA).
All the human subcellular fractions represented mixed-gender donors. Crude,
lyophilized hepatic porcine carboxylesterase was purchased from Sigma (less than 5%
buffer salts) (St. Louis, MO, USA). Rat microsomes and cytosol were prepared from the
liver and small intestinal tissues from adult, female Wistar rats (n=4) using our
previously published method (Roberts et al., 2011). Protein concentrations were
determined using the Bradford assay with a microplate reader (Bradford, 1976). TBB
(95% purity) and TBPH (99% purity) were purchased as neat solutions from
AccuStandard, Inc. (New Haven, CT, USA), and stock solutions were prepared in DMSO
at concentrations ranging from 0.0078–31.1 mM. The internal standard 4’-fluoro-2,3’,4,6-
tetramethylphenylether (FBDE 69) was purchased from Chiron (Trondheim, Norway). The internal standards 2,3,5-triiodobenzoic acid (TIBA) and monohexyl-2,3,4,5-tetrachlorophthalate (TCMHP) were purchased from Sigma. The metabolic product 2,3,4,5-tetrabromobenzoic acid (TBBA; estimated >98% purity by H1-NMR) was synthesized by the Duke Small Molecule Synthesis Facility (the detailed synthesis procedure is reported in the Supplemental Information). Mono(2-ethylhexyl) tetrabromophthalate (TBMEHP) was a gift from Dr. Kim Boekelhide’s group at Brown University. All solvents and other materials were HPLC grade.

B.2.2 Metabolic Incubations

All incubations were performed in 100 mM potassium phosphate incubation buffer (pH 7.4) in glass culture tubes in a shaking water bath at 37°C. The enzyme kinetics were evaluated using a microsomal protein concentration of 40 µg mL⁻¹ for 10 min incubations. The incubation conditions were chosen to minimize substrate depletion, especially in incubations with low concentrations, while producing sufficient product to measure using our LC/MS/MS assay. The incubations for kinetics analyses were performed with 9 TBB concentrations ranging from 0.0078–31.1 µM. The reactions were stopped by the addition of an equal volume of 1 M HCl. The incubations to assess the Phase II metabolism of TBBA and TBMEHP were conducted in the presence of the following cofactors: 50 µM PAPS (phosphoadenosine phosphosulfate) and 8 mM MgCl₂ for sulfation; 2 mM UDPGA (uridine diphosphate glucuronic acid), 25 µg mL⁻¹
alamethicin, and 8 mM MgCl\(_2\) for glucuronidation; and 10 \(\mu\)M reduced glutathione for glutathione conjugation. The Phase II incubations were performed for 90 min.

**B.2.3 Sample Preparation**

After stopping the reactions with HCl, 25 ng each of FBDE 69, TIBA, and TCMHP were added as internal standards for TBB and TBPH, TBBA, and TBMEHP, respectively. The reaction mixtures were extracted using Agilent SampliQ OPT cartridges (3 mL, 60 mg; Agilent Technologies, Inc., Santa Clara, CA, US) using a method similar to our previously published extraction procedure for thyroid hormones (Wang and Stapleton, 2010). After conditioning the SPE cartridges with 3 mL of methanol and 3 mL of water, the entire incubation mixture was added. The cartridges were washed with 3 mL of water, and the analytes were eluted with 2 mL of methanol and 2 mL of dichloromethane. The eluent fractions were combined, evaporated under nitrogen, and reconstituted in methanol.

**B.2.4 GC/MS Identification of Brominated Metabolites**

To identify potential metabolites using GC/MS, the extracts were reconstituted in hexane, derivatized using diazomethane (a methylating agent), and analyzed using full scan GC/ECNI (electron capture negative ionization)-MS and GC/EI-MS using our lab’s previously published GC conditions (Stapleton et al., 2008b). The mass spectra of peaks not present in control incubations (without microsomes) were analyzed to determine the
structural characteristics of the potential metabolites based on fragmentation patterns and bromine isotope signatures.

**B.2.5 LC/MS/MS Quantification**

The TBB and TBPH dosing concentrations were evaluated using LC/MS/MS with negative APCI (atmospheric pressure chemical ionization) using previously published MRMs and operating parameters (Zhou et al., 2010). Isochronic LC separation was performed using a Synergy XB-C18 column (100 × 2.10 mm, 2.6 μm; Phenomenex, Torrance, CA, US) with 98% methanol and 2% water as the mobile phase for 9 min. The method detection limits for TBB and TBPH were 5.05 ng mL⁻¹ and 2.41 ng mL⁻¹, respectively.

An LC/MS/MS method using negative ESI (electrospray ionization) was developed to quantify TBBA without derivatization and to confirm the GC/MS results. Separation of a 15-μL injection was performed on a Synergi Polar-RP column (50 × 2.0 mm, 2.5 μm; Phenomenex) with water and acetonitrile mobile phases with 5 mM acetic acid. A gradient method at a flow rate of 0.4 mL min⁻¹ was used to separate TBBA, TBMEHP, and their surrogate standards, TIBA and TCMHP, starting with 30% acetonitrile, increasing to 98% acetonitrile over 5 min, and returning to 30% acetonitrile over 3 min. TBBA and TIBA ionized and fragmented similarly, forming [M-H]-precursor ions and [M-CO2]⁻ and [M-X]⁻ product ions (X= Br or I; Table 9). TBMEHP formed an [M-CO2]⁻ parent ion (m/z=549.0) using ESI and fragmented to form a [Br]⁻
product ion (80.9). Multiple reaction monitoring (MRM) was used with the transitions and conditions shown in Table 9. The mean recoveries of 10 ng of the analytes and 50 ng of the internal standards spiked into buffer containing 50 µg of bovine albumin were calculated and are shown in Table 9. The calibration curves for TBBA and TBMEHP were linear (R²>0.99) over a calibration range of 1.00–1,000 ng mL⁻¹. Phase II metabolites (i.e., glucuronide- and sulfate-conjugated metabolites) were screened using a combination of techniques including neutral loss scans (176 Da for glucuronide cleavage) and predicted MRM analyses as described in a previous study (Clarke et al., 2001).

**Table 9: LC/MS/MS conditions, recovery, and detection limits for negative electrospray ionization for the metabolites, TBBA and TBMEHP, and the internal standards used for their quantification, 2,3,5-triiodobenzoic acid (TIBA) and monohexyl-2,3,4,5-tetrachlorophthalate (TCMHP), respectively.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Fragmentor Voltage</th>
<th>Gas Temp (°C)</th>
<th>MRM Transition</th>
<th>Mean Recovery</th>
<th>Method Detection Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBBA</td>
<td>75</td>
<td>250</td>
<td>436.6 → 392.6</td>
<td>63 ± 5%</td>
<td>1.25 ng mL⁻¹</td>
</tr>
<tr>
<td>TIBA</td>
<td>75</td>
<td>250</td>
<td>498.7 → 454.8</td>
<td>63 ± 6%</td>
<td></td>
</tr>
<tr>
<td>TBMEHP</td>
<td>120</td>
<td>250</td>
<td>548.8a → 80.9</td>
<td>110 ± 3%</td>
<td>1.20 ng mL⁻¹</td>
</tr>
<tr>
<td>TCMHP</td>
<td>80</td>
<td>250</td>
<td>343.0a → 215.0</td>
<td>108 ± 1%</td>
<td></td>
</tr>
</tbody>
</table>

a The parent ion in the MRM transition represents [M-CO₂]⁻  
b Recovery of 10 ng of TBBA and TBMEHP (n=6) and 50 ng of TIBA and TCMHP (n=3)
B.2.6 Quality Assurance

The results in this study are reported as the means ± SE of at least 2 independent experiments including 2–4 samples in each experiment along with controls containing 50 μg of bovine albumin as lab blanks. The mean concentrations of TBBA, TBMEHP, TBB, TBPH in the lab blanks were 0.474 ± 0.092, 0.288 ± 0.125, 2.77 ± 0.29, and 0.938 ± 0.186 ng mL⁻¹, respectively. Method detection limits were defined as the mean of the blank measurements plus 3 times the SD of the blank measurements. Statistical analyses and enzyme kinetics were calculated using JMP 9 (Cary, NC, USA). A Michaelis-Menten model was used to calculate enzyme kinetics as follows:

\[ v = \frac{V_{\text{max}} \times S}{K_m + S} \]

where \( v \) is the initial velocity at a given substrate concentration (S). The goodness of fit of the Michaelis-Menten models is reported using the coefficient of determination \( R^2 \). ANOVA was used to determine whether significant interactions existed between the formation rates and the tissue preparations. Tukey’s post-hoc test was used to compare TBBA formation rates among all the tissues with a significance level of \( p<0.05 \).

B.3 Results and Discussion

B.3.1 In Vitro Metabolism of TBB

The results from our experiments indicated that TBB was rapidly metabolized in both HLM and rat microsomes. To initially identify the potential metabolites of TBB, 60-min incubations were performed with HLM. The sample extracts were analyzed before
and after methyl-derivatization with diazomethane. Using full-scan GC/MS with ECNI and EI ionization, no potential debrominated or other metabolites were detected in the nonderivatized samples for TBB, but a 79.9 ± 4.6% reduction in the concentration of TBB was observed. In samples treated with diazomethane, TBBA was identified as the major metabolite of TBB based on both ECNI and EI mass spectra of the methyl-derivative of TBBA, as shown in Figure 38, and no other potential metabolites were identified. A peak with an m/z of 452.0 was present in both ECNI and EI mass spectra that corresponded to the molecular ion of the TBBA-methyl derivative along with other fragment ions (Figure 38). To further confirm the identification, an analytical standard for TBBA was synthesized, and a more robust LC/MS/MS method using negative ESI was developed using 2,3,5-triiodobenzoic acid (TIBA) as an internal standard. Comparison of the metabolite with the synthesized TBBA confirmed that TBBA was the primary metabolite of TBB.
Figure 38: Mass spectra of the TBBA methyl-derivative obtained using GC/MS operated in EI (top) and ECNI (bottom) ionization modes with labeled fragments.

**B.3.2 Carboxylesterase-Mediated Metabolism**

To determine whether cytochrome P450 metabolizing enzymes were involved in the metabolism of TBB, incubations were performed in the presence and absence of the necessary cofactor, NADPH. The addition of NADPH did not significantly affect the formation rate of TBBA. Based on the observed mechanism of ester hydrolysis in the
absence of cofactors, we hypothesized that carboxylesterases were responsible for the metabolism of TBB. Esterases represent an extensive class of cytosolic and microsomal enzymes with broad substrate specificity and widespread expression in mammalian tissues (Sogorb and Vilanova, 2002). Furthermore, the nonhalogenated analog of TBB, 2-ethylhexyl benzoate, is a well-characterized substrate of carboxylesterases (Albro et al., 1976). Therefore, the metabolism of TBB was also examined in a purified preparation of hepatic porcine carboxylesterase (PCE). Although PCE may demonstrate some species-specific differences from human carboxylesterases, previous studies have shown that human and porcine carboxylesterases catalyze similar metabolic reactions with differences only in the observed reaction rates (Huang et al., 1996). The PCE enzyme preparation rapidly metabolized TBB to TBBA, and no other metabolites were detected. Because various esterases are widely expressed in vertebrates and bacteria, TBB hydrolysis may occur in many different organisms, and bacterial degradation may occur in the environment or during wastewater treatment (Sogorb and Vilanova, 2002).

**B.3.3 Enzyme Kinetics**

The hydrolysis of TBB in the liver to form TBBA may influence the distribution and potential toxicity of TBB *in vivo*. To determine the maximum rate of metabolism and the enzyme specificity for TBB in HLM and PCE, we determined the Michaelis-Menten kinetics. To optimize the incubation conditions for the kinetics experiments, the time courses of both HLM- and PCE-mediated TBB hydrolysis were evaluated over 60 min.
with either 40 μg protein mL⁻¹ of HLM or 2 μg protein mL⁻¹ of PCE at a TBB concentration of 27.1 ± 1.3 μM. TBBA formation continued at an approximately linear rate for the entire 60-min incubation for both preparations, as shown in Figure 39.

Figure 39: Formation of TBBA over time in (a) human liver microsomes (40 μg protein mL⁻¹) and (b) porcine hepatic carboxylesterase (2 μg protein mL⁻¹) at a TBB concentration of 27.1 ± 1.3 μM showing approximately linear formation for 60 min. The values represent the mean of 4 experiments.
Attempts were made to characterize the rate of TBBA formation relative to the metabolic loss of TBB (i.e., a mass balance). TBBA increased over time during the incubation, producing approximately 1 nmol during the 6-min incubation. TBB, on the other hand, decreased by approximately 11% (e.g., 2.9 nmol) during the 60-min incubation; therefore, the formation of TBBA appeared to account for approximately 30% of the substrate loss of TBB over the 60-min incubation with HLM. However, given the standard error in the measurement of TBB, this loss was not statistically significant.

The kinetics of TBBA formation in HLM and PCE were evaluated by performing incubations with a range of TBB concentrations. The results of the kinetics experiments are shown in Figure 40. Using a Michaelis-Menten model, the $K_m$ and $V_{max}$ for TBBA formation in HLM were determined to be $11.1 \pm 3.9 \mu M$ and $0.644 \pm 0.144 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$, and the $K_m$ and $V_{max}$ in PCE were $9.3 \pm 2.2 \mu M$ and $24.4 \pm 2.6 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$, respectively. The $K_m$ was approximately 10 µM in both HLM and PCE, which indicates similar enzyme specificity among the enzymes in both preparations. Because of the limited solubility of TBB in the incubation buffer, approximately 30 µM was the highest concentration that could be used to evaluate the kinetics without introducing unacceptably high variability in the substrate concentrations and, thus, the reaction velocities.
Figure 40: Initial velocity of TBBA formation at various substrate concentrations fit to a Michaelis-Menten model using 10-min incubations with human liver microsomes and porcine hepatic carboxylesterase with $R^2$ values of 0.782 and 0.862, respectively. The values represent the mean of 4 experiments.

**B.3.4 Multiple Species and Tissue Comparison**

TBB metabolism was compared among human tissues using pooled samples of HLM from two different suppliers and human liver cytosol and intestinal microsomes. TBB metabolism was evaluated in simultaneous 10-min incubations with 40 $\mu$g protein mL$^{-1}$ of each subcellular fraction at a nominal TBB concentration of 20 $\mu$M (the actual
mean concentration was measured to be $5.25 \pm 0.31 \mu\text{M}$, which was likely due to the limited solubility in the DMSO dosing solvent. The results of these experiments are shown in Figure 41. TBBA formation rates observed in the two commercial preparations of HLM were not significantly different. TBB metabolism was slightly lower in the cytosol ($0.207 \pm 0.020 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$) than the intestinal and liver microsomes ($0.297 \pm 0.037$ and $0.259 \pm 0.033 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$, respectively), but these differences were also not statistically significant.

Potential TBB metabolism was compared among rat tissues to evaluate species-specific differences between rats and humans and to provide knowledge of metabolism in rats for future *in vivo* studies. TBB metabolism was investigated in rat liver microsomes and cytosol, intestinal microsomes, and serum. TBB was metabolized to form TBBA in all the rat tissues at formation rates ranging from $0.203 \pm 0.004$ and $0.422 \pm 0.093 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$ in intestinal microsomes and liver cytosol, respectively, to $6.25 \pm 0.58 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$ in liver microsomes, as shown in Figure 41. TBB metabolism also occurred in rat serum but at a much slower rate than in the other tissues ($0.0418 \pm 0.0090 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$). The formation of TBBA in rat liver microsomes occurred at a significantly faster formation rate than all the other rat and human tissues, but was similar to the rate of TBBA formation in PCE ($6.29 \pm 0.92 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$). Unlike human tissues, TBB metabolism was significantly slower in rat intestinal microsomes than in rat liver microsomes. While the metabolism of TBB was similar
among human liver and intestinal microsomes and human cytosol, TBB metabolism in rat liver microsomes occurred much faster. This species-specific difference in TBB metabolism should be considered in toxicity studies performed using rats due to the more rapid elimination of TBB in rat livers. However, the rat data presented here are representative of a small number of female rats (n=4), and further work is necessary to confirm whether these values are representative of male rats and other rat strains.

![Graph showing TBBA formation rates](image)

**Figure 41:** TBBA formation rates for incubations performed with human and rat tissues normalized to protein content. In pooled samples, n equals the number of individual experiments performed with the pooled sample. In the rat tissues, n equals the number of animals from which individual microsomal or serum samples were prepared. The asterisk indicates significant difference from samples without an asterisk using Tukey’s post-hoc test (p<0.01) after ANOVA indicated a main effect of tissue on formation rate (p<0.001).
**B.3.5 TBPH Metabolism**

Unlike TBB, in experiments with HLM, a significant loss of TBPH was not observed, and no metabolites were detected by GC/MS analysis of the sample extracts. An LC/MS/MS method was developed to monitor tetrabromo-MEHP (TBMEHP), a potential hydrolysis metabolite of TBPH (Figure 37). After a 6-h incubation in HLM, TBMEHP was not detected as a metabolite of TBPH and no significant loss of TBPH was observed. However, TBPH was slowly metabolized to form TBMEHP in the presence of 0.1 mg mL⁻¹ of PCE. This reaction was monitored at multiple time points up to 6 h and maintained linearity at an approximate rate of 1.08 pmol min⁻¹ mg esterase⁻¹ as shown in Figure 39. In a previous study with PCE, DEHP (50 μM) was metabolized to form MEHP at a rate of 127 pmol min⁻¹ mg protein⁻¹ (Niino et al., 2003). This rate was approximately 100 times faster than the hydrolysis of TBPH observed in this study (1.08 pmol min⁻¹ mg protein⁻¹). The prominent difference between the metabolic hydrolysis of DEHP and TBPH may be a result of steric hindrance by the fully brominated phenyl ring of TBPH.

**B.3.6 Phase II Metabolism**

The potential Phase II metabolism of TBBA and TBMEHP was evaluated in HLM and cytosol. Potential sulfation and glutathione conjugation were evaluated in cytosol, and glucuronidation was evaluated in microsomes. Using a combination of LC/MS/MS techniques, no Phase II metabolites were detected for either compound.
Furthermore, there were no significant losses of the parent compounds, TBBA or TBMEHP, during the incubations.

TBBA may prove to be a useful biomarker of human exposure to TBB. Although this study shows that TBBA is likely formed in vivo via metabolism, the mechanism of in vivo TBBA excretion has not been studied. No Phase II metabolites of TBBA were detected in this study after performing incubations with cofactors specific for sulfation, glucuronidation, and glutathione conjugation. If TBBA is excreted in the urine, it may be an important indicator of human TBB exposure; therefore, future studies should evaluate the distribution and excretion of TBBA.

The results for TBPH provide some insight into the fate and potential toxicity of TBPH. Because TBPH is apparently more recalcitrant to metabolism than TBB, TBPH may have a longer half-life after absorption in vivo. A study analyzing marine mammals from the Pearl River Delta in China reported mean concentrations of 5.6 ± 17 and 342 ± 883 ng g lipid⁻¹ for TBB and TBPH, respectively (Lam et al., 2009), even though the approximate ratio of TBB to TBPH in the Firemaster 550 commercial mixture is 4:1 (Stapleton et al., 2008a). Metabolism of TBB could be an important factor in these observed differences in TBB and TBPH concentrations and in the ratios of TBB and TBPH in other environmental matrices.

The metabolism of TBB to form TBBA has several toxicological implications for TBB. While metabolism apparently reduces the potential for bioaccumulation of TBB, it
introduces a metabolite, TBBA, with unknown toxicity and fate. This metabolite may be used as a biomarker of TBB exposure for public health and exposure studies once its excretion pathway has been characterized. The metabolism of TBPH to form TBMEHP may have implications on the toxicity of TBPH, but may not be rapid enough to affect the bioaccumulation of TBPH. However, its persistence in tissues and the ubiquity of carboxylesterases in other organs and tissues may facilitate TBPH metabolism in mammalian tissues. *In vivo* toxicological studies should assess the *in vivo* accumulation and metabolism of both TBB and TBPH in mammals and evaluate the toxicity of both TBBA and TBMEHP.
Appendix C. Supplemental Statistical Results

Figure 42: Two-way ANOVA and Tukey's post-hoc results for the time-course effects on DIO2 activity shown in Figure 10.
Figure 43: One-way ANOVA and Tukey’s post-hoc results for the dose-response effects on DIO2 activity shown in Figure 11.
Figure 44: One-way ANOVA and Tukey’s post-hoc results for the dose-response effects on DIO2 mRNA expression shown in Figure 13.
Figure 45: Two-way ANOVA and Tukey’s post-hoc results for the T3 concentration in the cell culture medium of co-cultured cells and SK-N-AS cells cultured alone as shown in Figure 19.
References


Hallgren, S., Sinjari, T., Håkansson, H., and Darnerud, P. (2001). Effects of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) on thyroid hormone and vitamin A levels in rats and mice. *Arch. Toxicol.*, **75**, 200–208.


217


Biography

Simon Roberts was born in Crossville, Tennessee and lived in Tennessee until he received a Bachelor’s of Science in Environmental Health at East Tennessee State University in 2009. Simon started his doctoral studies at Duke University in 2009 under the supervision of Heather Stapleton. Simon received the Otto Hutzinger best student presentation award at the 2010 Dioxin International Symposium, the best student poster award at the 2011 Annual Workshop on Brominated Flame Retardants, and an EPA STAR fellowship in 2012. During his graduate studies, Simon has published 2 first-author publications and has collaborated with several researchers to contribute as an author in 6 published articles:


