The Effects of Brominated Flame Retardants on Thyroid Hormone Homeostasis in Human Placenta Tissues and Cell Culture

by

Christopher Philip Leonetti

Environment
Duke University

Date:_______________________
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Rebecca C. Fry

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Joel N. Meyer

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Marie Lynn Miranda

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

2016
ABSTRACT

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Polybrominated diphenyl ethers (PBDEs) are a class of brominated flame retardants (BFRs) that have been heavily used in consumer products such as furniture foams, plastics, and textiles since the mid-1970’s. BFRs are added to products in order to meet state flammability standards intended to increase indoor safety in the event of a fire. The three commercial PBDE mixtures, Penta-, Octa-, and DecaBDE, have all been banned in the United States, however, limited use of DecaBDE is still permitted. PBDEs were phased out of production and added to the Stockholm Convention due to concerns over their environmental persistence and toxicity. Human exposure to PBDEs occurs primarily through the inadvertent ingestion of contaminated house dust, as well as through dietary sources. Despite the phase-out and discontinued use of PBDEs, human exposure to this class of chemicals is likely to continue for decades due to the continued use of treated products and existing environmental reservoirs of PBDEs. Extensive research over the years has shown that PBDEs disrupt thyroid hormone (TH) levels and neurodevelopmental endpoints in rodent and fish models. Additionally, there is growing epidemiological evidence linking PBDE exposure in humans to altered TH homeostasis and neurodevelopmental impairments in children. Due to the importance of THs throughout gestation, there is a great need to understand the effects of BFRs on the developing fetus. Specifically, the placenta plays a critical role in the transport,
metabolism, and delivery of THs to the fetal compartment during pregnancy and is a likely target for BFR bioaccumulation and endocrine disruption. The central hypothesis of this dissertation research is that BFRs disrupt the activity of TH sulfotransferase (SULT) enzymes, thereby altering TH concentrations in the placenta.

In the first aim of this dissertation research, the concentrations of PBDEs and 2,4,6-TBP were measured in a cohort of 102 placenta tissue samples from an ongoing pregnancy cohort in Durham, NC. Methods were developed for the extraction and analysis of the BFR analytes. It was found that 2,4,6-TBP was significantly correlated with all PBDE analytes, indicating that 2,4,6-TBP may share common product applications with PBDEs or that 2,4,6-TBP is a metabolite of PBDE compounds. Additionally, this was the first study to measure 2,4,6-TBP in human placenta tissues.

In the second aim of this dissertation research, the placenta tissue concentrations of THs, as well as the endogenous activity of deiodinase (DI) and TH SULT enzymes were quantified using the same cohort of 102 placenta tissue samples. Enzyme activity was detected in all samples and this was the first study to measure TH DI and SULT activity in human placenta tissues. Enzyme activities and TH concentrations were compared with BFR concentrations measured in Aim 1. There were few statistically significant associations observed for the combined data, however, upon stratifying the data set based on infant sex, additional significant associations were observed. For example, among males, those with the highest concentrations of BDE-99 in placenta had
T3 levels 0.80 times those with the lowest concentration of BDE-99 (95% confidence interval (CI): 0.59, 1.07). Whereas females with the highest concentrations of BDE-99 in placenta had T3 levels 1.50 times those with the lowest concentration of BDE-99 (95% CI: 1.10, 2.04). Additionally, all BFR analyte concentrations were higher in the placenta of males versus females and they were significantly higher for 2,4,6-TBP and BDE-209. 3,3’-T2 SULT activity was significantly higher in female placenta tissues, while type 3 DI activity was significantly higher in male placenta tissues. This research is the first to show sex-specific differences in the bioaccumulation of BFRs in human placenta tissue, as well as differences in TH concentrations and endogenous DI and SULT activity. The underlying mechanisms of these observed sex differences warrant further investigation.

In the third aim of this dissertation research, the effects of BFRs were examined in a human choriocarcinoma placenta cell line, BeWo. Michaelis-Menten parameters and inhibition curves were calculated for 2,4,6-TBP, 3-OH BDE-47, and 6-OH BDE-47. 2,4,6-TBP was shown to be the most potent inhibitor of 3,3’-T2 SULT activity with a calculated IC$_{50}$ value of 11.6 nM. It was also shown that 2,4,6-TBP and 3-OH BDE-47 exhibit mixed inhibition of 3,3’-T2 sulfation in BeWo cell homogenates. Next, a series of cell culture exposure experiments were performed using 1, 6, 12, and 24 hour exposure durations. Once again, 2,4,6-TBP was shown to be the most potent inhibitor of basal 3,3’-T2 SULT activity by significantly decreasing activity at the high and medium dose (1 µM and 0.5 µM, respectively) at all measured time points. Interestingly, BDE-99 was also shown to
inhibit basal 3,3′-T2 SULT activity in BeWo cells following the 24 hour exposure, despite exhibiting no inhibitory effects in the BeWo cell homogenate experiments. This indicates that BDE-99 must act through a pathway other than direct enzyme inhibition. Following exposures, the TH concentrations in the cell culture growth media and mRNA expression of TH-related genes were also examined. There was no observed effect of BFR treatment on these endpoints. Future work should focus on determining the downstream biological effects of TH SULT disruption in placental cells, as well as the underlying mechanisms of action responsible for reductions in basal TH SULT activity following BFR exposure.

This was one of the first studies to measure BFRs in a cohort of placenta tissue samples from the United States and the first study to measure THs, DI activity, and SULT activity in human placenta tissues. This research provides a novel contribution to our growing understanding of the effects of BFRs on TH homeostasis within the human placenta, and provides further evidence for sex-specific differences within this important organ. Future research should continue to investigate the effects of environmental contaminants on TH homeostasis within the placenta, as this represents the most critical and vulnerable stage of human development.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ARS</td>
<td>Arylsulfatase</td>
</tr>
<tr>
<td>BDE-47</td>
<td>$2,2',4,4'$-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-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-209</td>
<td>Decabromodiphenyl ether</td>
</tr>
<tr>
<td>BPA</td>
<td>Bisphenol A</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>DecaBDE</td>
<td>Commercial DecaBDE mixture</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary DNA</td>
</tr>
<tr>
<td>CI</td>
<td>Chemical ionization</td>
</tr>
<tr>
<td>$C_t$</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>CYP450</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>DDT</td>
<td>Dichlorodiphenyltrichloroethane</td>
</tr>
<tr>
<td>DI</td>
<td>Deiodinase</td>
</tr>
<tr>
<td>DIO1</td>
<td>Type 1 iodothyronine deiodinase</td>
</tr>
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<td>Type 2 iodothyronine deiodinase</td>
</tr>
<tr>
<td>DIO3</td>
<td>Type 3 iodothyronine deiodinase</td>
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<td>Dithiothreitol</td>
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<td>ECNI</td>
<td>Electron capture negative ionization</td>
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<td>Electron Impact</td>
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<td>EPA</td>
<td>Environmental Protection Agency</td>
</tr>
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<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>ESULT</td>
<td>Estradiol sulfotransferase</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>E-waste</td>
<td>Electronic waste</td>
</tr>
<tr>
<td>GC/MS</td>
<td>Gas chromatography/mass spectrometry</td>
</tr>
<tr>
<td>hCG</td>
<td>Human chorionic gonadotropin</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IUGR</td>
<td>Intrauterine growth restriction</td>
</tr>
<tr>
<td>Kₘ</td>
<td>Michaelis-Menten constant (1/2 Vₘₐₓ)</td>
</tr>
<tr>
<td>Kₐₜₜ</td>
<td>Octanol-water partition coefficient</td>
</tr>
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<table>
<thead>
<tr>
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<th>Description</th>
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<tr>
<td>LAT</td>
<td>L-amino acid transporter</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>MIT</td>
<td>Monoiodotyrosine</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple reaction monitoring</td>
</tr>
<tr>
<td>MVM</td>
<td>Microvillous membrane</td>
</tr>
<tr>
<td>OATP</td>
<td>Organic anion transport protein</td>
</tr>
<tr>
<td>OH-BDE</td>
<td>Hydroxylated PBDE</td>
</tr>
<tr>
<td>PBDE</td>
<td>Polybrominated diphenyl ether</td>
</tr>
<tr>
<td>PCB</td>
<td>Polychlorinated biphenyl</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
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<td>Perfluoroalkyl substances</td>
</tr>
<tr>
<td>PFHxS</td>
<td>Perfluorohexane sulfonate</td>
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<td>Perfluorooctanoate</td>
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<td>PFOS</td>
<td>Perfluorooctane sulfonate</td>
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<td>PXR</td>
<td>Pregnane X receptor</td>
</tr>
<tr>
<td>rT3</td>
<td>3,3',5'-Triiodothyronine</td>
</tr>
<tr>
<td>rT3S</td>
<td>rT3 sulfate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</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>T3S</td>
<td>T3 sulfate</td>
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<td>Thyroxine</td>
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<td>TBBPA</td>
<td>Tetrabromobisphenol A</td>
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<tr>
<td>TH</td>
<td>Thyroid hormone</td>
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<tr>
<td>THHTP</td>
<td>Thyroid hormone transporter protein</td>
</tr>
<tr>
<td>TR</td>
<td>Thyroid receptor</td>
</tr>
<tr>
<td>TRE</td>
<td>Thyroid response element</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid stimulating hormone</td>
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<td>TTR</td>
<td>Transthyretin</td>
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<tr>
<td>UDPGT</td>
<td>Uridine diphosphate glucuronosyl transferase</td>
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<tr>
<td>V_{max}</td>
<td>Maximum enzyme rate at substrate saturation</td>
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1. Introduction

1.1 Flame Retardant Overview

Many indoor and household products are constructed with highly flammable materials such as polyurethane foam, plastics, and other synthetic materials. These products include mattresses, upholstered furniture, electronics, building materials, and a variety of textiles and infant products. These products present a potential fire hazard within the home environment as they can act as combustion sources in the case of a fire incident. According to a 2015 report from the National Fire Protection Association, US fire departments responded to approximately 1,298,000 fires in 2014. These fires caused 3,275 civilian deaths and an estimated $11.6 billion in direct property loss (Haynes 2015). Clearly there exists great concern for minimizing the incidence of indoor fires in order to protect human safety and damage to property. Efforts to reduce the incidence of residential fires began in 1975 with the passing of Technical Bulletin 117 (TB117) by the California Bureau of Electronic and Appliance Repair, Home Furnishings, and Thermal Insulation. The passing of this legislation was largely driven by the increasing incidence of household fires caused by unattended cigarettes in the early 1970s, with 60-70% of residential furniture fires being caused by the careless use of cigarettes (Damant and Nurbakhsh 1995). As a result, TB117 was established as the first set of flammability standards for upholstered furniture in an effort to reduce the occurrence of household fires and increase public safety. TB117 required furniture manufacturers to perform and
meet flammability tests before they could sell their products in the state of California. TB117 was a mandatory test that required the polyurethane foam cushioning of upholstered furniture to pass a 12-second open flame test meant to simulate ignition from a cigarette source (Damant and Nurbakhsh 1995). Following these new California flammability requirements, the majority of furniture produced and sold in the US after 1975 were compliant with TB117 because it was easier for manufacturers to maintain a single production line of compliant furniture, rather than a separate production line solely for the state of California (Weil and Levchik 2004). As a result, TB117 compliant furniture was sold in the US marketplace from 1975 through 2014.

The introduction of TB117 was significant in changing the furniture manufacturing market, as well as the chemical flame retardant industry. TB117 did not specify how furniture manufacturers were required to meet the new flammability standards, thus companies often chose the most economical solution. Generally, the cheapest and most efficient way to meet TB117 guidelines was by use of liquid formulations of chemical flame retardants that could be directly applied to the polyurethane foam during production. Halogenated flame retardants soon became the primary chemical treatment for furniture foams because they were readily available, easy to apply, and effective in meeting the TB117 guidelines.

Halogenated flame retardants act in the gaseous phase during combustion, wherein low energy halogen radicals such as Br• and Cl• are released from the polyurethane
foams once the temperature is sufficient for volatilization. During a fire, increasing heat causes the halogenated flame retardants to decompose before the matrix of the base polymer, due to the relatively weak carbon-bromine and carbon-chlorine bonds that break at approximately 400°C. The halogens, bromine and chlorine, have suitable enthalpies of dissociation for use as effective flame retardants, in that they have strong enough bonds with carbon at room temperature in order to resist degradation over the life-span of consumer product applications, but at the same time these same bonds will break down at combustion temperatures. The newly released halogen radicals disrupt the combustion process by quenching the high energy radicals HO• and H• that are present in the exothermic fire cycle. The net effect of halogenated flame retardants is a reduction in fire propagation via radical quenching in the gaseous phase of the combustion cycle, thus leading to an increased time to flashover and a longer time for people to evacuate from a fire event (Rahman et al. 2001).

There is a great diversity in the chemical structures of halogenated flame retardants, however they typically contain bromine or chlorine atoms because these are the most effective halogens for use in flame retardant chemicals. The majority of the flame retardants used in consumer products are additive flame retardants. This class of flame retardants are not chemically bound to the base polymer following application in the manufacturing process. These chemicals tend to be less stable within the product and often end up migrating out of the products over their life span and accumulating in the
indoor environment (Zhang et al. 2011b). In contrast, reactive flame retardants are chemically bound to the base polymer. Despite their increased effectiveness over product life span, reactive flame retardants are not widely used due to their higher cost and effects on base polymer properties (Camino and Costa 1988).

Since the 1975 legislation, an updated flammability standard known as TB117-2013 was introduced in 2014. This updated legislation came in response to the growing scientific evidence that flame retardant chemicals were not as effective at preventing/slowing fires as they were thought to be, as well as due to concerns over the persistence and toxicity of these chemicals. The updated standard no longer requires open flame testing and now allows the use of a barrier material between the polyurethane foam and the outermost fabric that is used on the final product. This amendment has the potential to maintain fire safety, as well as reduce the use of flame retardant chemicals in furniture items due to the more realistic testing protocols. However, the use of chemical flame retardants in US furniture items and consumer products will continue for some time.

### 1.2 Brominated Flame Retardants

#### 1.2.1 Polybrominated Diphenyl Ethers (PBDEs)

As reported in 2001, the production volume of brominated flame retardants (BFRs) was 53,900 metric tons in the Americas (26.5% of worldwide) and 203,740 metric tons worldwide. BFRs include the widely used class of chemicals known as polybrominated
diphenyl ethers (PBDEs), which made up 61.4% of the production volume in the Americas in 2001 (Birnbaum and Staskal 2004). PBDEs are a class of chemicals with a chemical structure consisting of two aromatic rings joined by an ether linkage. The hydrogen atoms on the aromatic rings can be substituted with between one and ten bromine atoms, resulting in potentially 209 possible congeners. PBDEs are structurally similar to the industrial dielectric and coolant chemical class, polychlorinated biphenyls (PCBs), and follow the same congener naming scheme. PBDE chemical properties vary by their degree of bromination and can be further subdivided into groups based on the number of bromine substitutions (tri-, penta-, octa-, deca-). There are three main PBDE commercial mixtures that have been manufactured and used since the 1970’s: PentaBDE, OctaBDE, and DecaBDE. As of 2001, the worldwide market demand for DecaBDE was 83.3% followed by PentaBDE and OctaBDE at 11.1% and 5.6%, respectively (LaA Guardia et al. 2006). PentaBDE was most widely used in polyurethane furniture foams beginning in the 1970s with the passing of California TB117. One formulation of the PentaBDE technical mixture, DE-71 (Great Lakes Chemical Corp., Indiana), is composed of 23 PBDE congeners, with BDE-99 making up 48.6% of the mixture, followed by BDE-47 with 38.2% and BDE-153 with 5.4%. OctaBDE was widely used in acrylonitrile butadiene styrene (ABS) in plastics and electronic products. The OctaBDE technical mixture DE-79 (Great Lakes Chemical Corp., Indiana) is composed of 13 PBDE congeners, with BDE-183 making up 42% of the mixture, followed by BDE-197 with
22.2% and BDE-207 with 11.5%. DecaBDE is the main commercial PBDE mixture used in electronics housing and textile backcoatings. The DecaBDE commercial mixture, Saytex 102E (Albermarle Corp., Louisiana), is composed of four PBDE congeners, with BDE-209 making up 96.8% of the mixture, followed by BDE-209 with 2.2% (LaA Guardia et al. 2006). An overview of the PBDE commercial mixtures is provided below in Table 1.

PBDEs exhibit a wide range of physicochemical properties based on their pattern and degree of bromination. For example, the range of log $K_{OW}$ values for all PBDE congeners is between 5.5 – 10.0 and the range of vapor pressures (log $P_L$) is -0.79 – 6.33 (Yue and Li 2013). This leads to congener-specific differences in environmental fate, biological uptake properties, and bioaccumulation rates. For example, some PBDE congeners such as BDE-209 have a biological half-life of approximately 15 days, while the biological half-life of BDE-153 is estimated to be 5 – 7 years (Thuresson et al. 2006).
Table 1: Overview of Congener Profiles in PBDE Commercial Mixtures

<table>
<thead>
<tr>
<th>Commercial Mixture</th>
<th>Congener</th>
<th>% of Commercial Mixture</th>
<th>Product Application</th>
<th>Log ( K_{ow} )</th>
<th># of Bromines</th>
<th>% of World Demand in Americas</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PentaBDE (DE-71)</strong></td>
<td>BDE-99</td>
<td>48.6</td>
<td>Polyurethane foams</td>
<td>8.19</td>
<td>5</td>
<td>95%</td>
</tr>
<tr>
<td></td>
<td>BDE-47</td>
<td>38.2</td>
<td>Furniture cushioning</td>
<td>6.77</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-100</td>
<td>13.1</td>
<td>Mattresses</td>
<td>8.03</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-153</td>
<td>5.44</td>
<td>Carpet padding</td>
<td>8.98</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-154</td>
<td>4.54</td>
<td></td>
<td>8.83</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-85</td>
<td>2.96</td>
<td></td>
<td>8.02</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-49</td>
<td>0.74</td>
<td></td>
<td>7.47</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><strong>OctaBDE (DE-79)</strong></td>
<td>BDE-183</td>
<td>42.0</td>
<td>Acrylonitrile-butadiene styrene</td>
<td>9.49</td>
<td>7</td>
<td>40%</td>
</tr>
<tr>
<td></td>
<td>BDE-197</td>
<td>22.2</td>
<td>Molded plastics</td>
<td>10.00</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-207</td>
<td>11.5</td>
<td>Televisions</td>
<td>10.62</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-196</td>
<td>10.5</td>
<td>Computer casings</td>
<td>10.08</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-153</td>
<td>8.66</td>
<td></td>
<td>8.98</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-203</td>
<td>4.40</td>
<td></td>
<td>10.11</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-171</td>
<td>1.81</td>
<td></td>
<td>9.50</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-180</td>
<td>1.70</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>BDE-206</td>
<td>1.38</td>
<td></td>
<td>10.70</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-209</td>
<td>1.31</td>
<td></td>
<td>11.24</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-154</td>
<td>1.07</td>
<td></td>
<td>8.83</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td><strong>DecaBDE (Saytex 102E)</strong></td>
<td>BDE-209</td>
<td>96.8</td>
<td>High-impact polystyrene Epoxy-resin</td>
<td>11.24</td>
<td>10</td>
<td>44%</td>
</tr>
<tr>
<td></td>
<td>BDE-206</td>
<td>2.19</td>
<td>Back covers</td>
<td>10.70</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-207</td>
<td>0.24</td>
<td>Circuit boards</td>
<td>10.62</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BDE-208</td>
<td>0.06</td>
<td></td>
<td>10.70</td>
<td>9</td>
<td></td>
</tr>
</tbody>
</table>

### 1.2.2 2,4,6-Tribromophenol (2,4,6-TBP)

2,4,6-tribromophenol (2,4,6-TBP) is the most widely produced bromophenol in the world, with an estimated annual production volume of 9500 tons in 2001 (Haldén et
The primary use of 2,4,6-TBP is as an intermediate in the production of other brominated flame retardants and as a fungicide and wood preservative as a replacement to pentachlorophenol (Savory et al. 1970). 2,4,6-TBP also has several natural sources in the environment, primarily from marine fauna. Several species of marine algae and many marine benthic animals produce and excrete large amounts of bromophenols such as 2,4,6-TBP (Gribble 2000; Sim et al. 2009). An evaluation of the physicochemical properties of 2,4,6-TBP show that this chemical will be relatively immobile in the environment, highly resistant to biodegradation, capable of bioaccumulation, and found primarily in lipid-rich tissues (Howe et al. 2005).

There are currently few studies that have evaluated the toxicity and health effects of 2,4,6-TBP. However, one in vitro study suggests that 2,4,6-TBP disrupts TH homeostasis via competitive binding to transthyretin (TTR) at a higher potency than the natural ligand, T4, as well as exhibits antiestrogenic activity by inhibiting estradiol sulfotransferase (ESULT) activity (Hamers et al. 2006). An additional study conducted in Danio rerio showed that 2,4,6-TBP disrupts reproductive development and fertility following dietary exposure (33,000 µg/g dry) (Haldén et al. 2010).

1.3 Sources and Human Exposure to BFRs

1.3.1 PBDEs

PBDEs are ubiquitous environmental contaminants that have been detected worldwide in biological tissues and environmental compartments. Due to their primary
use as additive flame retardants, in addition to their physicochemical properties (ie. volatility), PBDEs can migrate out of the products to which they were applied. As a result, PBDEs are released from polyurethane foams, computers, televisions, and a variety of other household items, where they accumulate within the indoor environment over time. The two primary routes of exposure for PBDEs are through exposures in the indoor environment (house dust) and through dietary exposures, and are discussed below.

PBDE exposure can occur from ingestion of contaminated food items. Fish, meats, and dairy products contain the highest concentrations of PBDEs, with fish having the highest PBDE concentrations in the US. Given the high consumption of meat and dairy within the US, diet is thought to be a significant route of exposure for PBDEs in humans, accounting for approximately 16.8% of daily exposure depending on an individual’s diet (Lorber 2008; Schecter et al. 2004, 2006, 2008). Dietary exposure to PBDEs is estimated to be between 60-84 ng/day for the US general population, and approximately 38-97 ng/day for Europeans (Gómara et al. 2006; Schecter et al. 2006). The primary route of pentaBDE congener exposure for Europeans is from contaminated food items. Since the overall use of lower brominated PBDEs (tri-octa) is much lower in Europe than in the US, a greater proportion of these compounds come from dietary sources instead of from treated furniture items (use and exposure to BDE-209 is likely similar between regions).
However, for the US population, the primary route of PBDE exposure is from ingestion of contaminated house dust, with approximately 82% of daily exposure coming from indoor dust. Indoor dust has been shown to be a repository for a variety of environmental contaminants, including PCBs, PAHs, phthalates, and lead (Butte and Heinzow 2002). Numerous studies have measured PBDEs in indoor house dust and air, indoor office space dust and air, and within indoor air intake filters (Johnson-Restrepo and Kannan 2009; Mandalakis et al. 2009; Stapleton et al. 2005, 2006). Median dust concentrations of ΣBDEs in the US range from 2,865.5 ng/g dust to 7,142 ng/g dust (Allen et al. 2008; Stapleton et al. 2005; Whitehead et al. 2013). Median dust concentrations in Europe range from 207 ng/g dust (Sweden) to 979.1 ng/g dust (Germany) (Fromme et al. 2014; Newton et al. 2015; Sahlström et al. 2015). The estimated US adult intake dose of PBDEs is 7.7 ng/kg body weight/day, while the estimated intake dose for US children ages 1-5 is 49.3 ng/kg/day (Lorber 2008). The estimated intake dose for US children is higher than that for adults because child-specific behaviors such as crawling and frequent hand-to-mouth contact more than double their ingestion rates of contaminated house dust and thus increases exposure to contaminants in dust (Stapleton et al. 2008a). The median blood concentrations of tetra- to decaBDEs measured in US and Canadian samples ranges from 12 – 43 ng/g lipid (Foster et al. 2011; Mazdai et al. 2003; Rawn et al. 2014). The median blood concentrations of tetra- to decaBDEs measured in European samples ranges from 0.9 – 11.5 ng/g lipid (Fromme et al. 2015). North American tissue
concentrations of pentaBDEs are significantly higher than the rest of the world due to the higher historical use of these commercial mixtures in consumer products that have led to high exposure rates via contaminated house dust in the indoor environment.

In addition to indoor exposure from PBDE-treated consumer products, there are high levels of occupational exposures and large environmental inputs of PBDEs from electronic products dismantling facilities. There is a growing need for electronics waste (e-waste) processing worldwide, and the dominant market for this practice is in southeastern China (Chi et al. 2011). These e-waste dismantling and recycling facilities often use primitive and improper methods for extracting precious metals and other valuable materials during the recycling process. For example, Chinese e-waste recycling facilities employ crude techniques such as open-air burning, melting circuit boards over coal grills, and open-pit acid baths, all performed by workers that do not have access to the necessary personal protective equipment (Wong et al. 2007). These practices lead to very high environmental contamination in the areas surrounding the e-waste facilities and also very high exposures for the employees and nearby inhabitants. For example, the median ΣPBDE serum concentrations of one cohort of individuals from a Chinese e-waste site was 753 ng/g lipid (range: 105-4099 ng/g lipid) (Zheng et al. 2014). Another cohort study measured serum concentrations of the hepta- through decaBDE congeners at 11-20 times that of the reference population and measured 3,436 ng/g lipid of BDE-209 in an e-waste dismantling worker’s serum (Qu et al. 2007). Additional occupational
exposures that are more common in developed countries include carpet installers and foam recyclers, who were found to have serum PBDE concentrations one full order of magnitude higher than the general US population (Stapleton et al. 2008b). It remains important to consider these high exposure populations when evaluating the human health effects of PBDEs.

1.3.2 2,4,6-Tribromophenol

Despite the natural sources of 2,4,6-TBP from marine sources, human exposure to 2,4,6-TBP is thought to occur primarily within the indoor environment via anthropogenic sources from its various consumer product applications. This is because the human tissue concentrations of 2,4,6-TBP is much higher than that of naturally occurring MeO-PBDEs and OH-BDEs, indicating that natural sources represent a small contribution to 2,4,6-TBP exposure in humans (Gao et al. 2015). Other indirect sources of 2,4,6-TBP have also been considered. For example, the abiotic production of 2,4,6-TBP and other bromophenol compounds from degradation of brominated flame retardants (e.g. PBDEs) has been shown to occur following UV irradiation (Bendig and Vetter 2013). This study exposed individual PBDE congeners, as well as the technical commercial mixture, OctaBDE (DE-79), to both simulated and natural sunlight irradiation and observed the production of 0.4 – 4.0% by mass bromophenols. However, the concentrations of 2,4,6-TBP measured in human tissues is comparable to or higher than PBDEs, so bromophenol formation via UV irradiation is most likely not the
dominant source of 2,4,6-TBP for humans (Gao et al. 2015; Leonetti et al. 2016). Further research was conducted to determine if oxidative metabolism of BDE-209 in human microsomes was a potential source of 2,4,6-TBP, given the strong associations observed between these two analytes measured in human tissues (Gao et al. 2015). Following incubation of BDE-209 in human liver microsomes, there was no observed measurement of 2,4,6-TBP or any bromophenol compounds, indicating that BDE-209 is likely not a significant precursor for 2,4,6-TBP production; however, it is not clear whether metabolism may be different in vivo. Future studies should focus on assessing metabolite formation from other PBDE and BFR compounds (ex. tetrabromobisphenol A or BTBPE) as potential sources of 2,4,6-TBP, as well using different in vitro and in vivo models. This same study evaluated the presence of bromophenols in the DecaBDE commercial mixture and did not detect 2,4,6-TBP as a manufacturing byproduct. However, 2,4,6-TBP is used as an intermediate in the production of other flame retardants such as 1,2-bis (2,4,6-tribromophenoxy) ethane (BTBPE) and tetrabromobisphenol A (TBBPA), and it is likely an impurity within these chemical flame retardant solutions (Suzuki et al. 2008). The role of oxidative metabolism in the production of bromophenols and OH-BDEs in human liver microsomes was also investigated using BDE-99 as a substrate (Erratico et al. 2012). Following microsomal incubation, 10 hydroxylated products were produced and identified, including five identified OH-BDEs and 2,4,5-TBP, however, no detection of 2,4,6-TBP was observed. These experiments also confirmed the primary role of
CYP2B6 in the biotransformation of BDE-99. CYP2B6 was also confirmed as the predominant CYP involved in the metabolism of BDE-47 in human liver microsomes, forming six OH-BDEs following incubation (Feo et al. 2013). Additional research using BDE-47, BDE-99, and BDE-153 in non-pooled human liver microsomes showed differences in the extent of each individual’s metabolism, as well as differences in the metabolites formed from each parent compound (Lupton et al. 2009). Briefly, BDE-47 was metabolized to a dihydroxylded BDE-47 and 2,4,-dibromophenol, BDE-99 was metabolized to a dihydroxylated BDE-99, 2,4,5-tribromophenol, and 1,3,- dibromobenzene, and BDE-153 showed no sign of metabolism. Finally, human hepatocytes were exposed to BDE-99 and BDE-209, and the formation of 2,4,5-tribromophenol and two monohydroxylated pentabrominated diphenyl ether metabolites were measured in cells following exposure to BDE-99, while no hydroxylated or debrominated metabolites were measured in cells exposed to BDE-209 (Stapleton et al. 2009). Based on the pattern of bromination, BDE-100, BDE-154, and even BDE-209 could theoretically be suitable parent compounds for the production of a 2,4,6-TBP metabolite; however, the formation of 2,4,6-TBP via oxidative metabolism of PBDEs has yet to be shown. The results from these PBDE metabolic studies indicate that the sources of exposure to 2,4,6-TBP remain to be fully understood and that more research needs to be conducted on the measurement of 2,4,6-TBP, as well as determining the
direct sources of 2,4,6-TBP and the indirect sources via metabolic and/or abiotic degradation pathways.

Only a few studies have examined environmental levels and human exposure to 2,4,6-TBP. 2,4,6-TBP has been measured in marine sediments at an average concentration of 3.02 ng/g dry weight and in riverine systems at 0.66 ng/g dry weight (Sim et al. 2009). 2,4,6-TBP has also been measured in the indoor environment of Japanese homes, with indoor house dust concentrations ranging from 15-30 ng/g and indoor air concentrations between 220-690 pg/m³ (Takigami et al. 2009). Very few biomonitoring studies have included 2,4,6-TBP in the analyses of human tissues such as serum, cord blood, and/or breast milk. One Japanese study collected maternal serum and umbilical cord blood from a cohort of 16 mothers in 2006 for analysis of BFRs and PCBs. This study measured 2,4,6-TBP in maternal blood at a concentration of 22 pg/g wet weight and in cord blood at a concentration of 37 pg/g wet weight (Kawashiro et al. 2008). BFRs were also evaluated in Norwegian individuals working in electronics dismantling facilities, where 2,4,6-TBP was measured in plasma ranging from 0.17 to 81 ng/g lipid (ΣPBDE range: 1.6 – 24 ng/g lipid) (Thomsen et al. 2001). In a study measuring BFRs in a Canadian Inuit population from Nunavik, Quebec, plasma samples contained a geometric mean 2,4,6-TBP concentration of 9.4 µg/kg lipid, however, these concentrations were not correlated with PBDE concentrations (Dallaire et al. 2009). Qiu et al. measured mean 2,4,6-TBP concentrations of 5.6 ng/g lipid in fetal plasma and 0.8 ng/g lipid in maternal plasma
A mean concentration of 5.05 ng/g lipid of 2,4,6-TBP was measured in 47 adipose tissue samples from individuals in New York City, NY (Gao et al. 2015). Finally, a geometric mean concentration of 19.2 ng/g lipid of 2,4,6-TBP was measured in 43 maternal serum samples from a pregnancy cohort in Durham, NC (Butt et al. 2016).

**1.4 Toxicity of BFRs**

**1.4.1 Overview**

PBDEs were widely used for decades before their full safety and human health risks were considered. Initial concerns regarding PBDE exposures began due to their environmental persistence and detection in human adipose tissues and breast milk (Meironyté Guvenius et al. 2001; Meironyté et al. 1999). In 2006, the US EPA released the 2006 PBDE Project Plan, which summarized results from various animal studies using the PBDE commercial mixtures as well as individual congeners. The EPA summary identified concerns for liver toxicity, thyroid toxicity, developmental toxicity, and developmental neurotoxicity (Linares et al. 2015; Meeker et al. 2009; Turyk et al. 2008). Over the past two decades a number of research studies have investigated acute and chronic toxicity of PBDEs in a variety of animal models including mice, rats, American kestrels, zebrafish, and fathead minnows (Chen et al. 2012; Eriksson et al. 2002; Hallgren et al. 2001; Noyes et al. 2013a; Sullivan et al. 2013). The overall results of these animal studies suggest that PBDEs do not exhibit acute toxicity at environmentally relevant concentrations, however, they cause impaired neurodevelopment, decreased thyroid
hormone (TH) concentrations, reproductive deficits, reduced immunological response, and upregulation of various Phase I and II metabolic pathways. Despite the wide variety of biological endpoints impacted by PBDEs, the effects on the thyroid system are the most well studied and documented and will be discussed in detail in the following sections.

1.4.2 Thyroid Hormone Effects

THs play a significant role in the human endocrine system through the regulation of development, growth, neural differentiation, and metabolism (Zoeller et al. 2007). An overview of the hypothalamic-pituitary-thyroid (HPT) axis is provided below.

The primary THs, thyroxine (T4) and triiodothyronine (T3), are produced in the thyroid gland and secreted into systemic circulation in response to an upregulation of thyroid stimulating hormone (TSH). TSH is produced and secreted by the anterior pituitary in response to feedback from circulating TH concentrations, and binds TSH-receptors located in the thyroid follicular cell basolateral membranes (Chiamolera and Wondisford 2009). TSH mediates the uptake of iodide by the sodium-iodide symporter (NIS), as well as other steps necessary for TH synthesis such as iodine organification and the production of the TH precursor molecules, thyroglobulin (Tg) (Cheng et al. 2010). Upon release into the bloodstream, a majority of the THs bind to serum transporters and are circulated throughout the body to facilitate delivery to peripheral tissues.

Approximately 70% of THs are bound to thyroid binding globulin (TBG), 10-15% are
bound to TTR) and 15-20% are bound to albumin, while only 0.3% of T3 is unbound (free) and 0.03% of T4 is unbound (bound THs are in the nM range and free THs are in the pM range in human serum). Free THs are transported into cells via active uptake by various TH transporters, including monocarboxylate transporters (MCTs), L-amino acid transporters (LATs), and organic-anionic transport proteins (OATPs). Once transported into cells, THs undergo a variety of metabolic pathways that can be activating, inactivating, or temporarily inactivating. Notably, approximately 80% of the T3 in peripheral tissues is a product of deiodination from T4 molecules. T3 is considered to be the genomically active hormone due to its high affinity for thyroid hormone receptor alpha (TRα) and thyroid hormone receptor beta (TRβ). TRs are type II nuclear receptors, meaning that they are always located within the cell nucleus regardless of their ligand binding status. TRs heterodimerize with the retinoid X receptor (RXR) within the nucleus, and this heterodimer unit binds to a thyroid response element (TRE) along with a nuclear receptor co-repressor, and this results in the active repression of TRE gene expression. However, upon binding T3, a conformational change occurs resulting in the release of the co-repressor and the promotion of coactivator binding. With T3 bound to the ligand-binding domain of the TR/RXR heterodimer, along with the coactivator, recruitment of polymerase machinery and the initiation of gene transcription begins (Brent 2012). An overview of the HPT axis is shown below in Figure 1.
As described above, there are many different mechanisms of action along the HPT axis that are susceptible to disruption by endocrine disrupting environmental contaminants. Disruptions in circulating TH levels can represent a serious concern for human health, especially for the developing fetus and young children. PBDEs and their hydroxylated metabolites share similarities in their chemical structures such as a
diphenyl ether backbone, and halogenated substituents. As a result of this structural similarity, many studies have focused on the effects of PBDEs on different aspects of thyroid homeostasis, such as effects on serum TH concentrations, expression and function of TH membrane transport proteins, TH serum transport proteins, TH nuclear receptors, and TH metabolizing enzymes such as deiodinase (DI) and sulfotransferase (SULT).

1.4.2.1. Thyroid Hormone Effects in Animal Models

A number of animal studies conducted over the last few decades have investigated the effects of PBDEs on TH homeostasis. The results of these experiments have led to the conclusion that PBDEs disrupt TH homeostasis in vivo and often lead to hypothyroidism in exposed animals. Studies consistently show that there is an observed decrease in the free and bound concentrations of serum T4 and T3 concentrations following PBDE exposure (Bansal et al. 2014; Blanco et al. 2013; Bowers et al. 2015; Ernest et al. 2012; Kuriyama et al. 2007; Lema et al. 2008; Zhou et al. 2001). It has been shown that PBDEs induce an upregulation in the expression and activity of many TH metabolizing proteins such as uridine diphosphate glucuronosyl transferases (UDPGTs), SULTs, and TH membrane transporters, while simultaneously inducing a downregulation in the expression and activity of DIs and TTR. As a result, it is thought that these alterations in the metabolic and clearance capacities of exposed rodents leads to an increase in the elimination of PBDEs and, inadvertently, THs, resulting in a
hypothyroidal state following PBDE exposure (Richardson et al. 2008; Szabo et al. 2009; Zhou et al. 2001).

1.4.2.2 Thyroid Hormone Effects in Humans and Epidemiological Studies

Many epidemiological studies have reported on associations between PBDEs and THs in normal adults, as well as in women during pregnancy. However, many of these results have been inconsistent. For example, in an analysis of 52 healthy adults from Boston, Massachusetts, PBDEs were found to be inversely associated with TT4 but not TT3, free T4, or TSH (Makey et al. 2015). A different study measured 124 serum samples from the general population in Northern China and found that T3 and TSH both showed positive and inverse associations with different PBDE congeners, while FT4 showed no associations with PBDEs (Huang et al. 2014). Finally, in an assessment of 308 adult male sport fish consumers, PBDEs were shown to have a positive associations with T4 and rT3, and an inverse relationship with TT3 and TSH (Turyk et al. 2008).

For pregnancy cohorts, many studies have measured inverse relationships between PBDEs and THs (Abdelouahab et al. 2013; Herbstman et al. 2008; Zhang et al. 2011a), while others have measured positive associations (Roze et al. 2009; Stapleton et al. 2011; Vuong et al. 2015). Additional studies have even measured no associations between PBDEs and THs during pregnancy (Kim et al. 2009; Mazdai et al. 2003; Zhang et al. 2010). A summary of these pregnancy studies is provided below in Table 2.
Table 2: Summary of PBDE and TH Associations from Pregnancy Studies

<table>
<thead>
<tr>
<th>Reference</th>
<th>Study Size</th>
<th>Time of Sampling (weeks of gestation)</th>
<th>PBDEs</th>
<th>TSH</th>
<th>TT4</th>
<th>TT3</th>
<th>FT4</th>
<th>FT3</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>PBDEs</td>
<td>THs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abdelouahab et al., 2013</td>
<td>380</td>
<td>10±3</td>
<td>BDE-47, BDE-99, BDE-100, BDE-153</td>
<td>NS</td>
<td>↓ (ΣPBDEs, BDE-47, -99)</td>
<td>↓ (ΣPBDEs, BDE-47, -99)</td>
<td>↑ (ΣPBDEs, BDE-47, -99)</td>
<td>↑ (ΣPBDEs, BDE-99)</td>
</tr>
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<td></td>
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</tr>
<tr>
<td></td>
<td>260</td>
<td>10±3</td>
<td>Delivery</td>
<td>NS</td>
<td>↓ (BDE-99)</td>
<td>↓ (ΣPBDEs, BDE-47)</td>
<td>↓ (BDE-99)</td>
<td>↓ (ΣPBDEs, BDE-47)</td>
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<tr>
<td></td>
<td>260</td>
<td>10±3</td>
<td>Cord serum</td>
<td>NS</td>
<td>↓ (ΣPBDEs, BDE-47)</td>
<td>NS</td>
<td>↓ (ΣPBDEs, BDE-47)</td>
<td>-</td>
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<tr>
<td></td>
<td>256</td>
<td>16±3</td>
<td>Cord serum</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>↓ (BDE-28)</td>
</tr>
<tr>
<td>Zota et al., 2011</td>
<td>25</td>
<td>19-12</td>
<td>BDE-28, BDE-47, BDE-85, BDE-99, BDE-100, BDE-153, BDE-207</td>
<td>↑ (BDE-85)</td>
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<tr>
<td></td>
<td>270</td>
<td>27.3±3.1</td>
<td>BDE-28, BDE-47, BDE-99, BDE-100, BDE-153</td>
<td>↓</td>
<td>NS</td>
<td>-</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Chevrier et al., 2010</td>
<td></td>
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<tr>
<td></td>
<td>136</td>
<td>&gt;34</td>
<td>BDE-47, BDE-99, BDE-100, BDE-153</td>
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<td>↑ (ΣPBDEs, BDE-47, -99, -100)</td>
<td>↑ (BDE-47)</td>
<td>↑ (ΣPBDEs, BDE-47, -153)</td>
<td>NS</td>
</tr>
<tr>
<td>Study</td>
<td>Sample</td>
<td>No.</td>
<td>Tissue</td>
<td>Concentrations</td>
<td>Significance</td>
<td>t-Value</td>
<td>p-Value</td>
<td>Notes</td>
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<tr>
<td>Roze et al., 2009</td>
<td>51</td>
<td>35</td>
<td>Cord serum</td>
<td>BDE-47, BDE-99, BDE-100, BDE-153</td>
<td>NS</td>
<td>NS</td>
<td>↑ (BDE-47, -99, -100)</td>
<td>NS</td>
</tr>
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<td>Mazdai et al., 2003</td>
<td>9</td>
<td></td>
<td>Delivery</td>
<td>BDE-47, BDE-99, BDE-100, BDE-153</td>
<td>-</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Herbstman et al., 2008</td>
<td>289</td>
<td></td>
<td>Cord serum</td>
<td>BDE-47, BDE-100, BDE-153</td>
<td>NS</td>
<td>↓ (BDE-100)</td>
<td>-</td>
<td>NS</td>
</tr>
<tr>
<td>Kim et al., 2009</td>
<td>108</td>
<td></td>
<td>Cord serum</td>
<td>BDE-28, BDE-47, BDE-99, BDE-100</td>
<td>NS</td>
<td>-</td>
<td>-</td>
<td>NS</td>
</tr>
<tr>
<td>Zhang et al., 2010</td>
<td>50</td>
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<td>Cord serum</td>
<td>ΣPBDEs</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>-</td>
</tr>
<tr>
<td>Kim et al., 2011</td>
<td>90</td>
<td></td>
<td>Cord serum</td>
<td>BDE-28, BDE-47, BDE-99, BDE-100, BDE-153</td>
<td>NS</td>
<td>-</td>
<td>NS</td>
<td>↓ (BDE-28)</td>
</tr>
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</table>
1.4.3 Sulfotransferase Enzymes

1.4.3.1 Roles of Sulfotransferase Enzymes

SULT enzymes are a supergene family of proteins that catalyze the sulfation of numerous substrates, including xenobiotics, drugs, and endogenous compounds such as hormones, bile acids, neurotransmitters, peptides, and lipids (Gamage et al. 2006). Sulfation is an important metabolic reaction that generally acts to increase a substrate’s hydrophilicity to enhance renal and biliary clearance, as well as decrease or otherwise modify its biological activity. SULTs play an important role in detoxification pathways, as well as balancing the conjugated/unconjugated ratios of endogenous compounds such as hormones, lipds, bile acids, peptides, and neurotransmitters. Additionally, sulfation can also bioactivate many procarcinogens such as N-hyrdoxy heterocyclic amines, N-hydroxy arylamines, and hydroxymethyl polycyclic aromatic hydrocarbons (PAHs) into highly reactive electrophiles that are mutagenic and carcinogenic (Falany 1997a; Weinshilboum et al. 1997). In humans, there are three SULT families, SULT1, SUL, and SULT4, with 13 distinct isoforms. SULTs are widely expressed in human tissues and are localized in many tissues that act as portals of entry, such as the intestines and lungs, where they play a significant role in the extrahepatic detoxification and metabolic activation of many chemicals (Gamage et al. 2006). SULTs have broad and overlapping substrate specificities due to their plastic substrate binding sites, however, many isoforms exhibit strict substrate preferences in addition to general binding. SULTs are
globular proteins with highly conserved core catalytic residues, a 3′-phosphoadenosine 5′-phosphosulfate (PAPS) binding site, and a highly variable covered hydrophobic substrate binding pocket (Gamage et al. 2006). Generally, the SULT binding sites are highly plastic and capable of adapting varying binding architectures to accommodate a wide range of substrates. Transfer of the sulfonate group (SO$_3^-$) from the universal sulfonate donor, PAPS, occurs via a single reaction (no intermediate product formation) upon binding of both substrate and PAPS cofactor to their appropriate binding sites (Whittemore et al. 1986). There are two main classes of SULTs: membrane-bound SULTs and cytosolic SULTs. Membrane-bound SULTs are located in the Golgi apparatus of cells and catalyze the sulfation of peptides, proteins, lipids, and glycosaminoglycans (Negish et al. 2001). Cytosolic SULTs are located in the cytoplasm of cells and catalyze the sulfation of xenobiotics, steroid, bile acids, neurotransmitters, and iodothyronines (Falany 1997b). Due to their relevance to this dissertation, this section will focus on the class of cytosolic SULTs and their metabolism of THs.

THs undergo phase II metabolism via conjugation of the phenolic hydroxyl group by UDPGTs and SULTs. Sulfation of THs acts as an inactivation pathway in TH metabolism. For example, T4 is irreversibly sulfated to form T4 sulfate (T4S), which is then further metabolized exclusively by DI enzymes. The sulfation of T4 acts to increase the rate of DIO1-mediated inner ring deiodination (IRD) by approximately 200-fold, forming the inactive metabolite, rT3 sulfate (rT3S), and completely blocking outer ring
deiodination (ORD) reactions (Visser 1994). However, the genomically active TH, T3, can be reversibly sulfated to form T3 sulfate (T3S), which is an inactive conjugate not capable of binding the TH receptor. The rate of IRD is also enhanced for T3S, however, this metabolite can act as an inactive reservoir of T3 that can be converted back into its active form via arylsulfatase (ARS) enzymes in a tissue-specific and developmental stage-dependent manner (Kester et al. 2002b). As a result, SULTs work in concert with DI enzymes in order to maintain localized tissue-specific TH homeostasis. Of the 13 different SULT isozymes expressed in humans, eight have been shown to perform TH sulfation in a wide variety of peripheral tissues: SULT1A1, SULT1A3, SULT1A5, SULT1B1, SULT1B2, SULT1C1, SULT1E1, and SULA1 (Kester et al. 1999; Wu et al. 2005). There is a general substrate preference for 3,3'-T2, however, the biologically relevant sulfation reactions involve T4 and T3 (Wu et al. 2005). Of interest to note, there is a high degree of inter-individual variability in isoform expression and activity levels among humans (Hildebrandt et al. 2007; Lindsay et al. 2008). For example, there is a common polymorphism in the SULT1A1 gene that results in a single amino acid substitution and a corresponding decrease in enzyme activity and expression levels (Jones et al. 1993; Raftogianis et al. 1997). This polymorphism leads to the production of the allozyme, SULT1A1*2, and individuals who are homozygous for this allele express approximately 15% of the SULT1A1 activity compared to wild type homozygotes (Raftogianis et al. 1997). This polymorphism in SULT1A1 also leads to significantly lower thermal stability
of the enzyme, and coupled with the lower rates sulfation activity, contributes to the interindividual variation in drug and toxicant metabolism by SULTs. Additionally, it is also thought that environmental factors such as cigarette smoke and other environmental stressors will contribute to the observed variability in SULT enzyme expression and activity in a manner similar to CYPs (Boccia et al. 2015).

SULT enzymes are expressed within the placenta during pregnancy and contribute to the maintenance of TH homeostasis within the tissue prior to delivery to the fetal compartment. The expression of SULT1A1 and SULT1A3 within the human placenta have been confirmed and measured as early as 13 weeks gestation (Stanley et al. 2001). This study also confirmed SULT1A1 as the primary isoform responsible for catalyzing the sulfation of 3,3’-T2, and showed that the human placenta has a very low capacity for the sulfation of T4, T3, and rT3, suggesting that the placenta is not the primary source of TH sulfoconjugates within the fetal compartment.

In addition to the placenta, SULTs are widely expressed within the developing fetus. During gestation, the fetus is continuously exposed to a broad variety of chemicals with biological, pharmacological, and toxicological properties, either from maternal transfer via the placenta or produced by the fetus itself. The fetus lacks the complete set of detoxification enzymes and mechanisms that are present in adults, and thus, is susceptible to increased risk of chemical insult and toxicity. For example, Phase I and Phase II metabolic enzymes such as cytochrome P450s (CYPs) and UDPGTs are absent
or expressed at very low levels during fetal development (Hines and McCarver 2002; McCarver and Hines 2002). However, the fetus expresses high levels of SULT enzymes throughout gestation and sulfation serves as the major detoxification pathway that protects the fetus from chemical exposures. The expression of SULT proteins and mRNAs, as well as SULT enzyme activities were measured in fetal tissues between 14 and 18 weeks gestation in a study by Stanley et al. This study showed that SULTs are expressed in human fetal tissues at levels equivalent to or higher than adults, supporting the hypothesis that sulfation is the primary detoxification pathway in human fetuses. For example, SULT1A1 was measured in all fetal tissues examined in this study (lung, liver, adrenal, kidney, small bowel, and thyroid gland), with the liver being the primary site of expression, at levels equivalent to adult hepatic SULT1A1 expression. Additionally, SULT1E1 which has a high affinity for estrogens, was measured in fetal tissues at higher levels of expression than in adult tissues. SULT1E1 was expressed highest in fetal kidney and brain (choroid plexus). Since SULT1E1 also metabolizes THs, and THs play a prominent role in fetal neurodevelopment, this isoform is likely important in modulating TH bioavailability within the developing fetal brain (Stanley et al. 2005). Additionally, the choroid plexus is the most highly vascularized tissue in the developing brain and since it serves as a potential portal of entry for circulating toxins, most likely of maternal origin, the high expression of SULT enzymes present in this tissue can provide a high degree of chemical detoxification as a defense (Richard et al.
Furthermore, the expression and activity of various SULT isoforms, as well as ARS enzymes has been examined in fetal tissues. Richard et al. measured similar SULT isoform tissue expression levels and patterns as previous studies and also confirmed the role of SULT1A1 in the sulfation of THs, specifically 3,3′-T2 using human fetal tissues (10-22 weeks gestation) (Richard et al. 2001). One novel finding from this study was that arylsulfatase C (ARSC) was expressed in fetal liver, lung, and brain tissues and exhibited significant TH sulfatase activity (using 3,3′-T2 sulfate as a substrate). There are very high levels of sulfated THs (T4S, T3S, and rT3S) present in fetal circulation, which potentially serve as a large pool of inactivated THs that can be reactivated to their parent compounds via hydrolysis by ARS enzymes (Hume et al. 2004).

Overall, the actions of SULTs and ARSs within the developing human placenta and fetus are complex and highly regulated, with tissue-specific and developmental time point sensitive expression and activity patterns that contribute to maintaining TH homeostasis and bioavailability, as well as protecting the fetus from the toxic effects of endogenous and xenobiotic compounds.

1.4.3.2 Effects of Environmental Contaminants on Sulfotransferase Enzymes

The inhibition of SULT enzymes is of great interest due to the ubiquitous expression of these important Phase II metabolic proteins. As described above, SULTs play a critical role in the detoxification of xenobiotics, as well as in their metabolism, whether it be bioactivation or inactivation of endobiotics such as hormones and neurotransmitters.
Sulfation is an important conjugation pathway in human adults, however, the role of sulfation in the developing fetus is highly significant due to the dominant role of SULTs in fetal Phase II metabolism. As a result, the inhibitory effects of various chemicals on the enzyme activity of different SULT enzymes has been widely researched.

The ability of a chemical compound to bind to and/or interact with the PAPS binding region or substrate binding region of a SULT enzyme will lead to a degree of inhibition within that enzyme. A huge variety of chemicals are capable of interacting with SULT enzymes due to the plasticity of SULT substrate-binding pockets and their ability to accommodate different binding architectures.

There are many dietary chemicals that act as potent inhibitors of SULTs. Some of these chemicals include cyaniding 3-rutinoside (anthocyanin found in red wine), (+)-catechin (flavonal found in red wine), carmoisine (synthetic food colorant), quercetin (flavonoid in vegetables, fruit, and wine), diadzein (phytoestrogen), and genistein (phystoestrogen) (De Santi et al. 2002; Ebmeier and Anderson 2004; Gibb et al. 1987). Some of these chemicals may potentially act on SULTs following dietary exposure. For example, children who often consume food items that contain high amounts of artificial food colorings or infants that are nursed on soy-based infant formula may experience inhibitory effects on SULT activity following ingestion (Wang and James 2006).

In addition to naturally occurring plant compounds and common food additive chemicals, there are many environmental chemicals that are known SULT inhibitors. The
first class of environmental contaminants shown to inhibit SULT enzymes were polychlorinated biphenyls (PCBs) and their hydroxlyated metabolites (OH-PCBs) (Schuur et al. 1998a, 1998c). PCBs were widely used in the US as dielectric and coolant fluids in various electrical applications; however, due to their toxicity and persistence, they were banned by the US Congress in 1979 (Grimm et al. 2015). Multiple OH-PCB congeners were found to be potent inhibitors of human SULT1E1 activity with sub-nM half maximal inhibitory concentration (IC$_{50}$) values (Kester et al. 2000). Next, many hydroxylated metabolites of polyhalogenated aromatic hydrocarbons (PHAHs), polychlorinated dibenzo-p-dioxins (PCDDs), and polychlorinated dibenzo-furans (PCDFs) were found to be potent SULT inhibitors in a variety of assays. One study looked at the effects of OH-PCDDs and OH-PCDFs on 3,3′-T2 sulfation activities in rats, and measured IC$_{50}$ values in the low micromolar range (0.2 – 3.0 µM) (Schuur et al. 1998b). The commonly used wood treating fungicide and disinfectant, pentachlorophenol (PCP), has also been shown to be a potent inhibitor of phenolic SULT activity in vivo using a rat model (Koster et al. 1979). The commonly used antimicrobial agent, triclosan, has also been shown to inhibit 3,3′-T2 SULT activity in rat liver cytosol with an IC$_{50}$ value of 3.1 µM (Schuur et al. 1998b).

A comprehensive evaluation of the inhibition of TH SULT activity by BFRs and halogenated phenolics was previously conducted using pooled human liver cytosol and 3,3′-T2 as the substrate (Butt and Stapleton 2013). In this study, 14 OH-BDEs, BDE-47,
triclosan, and the brominated, fluorinated, and chlorinated analogues of 2,4,6-trihalogenated phenols and bisphenol A (BPA) were analyzed for their inhibitory effects. Inhibition of TH SULT activity was observed for all analytes tested except for BDE-47. The 2,4,6-trihalogenated phenols were the most potent inhibitors of SULT activity, followed by the OH-BDEs and halogenated BPAs. The measured IC$_{50}$ value for 2,4,6-TBP was 8.3 nM, while the OH-BDEs were between 24-640 nM. The presence of an –OH group appears to be necessary for TH SULT inhibition as reflected by the fact that BDE-47 did not inhibit SULT activity, while all of the hydroxylated versions of BDE-47 did cause inhibition. These results are similar to that of previous studies which did not measure any SULT inhibition from PCBs, but did show potent inhibition from OH-PCBs (Schuur et al. 1998a). Finally, BDE-47 and BDE-99 were shown to have SULT inhibitory effects only after incubation with CYP-enriched microsomes (Schuur et al. 1998b). The oxidative metabolism of the PBDEs by the CYP enzymes present in microsomes leads to the formation of OH-BDE metabolites, and these compounds are potent inhibitors of SULTs due to their favorable binding affinities with SULT substrate-binding pockets.

Overall, it has been shown that SULTs are very sensitive to enzyme inhibition by a wide variety of chemicals, including many common environmental contaminants and their metabolites. Specific to TH metabolism, SULTs are approximately an order of magnitude more sensitive to inhibition than DI enzymes, and greater emphasis should
be placed on studying the downstream effects of SULT inhibition *in vivo* (Butt et al. 2011).

### 1.4.4 Deiodinase Enzymes

#### 1.4.4.1 Role of Deiodinase Enzymes

Iodothyronine deiodinases are selenoproteins that contain a single selenocysteine residue in the core catalytic center (Kuiper et al. 2005). There are three main DI isoforms known as type 1, type 2, and type 3 (DIO1, DIO2, and DIO3) that all vary in their tissue localization as well as their relative catalytic capacities. DI enzymes catalyze the reductive deiodination of THs via IRD and/or ORD. IRD is an inactivating pathway for THs, catalyzing the biotransformation of T4 to rT3 and T3 to 3,3'-T2. ORD is an activating pathway for THs, catalyzing the biotransformation of T4 to T3. Therefore, DI enzymes play a critical role in the metabolism and regulation of TH concentrations in peripheral tissues. Specifically, within the placenta tissue, DIO3 is the predominant isoform expressed throughout gestation, although there is a small amount of DIO2 expression and activity as well. DIO3 is thought to play an important role in the regulation of maternally-derived THs within the placenta tissue. For example, the inactivating pathway mediated by DIO3 is thought to effectively buffer the amount of THs available for transport to the fetal circulatory compartment. Additionally, the DI enzyme activity within the placenta is also thought to provide a source of iodide to the fetal compartment to facilitate fetal TH synthesis during pregnancy. Total DIO3 activity
increases during gestation, however, DIO2 activity does not and remains approximately 200 times lower than DIO3 activity (Koopdonk-Kool et al. 1996).

### 1.4.4.2 Effects of Environmental Contaminants on Deiodinase Enzymes

The inhibition of DI activity by environmental contaminants is of great interest due to the important role these enzymes play in the bioactivation and inactivation of THs in peripheral tissues. A comprehensive *in vitro* analysis of the iodotyrosine DI inhibitory effects of 44 halogenated compounds was previously conducted using microsomes of HEK-293 T cells (Shimizu et al. 2013). Seventeen PCB compounds were tested and only five compounds showed DI enzyme inhibition, with 4-OH-2’,3’,4’,5,6’-PCB exhibiting the most potent inhibition with an IC$_{20}$ value of 17 µM. Fifteen PBDE compounds were tested and only six compounds showed DI enzyme inhibition, with 4’-OH-BDE-47 and 4-OH-BDE-42 exhibiting the most potent inhibition with an IC$_{20}$ value of 16 µM. None of the PBDE parent compounds or methoxylated compounds showed any effects on DI activity. Finally, a variety of agrichemicals, antiparasitics, pharmaceuticals and food colorants were tested, and the food colorants exhibited the most potent inhibition with IC$_{20}$ values between 0.2 – 0.8 µM. Overall, it appears that the structural requirements for DI enzyme inhibition include the presence of a halogen atom and a hydroxyl group substitution on a phenyl ring.

The *in vitro* inhibition of DI activity by several halogenated phenolic contaminants was also previously evaluated using pooled human liver microsomes.
(Butt et al. 2011). 5’-OH BDE-99 was the most potent inhibitor of ORD with an IC₅₀ value of 0.4 µM, followed by tetrabromobisphenol A (TBBPA) with an IC₅₀ value of 2.1 µM. Amongst the trihalogenated phenols, 2,4,6-TBP was the second most potent inhibitor of ORD with an IC₅₀ value of 40 µM. Finally, there was no inhibitory effect observed for BDE-99 or any of the other OH-BDEs. These in vitro studies confirm that some halogenated phenolics are capable of disrupting DI enzyme activity in the low µM range, and future work should determine the downstream effects of DI inhibition in vivo.

1.4.4.3 In Vitro Studies

The endocrine-disrupting potency of BFRs has been extensively studied, with special attention given to TH-related endpoints. In vitro binding and transactivation assays with TRα and TRβ have been used to evaluate the binding affinity and receptor activation potential of BFRs in an isoform-specific manner. For example, 4-OH BDE-47 was found to be a TRα agonist but unable to bind TRβ in a reporter gene assay, whereas BDE-206 was found to be an antagonist for both receptor isoforms (Schriks et al. 2007). This study highlights the importance of evaluating individual compounds for their receptor binding and activation characteristics using both TR types, and further demonstrates the complexity of in vivo exposures in which multiple chemical exposures will have different biological activities that lead to unpredictable net effects. The TR binding of OH-BDEs is much greater than that of the parent compounds due to the presence of the hydroxyl group that increases the structural similarity of PBDEs to THs.
However, PBDEs can exert indirect effects on TRs. For example, the effects of BDE-47 on the human pregnane X receptor (PXR) were evaluated in HepG2 cell model. The PXR is a nuclear transcription factor that can be activated by a wide variety of chemicals and controls xenobiotic-metabolizing enzymes such as CYPs, SULTs, and UDPGTs. This study showed the BDE-47 was a strong PXR agonist that led to the downregulation of TRα and TRβ mRNA and protein expression, and the upregulation CYPs, SULTs, and UPDGT expression (Hu et al. 2014).

In addition to TR binding, BFRs are capable of disrupting TH homeostasis at the level of hormone transport. THs are transported in the blood by TTR, TBG, and albumin, and the ability of environmental contaminants to competitively bind these transporters may lead to a disruption of TH transport and delivery. Multiple studies have profiled the binding affinities of PBDEs and OH-BDEs with TTR and TBG. These studies are consistent in showing that OH-BDEs are more potent competitors of TH binding proteins than PBDEs, and that some congeners have greater binding affinities than the endogenous ligands, T4 and T3 (Hamers et al. 2006; Harju et al. 2007; Ren et al. 2013; Yang et al. 2011). For example, 3-OH BDE-47, 4-OH BDE-47, and 4’-OH BDE-47 were found to have a TTR-binding relative potency values of 4.0, 3.5, and 3.5, respectively (Hamers et al. 2008). This indicates that these OH-BDEs have a greater binding affinity for TTR than the endogenous ligand, T4. Interestingly, BDE-47 and 6-OH BDE-47 had relative potency values of 0.0025 and 0.39, indicating that both the presence of a
hydroxyl group, as well as its positioning on the phenolic ring are important determinants for TTR-binding affinities.

1.4.5 Neurodevelopmental Effects of BFRs

The neurodevelopmental toxicity of a wide range of chemicals have been studied extensively over the years. Some prominent examples include lead, methylmercury, arsenic, chlorpyrifos, and dichlorodiphenyltrichloroethane (DDT), all of which have been directly implicated as developmental neurotoxicants. In addition to these more widely studied chemicals, PBDEs and their hydroxylated metabolites have seen growing evidence of neurodevelopmental toxicity over the years. Through experimental studies in animals and epidemiological observations in human study cohorts, the link between PBDEs and adverse neurodevelopmental outcomes continues to strengthen. However, the exact mechanisms of action underlying these neurotoxic effects are not known at this time. There are two main, and not mutually exclusive, hypotheses concerning the mode of action of PBDE-mediated neurotoxicity. First, an indirect mode of action in which PBDEs impair TH homeostasis through multiple points of action along the HPT axis. Secondly, a direct effect of PBDEs on nervous system cells, such as oxidative stress-related damage via DNA damage, mitochondrial dysfunction, apoptosis, interference with calcium signal transduction, and inhibition/disruption of neurotransmitter systems (Costa et al. 2013). The indirect effects of PBDEs on the TH system, and the various
effects observed in animal studies, *in vitro* studies, and human epidemiological studies will be summarized below.

1.4.5.1 Animal Exposure Studies

There have been many studies performed using rodent animal model systems to evaluate the neurotoxic effects of PBDEs. The standard for PBDE dosing in mice was established in a 2001 study from Eriksson et al., in which mice were exposed to a single oral dose of BDE-47 and BDE-99 on postnatal day 10 (PND 10). Following exposure, a series of dose-dependent effects on spontaneous behavior, including locomotion, rearing, and total activity, as well as in re/learning abilities and habituation capacity of two- and four-month old mice was observed (Eriksson et al. 2002). Similar and supporting results have been observed in subsequent mouse studies using BDE-153, BDE-183, BDE-203, BDE-206, and BDE-209 (Johansson et al. 2008; Viberg et al. 2003, 2006). These studies strongly suggest that PBDEs cause adverse neurobehavioral effects following early-life exposure, and that PBDEs can alter the physiology of the developing brain, leading to permanent later-life effects on behavior and cognition in rodents.

In addition to rodent studies, some research has focused on neurodevelopmental effects of PBDEs in aquatic species. For example, adult zebrafish were exposed via water to environmentally relevant concentrations of DE-71 (the PentaBDE commercial mixture), and effects were measured in the F1 larvae. Various genes related to central nervous system development (myelin basic protein, synapsin IIa, and α1-tubulin) were
found to be significantly downregulated in the larvae and some of the protein expression levels of these genes were also found to be reduced. Additionally, a significant decrease in total locomotor activity was observed in the F1 larvae, providing evidence for neurodevelopmental toxicity in zebrafish following prenatal exposures (Chen et al. 2012). Another recent study exposed zebrafish to various concentrations of DE-71 (0-100 µg/L) and measured the effects of exposure on the dopaminergic system in F1 larvae. This study measured reductions in whole-body content of dopamine, a significant decrease in the expression of genes necessary for dopaminergic neuron development, and a decrease in larval locomotor activity (Wang et al. 2015). These two studies provide valuable insight into the prenatal effects of PBDE exposure in zebrafish; however, their use of DE-71 as a dosing agent may complicate their results because the commercial mixtures contain trace concentrations of brominated dioxins and furans (Hanari et al. 2006). However, one study evaluated the effects of dietary BDE-209 exposure in Fathead minnows and found that 28 day exposures led to decreases in TT4, TT3, and brain DI activity, and induced increases in mRNA expression of DI, TRs, and membrane transporters in the brain and liver (Noyes et al. 2013b). The effects of OH-BDEs has also been evaluated in zebrafish, where 6-OH BDE-47 was shown to impact activity levels, habituation, and fear responses following embryonic and larval exposure (Macaulay et al. 2015a). Furthermore, 6-OH BDE-47 exposure was also shown to be a developmental toxicant by inducing altered developmental morphology such as
decreases in head trunk angle, increases in otic vesicle length, and decreases in eye pigmentation (Macaulay et al. 2015b).

1.4.5.2 Human Epidemiological Studies

There are numerous human epidemiological studies that have examined the associations between maternal and cord blood PBDE concentrations and various neurodevelopmental outcomes in children. As expected, these results are often difficult to interpret and are further complicated by confounding variables such as interindividual variations, the subtlety of such endpoints, and concomitant exposure to other chemicals. Despite these challenges in epidemiology, the large majority of studies have observed associations between PBDEs and adverse neurodevelopmental outcomes in children.

The majority of studies utilize various standardized developmental tests for assessing children between 1-9 years of age. The results of these tests can be grouped based on the neurobehavorial endpoint and have been combined and organized here for summary purposes. PBDEs have been shown to be significantly associated with impaired fine motor coordination (Eskenazi et al. 2013; Gascon et al. 2011), impaired attention (Cowell et al. 2015; Eskenazi et al. 2013; Gascon et al. 2012; Roze et al. 2009; Sagiv et al. 2015), decreased full-scale IQ (Chen et al. 2014a; Eskenazi et al. 2013; Herbstman et al. 2010), impaired psychomotor development (Herbstman et al. 2010), and decreased adaptive behavior (Shy et al. 2011).
In addition to using early-life behavioral and cognitive evaluations as an endpoint for neurodevelopmental effects, physical parameters, such as head circumference, have been used as a neurodevelopmental measure in pregnancy cohort studies. For example, in a pregnancy cohort of predominantly African American women from Durham, NC, maternal serum PBDE concentrations, specifically BDE-153, were associated with an average decrease in head circumference of 0.32 cm (Miranda et al. 2015). Similar results were found in a Spanish study that measured PBDE concentrations in maternal serum and cord blood (670 mothers; 2003-2008 collection) and found inverse associations between fetal abdominal circumference, estimated fetal weight, biparietal diameter at weeks 20-34 (measurements made by ultrasound), and with head circumference at birth (Lopez-Espinosa et al. 2015). Finally, in a prospective birth cohort of 215 mothers from rural China (2010-2012 collection) serum PBDE concentrations were inversely associated with birth length and birth weight in males. Overall, there is growing evidence suggesting effects of PBDEs on birth outcomes, in addition to effects on neurodevelopment. A summary of recent PBDE and birth outcomes studies is provide below in Table 3.
Table 3: Summary of Birth Outcome Results Related to PBDE Tissue Concentrations (all associations shown found to be statistically significant).

<table>
<thead>
<tr>
<th>Study Dates</th>
<th>Tissue Measured</th>
<th>Birth Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maternal Serum</td>
<td>Cord Blood</td>
</tr>
<tr>
<td>2008-2010</td>
<td>140; 3rd Trimester</td>
<td>140; Birth</td>
</tr>
<tr>
<td>2003-2008</td>
<td>670; Week 12</td>
<td>534; Birth</td>
</tr>
<tr>
<td>2010-2012</td>
<td>215</td>
<td>-</td>
</tr>
<tr>
<td>2012</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2005-2009</td>
<td>234 couples (includes paternal serum)</td>
<td>-</td>
</tr>
<tr>
<td>1999-2000</td>
<td>286; Week 26</td>
<td>-</td>
</tr>
</tbody>
</table>
1.5 The Role of the Placenta

The placenta is a highly specialized organ that facilitates the transport of nutrients, gases, and waste products between the maternal and fetal compartments throughout pregnancy. Normal human placental developments begins immediately after conception and occurs via uterine implantation followed by extensive proliferation by placental trophoblast cells (Huppertz 2008). By five days post conception, the blastocyst consists of an inner cell mass (future embryo) that is surrounded by a single layer of trophoblasts. This outer layer of trophoblasts are the first cell type to differentiate following fertilization and later develop into the placenta organ. The inner cell mass positions itself within the blastocyst such that the trophoblasts directly in line with the inner cell mass, known as the polar trophoblasts, initiate implantation and attachment to the uterine epithelium. Immediately following attachment, the polar trophoblasts begin differentiating into multinucleated syncytiotrophoblasts which are responsible for initiating the invasive phenotype of the placenta. Post-conception days eight through 12 consist of further placental invasion and the finalizing of the blastocyst implantation with the embryo becoming completely embedded within the endometrium and the establishment of the chorionic plate and villous tree network. The cytotrophoblasts are mononucleated cells responsible for maintaining the syncytiotrophoblast cell populations. The apical side of cytotrophoblasts are always in contact with syncytiotrophoblasts and never in direct contact with maternal circulation.
The syncytiotrophoblast is an apical, maternal-facing membrane with a basal membrane that is always in contact with cytotrophoblasts. Syncytiotrophoblasts compose a single layer around the placenta unit and make up the apical surface of the villous trees. These cells are much larger, have less cellular organelles, are multinucleated, and do not show any proliferative activity. As a result, syncytiotrophoblasts rely solely on the underlying cytотrophoblast layer for cellular maintenance and proliferation. Following establishment of the villous trees, the placenta continues to increase in size during gestation to an average full-term diameter of 22 cm, central thickness of 2.5 cm, and average weight of 270 g (Huppertz 2008).

After the initial implantation steps and throughout the first trimester of pregnancy, the placenta is primarily composed of unicellular, mononucleated cytотrophoblast cells. By the end of the first trimester, many of these cytотrophoblasts have differentiated into the larger multinucleated syncytiotrophoblasts. At this point in gestation, the placenta forms a hemochorial plate which acts as the direct interface between maternal and fetal circulation. The apical (maternal-facing) side of the placenta is composed of a dense bed of chorionic villa that are composed of mainly syncytiotrophoblasts. These villi are in direct contact with maternal blood and create a tight barrier between the circulatory compartments, with the syncytiotrophoblasts controlling the majority of transcellular delivery (Schneider 1991). Throughout gestation,
the cellular composition of the apical and basal membranes of the placenta changes, and these shifts in cellular morphology may lead to differences in placental transport.

While the placenta plays many important roles in fetal development and materno-fetal transport, its role in transporting THs to the fetal compartment is very significant, especially during the first trimester of pregnancy. The fetal thyroid gland is the first endocrine organ to develop (5-6 weeks gestation), but does not begin synthesizing THs until 14-16 weeks gestation, with significant production not beginning until 20 weeks gestation (Burrow et al. 1994). As a result, the fetus has no endogenous source of THs for the first trimester of pregnancy and must rely solely upon maternally-derived THs, primarily T4, during this time period. Following the onset of fetal TH production, the contribution of maternal T4 to the fetus declines to 40% and the fetal thyroid gland produces the remaining 60% of THs (Chan et al. 2009).

Beginning at conception, maternal production of THs increases by 50% and dietary iodide requirements increases from 100-150 µg/day to 250-300 µg/day (Moleti et al. 2014). The more than doubling of daily dietary iodide requirements in mothers is the result of three main events that occur during pregnancy. First, there is an increased need for iodide for the increased synthesis of THs by the maternal thyroid gland. Iodide is an essential micronutrient used in the production of monoiodotyrosine (MIT) and diiodotyrosine (DIT), the amino acid derivative precursors to T4 and T3. There is no other known function or use of iodine in humans, and due to the 50% increase in the TH
synthesis during pregnancy, there is an increased demand for dietary iodide in order to maintain TH production for delivery to the fetus. Second, the renal clearance of iodide increases significantly during pregnancy as a result of increased renal blood flow and glomerular filtration (Liberman et al. 1998). Finally, maternal iodine is transferred to the fetus during pregnancy to facilitate fetal TH production beginning at 14 weeks. This transport is facilitated by the expression of sodium iodide symporters (NIS), which are upregulated within the syncytiotrophoblasts by increases in human chorionic gonadotropin (hCG) levels. Iodide is then transported and stored in the placental unit during the first trimester as a reservoir for the fetus when it begins endogenous TH production (Burns et al. 2011).

The transplacental passage of THs from mother to fetus in humans has long been known to be important to normal fetoplacental development. Many studies have highlighted the importance of a healthy maternal TH status by showing associations between abnormal maternal thyroid function and later-life neurodevelopmental effects in offspring (Haddow et al. 1999; Li et al. 2010; Pop et al. 2003). The results of these studies has led to the understanding that transplacental passage of maternal THs is critical to normal human fetal central nervous system development, especially during the first trimester of pregnancy. Additionally, the importance of early gestation transplacental transport of THs is underlined by the presence of THs within the fetal compartment, as well as in fetal tissues before the onset of endogenous fetal TH
production. For example, T4 has been measured in the exocoelomic cavity beginning at five weeks gestation. T4 and T3 have been measured in fetal limbs, brain, and liver between 6-12 weeks gestation, and finally, the fetal cerebral cortex expresses functional TH transporters, receptors, and DI enzymes beginning at seven weeks gestation (Calvo et al. 2002; Chan et al. 2002, 2011; Costa et al. 1991). Not only are maternally-derived THs present within the fetal compartment, but the fetus, specifically the fetal brain, contains the necessary molecular systems to transport, metabolize, and respond to THs.

Maternal THs are transported to the fetus by way of a complex network of membrane transporters, TH-metabolizing enzymes, and binding proteins. First, numerous studies have reported on the transport of THs by three main classes of TH-transporting proteins (THTPs): MCTs, LATs, and OATPs (Fujiwara et al. 2001; Visser et al. 2008). The expression of THTPs was investigated by Loubiere, et al. using the apical microvillous plasma membrane (MVM) of human term syncytiotrophoblasts. This study identified the expression of various THTPs including LAT1, MCT8, MCT10, OATP1A2, and OATP4A1. This study further went on to show that these THTPs are capable of rapid, saturable T4 and T3 uptake, and that no single transporter plays a dominant role in TH uptake (Loubière et al. 2012). The Michaelis constant values (K_m) for T4 and T3 are between 0.8 and 8 µM, for these transporters, while the K_m values for the other endogenous competing molecules that use the same transporters are between 10- and 100- fold greater than for THs, meaning that THs can outcompete other molecules for
transport within the MVM. (Friesema et al. 2001, 2003, 2008). In addition to membrane-bound TH transport proteins, the delivery of maternal THs to the fetal compartment can occur by way of TTR. TTR is a homotetrameric protein that is synthesized and secreted by the liver, choroid plexus, yolk sac, intestine, and placenta (Gitlin and Perricelli 1970; Herbert et al. 1986; Jacobsson 1989; Loughna et al. 1995; McKinnon et al. 2005). The primary function of TTR is to transport and ensure equal distribution of TH and retinol throughout the body and brain. TTR is one of three TH binding proteins that circulate in the blood, the others being TBG and albumin. TBG has the highest affinity for T4 with an affinity constant (K_a) of 1.0 x 10^{10} M^{-1}, followed by TTR with a K_a of 7.0 x 10^{7} M^{-1}, and albumin with a K_a of 7.0 x 10^{3} M^{-1}. Together, TH binding proteins maintain serum concentrations of free and bound THs and ensure that only 0.03% of circulating T4 and 0.3% of T3 are free/unbound (Refetoff et al. 1970). Within systemic circulation, TBG carries 65% of circulating T4 and TTR carries 15% of T4 (Schreiber 2002). However, TTR plays a significant role in TH transport within the placenta. The synthesis and expression of TTR by trophoblasts was not recognized until a 2005 study that measured TTR mRNA and protein expression in 38-40 week old human placenta (McKinnon 2005). Following this discovery, numerous follow-up studies have examined the synthesis and role of TTR within the human placenta. Notably, it has been determined that TTR is synthesized in placental syncytiotrophoblasts and secreted into maternal circulation. This creates a locally high concentration of TTR at the materno-placental interface that
binds maternally-derived T4 which can then be selectively taken back up by trophoblasts and delivered to the fetal compartment (Mortimer et al. 2012). In effect, this creates a TTR shuttling system for TH uptake and transport. Additionally, TTR may play a role in TH transport within the placenta by acting as a delivery shuttle to other TH membrane transport proteins, such as those discussed above. Once translocated from maternal circulation into placental tissues, TTR-bound T4 may be protected by catabolic deiodination by DIO3 and/or conjugation by UDPGTs/SULTs, allowing for the delivery of intact T4 to the fetal circulatory compartment. Finally, TTR could also play a role in the transport of other lipophilic environmental contaminants such as halogenated flame retardants, especially those molecules with chemical structures similar to that of THs such as hydroxylated PBDEs. Binding of these chemicals to TTR may lead to their transport from maternal to fetal circulation, especially if they have higher affinity constants than T4 and can compete for TTR binding.

An overview of potential DI and SULT disruption within the placenta is shown below in Figure 2.
1.6 Thesis Research Aims

The objective of this thesis research is to assess the effects of PBDEs, OH-BDEs, and 2,4,6-TBP on TH regulation within the placenta, using human tissue subsamples and a placenta cell line. Given that BFRs have been measured in human placenta tissues and that these chemicals have been shown to disrupt TH homeostasis, there exists the possibility that BFRs can disrupt TH homeostasis within the placenta during pregnancy. The disruption of TH metabolism or transport within the placenta during pregnancy can potentially lead to a disruption in the concentration of THs that are delivered to the fetal compartment. Since THs are critically important to fetal development, specifically early neurodevelopment, any perturbations in TH homeostasis within the fetal compartment
can disrupt TH-mediated processes and lead to later-life effects. The investigation of TH regulation within the human placenta, as well as within a placenta cell culture model system, is critical to improving our understanding of this highly vulnerable window of exposure for the developing fetus.

The main hypothesis of this research is that PBDEs and OH-BDE metabolites disrupt TH metabolism in placental tissues by disrupting TH SULT activity. The following research aims were conducted to test this hypothesis:

**Aim 1: Measure PBDEs, 2,4,6-TBP, as well as thyroid hormones (T4, T3, and rT3) in human placenta tissue and examine their associations [Chapter 2].** Placenta tissue subsamples (n=102) from an existing pregnancy cohort were analyzed for PBDEs, 2,4,6-TBP, T4, T3, and rT3 concentrations. Analytical and extraction methods were developed for BFR and TH analytes. BFRs and THs were measured in all samples and 2,4,6-TBP was found to be associated with all PBDE congeners. Additionally, this is the first study to measure 2,4,6-TBP in human placenta tissue.

**Aim 2: Measure endogenous thyroid hormone SULT and DIO3 activity in human placenta samples and determine if they are associated with flame retardant levels and thyroid hormones measured in placental tissues [Chapter 3].** SULT and DI enzyme activities were measured and quantified using methods developed in our laboratory. These endogenous enzyme activities were compared to BFR and TH analyte
concentrations measured in Aim 1. Significant associations were observed for BFRs, THs, and DIO3/SULT activity and were found to be dependent on the sex of the infant.

**Aim 3: Evaluate the effects of flame retardant chemicals on thyroid hormone SULT activity, expression of genes involved in thyroid hormone metabolism, and thyroid hormone concentrations in a cultured human choriocarcinoma placental cell line (BeWo) [Chapter 4].** Cultured human placental cells were exposed (1, 6, 12, and 24 h) to BDE-47, BDE-99, 3-OH BDE-47, 6-OH BDE-47, and 2,4,6-TBP to determine the effects on 3,3’-T2 SULT activity. Additional experiments were performed to further investigate the mechanisms of action within the exposed cells. For example, Michaelis-Menten parameters and inhibition profiles were determined using BeWo cell homogenates. 2,4,6-TBP was shown to be the most potent inhibitor of 3,3’-T2 SULT activity in BeWo cells.

**Chapter 5** summarizes the major findings of this thesis research and integrates the discussion of these findings with current research on BFRs and TH metabolism within the placenta during pregnancy. Additionally, the challenges and limitations of this research will be discussed in detail.
2. Concentrations of Polybrominated Diphenyl Ethers (PBDEs) and 2,4,6-Tribromophenol in Human Placenta Tissues

This chapter was published under the title “Concentrations of polybrominated diphenyl ethers (PBDEs) and 2,4,6-TBP in human placental tissues” in Environment International in 2016. The authors are Christopher Leonetti, Craig Butt, Kate Hoffman, Marie Lynn Miranda, and Heather Stapleton.

2.1 Introduction

Polybrominated diphenyl ethers (PBDEs) have been used as additive flame retardants for decades in a variety of applications from polyurethane foams to high-impact polystyrene (HIPS). The presence of PBDEs in consumer products has led to their accumulation in indoor environments, and subsequent human exposure via inadvertent ingestion and/or inhalation of dust particles (Johnson et al. 2010; Stapleton et al. 2012a) Particular attention has been given to a PBDE commercial mixture known as pentaBDE, which had a primary application in polyurethane foam used in residential furniture (Stapleton et al. 2012b; Zhang et al. 2011b). Studies have documented higher serum concentrations of PBDEs associated with pentaBDE in the US population relative to other regions of the world, likely due to the higher use of this mixture in residential furniture to meet a regional (state of California) flammability standard (Hites 2004). While the use of pentaBDE has now been banned or phased-out throughout the world, many older products in the home still contain these flame retardants, which will
continue to leach into the indoor environment during the product lifetime. As a result, human exposure to PBDEs will continue for years to come, especially with the use of recycled foams and plastics in consumer products that may contain these phased-out chemicals. As such, PBDEs continue to be measured in human tissues such as serum, breast milk, umbilical cord blood, and placental tissues, suggesting that prenatal exposures to PBDEs occurs during pregnancy, and continues during infancy via breast feeding (Abdelouahab et al. 2013; Adgent et al. 2014; Nanes et al. 2014; Zota et al. 2013).

In contrast, 2,4,6-tribromophenol (2,4,6-TBP) is widely used as an industrial chemical with an estimated US production volume of 4500 to 23,000 tonnes in 2006 (Covaci et al. 2011). 2,4,6-TBP has multiple applications, including use as an antifungal agent (e.g. as a replacement for pentachlorophenol) in wood applications, as a reactive brominated flame retardant (BFR), and as an intermediate in the production of other BFRs. 2,4,6-TBP can also be formed as a result of the photolytic degradation of tetrabromobisphenol-A (TBBPA), a widely used reactive BFR, and during the synthesis of 1,2-bis (2,4,6-tribromophenoxy) ethane (BTBPE) (Suzuki et al. 2008). In addition to the anthropogenic sources of 2,4,6-TBP, there are natural sources of 2,4,6-TBP and other bromophenols from marine organisms and algae (Gribble 2000). Few toxicity studies have examined the effects of 2,4,6-TBP in animal models. One study examined oral exposure to 2,4,6-TBP in adult zebrafish and observed reproductive toxicity in addition to perturbed gonadal morphology when exposed to spiked food at concentrations of
3300 ug/g dw (Haldén et al. 2010). Only a few studies have examined environmental levels and human exposure to 2,4,6-TBP. It has been measured in marine sediments at an average concentration of 3.02 ng/g dry weight and in riverine systems at 0.66 ng/g dry weight (Sim et al. 2009). 2,4,6-TBP has also been measured in the indoor environment of Japanese homes, with indoor house dust concentrations ranging from 15-30 ng/g and indoor air concentrations between 220-690 pg/m³ (Takigami et al. 2009). Very few biomonitoring studies have included 2,4,6-TBP in the analyses of human tissues such as serum, cord blood, and/or breast milk. One Japanese study collected maternal serum and umbilical cord blood from a cohort of 16 mothers in 2006 for analysis of BFRs and PCBs. This study measured 2,4,6-TBP in maternal blood at a concentration of 22 pg/g wet weight and in cord blood at a concentration of 37 pg/g wet weight (Kawashiro et al. 2008). BFRs were also evaluated in Norwegian individuals working in electronics dismantling facilities, where 2,4,6-TBP was measured in plasma ranging from 0.17 to 81 ng/g lipid (Thomsen et al. 2001). In a study measuring BFRs in a Canadian Inuit population from Nunavik, Quebec, plasma samples contained a geometric mean 2,4,6-TBP concentration of 9.4 µg/kg lipid, however, these concentrations were not correlated with PBDE concentrations(Dallaire et al. 2009). Finally, Qiu et al. measured mean 2,4,6-TBP concentrations of 5.6 ng/g lipid in fetal plasma and 0.8 ng/g lipid in maternal plasma (Qiu et al. 2009).
PBDEs and 2,4,6-TBP share a chemical structure that is similar to endogenous thyroid hormones (THs), and have been demonstrated to disrupt TH homeostasis either in vitro or in animal exposure studies (Noyes et al. 2013b; Szabo et al. 2009). Concentrations of PBDEs in human serum have also been found to be significantly correlated with circulating levels of THs in adults, and are associated with adverse neurodevelopmental outcomes in children (Eskenazi et al. 2013; Herbstman et al. 2010). Early childhood represents a developmental period that is vulnerable to endocrine disruption. Development is a hormonally-regulated growth process that is sensitive to perturbations by environmental contaminants, like PBDEs and 2,4,6-TBP. The in utero stage of development also represents a highly vulnerable period of fetal growth that may be even more sensitive to endocrine disruption due to the underdeveloped nature of the fetus’ detoxification pathways, in addition to the myriad different growth and developmental processes that are occurring throughout gestation.

The placenta acts to facilitate the materno-fetal transfer of nutrients, gas, waste, and hormones throughout gestation and can act as a protective barrier against toxins and environmental contaminants (James et al. 2007). In the case of PBDEs, passive diffusion and/or active uptake of these chemicals into the placenta occurs, and the placenta can act as a repository for these lipophilic chemicals. For example, one study looked at mother-child pairs in China and compared the placental transfer characteristics of various environmental endocrine disruptors, including PBDEs. Their
results indicated that PBDEs can be transferred across the placenta from maternal circulation, and eventually reach the fetus (Li et al. 2013). Additionally, Frederiksen et al. used an experimental ex vivo human placenta perfusion system to show the differences in transplacental transfer of PBDEs based on their degree of bromination (Frederiksen et al. 2010b). Thus there is a need to better understand the accumulation of these contaminants in placental tissues, in order to understand fetal exposures. In this study, we present our findings from the analysis of 102 human placental tissues that were collected in North Carolina, USA. Tissue samples were analyzed for a suite of PBDEs and 2,4,6-tribromophenol in order to increase our understanding of these contaminants within the placenta.

2.2 Materials and Methods

2.2.1 Participant Recruitment

Participants were recruited from within an observational prospective cohort study assessing the joint effect of social, environmental, and host factors on pregnancy outcomes (the Healthy Pregnancy, Healthy Baby (HPHB) Study conducted by the Children’s Environmental Health Initiative) (Miranda et al. 2010; Swamy et al. 2011). The HPHB study enrolled pregnant women from the Duke Obstetrics Clinic and the Durham County Health Department Prenatal Clinic at the Lincoln Community Health Center in Durham, NC. Our analyses included a subset of women from the HPHB study that delivered at the Duke University Medical Center between March 2010 and December
2011. The intentional study design was to oversample women attending the Lincoln Community Health Clinic, in order to explore disparities in pregnancy outcomes by comparing African-American women with good outcomes to those with adverse pregnancy outcomes. As a result, the study population is predominantly African-American women with a lower socioeconomic standing and low educational attainment relative to the general US population. All aspects of this study were carried out in accordance with a human subjects research protocol approved by the Duke University Institutional Review Board.

2.2.2 Sample Collection

Consenting women had placenta tissue subsamples taken at the time of delivery at the Duke University Medical Center. Tissues (approximately 5-20 g) were stored in screwtop cryovials at -80°C until analysis.

2.2.3 Chemicals

All solvents used for the analysis were HPLC-grade or better. A fluorinated BDE standard, 2,3’,4,4’,6-tetrabromodiphenyl ether (FBDE-69) (Chiron Inc., Trondheim, Norway), $^{13}$C labeled 2,2’,3,4,5,5’-hexachlorinated diphenyl ether (CDE-141) (Cambridge Isotope Laboratories, Andover, MA), and labeled $^{13}$C-2,2’,3,3’,4,4’,5,5’,6,6’-decabromodiphenyl ether (BDE-209) were used as internal and recovery standards for the BFR extractions. PBDE calibration standards were purchased from Accustandard
and 2,4,6-tribromophenol was purchased from Cambridge Isotope Laboratories, Andover, MA.

### 2.2.4 BFR Analysis and Lipid Determination

Extractions were performed using between 2 and 17 grams of placenta tissue, depending on the sample and the amount collected during delivery. Tissues underwent 24 hours of lyophilization in order to completely dry the samples. The freeze-dried tissue samples were then homogenized into a fine powder with a pre-cleaned mortar and pestle before adding 15 mL of 1:1 hexane/dichloromethane (DCM) and letting the samples sit overnight, in order to allow for full solvent penetration. Samples were spiked with 1 ng of FBDE-69 and 13C-BDE-209 as internal standards. All glassware used for BFR analysis were cleaned by muffle furnace, in addition to triple-rinsing with hexane, DCM, and methanol solvents in order to minimize background contamination. Samples then underwent 20 minutes of water bath sonication followed by centrifugation, after which the solvent was decanted to a separate tube. The extraction step was then repeated twice (three times total), and the solvent extracts were combined in a clean 50 mL glass centrifuge tube. Following extraction, the samples were blown down under a gentle stream of N2 to a volume of 1 mL. A small aliquot of the extract was used for gravimetric lipid analysis and the remaining extract was passed through acidified silica columns for sample clean-up. Deactivated silica (4.0 g) was acidified using 40% by mass H2SO4, shaken, and loaded into a glass chromatography column.
The columns were pre-cleaned by rinsing with hexane and acetone and then conditioned with 15 mL of the elution solvent mix. The extract was then loaded on to the column and eluted using 30 mL of 80:20 hexane/DCM. Sample extracts were then blown down under a gentle stream of N2 gas to a final volume of 100 uL. Samples were transferred to 200 uL glass vial inserts and spiked with 1 ng of 13C-CDE-141 as a recovery standard. Finally, PBDEs and 2,4,6-TBP were identified and quantified using authenticated standards and gas chromatography with electron capture negative ion mass spectrometry (GC/ECNI-MS).

2.2.5 Quality Control/Quality Assurance

Laboratory blanks (e.g. sodium sulfate) were included with each batch of tissue sample extractions beginning with lyophilization (one batch includes 10 tissues samples plus two lab blanks). All sample values were blank subtracted and MDLs were calculated as three times the standard deviation of the lab blank values for each analyte. Individual values were normalized to the measured lipid content of each tissue sample used for the extraction procedure to yield a final value in ng/g lipid.

Labeled internal standards were used as surrogates and internal standards in all samples and included F-BDE-69 and 13C-BDE-209 as internal standards (spiked prior to extraction) and 13C-CDE-141 as a recovery standard (spiked prior to GC/MS analysis). The recovery of the internal standards was calculated for all tissue samples and laboratory blanks in order to assess the recovery efficiency of the extraction methods.
The mean recovery in the lab blanks for FBDE-69 was 82.5 ± 14%, while mean sample recovery was 60 ± 12%.

Additionally, the BFR extraction method was validated using Standard Reference Material (SRM) 1947 (NIST, Gaithersburg, MD). SRM 1947 is a Lake Michigan fish homogenate with certified concentrations of PBDEs. The BFR extraction procedure previously described was used on a triplicate set of SRM 1947 samples. The concentrations of PBDE congeners of interest (BDE-47, -66, -99, -100, -153, and 154) were measured at 99%-116% of the certified values. Recovery of 2,4,6-TBP was evaluated by spiking 10 ng into a laboratory blank (in triplicate) and carrying it through the method. Recoveries averaged 87% (± 29%).

2.2.6 Statistical Analysis

Statistical analyses were performed using JMP Pro 11. ΣBDE was calculated by summing all PBDE congeners including BDE-47, -99, -100, -153, -154, and -209, while ΣBFR includes all PBDE congeners plus 2,4,6-TBP. Only analytes with ≥50% detection frequency were included in statistical analyses. Values below MDL were assigned a value equal to one-half the detection limit for statistical analyses. Preliminary analyses (Shapiro-Wilkes Test) indicated that the PBDE data were not normally distributed. As such, Spearman rank sum correlation analyses were used to assess the relationships between PBDE congeners in placenta and to assess their relationship with maternal age. It is important to note that the BFR concentrations were not significantly and positively
associated with lipid content; however, we conducted all statistical analyses with both wet weight and lipid normalized concentrations. Results were similar using both methods. We present statistics using lipid normalized concentrations to facilitate comparison with other studies. Alpha < 0.05 was considered statistically significant.

2.3 Results

2.3.1 Population Characteristics

Participant demographics are summarized in Table 4. Sixty-eight percent of the women in the study were non-Hispanic black. Most (58%) women were relatively young, between the ages of 18-24 years old (range 18-40). This was the first pregnancy for 45.5% of the women. Of all participants, 43.6% reported completing high school, and less than 10% of the women had private health insurance. Recruitment for this study used English literacy as an exclusion criteria, so the demographics of this study population are not entirely reflective of the population of women visiting the Prenatal Clinic at the Lincoln Community Health Center in Durham, NC. The population of women who most commonly use this prenatal clinic are Hispanic, while the women included in this study are predominantly non-Hispanic black women with a lower socioeconomic standing.
### Table 4: Cohort Characteristics (n=101*)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal race</td>
<td></td>
</tr>
<tr>
<td>Non-Hispanic white</td>
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</tr>
<tr>
<td>Non-Hispanic black</td>
<td>69 (68.3)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>12 (11.9)</td>
</tr>
<tr>
<td>Other</td>
<td>4 (4.0)</td>
</tr>
<tr>
<td>Maternal age</td>
<td></td>
</tr>
<tr>
<td>18-19</td>
<td>21 (20.8)</td>
</tr>
<tr>
<td>20-24</td>
<td>38 (37.6)</td>
</tr>
<tr>
<td>25-40</td>
<td>42 (41.6)</td>
</tr>
<tr>
<td>Parity</td>
<td></td>
</tr>
<tr>
<td>First birth</td>
<td>46 (45.5)</td>
</tr>
<tr>
<td>Male infant</td>
<td>52 (51.5)</td>
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<tr>
<td>Maternal education</td>
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</tr>
<tr>
<td>Less than high school</td>
<td>25 (24.8)</td>
</tr>
<tr>
<td>High school diploma</td>
<td>32 (31.7)</td>
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<tr>
<td>More than high school</td>
<td>44 (43.6)</td>
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<tr>
<td>Not married</td>
<td>82 (81.2)</td>
</tr>
<tr>
<td>Smoked during pregnancy</td>
<td>22 (21.8)</td>
</tr>
<tr>
<td>Private health insurance*</td>
<td>9 (9.3)</td>
</tr>
</tbody>
</table>

*Demographic data was missing for one individual
*No data available on health insurance for four of the individuals

#### 2.3.2 BFRs

Detection frequencies for BDE-47, -100, -99, -154, -153, -209, and 2,4,6-TBP were all greater than 50% and are presented in Table 5 along with the range and distribution of concentrations measured. It is interesting to note that 2,4,6-TBP was detected in 100% of the samples and constituted 47.8% of the ΣBFR concentration measured in tissues.

The most prominent PBDE measured was BDE-47, representing 34% of ΣBDE burden.

The geometric mean concentration of 2,4,6-TBP was 15.4 ng/g lipid (range: 1.31 - 316
ng/g lipid), while the geometric mean concentration of BDE-47 was 5.09 ng/g lipid (range: 0.12 - 141 ng/g lipid). The PBDE congener ranking profile from highest geometric mean concentration to lowest geometric mean concentration is: BDE-47, -209, -153, -99, -100, -154 (Figure 3). Given the relatively homogenous distribution of our population, we were underpowered to examine associations between BFR exposures and race/ethnicity. However, we did examine associations with age. Interestingly, BDE-209 and 2,4,6-TBP were negatively associated with maternal age, $r_s = -0.16$ ($p = 0.10$) and $r_s = -0.17$ ($p = 0.08$), respectively, although again the associations did not reach statistical significance at $p < 0.05$. The remaining PBDE congeners showed no suggestion of associations with maternal age ($p > 0.20$).
Table 5: BFR concentrations (ng/g lipid) measured in placental tissues

<table>
<thead>
<tr>
<th>Variable</th>
<th>MDL</th>
<th>Detection Frequency (%)</th>
<th>Geometric Mean</th>
<th>Min</th>
<th>Max</th>
<th>25th</th>
<th>50th</th>
<th>75th</th>
<th>95th</th>
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<tbody>
<tr>
<td><strong>PBDEs (n = 102)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>BDE-47</td>
<td>0.07</td>
<td>91.2</td>
<td>5.09</td>
<td>0.12</td>
<td>141</td>
<td>2.12</td>
<td>5.05</td>
<td>12.4</td>
<td>37.5</td>
</tr>
<tr>
<td>BDE-99</td>
<td>0.07</td>
<td>68.6</td>
<td>1.95</td>
<td>0.09</td>
<td>223</td>
<td>0.62</td>
<td>1.95</td>
<td>4.43</td>
<td>17.1</td>
</tr>
<tr>
<td>BDE-100</td>
<td>0.02</td>
<td>88.2</td>
<td>1.45</td>
<td>0.03</td>
<td>50.1</td>
<td>0.62</td>
<td>1.65</td>
<td>3.25</td>
<td>11.1</td>
</tr>
<tr>
<td>BDE-153</td>
<td>0.01</td>
<td>93.1</td>
<td>2.06</td>
<td>0.02</td>
<td>513</td>
<td>1.21</td>
<td>2.36</td>
<td>4.15</td>
<td>16.9</td>
</tr>
<tr>
<td>BDE-154</td>
<td>0.01</td>
<td>83.3</td>
<td>0.63</td>
<td>0.01</td>
<td>20.2</td>
<td>0.33</td>
<td>0.74</td>
<td>1.41</td>
<td>3.41</td>
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<tr>
<td>BDE-209</td>
<td>0.17</td>
<td>52.9</td>
<td>2.86</td>
<td>0.16</td>
<td>50.4</td>
<td>1.55</td>
<td>2.64</td>
<td>6.83</td>
<td>17.3</td>
</tr>
<tr>
<td>ΣPBDEs</td>
<td></td>
<td></td>
<td>17.6</td>
<td>0.54</td>
<td>528</td>
<td>8.71</td>
<td>19.10</td>
<td>34.7</td>
<td>98.7</td>
</tr>
<tr>
<td><strong>Phenolic compound (n = 102)</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,4,6-TBP</td>
<td>0.05</td>
<td>100</td>
<td>15.4</td>
<td>1.31</td>
<td>316</td>
<td>6.25</td>
<td>15.0</td>
<td>32.7</td>
<td>171</td>
</tr>
<tr>
<td>ΣBFRs</td>
<td>37.3</td>
<td>2.18</td>
<td>568</td>
<td>18.3</td>
<td>38.1</td>
<td>75.6</td>
<td>317</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ΣPBDE was calculated by summing all PBDE congeners (i.e. BDE-47, -99, -100, -153, -154, and -209), while ΣBFR includes the sum of all PBDE congeners plus 2,4,6-TBP.
Figure 3: BFR analyte concentrations measured in human placenta tissues ($n = 102$; error bars represent ±SEM).
2.3.3 Associations Between PBDEs and 2,4,6-TBP

Correlation analyses are summarized in Table 6. All BFRs were significantly \( (p < 0.001) \) and positively correlated with each other and with \( \Sigma \)BFR concentrations. BDE-100 showed the strongest correlation \( (r_s = 0.89) \) with \( \Sigma \)BDE content followed by BDE-47 \( (r_s = 0.84) \). Interestingly, 2,4,6-TBP was significantly associated with all PBDE congeners. For example, 2,4,6-TBP showed a moderately strong correlation with BDE-209 \( (r_s = 0.58; p < 0.001; \text{Figure 4}) \).
Table 6: Spearman correlation matrix for BFRs (ng/g lipid)

<table>
<thead>
<tr>
<th>BFR</th>
<th>BDE-47</th>
<th>BDE-99</th>
<th>BDE-100</th>
<th>BDE-153</th>
<th>BDE-154</th>
<th>BDE-209</th>
<th>2,4,6-TBP</th>
<th>ΣPBDEs</th>
<th>ΣBFRs</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDE-47</td>
<td>1.00</td>
<td>0.48(^i)</td>
<td>0.88(^i)</td>
<td>0.58(^i)</td>
<td>0.61(^i)</td>
<td>0.49(^i)</td>
<td>0.50(^i)</td>
<td>0.84(^i)</td>
<td>0.73(^i)</td>
</tr>
<tr>
<td>BDE-99</td>
<td>1.00</td>
<td>0.52(^i)</td>
<td>0.43(^i)</td>
<td>0.52(^i)</td>
<td>0.60(^i)</td>
<td>0.66(^i)</td>
<td>0.68(^i)</td>
<td>0.72(^i)</td>
<td></td>
</tr>
<tr>
<td>BDE-100</td>
<td>1.00</td>
<td>0.71(^i)</td>
<td>0.71(^i)</td>
<td>0.50(^i)</td>
<td>0.48(^i)</td>
<td>0.89(^i)</td>
<td>0.77(^i)</td>
<td>0.66(^i)</td>
<td></td>
</tr>
<tr>
<td>BDE-153</td>
<td>1.00</td>
<td>0.71(^i)</td>
<td>0.50(^i)</td>
<td>0.38(^i)</td>
<td>0.77(^i)</td>
<td>0.66(^i)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BDE-154</td>
<td>1.00</td>
<td>0.54(^i)</td>
<td>0.50(^i)</td>
<td>0.77(^i)</td>
<td>0.72(^i)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BDE-209</td>
<td>1.00</td>
<td>0.58(^i)</td>
<td>0.73(^i)</td>
<td>0.72(^i)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,4,6-TBP</td>
<td>1.00</td>
<td>0.58(^i)</td>
<td>0.85(^i)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ΣPBDEs</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>ΣBFRs</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

\(^i\) p < 0.001
2.4 Discussion

This is the first study to measure both PBDEs and 2,4,6-TBP in human placenta tissues, and observe a suggestive negative association with maternal age. It has often been assumed that the relative tissue concentrations of environmental contaminants within the placenta can be representative of fetal exposures for some contaminants (Frederiksen et al. 2010a). In fact, numerous studies have examined the relationships of
environmental contaminants, such as PBDEs, within maternal serum, umbilical cord blood, and placenta tissues. The results of these studies indicate that transplacental transfer (TPT) of PBDEs does occur, and leads to fetal exposure during gestation (Chen et al. 2013; Gómara et al. 2007; Kim et al. 2015; Vizcaino et al. 2014). For example, a study by Frederiksen et al. found that PBDE exposure in the indoor environment, specifically from house dust ingestion, is linked to PBDE concentrations in maternal and umbilical cord plasma, which are additionally correlated with the PBDE concentrations measured in the paired placental tissues. This study also showed a decreased rate of transport of PBDE congeners across the placenta with increasing degree of bromination (Frederiksen et al. 2010a). These results illustrate the placental transfer of PBDEs following maternal exposure to house dust and/or dietary sources of PBDEs, and also show that PBDEs are transferred to the fetal compartment during gestation. However, to our knowledge, no studies have examined TPT of 2,4,6-TBP, which should be addressed in future studies.

Additional research has also been conducted using a human ex vivo placenta perfusion system to study the kinetics and placenta transfer characteristics of BDE-47, -99, and -209. Significant accumulation was observed for all PBDE congeners tested, with placental transfer of BDE-47 being faster and more extensive than BDE-99 and BDE-209 (Frederiksen et al. 2010b). These results indicate that in utero exposure to PBDEs occurs during gestation as a result of placental transfer, with higher rates of transfer and exposure for the lower brominated congeners. In contrast to these results, Chen et al.
measured higher ratios between fetal cord blood and maternal placenta (F/M ratio) for PBDEs with a higher degree of bromination, suggesting that TPT increases with increasing degree of bromination (Chen et al. 2014b). In addition, the ability of a chemical to bind to plasma transporter proteins will likely affect TPT characteristics. In the case of PBDEs, which have chemical structures similar to that of THs, their ability to bind TH transport proteins such as TTR and/or TH membrane transporters such as OATPs, MCTs, and LATs, may affect their TPT properties (Patel et al. 2011a). Different compounds exhibit different partitioning and transport behaviors, and the exact mechanisms of TPT are not fully understood, however, the presence of contaminants found in both maternal and fetal circulation is clearly indicative of fetal exposure.

To date, only two other studies have measured PBDEs in placenta tissue samples from US populations (Dassanayake et al. 2009; Nanes et al. 2014). Additionally, placenta tissues from China, Japan, and European countries including Spain, Denmark, and Finland have been evaluated for PBDEs (Chen et al. 2014b; Frederiksen et al. 2009a; Gómar et al. 2007; Main et al. 2007; Xu et al. 2015a). The results from these studies are summarized in Table 7. The median value for ΣPBDEs4-7 (tetra- through hepta-substituted congeners) in this present study was 13.8 ng/g lipid, and is similar to levels reported for placentae from individuals living near a Chinese e-waste site (19.5 ng/g lipid and 19.4 ng/g lipid), as well as in another US cohort that had a smaller sample size (n = 42; 23.7 ng/g lipid). However, these values are much higher than the average
ΣPBDE concentrations found in European samples (1.09 ng/g lipid), as well as placentae from Japan (0.25 ng/g lipid) (Takasuga et al. 2006).

These current findings align with previous studies that have reported higher concentrations of PBDE congeners associated with the pentaBDE mixture in human samples from North America. North American concentrations are generally one to two orders of magnitude higher than those measured in European and Asian populations as a result of differences in fire regulatory standards, chemical regulatory and policy frameworks, and overall use and exposure to PBDEs (Frederiksen et al. 2009b). In Europe, Japan, and China, BDE-209 is often found to be the most abundant congener, accounting for more than 50% of the total concentrations in human placentae (Frederiksen et al. 2009a; Gómara et al. 2007; Zhao et al. 2013). In the US, however, BDE-47 is the most prevalent congener measured in human and other biological tissues, and this is consistent with the higher concentrations of BDE-47 measured in indoor dust in the US compared to other countries. It is interesting to note that in this study, BDE-209 concentrations measured in placental tissues were approximately equal to measurements made in Chinese samples, twice as high as the Danish samples, and eight times higher than Japanese samples. Furthermore, the measured values for ΣPBDEs in American placentae from this study are relatively similar to those measurements found in samples from individuals living and working in Chinese e-waste recycling towns. E-waste dismantling and processing is an occupation that typically involves significant
human exposure to flame retardants due to their higher contact with electronic components containing flame retardant chemicals. The concentration of PBDEs measured in placenta tissues from both populations suggests that the same level of PBDE exposure and accumulation occurs between the general US population and Chinese e-waste recycling town inhabitants, despite the stark discrepancy in their exposure scenarios, and likely exposure pathways. However, lower brominated PBDEs that are more commonly found in pentaBDE applications such as polyurethane foams sold in North America, were measured in high concentrations in the Chinese e-waste worker samples, despite the fact the pentaBDE has limited use in electronics. These may be the result of metabolic and/or abiotic debromination of BDE-209 and other higher brominated PBDEs that are more widely used in electronics and plastics.
<table>
<thead>
<tr>
<th>Population Location</th>
<th>Year</th>
<th># of placentae</th>
<th># of BDE congeners</th>
<th>Median $\Sigma_{3-7}$ BDEs (ng/g lw*)</th>
<th>Median BDE-209 (ng/g lw*)</th>
<th>Lipid (%)</th>
<th>Congener concentration ranking</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>USA</td>
<td>2010-2012</td>
<td>42</td>
<td>10</td>
<td>23.7</td>
<td>NA</td>
<td>NA</td>
<td>47&gt;153=99&gt;209</td>
<td>Nanes et al., 2014 (Nanes et al. 2014)</td>
</tr>
<tr>
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<td>2008-2010</td>
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<td>7</td>
<td>13.8</td>
<td>2.64</td>
<td>1.09</td>
<td>47&gt;209&gt;153=99&gt;100</td>
<td>This study</td>
</tr>
<tr>
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<td>2007-2008</td>
<td>5</td>
<td>42</td>
<td>NA</td>
<td>NA</td>
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<td>47=99&gt;153</td>
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<td>47&gt;153=99</td>
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<td>B</td>
<td>C</td>
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* lw = lipid weight based
In the current study, we also observed a suggestive negative association with maternal age for BDE-209 and 2,4,6-TBP, which to our knowledge, has not been observed previously. The explanation for this negative relationship is unclear, but may relate to differences in exposure based on difference in behavior with age (e.g. time spent in various micro-environments). The fact that both BDE-209 and 2,4,6-TBP were negatively associated with maternal age, and that both were correlated with each other, suggests that they may share a similar source (e.g. electronics). Usually, PBDE congeners within a single commercial mixture are more strongly correlated with one another than between commercial mixtures. However, our results are partially in agreement with a recent assessment of placental PBDE concentrations that measured significant correlations between BDE-209 and BDE-28, -47, -99, and -183, but not between BDE-209 and BDE-100, -153, and -154 (Xu et al. 2015b). There are currently no other studies that have measured 2,4,6-TBP in human placenta tissue. The specific applications of 2,4,6-TBP as a reactive flame retardant remain unclear; however it appears that exposure to 2,4,6-TBP is common within our study population since it was detected in 100% of samples. 2,4,6-TBP was found to have a positive correlation with all PBDEs quantified, but was strongest for BDE-99 and BDE-209. 2,4,6-TBP and BDE-209 are not commonly analyzed together in biological tissues, likely due to the lower awareness of 2,4,6-TBP as an environmental contaminant of interest. As stated earlier, this unique relationship may be
a result of these chemicals having related sources of exposure, or it may be indicative of a metabolic pathway that transforms BDE-209 into 2,4,6-TBP.

Previous work has explored the *in vitro* endocrine-disrupting potency of 2,4,6-TBP, as well as other BFRs, using a wide variety of assays. 2,4,6-TBP was found to be a potent inhibitor of ESULT activity along with TBBPA and 6-OH-BDE-47, while the PBDEs showed much higher IC$_{50}$ value (half maximal inhibitory concentration, or the concentration at which the enzyme activity is diminished by 50%) and/or no ESULT inhibition, indicating that ESULT inhibition potency is determined largely in part by the presence of a hydroxylated aromatic group. 2,4,6-TBP was also shown to be a very potent T4 competitor in the TTR-binding assay, with a TTR-binding affinity 10.2 greater than the natural ligand, T4 (Hamers et al. 2006). Additionally, the ability of 2,4,6-TBP to inhibit thyroid hormone SULT activity in pooled human liver cytosol was evaluated, and 2,4,6-TBP was shown to have an IC$_{50}$ value of 8.3 nM, which was more potent than any of the hydroxylated PBDEs profiled (Butt and Stapleton 2013). However, more research is necessary to understand the sources of 2,4,6-TBP in the indoor environment, as well as to examine potential adverse effects from exposure to 2,4,6-TBP among the general population.

It is well known that the first trimester of pregnancy is a critical period for fetal neurodevelopment, and that these neurodevelopmental processes are largely driven by the action of THs (Préau et al. 2014). Animal exposure studies with PBDEs have shown
permanent effects on spontaneous motor behavior (eg. hyperactivity) and decreased performance in learning and memory tests, implicating PBDEs as developmental neurotoxicants and endocrine disruptors (Costa and Giordano 2007; Williams and DeSesso 2010). Additionally, PBDEs have been shown to disrupt TH homeostasis; therefore, the presence of PBDEs in the placenta may impact the materno-fetal transfer of THs during gestation, leading to the disruption of TH-mediated processes in the fetal compartment. Furthermore, growing epidemiological evidence show associations between prenatal exposure to PBDEs and subsequent neurodevelopmental deficits measured in children (Chen et al. 2014a; Eskenazi et al. 2013; Herbstman and Mall 2014).

Overall, flame retardant levels should continue to be closely monitored in the placenta, as well as their potential effects on fetal TH status and neurodevelopment.

One potential shortcoming of this study is the subsampling technique used in the collection of the placental tissue samples. Placental samples were collected at delivery and then sub-sampled to share among various studies. Therefore it was impossible to collect a whole placenta and homogenize the sample prior to sub-sampling. The placenta is a large, highly vascularized, heterogeneous organ. As a result, inconsistent or non-standardized subsampling techniques of the placenta organ may result in differences in our measurements of BFRs. For example, subsamples taken from the highly vascularized central region of the organ may contain different concentrations of BFRs than a peripheral subsample that has different vasculature and adipose composition.
Normalization to lipid content may help control for some of these differences. However, we observed no significant correlations between BFR concentrations on a wet weight basis (ng/g ww) with percent lipid. But despite the inconsistencies in subsampling, the median values of PBDE concentrations from this study agree with PBDE measurements from previous studies (Chen et al. 2014b; Nanes et al. 2014; Xu et al. 2015b).

Results from this study indicate that PBDEs and 2,4,6-TBP bioaccumulate in human placenta tissues, and provide insight into fetal BFR exposure during pregnancy. This study also characterizes BFR exposures in a population of women from low socioeconomic backgrounds and represents a unique subpopulation of understudied women in the US that are not typically represented in other exposure studies. These data may provide useful comparisons to other study populations from different regions with different ethnic and socioeconomic backgrounds, and further our understanding of the exposure patterns across the US. Future studies should also consider investigating associations between adverse health outcomes and exposures to these mixtures of BFRs given their reported effects on endocrine function.
3. Associations between Brominated Flame Retardants, Thyroid Hormone Levels, and Thyroid Hormone Regulating Enzyme Activity in Human Placental Tissues

3.1 Introduction

Brominated flame retardants (BFRs) such as polybrominated diphenyl ethers (PBDEs) and 2,4,6-tribromophenol (2,4,6-TBP) have been applied to numerous types of furniture and electronic items in order to meet state and federal flammability standards (Costa et al. 2008). PBDE mixtures were phased out of use and production because of their persistence, bioaccumulation and potential toxicity beginning in 2004 (de Wit et al. 2010; Linares et al. 2015). Despite the phase out, many older products containing PBDEs remain in use in the home environment, and thus human exposure to PBDEs will continue for some time. Additionally, 2,4,6-TBP currently remains in use as fungicide/wood preservative, an intermediate in the production of other BFRs, and as a reactive flame retardant (Mardones et al. 2003; Suzuki et al. 2008).

PBDEs and 2,4,6-TBP have chemical structures similar to thyroid hormones (THs), and several studies have demonstrated that they can impact thyroid regulation, endocrine function, and neurodevelopment in animal models such as birds, rodents, and fish (Deng et al. 2010; Fernie et al. 2005; Noyes et al. 2013b; Viberg and Eriksson 2011). Epidemiological studies have also observed associations between maternal or cord sera PBDE concentrations and various negative developmental, behavioral, and cognitive effects in young children (Chen et al. 2014a; Cowell et al. 2015; Eskenazi et al. 2013;
Gascon et al. 2012; Roze et al. 2009). However, the impacts of early-life exposure to 2,4,6-TBP have not been evaluated. Proposed mechanisms of TH dysfunction include the aberrant binding to TH receptors, disruption of TH transport proteins such as TTR and TBG, or disruption of TH-metabolizing enzymes such as DI and SULT (Butt and Stapleton 2013; Szabo et al. 2009). Disruption of TH transport or metabolism can lead to alterations in serum or tissue concentrations of these hormones, which may in turn lead to downstream effects on TH-mediated processes, especially early neurodevelopmental pathways in developing children (Dingemans et al. 2011).

During pregnancy, the placenta, a highly specialized fetomaternal organ, functions as a regulatory transport barrier for a wide variety of biomolecules and contains numerous passive and active transport pathways, in order to regulate the materno-fetal transfer of nutrients, waste products, and hormones (Patel et al. 2011a). PBDEs and other environmental contaminants are also transported between the maternal and fetal circulatory compartments via the placenta (Vizcaino et al. 2014) In addition, the placenta facilitates the uptake of THs from maternal circulation and subsequent delivery to the fetal compartment throughout gestation as the fetus does not begin producing its own THs until the beginning of the second trimester and relies solely on maternally-derived THs during the first trimester (Chan et al. 2009). During this period, the fetus is extremely sensitive to perturbations in TH homeostasis, and
disruption of TH regulation can lead to effects that may manifest later in life as cognitive and behavioral deficits (Costa et al. 2013).

To our knowledge, the effects of BFRs on TH function in human placenta tissues has not been investigated. Thus, the primary goal of this study was to examine associations between BFRs (PBDEs and 2,4,6-TBP), TH concentrations, and measures of TH metabolic enzyme activity in placental tissues. We hypothesized that BFR exposure may be associated with decreased TH enzyme function which may result in increased (e.g., T4) or decreased (e.g., rT3) thyroid hormone levels in placental tissues. Such changes may be important for understanding life-long health risks of exposure to PBDEs and 2,4,6-TBP.

3.2 Materials and Methods

3.2.1 Study Population

Participants were recruited within a then-ongoing prospective cohort study assessing the joint effect of social, environmental, and host factors on pregnancy outcomes (the Healthy Pregnancy, Healthy Baby (HPHB) Study). The HPHB study enrolled pregnant women from the Duke Obstetrics Clinic and the Durham County Health Department Prenatal Clinic (Maxson and Miranda 2011; Miranda et al. 2011; Stapleton et al. 2011). Our analyses included a subset of women from the HPHB study who were seen at the latter clinic, and delivered at the Duke University Medical Center between March 2010 and December 2011 and from whom sufficient placenta tissue was
available for this study (n = 102). Because we anticipated that gestational age may be related to TH concentrations in placenta, we further restricted analyses to women giving birth to term infants (37 or more weeks gestation (n = 95)). Reflective of the population of women receiving services at the clinic used in recruitment, the HPHB cohort is predominantly African-American with the majority of mothers having a high school diploma or less education and few having private health insurance (Leonetti et al. 2016). All aspects of this study were carried out in accordance with a human subjects research protocol approved by the Duke University Institutional Review Board and all women provided informed consent prior to participation.

### 3.2.2 Reagents and Materials

Isotopically labeled thyroid hormone standards ($^{13}$C$_{12}$- T4, $^{13}$C$_{6}$- T4, $^{13}$C$_{6}$-T3, and $^{13}$C$_{6}$-rT3) were used as internal and recovery standards for the TH extractions were purchased from Isotec (Miamisburg, OH). Dithiothreitol (DTT, > 99%), 3’-phosphoadenosine 5’-phosphosulfate (PAPS), T4 (> 98%), T3 (> 95%), and rT3 (> 95%), were purchased from Sigma-Aldrich (St. Louis, MO). 3,3’,5-triiodothyronine sulfate (> 95%) was purchased from Toronto Research Chemicals (Toronto, Ontario). $^{13}$C$_{6}$-3,3’-T2 was purchased from Isotec (Miamisburg, OH). 3,3’-T2 sulfate (3,3’-T2S, 98%) was custom synthesized by the Duke University Small Molecule Synthesis Facility (Durham, NC). 3,3’,5- T3 sulfate (T3S, > 98%) was purchased from Toronto Research Chemicals
(Toronto, ON). All solvents and other reagents were purchased from VWR (Radnor, PA).

### 3.2.3 Placenta BFR and Thyroid Hormone Analysis

Placenta tissue subsamples were taken at the time of delivery at the Duke University Medical Center. Tissues (approximately 5 - 20 g) were stored in screwtop cryovials at -80°C until analysis. BFRs were measured using previously described methods (see Leonetti et al. 2016).

Placenta tissue samples (approximately 200 milligrams) were analyzed for THs were using a modified extraction protocol from our laboratory. Briefly, tissues were homogenized using 0.5 mm glass beads and a BBX24 Bullet-Blender, prior to a 16 hour protein/tissue digestion in a 37°C water bath. The pronase digestion solution contained protease (*Streptomyces griseus*), L-glutathione, n-phenylthiourea, and tris hydroxymethyl aminomethane. Samples were spiked with a mixture of 5 ng of $^{13}$C$_{12}$-T4, $^{13}$C$_{6}$-T3, and $^{13}$C$_{6}$-rT3 as internal standards. Following digestion, 40 µL of an antioxidant solution containing 37.5 mg/mL of citric acid, ascorbic acid, and dithiothreitol were added to each sample, followed by 500 µL of cold acetone. Samples then undergo a series of solvent extractions and centrifugation in order to separate THs and remove lipids and other competing biomolecules. Samples are extracted sequentially using acetone, cyclopentane, and ethyl acetate. After extraction, the samples are concentrated by reducing the solvent volume to 50 uL under a stream of N$_2$ gas and resuspended in 3 mL
of 0.01 M HCl with 10% MeOH. Next, samples undergo clean-up/extraction via solid-phase extraction (SPE) using SampliQ OPT cartridges to further remove competing biomolecules and isolate the analytes of interest (T4, T3, and rT3). Cartridges are conditioned with 3 mL of MeOH, followed by 3 mL of H2O, and then samples are loaded. Cartridges are then washed with 20% MeOH in H2O and analytes were eluted in 4 mL of 0.1% acetic acid in MeOH and blown down under a stream of N2 gas to a final volume of 50 uL. Samples were resuspended in 400 µL of 1:1 H2O/MeOH, transferred to glass screw-top ASV vials, and spiked with 5 ng of 13C6-T4 as a recovery standard. Finally, THs are identified and quantified using authenticated standards and liquid chromatography tandem mass spectrometry (LC-MS/MS) with electrospray ionization (ESI).

For the TH data, MDLs are calculated as three times the standard deviation of the blank values for each analyte (T3, T4, and rT3). Individual values are then normalized to the wet tissue mass used for the extraction procedure to yield a final value of ng/g wet weight (ww).

### 3.2.4 Preparation of Placental Microsomes

For each placenta sample, approximately 4 g of tissue was sub-sampled, avoiding the fatty tissue, minced and homogenized using a Bullet Blender (Next Advance, Averill Park, NY) with stainless stain beads (1:2:1 ratio of 0.2 mm:0.5 mm: 1.4 mm diameter beads). Microsomal suspensions were prepared from the homogenized
placental tissues using methods adapted from McKinney et al (McKinney et al. 2004).

Briefly, the homogenization buffer consisted of 0.25 M sucrose, 0.1 M KPO4, 1 mM EDTA, 10 mM DTT (pH 7.4). The microsome resuspension buffer was prepared identically to the homogenization buffer with the addition of 20% glycerol. All solution were prepared fresh daily. Care was taken to ensure that all preparations were done on ice to preserve enzyme integrity. Protein concentrations were determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) with bovine serum albumin as the protein standard. Prepared microsomes were kept frozen at -80°C until use in the experimental assays.

3.2.5 Deiodinase Activity Assays

DI activity assays were performed using in vitro techniques and LC-MS/MS analysis as described by Butt et al., 2011. Briefly, human placenta microsomes were diluted to approximately 1 mg protein/ml in 0.1 M potassium phosphate buffer (pH 7.4) with 10 mM DTT and 100 µM NADPH (total volume = 1 ml). In addition, buffer controls were prepared to correct for substrate impurities and abiotic degradation. Assays were predominately performed in triplicate (n=75) with the remaining run in duplicate (n=11) or single measurement (n=16). Reactions were initiated by the microsome addition and incubated at 37°C for 1 hr in a shaking water bath. Assays were stopped with 1 ml of ice-cold methanol, spiked with a suite of mass-labelled internal standards (25 ng each of $^{13}$C$_{12}$-T4, $^{13}$C$_{6}$-T3, $^{13}$C$_{6}$-rT3 and $^{13}$C$_{6}$-3,3'-T2) and extracted using liquid-liquid extraction.
The organic solvent was evaporated under a gentle stream of nitrogen gas and the extracts were cleaned and concentrated using solid-phase extraction (SPE) techniques. The SPE eluent was reduced to approximately 1 ml and analyzed for THs by LC-MS/MS. Analyte responses were normalized to the corresponding internal standard responses.

Prior to performing the placental DI activity study, the experimental conditions were optimized by varying the microsomal protein concentration and incubation time. The results showed that the optimized in vitro parameters (i.e. 1 hr incubation time, 1 mg/ml protein concentration) were within the linear range of the variables.

### 3.2.6 Sulfotransferase Assays

Placenta tissue cytosol was created during microsome preparation. Approximately 6 mL of cytosol were produced per tissue sample and stored at -80°C to ensure enzyme integrity until analysis. TH SULT activity was measured using a previously published SULT assay (Butt and Stapleton 2013). Briefly, placenta cytosol was combined with 0.1 M potassium phosphate buffer (pH=7.2) with 50 µM PAPS as the cofactor and 1 µM 3,3'-T2 or T3 as the substrate (total volume of 200 µL). Assay reactions were started with the addition of the cytosol. Vials were incubated at 37°C for 30 minutes in a shaking water bath, and reactions were stopped by the addition of 0.1 M HCl (800 µL). Samples were then spiked with $^{13}$C-3,3'-T2 (5.0 ng) or $^{13}$C-T3 (5 ng) as the internal standard. Sample extracts were cleaned using SampliQ OPT SPE cartridges (Agilent Technologies). The SPE columns were first conditioned with 3 mL of methanol.
and 3 mL of water, samples were loaded, and the column was rinsed with 3 mL of water. The THs and sulfate conjugate were eluted with 4 mL of methanol, and the extract was reduced to approximately 50 µL under a gentle stream of nitrogen gas. Samples were reconstituted in 1:1 MeOH/H₂O and transferred to Mini-UniPrep Syringeless Filters (GE Life Sciences) before analysis by LC-MS/MS. The protein content of cytosolic fractions was determined using the Bradford assay and SULT activity measurements were calculated as picomoles of 3,3’-T2 or femtomoles of T3 formed per minute per milligram of protein.

3.2.7 Instrumental Analysis

Instrumental analysis was performed by liquid chromatography with electrospray ionization (ESI) tandem mass spectrometry (LC-MS/MS) using conditions modified from our previously published methods. Monitored analytes included 3,3’-T2, 3,3’-T2S, and T3S. The sulfated THs was analyzed in electrospray ionization negative mode. MS/MS parameters for 3,3’-T2, 3,3’-T2S, and T3S were optimized using authentic standards. All analyte responses were normalized to the response of 13C₆-3,3’-T2.

3.2.8 Quality Control/Quality Assurance

For QA/QC in the TH extractions, lab blanks were run in each batch of placenta tissue sample extractions, and authenticated isotopically labeled internal standards were used in all samples. Lab blanks were used to assess background TH levels and to calculate method detection limits (MDLs). All samples were run with 5 ng 13C-T3, 13C-
rT3, and $^{13}$C-T4 as internal standards. The recovery of the $^{13}$C-labeled THs were calculated for all tissue samples and lab blanks in order to assess the recovery efficacy of the extraction and clean-up methods (mean blank recovery of THs is 101%; mean sample recovery of THs is 75%).

### 3.2.9 Statistical Analysis

Preliminary analyses (Shapiro-Wilkes Test) indicated that TH, BFR, and enzyme activity data were not normally distributed. Accordingly, non-parametric statistical tests were used or data were log$_{10}$ transformed prior to statistical analyses. We used Spearman correlation coefficients to assess associations between BFRs and THs. To assess factors associated with placenta BFRs and relationships between BFRs and THs, we used linear regression models (continuous outcome measures were log$_{10}$-transformed). To aid in the interpretation of regression results, we exponentiated beta coefficients ($10^\beta$), producing the multiplicative change in outcome. As predictors of THs and enzyme activity, BFR levels were categorized into tertiles in order to minimize the effect of skewed data and outliers. Given the lower detection frequency of BDE-209, data were dichotomized into those with detectable levels of BDE-209 and non-detects. Analyses were also adjusted for maternal age and gestational age at delivery, factors which we anticipated might confound associations between BFRs and THs. Additionally, we hypothesize that there may be important sex differences in associations between BFRs and THs. To explore these differences we conducted all analyses in the
full cohort (combined group), as well as stratified by infant sex. All sex-specific analyses were conducted using n = 94 due to missing data for one sample. Statistical analyses were performed using JMP Pro 11. A p-value of 0.05 was considered statistically significant.

### 3.3 Results

#### 3.3.1 Population Characteristics
Of the 95 women included in analyses, sixty-eight percent were non-Hispanic black, and 57% were under 25 years old. This was the first pregnancy for 45.5% of the women, and 43.6% of the women had completed more than high school education. Finally, less than 10% of the women have private health insurance. Additional information on the demographic of this population has been reported in Leonetti et al. 2016.

#### 3.3.2 Brominated Flame Retardants
Detailed information on the concentrations of the different PBDE congeners and 2,4,6-TBP are reported in Leonetti et al. 2016. Briefly, detection frequencies for BDE-47, -100, -99, -154, -153, -209, and 2,4,6-TBP were all > 50%. The most common PBDE measured was BDE-47, representing 34% of ΣBDE burden. The geometric mean concentration of 2,4,6-TBP was 15.4 ng/g lipid (range: 1.31 - 316 ng/g lipid), while the geometric mean concentration of BDE-47 was 5.09 ng/g lipid (range: 0.12 - 141 ng/g lipid). Concentrations were generally higher in placental tissues associated with male
infants compared to female infants (Figure 5), but these differences were statistically significant for BDE-209, 2,4,6-TBP, ΣBDE, and ΣBFR (PBDEs plus 2,4,6-TBP) concentrations ($p < 0.01$).

3.3.3 Thyroid Hormones

Thyroid hormones were detected and quantified in 100% of the placenta tissue samples. Descriptive statistics for the TH measurements are provided in Table 9. T4 was measured in the highest concentration with a geometric mean of 28.1 ng/g ww (range:
11.8 - 53.6 ng/g ww), followed by rT3 with a geometric mean of 2.64 ng/g ww (range: 0.73 - 7.59 ng/g ww), and T3 was measured in the lowest concentrations with a geometric mean of 0.37 ng/g ww (range: 0.10 - 0.84 ng/g ww).

In analyses including all placental tissues, BFRs were not correlated with placental T3 or T4 concentrations. However, because we anticipated that the impact of BFRs on TH might vary by infant sex, we stratified the data and examined associations within each group. Among males (n = 48), suggestive negative associations with T3 were observed for BDE-99, 2,4,6-TBP, and ΣBFRs. Conversely, among females (n = 46), a statistically significant positive association was observed between T3 and BDE-99, 2,4,6-TBP, and ΣBFRs, while BDE-47, -153, and ΣBDEs showed a suggestive positive association with T3. Among males, suggestive negative associations with T4 were observed for BDE-99 and BDE-100. Among females, there was no clear trend between T4 and BFR concentrations. Concentrations of rT3 in placental tissue were significantly inversely correlated with BDE-99 ($r_s = -0.34; p = 0.02$) for both males and females (Table 10), while among males, an inverse association was observed between rT3 and BDE-209 ($r_s = -0.35; p = 0.01$).

Adjusted regression analyses were also conducted to assess associations between BFRs and THs while adjusting for potential confounding by maternal age and gestational age at the time of delivery. As in correlation analyses, none of the BFRs were significantly associated with T3 in analyses including all participants. However,
although results generally did not reach statistical significance, BDE-47, -99, and -100 were negatively associated with T3 concentrations among males, while BDE-47, -99, -100, -153 and 2,4,6-TBP were positively associated with T3 among females. For example, among males, those with the highest concentrations of BDE-99 in placenta had T3 levels 0.80 times those with the lowest concentration of BDE-99 (95% confidence interval (CI): 0.59, 1.07). Whereas females with the highest concentrations of BDE-99 in placenta had T3 levels 1.50 times those with the lowest concentration of BDE-99 (95% CI: 1.10, 2.04).

We observed little evidence of association between any of the BFR analytes and T4 concentrations; beta estimates were generally small, imprecisely estimated, and not statistically significant.

For rT3 concentrations, males showed a suggestive negative association with BDE-47, -99, -209, and 2,4,6-TBP, with BDE-99 having the strongest association in the highest tertile ($10^\beta = 0.72$, CI: 0.51, 1.02). In females, there was a suggestive negative trend between rT3 and BDE-47, -99, -100, -153, and 2,4,6-TBP in the highest tertiles, with BDE-99 having the strongest association ($10^\beta = 0.68$, CI: 0.50, 0.92). Finally, there were no observed sex differences in the placenta tissue concentrations of T4, T3, or rT3.

Regression analyses results for THs data are summarized below in Figure 6 - 8.
Table 8: Brominated flame retardant concentrations (ng/g lipid), thyroid hormone levels (ng/g ww), DIO3 activity (pmol rT3/mg protein/min), 3,3’-T2 SULT activity (pmol T2S/mg protein/min), and T3 SULT activity (fmol T3S/mg protein/min) measured in placenta tissue (n = 95). MDL indicates method detection limit.

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3.3.4 DIO3 and SULT Activities

DIO3 activity was measured in all placenta tissue microsome samples. The geometric mean value of DIO3 activity was 0.74 pmol rT3/mg protein/min, with a range of 0.01–3.86 rT3/mg protein/min (RSD = 6.9%). Interestingly, DIO3 activity was significantly higher in placental tissues from male infants compared to female infants ($p < 0.01$). DIO3 activity was negatively correlated with T4 concentrations ($r_s = -0.21; p = 0.04$); however, Spearman correlations were generally not statistically significant. In regression analyses, DIO3 activity among males showed a suggestive positive association with BDE-99, -100, and -153; however, no relationships were statistically significant at the $p < 0.05$ level. In females, DIO3 activity showed a suggestive negative association in the highest tertile for BDE-47, -99, -100, -153, and 2,4,6-TBP, with BDE-99 having the strongest association ($10^{9β} = 0.49$, CI: 0.26, 0.91). These data are summarized below in Figure 9.

TH SULT activity was measured in all placenta tissue cytosol samples. Both 3,3′-T2 and T3 SULT activities were assessed in this study. The geometric mean value of 3,3′-T2 SULT activity was 3.32 pmol T2S/mg protein/min (range: 0.35 – 19.2 pmol T2S/mg protein/min; RSD = 5.7%), while the geometric mean value of T3 SULT activity was 9.50 fmol T3S/mg protein/min (range: 3.59 – 37.9 fmol T3S/mg protein/min; RSD = 6.8%). The 3,3′-T2 SULT enzyme activities were approximately one order of magnitude greater than the T3 SULT activities, which was expected based on their substrate preferences. 3,3′-T2
SULT activity was significantly higher in placental tissues from females compared to males ($p < 0.01$). There was no observed sex difference for T3 SULT activity in placental tissues.

We observed a suggestive negative correlation between BDE-209 and 3,3’-T2 SULT activity ($r_s = -0.19; p = 0.06$) in analyses using all samples. However, 3,3’-T2 SULT activity, was generally not associated with other BFRs. Among male infants, 3,3’-T2 SULT activity was positively associated with rT3 ($r_s = 0.29; p = 0.04$). In combined analyses (males and females) T3 SULT activity was not associated with any BFR analytes. However, among males, T3 SULT activity was positively associated with BDE-47 ($r_s = 0.29; p = 0.04$) and showed suggestive positive associations with BDE-100, -153, and ΣBDEs. There were no statistically significant associations found in the female group, however, there was a suggestive negative association between BDE-99 and T3 SULT. Regression analyses produced similar results. For example, T3 SULT activity among males was positively associated with BDE-47, -100, and -153, with BDE-153 having the strongest association ($10^\beta = 1.48$, CI: 1.05, 2.09 comparing the 3rd to 1st tertile).

As in correlations analyses, T3S activity in females showed a negative association with BDE-99 ($10^\beta = 0.67$, CI: 0.49, 0.91) in adjusted regression models. These data are summarized below in Figure 10 and Figure 11.
Table 9: Spearman correlation coefficients for BFRs, THs, and DIO3 and SULT activity in placenta tissue samples by infant sex (n = 94)

<table>
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<tr>
<th></th>
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<th>BDE-99</th>
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<th>BDE-153</th>
<th>BDE-154</th>
<th>BDE-209</th>
<th>2,4,6-TBP</th>
<th>ΣBFR</th>
<th>ΣBDE</th>
<th>T3</th>
<th>rT3</th>
<th>T4</th>
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<tr>
<td>T3</td>
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<td>0.27*</td>
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<td>-0.11</td>
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* < 0.05, † < 0.10
Table 10: Spearman correlation coefficients for BFRs, THs, and DIO3 and SULT activity in placenta tissue samples (n = 95; combined data, both male and female infants)

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<th>BDE-154</th>
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<th>ΣBFRs</th>
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<th>rT3</th>
<th>T4</th>
<th>DIO3</th>
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\# \(p < 0.001\); \* \(p < 0.05\)
Figure 6: Adjusted model (adjusted for maternal age and gestational age) results indicating the multiplicative change ($10^\beta$) of T3 concentrations measured in placenta separated by infant sex. Horizontal bars reflect the 95% CI and * indicates $p < 0.05$. 
Figure 7: Adjusted model (adjusted for maternal age and gestational age) results indicating the multiplicative change (10β) of rT3 concentrations measured in placenta separated by infant sex. Horizontal bars reflect the 95% CI and * indicates $p < 0.05$. 
Figure 8: Adjusted model (adjusted for maternal age and gestational age) results indicating the multiplicative change ($10^\beta$) of T4 concentrations measured in placenta separated by infant sex. Horizontal bars reflect the 95% CI and * indicates $p < 0.05$. 
Figure 9: Adjusted model (adjusted for maternal age and gestational age) results indicating the multiplicative change (10\(\beta\)) of DIO3 activity measured in placenta separated by infant sex. Horizontal bars reflect the 95% CI and \(*\) indicates \(p < 0.05\).
Figure 10: Adjusted model (adjusted for maternal age and gestational age) results indicating the multiplicative change (10β) of T3 SULT activity measured in placenta separated by infant sex. Horizontal bars reflect the 95% CI and * indicates $p < 0.05$. 
Figure 11: Adjusted model (adjusted for maternal age and gestational age) results indicating the multiplicative change (10β) of 3,3'-T2 SULT activity measured in placenta separated by infant sex. Horizontal bars reflect the 95% CI and * indicates \( p < 0.05 \).
3.4 Discussion

The goals of this study were to measure TH tissue concentrations and endogenous TH DIO3 and SULT activity in placenta tissue subsamples, and to examine their associations with BFRs. This is the first study to measure THs in human placental tissues using liquid chromatography tandem mass spectrometry (LC/MS-MS) and the first study to measure 3,3'-T2/T3 SULT activity in human placental tissues. THs were quantified using LC-MS/MS, which offers greater specificity and sensitivity over RIA-based methods (Wang and Stapleton 2010). To our knowledge, THs have only been measured in placenta in one previous study in a cohort of 16 women with normal pregnancies using an RIA-based method. This study reported values for T4, T3, and rT3 (18.8, 0.03, and 1.70 ng/g tissue, respectively) (Yoshida et al. 1987), similar to levels reported here. Serum T4/T3 ratios are widely used in the clinical setting to evaluate TH status in individuals; however, the measurement of THs in specific tissues is not widely used, and there is not much known about the TH status of specific tissues such as the placenta, nor how it changes during pregnancy.

In general, our results suggest that the concentrations of BFRs in the placenta may be associated with lower levels of rT3. Associations were also observed for T3, but differed by sex (negative associations for males and positive associations for females). Previous pregnancy studies have reported all combinations of positive, negative, and no associations between serum PBDE and TH concentrations. For example, in a recent
study by Abdelouahab et al., PBDEs and THs were measured in maternal blood and umbilical cord blood at two different time points during gestation. They study observed negative associations between PBDEs and Total T4 (TT4) and Total T3 (TT3), as well as positive associations between PBDEs and free T4 and free T3 in maternal serum collected at less than 20 weeks gestation. However, these associations reversed for free T4 and free T3 in maternal blood collected at the time of delivery (Abdelouahab et al. 2013). And a 2010 study by Stapleton et al., observed positive associations between PBDEs and TT3, TT4, and free T4 in maternal blood samples collected during the third trimester (Stapleton et al. 2011). Finally, there are several studies that found no associations between PBDEs and THs measured in maternal serum samples collected during the second trimester, collected at delivery, or in umbilical cord blood (Mazdai et al. 2003; Zhang et al. 2010; Zota et al. 2011). Human epidemiological studies provide important information; however, they may not be directly comparable to each other due to differences in their experimental designs and methods. Additionally, there exists a great deal of inter-individual variability in TH set-points around which TH homeostasis operates, which may contribute variability to these studies. These differences are magnified during pregnancy, and this contributes to the difficulty in determining a normal TH profile for a pregnant mother (serum concentrations), and perhaps within the placenta tissue itself (Glinoer 1999).
We observed statistically significant negative associations between rT3 and BDE-99 tissue concentrations in all samples. rT3 is the genomically inactive form of TH that is formed from the inner ring deiodination (IRD) of T4 by type 1 DI (DIO1) or DIO3. DIO3 is one of three DI enzyme isoforms that only performs IRD. The placenta exhibits high DIO3 expression in early pregnancy, and total DIO3 activity increases significantly throughout gestation until birth (Koopdonk-Kool et al. 1996). It is thought that DIO3 plays an important role in buffering the maternal supply of T4 within the placental tissue compartment before being transported to fetal circulation, as well as provides a source of iodide for the fetus via deiodination (Forhead and Fowden 2014; Kilby et al. 2004). As a result, DI enzyme inhibition by environmental contaminants such as PBDEs and their metabolites may lead to reductions in T4 deiodination, causing higher levels of T4 and lower levels of rT3 in placental tissues. Such inhibition has been shown using *in vitro* models with human liver cytosol and human glial cells (Butt et al. 2011; Roberts et al. 2015). This mechanism may contribute to the observations in this study, as higher concentrations of BDE-99 were associated with lower DIO3 activity and lower concentrations of rT3.

While many other studies have measured TH, dopamine, and 4-nitrophenol sulfation in other tissues (Mitra and Audus 2009; Richard et al. 2001), this work is the first to evaluate TH SULT activity in human placental tissues. We chose to evaluate SULT activity because *in vitro* work from our laboratory using pooled human liver
cytosol indicated that TH SULT enzymes are more sensitive to inhibition by BFRs compared to DIO activity (Butt and Stapleton 2013). However, 3,3’-T2 SULT activity did not show any statistically significant associations with BFRs. The variability in 3,3’-T2 SULT activity amongst males is much higher than among females, and may provide some insight into the sex differences in placental SULT expression and activity.

T3 SULT activity was also measured in the samples. The T3 SULT enzyme activity rates in this study were a full order of magnitude lower than that of 3,3’-T2. These results are consistent with other studies that have compared the sulfation rates of the different iodothyronines in various tissues types, with 3,3’-T2 being the preferred substrate for sulfation and having an activity rate 1-2 orders of magnitude greater than T3 (Kester et al. 1999; Stanley et al. 2001; Visser et al. 1998). As such, 3,3’-T2 is used as a surrogate model for T3 sulfation kinetics in in vitro experiments (Schuur et al. 1998a). The positive association between 3,3’-T2 and T3 SULT activity observed here supports this claim. However, T3 sulfation plays a greater biological role in regulating the availability of T3 for subsequent binding to thyroid receptors (Darras et al. 1999). Sulfation of T3 is a reversible reaction that inactivates the biological activity of T3 and is thought to create a reservoir of T3 that can be reactivated following hydrolysis by ARSs in a tissue-specific manner (Kilby et al. 2004). This metabolic pathway is a potentially important step for buffering the concentrations of T3 in the placenta before delivery to the fetal compartment. In this study, T3 SULT enzyme activities showed a significantly
positive association with BDE-47 in males ($r_s = 0.29$, $p = 0.04$), and a suggestive negative association with BDE-99 in females ($r_s = -0.25$, $p = 0.09$). Additionally, we observed a positive association for the highest tertile of BDE-153 exposure in males ($10^{\beta} = 1.48$, $p = 0.028$), and a negative association in the highest tertile of BDE-99 exposure in females ($10^{\beta} = 0.67$, $p = 0.013$) in our adjusted models. This may suggest that BDE-99 is inhibiting T3 sulfation in placental tissues and leading to an increase in T3; however, it is unclear why this was only observed in placental tissues from females.

One interesting finding from this study is that BFR levels in placenta, and their associated impacts may be dependent upon infant sex. Sex-specific effects have been previously observed for serum perfluoroalkyl substances (PFASs), such as perfluorooctanoate (PFOA) and perfluorooctane sulfonate (PFOS), which were differentially associated with serum TH levels. For example, one study examined the relationships between serum PFAS concentrations and testosterone (T), TSH, and free and total T3 and T4 among males and females measured in the 2011-2012 NHANES cycle. This study observed positive associations between PFASs and FT3, TT3, FT4 in adult women and no associations in adult men (Lewis et al. 2015). Additionally, Wen et al. examined PFASs and TH concentrations using NHANES data from 2007-2010 and observed positive associations between PFOA and perfluorohexane sulfonate (PFHxS) with TT3 in females, and inverse associations between PFHxS and FT4 in males (Wen et al. 2013). Finally, prenatal exposure to DDE, PFOS, and PFOA were also examined in a
Dutch prospective cohort and they found that mean T4 levels were highest in females in the highest DDE and PFOA exposure quartile, while males in the second quartile of exposure for PFOS and PFOA had lower levels for T4 (de Cock et al. 2014). These previous studies are similar to our current results in that we observe a negative trend for THs with BFRs in males, and a positive trend for THs in females. Although these are different classes of chemicals, BFRs and PFASs share similar endocrine disrupting capacities and have both been shown to impact the HPT axis. The mechanisms underlying the sex-specific effects of TH disruption are unknown; however, it is known that sex hormones can modulate TH levels. For example, the serum half-life of TBG is increased by the action of estrogen, by increasing the posttranslational sialylation of TBG and reducing its clearance rate (Ain et al. 1987). Higher serum TBG concentrations lead to a decrease in serum FT4 concentrations and a complementary increase in TSH production, which eventually leads to the increased production of T4 by the thyroid gland, raising total serum T4 levels (Tahboub and Arafah 2009). Sex differences have been observed in animal studies, in which the biological half-lives of some chemicals were found to be up to 70 times longer in males versus females (Kudo et al. 2002; Lee and Schultz 2010). Similar sex-differences have been shown with PFOS in humans, with 30% of the differences in half-lives being attributed to elimination via menstruation, however, the remaining underlying mechanisms are unknown at this time (Wong et al. 2014). The observed concentrations of all BFR analytes were higher in placenta tissues
from male infants than in placenta tissue from female infants (Figure 5). It is possible that this observation may be driven by differences in the lipid content of the placenta tissues, however, we observed no significant differences in lipid content of the placenta tissue samples by infant sex. The underlying mechanism of this sex-specific difference is not known at this time, however, the sex of the infant seems to play a role in the bioaccumulation potential, metabolism, or kinetic parameters of the placenta during pregnancy. It is possible that the sex-specific differences in placental morphology and structure may play a role in this organ’s ability to transfer and metabolize BFRs (Rosenfeld 2015).

One limitation of this study is the nature of the placenta tissue subsamples. First, the tissues were harvested from different sections of the organ following delivery. This means that some tissue subsamples were taken from the peripheral sections of the placenta, while others were taken from more central locations. Due to the heterogeneous nature of the placenta, as well as spatial differences in cell type, vasculature, and lipid content of the organ, the samples analyzed in this study may not be equally representative. Additionally, this current study only quantified PBDEs and 2,4,6-TBP in placental tissues. Future work should measure other environmental contaminants that have been shown to disrupt TH function (ie. PCBs, OH-BDEs, etc.) and evaluate the net effects of exposure and bioaccumulation of these complex mixtures within the placenta.
The current study is the first to compare placental tissue concentrations of THs (instead of serum) with BFR concentrations in a human cohort, as well as one of the first to measure placental tissue DIO3 and SULT activity. It is known that THs are transported in the blood bound to transport proteins such as TTR and TBG before uptake into cells in peripheral tissues via TH transport proteins such as MCTs, LATs, and OATPs (Gereben et al. 2008). However, little is known about the tissue concentrations of THs after uptake and subsequent metabolism by DIs or SULTs. The tissue concentrations of THs are important in facilitating TH-mediated genomic activity, and in the case of the placenta, important in regulating the maternal supply of THs before delivery to fetal circulation. The results of this study indicate that placental TH concentrations may be sensitive to BFR tissue concentrations in a sex-specific manner. Future studies should evaluate TH concentrations, as well as other TH-related endpoints within the placenta tissue in order to broaden our understanding of BFR effects on TH homeostasis during pregnancy.
4. Disruption of Thyroid Hormone Sulfotransferase Activity by Brominated Flame Retardant Chemicals in the Human Choriocarcinoma Placenta Cell Line, BeWo

4.1 Introduction

The examination of BFR effects on TH metabolism in human placenta tissue samples (Chapter 3) provides insight into the complex nature of TH biology in vivo. However, given the numerous confounding variables present in human studies, the use of a more controlled in vitro experimental system could potentially provide additional clarity into the underlying mechanisms of action following BFR exposures. For this research Aim, the human placenta cell line, BeWo, was used to specifically evaluate the effects of BFR exposure on TH SULT activity.

The BeWo cell line is a malignant human choriocarcinoma cell line derived from a methotrexate-resistant tumor from a male placenta (Pattillo et al. 1968). Approximately 96-99% of BeWo cells are cytotrophoblast-like cells while the remaining cell population consists of syncytiotrophoblast-like cells. The cytotrophoblast-like cells are mononucleated, moderate in size, and highly proliferative, while the syncytiotrophoblast-like cells are much larger, multinucleated, and nonproliferative. Studies have confirmed the BeWo cell line to be ultrastructurally similar to in utero trophoblast cells, indicating that they are an appropriate model for placenta cell culture experiments (Friedman and Skehan 1979). An overview of the different placental cell types is shown below in Figure 12. Additionally, because their cell type composition is
dominated by cytotrophoblasts, the BeWo cell line is more similar to an early-stage/first trimester placenta before subsequent differentiation and proliferation of syncytiotrophoblasts. This is important because the early-stage placenta represents the most vulnerable developmental window of exposure for the fetus. Thus the use of the BeWo cell line can provide insight into this difficult-to-study developmental period.

Figure 12: Overview of placental morphology showing the interface of placental and maternal tissues. Trophoblasts are in direct contact with maternal blood supply and are composed of an outer layer of syncytiotrophoblasts with an underlying layer of cytotrophoblasts (Gray, Henry. Anatomy of the Human Body. 1918.)

The BeWo cell line has been used to investigate several regulatory processes and endpoints related to TH homeostasis, such as the expression and kinetic parameters of
TH transporters (LATs and OATPs), the presence and activity of cytochrome P450 enzymes, iodide transport, expression of TH receptors, and the expression and activity of SULT enzymes (Avery et al. 2003; J and P 2001; Li et al. 2011; Mitra and Audus 2009; Vattai et al. 2015; Yang et al. 2014). With regard to SULT enzymes, the expression of various SULT isoforms was investigated by RT-PCR and it was shown that SULT1A1, 1A3, 1C1, 1C2, and 2A1 were expressed in BeWo cells, with SULT1A3 and SULT1C2 showing the highest levels of expression. Additionally, there were no detectable levels of SULT1B1, 2B1, or 1E1 in BeWo cells (Tamura et al. 2001). The expression of SULTs in BeWo cells and primary placenta cells was also investigated by RT-PCR in a more recent study, which measured SULT1A1, 1A3, 2A1 and very low levels of 1E1 (Mitra and Audus 2009). SULT1A1 mRNA was expressed more abundantly in BeWo cells compared to primary cytotrophoblast cells, SULT1E1 was equivalently expressed between the two cell types, and SULT2A1 was abundantly expressed in BeWo cells but not in the primary cytotrophoblast cells. This study also measured the sulfation kinetics of nitrophenol and dopamine in combination with the SULT inhibitors, 2,6-dichloro-4-nitrophenol and sodium chloride. However, to our knowledge, there have been no studies conducted using THs as a substrate in the study of SULT kinetics in the BeWo cell line.

Investigating the effects of BFRs on TH SULT activity in BeWo cells is of great interest due to the important role these enzymes play in the maintenance of THs in the
placenta during pregnancy. In addition to their important role in buffering and regulating tissue concentrations of THs, it has been shown that SULT enzymes are highly sensitive to enzyme disruption by environmental contaminants using *in vitro* models. For example, a comprehensive evaluation of 27 BFRs revealed that many compounds were capable of inhibiting estrogen sulfation, with TBBPA showing the highest potency ($IC_{50} = 0.016 \, \mu M$) (Hamers et al. 2006). One additional study used a crystallographic analysis to show that TBBPA and 3-OH BDE-47 are capable of mimicking estradiol and binding to SULT enzymes via a flexible substrate binding pocket (Gosavi et al. 2013). Furthermore, SULTs were shown to be much more sensitive to enzyme disruption than DI enzymes using human hepatic tissues. For example, the calculated $IC_{50}$ value for 2,4,6-TBP on TH DI activity in human liver microsomal fractions was 315 $\mu M$ (Butt et al. 2011). However, the calculated $IC_{50}$ value for 2,4,6-TBP on 3,3’-T2 SULT activity in pooled human liver cytosol was 8.3 nM (Butt and Stapleton 2013). This represents a significant difference in the potency of environmental contaminants on DI and SULT activities, and suggests that SULT enzymes may act as a more sensitive biomarker of BFR exposure in peripheral tissues such as the placenta. In Chapter 3 we observed significant associations between BFRs and endogenous SULT activity in human placenta tissue subsamples; however, it is impossible for us to determine if this is a direct or indirect effect. Thus, the goal of this study was to further investigate specific effects of BFRs on TH SULT activity using the BeWo cell line as a
surrogate for placental cytotrophoblast cells. I hypothesize that BFRs will inhibit TH SULT activity and lead to reductions in the abundance of T3 and T4 in cells, which may have downstream effects on development.

4.2 Materials and Methods

4.2.1 Reagents and Materials

2,4,6-TBP (99% pure), T4 (> 99%), T3 (> 99%), rT3 (> 99%), 3,3’-T2 (> 99%), and adenosine 3’- phosphate 5’-phosphosulfate lithium salt hydrate (> 60%) were purchased from Sigma-Aldrich (St. Louis, MO). BDE-47 (> 97%), BDE-99 (> 97%), 3-OH 2,2′,4,4′-BDE (3-OH BDE-47; 99%), and 6-OH 2,2′,4,4′-BDE (6-OH BDE-47; 100%) were purchased as neat standards from AccuStandard (New Haven, CT). [13C6]-3,3’-T2 was purchased from Isotec (Miamisburg, OH). 3,3’-T2 sulfate (3,3’-T2S; 98%) was custom synthesized by the Duke University Small Molecule Synthesis Facility (Durham, NC). T3 sulfate (T3S) was purchased from Toronto Research Chemicals (Toronto, ON; 98.6%).

Ham’s F-12K (Kaighn’s Modification) Medium cell culture media and other cell culture reagents were purchased from Life Technologies (Carlsbad, CA). All solvents and other reagents were purchased from VWR (Radnor, PA). BeWo cells (ATCC® CCL-98™) were obtained from the Duke University Cell Culture Facility (Durham, NC). All cells were grown in TC treated flasks (75 and 182 cm²) and dishes (36.3 cm²) from Genesee Scientific.
4.2.2 Cell Culture

BeWo cells were grown in Ham’s F-12K (Kaighn’s Modification) Medium cell culture media 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, and all replicate experiments were performed with cells from different aliquots of the same source of cells in order to ensure and evaluate reproducibility. Cells were thawed and plated in T-75 flasks, media was changed every 48 h, and then cells were passaged and transferred to T-182 flasks for growth (1:3 passage ratio used; passage at 80% confluency/every 96 h).

4.2.3 Dosing

Dosing of BeWo cells occurred after growing a sufficient number of cells for the exposure experiments (nine T-182 flasks). Cells were passaged and transferred to cell culture dishes and seeded at a density of 1.0 x 10\(^6\) cell cm\(^{-2}\). Cells were allowed to grow for 36 hours, after which point the growth media was replaced with the appropriate dosing media. After the allotted exposure period (1, 6, 12, or 24 h), the growth media was collected and stored for TH analysis and verification of dosing concentrations. Cell culture dishes were rinsed with Dulbecco’s Phosphate Buffered Saline without calcium chloride and magnesium chloride (Sigma-Aldrich). Cell culture dishes were then loaded with 1 mL of phosphate sucrose buffer (KPO\(_4\) buffer containing 0.25 M sucrose and 1
mM EDTA) and stored at -80°C for future analysis. An overview of the experimental
design for the cell culture exposure experiments is shown below in **Figure 13**.

The low dose for 2,4,6-TBP, BDE-47, and BDE-99 was 0.05 µM (50 nM) in order to
represent the 95th percentile of human serum concentrations measured in the United
States (Stapleton et al. 2008b). The low dose for 3-OH BDE-47 and 6-OH BDE-47 was
0.001 µM (1 nM) in order to represent the 95th percentile of human serum concentrations
measured in the United States (Chen et al. 2013). The high and medium doses were the
same for all BFR compounds tested (1.0 µM and 0.5 µM, respectively). All dosing
compounds were dissolved in DMSO at 1,000 times higher than the desired final
concentration in the cell culture media to ensure a concentration of < 0.1% DMSO. The
concentrations of the compounds in the dosing media were verified using GC-MS and
LC-MS/MS methods (Erratico et al. 2010; Stapleton et al. 2008a).
4.2.4 Cytotoxicity

Cytotoxicity was assessed using a resazurin cell viability assay. Cells were dosed for 24 hours and then the reduction of resazurin to resarufin over 60 minutes was analyzed. Control cells were grown with and without DMSO as a comparison to treated cells.
4.2.5 Basal Sulfotransferase Activity Assays

Basal SULT activity was measured in BeWo cells after exposure (1, 6, 12, or 24 h) to low, medium, or high doses of BFRs, as well as in DMSO controls. The high and medium dose used for all analytes was 1 µM and 0.5 µM. The low dose for 2,4,6-TBP, BDE-47, and BDE-99 used was 0.05 µM, while the low dose for 3-OH BDE-47 and 6-OH BDE-47 was 0.001 µM. A different low dose was used for the hydroxylated PBDEs in order to maintain environmental relevance of the low dose exposures, because these metabolites are measured at approximately 10x lower concentrations than the parent compounds. Cells were scraped from tissue culture dishes into 1 mL of KPO₄ buffer containing 0.25 M sucrose and 1 mM EDTA. The 1 mL of phosphate buffer with BeWo cells was split into three aliquots: 500 µL and two 250 µL aliquots. One 250 µL aliquot was frozen at -80°C for future mRNA extraction and the other 250 µL aliquot was centrifuged in order to pellet the cells and then resuspended in RNA isolation buffer (RNeasy, QIAGEN). The 500 µL aliquot was sonicated for 10 seconds on ice before being stored at -80°C for future use in the basal SULT activity assay. The 3,3′-T2 SULT assays were performed using whole cellular homogenates. The SULT assays were performed as previously described in Chapter 3. Assays were performed for 30 minutes, after which time 1 mL of 1 M HCl was added to stop the reaction mixture, and samples were spiked with 5 ng of stable isotope-labelled surrogate standard ([¹³C₆]-3,3′-T2). The reaction mixtures were extracted using Agilent OPT Solid Phase Extraction tubes and analyzed.
using LC-MS/MS following previously published analytical methods (Butt and Stapleton 2013). The total mass of 3,3'-T2-sulfate (T2S) formed in each sample was normalized to the value of DMSO controls within each experiment, and all values are presented as a 3,3'-T2 SULT activity percent relative to control.

4.2.6 Cell Culture Growth Media Analysis – THs and Sulfated/Hydroxylated Metabolites

The concentrations of T4, T3, rT3, and 3,3'-T2 were measured in 2 mL aliquots of cell culture media from each exposure dish. The cell culture media was acidified by adding 1 mL of 1 M HCl and a suite of mass-labeled TH standards were added (5 ng each of $^{13}$C$_{12}$-T4, $^{13}$C$_{6}$-T3, $^{13}$C$_{6}$-rT3, and $^{13}$C$_{6}$-T2). The sample extracts were then cleaned using SampliQ OPT SPE cartridges (Agilent Technologies). For clean-up and extraction, SPE columns were first conditioned with 3 mL of methanol and 3 mL of water and then the samples were loaded. The columns were rinsed with 3 mL of water and the THs were eluted in 4 mL of methanol. The final extract was reduced to 50 µL under a gentle stream of nitrogen gas. The samples were reconstituted in 250 µL of 1:1 methanol/water and transferred to UniPrep filter vials before analysis by LC-MS/MS. The data represent total concentrations of each TH measured (free and bound fractions; ng/mL).

OH-BDEs were extracted and analyzed using a previously developed method from our laboratory. Briefly, cell culture growth media was spiked with internal standards (2.5 ng of $^{13}$C-6-OH BDE-47), acidified with 2 mL of formic acid, and vortexed for 30 seconds. The acidified growth media was then vortexed again with the addition of
6 mL H₂O before 20 minutes of water bath sonication. Samples were then extracted using Water Oasis Hydrophilic-Lipophilic-Balanced (HLB) columns (500 mg, 6 mL). Columns were conditioned with 5 mL of DCM, MeOH, and H₂O before loading the sample. Samples were washed with 5 mL of H₂O and then the columns were completely dried with N₂ gas under positive pressure. Samples were then extracted with 10 mL of 50:50 DCM:ethyl acetate and extracts were blown down to near dryness and reconstituted in approximately 250 μL MeOH for analysis by LC-MS/MS.

Cell culture media was also screened for the presence of sulfated metabolites following exposure. Sulfated conjugates were extracted exactly as THs and analyzed by LC-MS/MS. There are currently no available standards for sulfated 2,4,6-TBP, 3-OH BDE-47, or 6-OH BDE-47, so quantitative analysis was not possible. The presence of sulfated metabolites was assessed by inspection of the chromatograms for a peak that had the molecular weight of a presumed sulfated conjugate. The following m/z transitions were used for 3-OH BDE-47 and 6-OH BDE-47: 578.8 > 420.6 and 578.8 > 81.3. For 2,4,6-TBP-sulfate, the following m/z transitions were used: 406.9 > 326.8 and 406.9 > 81.3.

4.2.7 Enzyme Kinetics and Inhibition Assays

Enzyme kinetic experiments were conducted using BeWo cell homogenates. Cells were grown under normal conditions and harvested after reaching > 80% confluency. To harvest, cells culture flasks were rinsed twice with 3 mL of Dulbecco’s
phosphate buffered saline (no calcium chloride, no magnesium chloride). Following the saline rinse, 2 mL of 0.25% trypsin-EDTA (gibco Life Technologies) was added to each flask and mixed to cover the entire flask bottom surface area. The flasks were then incubated at 37°C for five minutes to allow for adequate cell dissociation. Following trypsinization, flasks were rinsed with 10 mL of growth media and cells were transferred to a 15 mL centrifuge tube. Cells were pelleted at 200 g for three minutes, after which the remaining trypsin-media fraction was poured off. Cells were then resuspended in 6 mL of cold 0.1 M phosphate sucrose buffer. At this time, the suspended cells were separated into 1 mL aliquots and sonicated (7 seconds sonication, 3 second pause, 7 second sonication) on ice. The sonicated BeWo cell homogenates were then recombined and stored at -80°C for future use in the SULT assays.

Competition binding (inhibition) assays were performed using 0.1 M potassium phosphate buffer (pH 7.2) with 50 µM PAPS as the cofactor and 1 µM 3,3'-T2 as the substrate (total volume of 200 µL). Stock solutions of competitors were prepared in DMSO at concentrations 1000 times higher than the desired dosing concentrations in order to ensure a DMSO concentration of less than 0.1% of the incubation volume. For these experiments, the following BFR analytes were tested for SULT enzyme inhibition: 2,4,6-TBP, BDE-47, BDE-99, 3-OH BDE-47, and 6-OH BDE-47. A wide range of inhibitor concentrations were tested in order to fully examine inhibition potential. Briefly, 2,4,6-TBP was tested at 15 concentrations (range: 0.0001 µM – 100 µM), BDE-47 and BDE-99
were tested at ten different concentrations (range: 0.01 µM – 100 µM), 3-OH BDE-47 was tested at nine different concentrations (range: 0.001 µM – 2.0 µM), and 6-OH BDE-47 was tested at six different concentrations (range: 0.01 µM – 1.0 µM). All reactions were spiked with 1 µL of the corresponding inhibitor dosing stock (1 µL of DMSO spiked into control samples). All inhibitor concentration levels were performed in duplicate sets, while each set of DMSO control samples was performed in sets of four. Assay reactions were initiated with the addition of the BeWo cell homogenate (approximately 800 µg of protein/mL) and vials were incubated at 37°C for 30 minutes in a shaking water bath. Following incubation, the reactions were stopped with the addition of 800 µL of 0.1 M HCl and samples were then spiked with [13C6]-3,3′-T2 (5 ng) as the internal standard. Sample extracts were then cleaned using SampliQ OPT SPE cartridges (Agilent Technologies). For clean-up and extraction, SPE columns were first conditioned with 3 mL of methanol and 3 mL of water and then the samples were loaded, the columns were rinsed with 3 mL of water, and the THs and sulfated TH conjugate were eluted in 4 mL of methanol. The final extract was reduced to 50 µL under a gentle stream of nitrogen gas. The samples were reconstituted in 250 µL of 1:1 methanol/water and transferred to UniPrep filter vials before analysis by LC-MS/MS. The total 3,3′-T2-sulfate conjugate product formation (ng) was quantified for each vial. The mean value for the DMSO control samples (n = 4) was calculated for each experimental batch, and all inhibitor concentration levels were normalized to the control values to yield a final value of 3,3′-
T2-sulfate formation as a percent value relative to control for each vial. All experiments were repeated at least two times total using different batches of cells in order to evaluate reproducibility between different cell populations and day-to-day experimental variability. If no differences were observed among replicates, the replicate experiments were combined to produce a single inhibition curve, where each data point represents the mean value of percent inhibition \( (n = 4; \text{error bars represent SEM}) \). The inhibition curves and half maximal inhibitory concentrations (IC\(_{50}\)) for each analyte were calculated using Sigma Plot 12.0.

Michaelis-Menten kinetics of 3,3’-T2 sulfation were also investigated in BeWo cell homogenates. For these experiments, a fixed concentration of inhibitor (2,4,6-TBP or 3-OH-BDE-47) was used, while the substrate (3,3’-T2) concentration was varied. The inhibitor concentration for these analyses was chosen based on the results of the inhibition curve experiments. For example, the inhibitor concentration used for 2,4,6-TBP and 3-OH BDE-47 was based on the calculated IC\(_{50}\) values, in order to use a concentration that falls within the linear dynamic range of enzyme kinetics within the cell homogenate. A constant dose of 10 nM and 50 nM was used for 2,4,6-TBP and 3-OH BDE-47, respectively, in these experiments based on the previously calculated IC\(_{50}\) values. The reaction buffer was made before each experiment using 0.1 M potassium phosphate buffer (pH 7.2) and 50 µM PAPS as the cofactor, along with the appropriate dose of inhibitor or DMSO control spike (<0.1% DMSO). The reaction buffer was then
separated into seven aliquots and each was spiked with the appropriate substrate (3,3’-T2) concentration (range: 0.05 – 5 μM). All substrate concentration levels were performed in duplicate sets. Assay reactions were initiated with the addition of the BeWo cell homogenate and vials were incubated at 37°C for 30 minutes in a shaking water bath. Following incubation, the reactions were stopped with the addition of 0.1 M HCl (800 µL) and samples were then spiked with [13C6]-3,3’-T2 (5 ng) as the internal standard. Sample extracts were then cleaned using SampliQ OPT SPE cartridges as previously described. The total 3,3’-T2-sulfate conjugate product formation (ng) was quantified for each vial. Additionally, the protein concentration (mg) of the BeWo cell homogenate used in each experiment was determined using the Bradford assay. Finally, the data were calculated and expressed as pmol of 3,3’-T2-sulfate formed per milligram of protein per minute (pmol T2S/mg protein/min). The 3,3’-T2 sulfation showed typical Michaelis-Menten enzyme kinetics and the kinetics curves and parameters (Km and Vmax) were calculated using Sigma Plot 12.

4.2.8 mRNA Expression Analysis

Total RNA was extracted from a 250 µL aliquot of the scraped cells (25% of the total cells grown in the dish) using the Quick-RNA MicroPrep kit from Zymo Research (Irvine, CA) and RNA content was quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific). Total RNA was converted to cDNA using the High Capacity cDNA reverse transcription kit from Life Technologies (Carlsbad, CA)
according to the manufacturer’s methods. Approximately 10 ng of cDNA was analyzed
in 20 µL qPCR reactions using TaqMan Gene Expression Assays (Life Technologies) and
an Applied Biosystems 7300 Real-Time PCR system (Foster City, CA). The genes
investigated in this study were: Dio3 (Hs00956431_s1), TR-α (Hs00268470_m1), TR-β
(Hs00230861_m1), SULT1A1 (custom assay), SULT1C2 (Hs00602560_m1), SHBG
(Hs01050182_g1), RPL13A (Hs04194366_g1), and β-actin (Hs01060665_g1). The threshold
cycles (Ct) of RPL13A and β-actin were compared in an initial experiment to determine
the best reference gene, and β-actin was selected as the internal reference gene due to its
stable expression between control cells and different treatment groups. Expression
values are reported as the expression ratio relative to control samples within each
experiment normalized to β-actin using the $2^{\Delta\Delta Ct}$ method.

4.2.9 Statistics

ANOVA tests were performed using Sigma Plot 12 to test for significant effects
of treatment and experimental day. Significant effects were further tested using Tukey’s
post-hoc test. All experiments were performed with 2-4 samples and repeated on a
separate day. When there was no significant effect of the experimental day, the results
from the individual experiments were combined for a total of 4-8 samples per treatment
group. The results for each experiment were first normalized to the control values
within that experiment and then combined with the results from the corresponding
repeated experiment.
The mRNA expression results were analyzed using ΔΔCt values and reported graphically as the $2^{\Delta\Delta\text{Ct}}$ expression ratios.

4.3 Results

4.3.1 Competition Binding Assays

Inhibition assays were performed for BDE-47, BDE-99, 2,4,6-TBP, 3-OH BDE-47, and 6-OH BDE-47. However, inhibition curves and IC$_{50}$ values were only calculated for 2,4,6-TBP, 3-OH BDE-47, and 6-OH BDE-47 because BDE-47 and BDE-99 displayed no inhibition in the BeWo cell homogenates (Figure 14). The calculated IC$_{50}$ values for 2,4,6-TBP, 3-OH BDE-47, and 6-OH BDE-47 are 11.6 nM (95% CI: 9.22 – 14.0), 48.9 nM (95% CI: 46.6 – 51.2) and 395.5 nM (95% CI: 391.6 – 399.4), respectively.
Table 11: Comparison of measured IC₅₀ values (nM) between BeWo cell homogenates and pooled human liver cytosol for various BFR compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>BeWo Homogenate</th>
<th>Pooled Human Liver Cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀ (this study)</td>
<td>IC₅₀ (Butt et al., 2013)</td>
</tr>
<tr>
<td>BDE-47</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>BDE-99</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>2,4,6-TBP</td>
<td>11.6 nM</td>
<td>8.3 nM</td>
</tr>
<tr>
<td>6-OH BDE-47</td>
<td>395.5 nM</td>
<td>130 nM</td>
</tr>
<tr>
<td>3-OH BDE-47</td>
<td>48.9 nM</td>
<td>60 nM</td>
</tr>
</tbody>
</table>

Figure 14: Inhibition of 3,3'-T2S formation resulting from the incubation of BeWo cell homogenates with 1 μM 3,3-T2 and various BFR compounds. Data points represent the mean (n = 4) and error bars represent ±SEM.
4.3.2 Competition Binding Assay – Michaelis-Menten Curves

As 2,4,6-TBP and 3-OH-BDE-47 were found to be more potent inhibitors of 3,3’-T2 SULT activity, further experiments were conducted to examine the specific Michaelis-Menten kinetics and determine whether or not the competition was direct or indirect. Interestingly, inhibition was observed at the highest substrate concentration tested (5 µM 3,3’-T2). This type of substrate inhibition is characteristic of SULT enzyme kinetics (James 2014). In order to calculate the kinetic parameters, and for clarity, these data points were omitted from the figures presented below. The apparent $K_m$ for the 3,3’-T2 sulfation reaction in the control BeWo cell homogenates was $0.61 \pm 0.10$ µM and the $V_{max}$ was $4.52 \pm 0.27$ pmol T2S/mg protein/min. The apparent $K_m$ for the 3,3’-T2 sulfation reaction in the 2,4,6-TBP exposed cells was $1.46 \pm 0.20$ µM and the $V_{max}$ was $2.29 \pm 0.15$ pmol T2S/mg protein/min. The apparent $K_m$ for the 3,3’-T2 sulfation reaction in the 3-OH BDE-47 exposed cells was $1.29 \pm 0.16$µM and the $V_{max}$ was $3.42 \pm 0.20$ pmol T2S/mg protein/min. The type of inhibition (competitive vs noncompetitive) was investigated by comparing these calculated Michaelis-Menten parameters. The calculated $V_{max}$ and $K_m$ for 2,4,6-TBP and 3-OH BDE-47 showed a statistically significant difference from the control parameters. The $V_{max}$ for both compounds was significantly lower than the controls, while the $K_m$ for both compounds was significantly higher. This observed decrease in $V_{max}$ and increase in $K_m$ is indicative of mixed type inhibition in the BeWo
cell homogenates for 2,4,6-TBP and 3-OH BDE-47. These results are summarized below in Figure 15 and Table 12.

![Figure 15: Rate of formation (picomoles per minute per milligram of protein) of 3,3'-T2 sulfate resulting from the incubation of 3,3'-T2 in BeWo cell homogenates for 30 min. The Michaelis constant ($K_m$) and the maximal reaction rate ($V_{max}$) were obtained from nonlinear regression analysis. Each data point represents the mean ($n = 4$) and error bars represent ±SEM.](image)
Table 12: Michaelis-Menten parameters for BeWo cell homogenates ($V_{\text{max}}$ (pmol/T2S/mg protein/min) and $K_m$ (μM 3,3'-T2)).

<table>
<thead>
<tr>
<th>Michaelis-Menten Parameters for BeWo Cell Homogenates</th>
<th>$V_{\text{max}}$</th>
<th>$K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.52</td>
<td>0.61</td>
</tr>
<tr>
<td>3-OH BDE-47</td>
<td>3.42</td>
<td>1.29</td>
</tr>
<tr>
<td>2,4,6-TBP</td>
<td>2.29</td>
<td>1.46</td>
</tr>
</tbody>
</table>

4.3.3 Cell Culture Exposure Experiments

The following BFR analytes were evaluated for their effects on basal 3,3'-T2 SULT activity in cell cultures exposed for 24 hours: 2,4,6-TBP, BDE-47, BDE-99, 3-OH BDE-47, and 6-OH BDE-47. The cell culture exposure data are presented as the percent of 3,3'-T2-sulfate formation relative to control. All data presented are the combined data of two replicate experiments ($n = 6$) performed on different days and using different batches of BeWo cells. Each bar represents the measured basal 3,3'-T2 SULT activity at each dose relative to the control samples and the error bars represent ± SEM. Results from the 24 h cell culture exposure experiments are summarized below in Figure 16.

There was a statistically significant reduction in basal 3,3'-T2 SULT activity for all BFR analytes in the high and medium doses. Additionally, there was a statistically significant reduction in basal 3,3'-T2 SULT activity in the low dose for 2,4,6-TBP.
Treatment of the BeWo cells with 1.0 µM, 0.5 µM, and 0.05 µM 2,4,6-TBP for 24 hours significantly decreased the 3,3’-T2 SULT activity measured in the cell homogenate by 86.0 ± 0.28%, 70.6 ± 1.78%, and 17.0 ± 0.93%, respectively. Treatment of the BeWo cells with 1.0 µM and 0.5 µM BDE-99 for 24 hours significantly decreased the 3,3’-T2 SULT activity measured in the cell homogenate by 38.4 ± 2.4% and 26.4 ± 3.7%, respectively. Treatment of the BeWo cells with 1.0 µM and 0.5 µM 6-OH BDE-47 for 24 hours significantly decreased the 3,3’-T2 SULT activity measured in the cell homogenate by 41.5 ± 3.3% and 23.0 ± 3.49%, respectively. Treatment of the BeWo cells with 1.0 µM and 0.5 µM, 3-OH BDE-47 for 24 hours significantly decreased the 3,3’-T2 SULT activity measured in the cell homogenate by 35.8 ± 7.5% and 26.0 ± 4.9%, respectively. There was no observed effect on basal 3,3’-T2 SULT activity in BeWo cells following exposure to BDE-47.
Similar experiments were also performed with the same BFRs except the exposure period was reduced to 12 hours compared to the previous 24 hour period. Following the 12 hour exposure period, a statistically significant reduction in basal 3,3'-T2 SULT activity was observed only in the high doses for 3-OH BDE-47 and 2,4,6-TBP, as well as in the medium dose for 2,4,6-TBP. There was no observed change in basal 3,3'-T2 SULT activity following 12 hour exposure to BDE-99 or 6-OH BDE-47. Treatment of
the BeWo cells with 1.0 µM and 0.5 µM 2,4,6-TBP for 12 hours significantly decreased the 3,3’-T2 SULT activity measured in the cell homogenate by 75.0 ± 1.5% and 58.3 ± 4.4%, respectively. Treatment of the BeWo cells with 1.0 µM 3-OH BDE-47 for 12 hours significantly decreased the 3,3’-T2 SULT activity measured in the cell homogenate by 36.4 ± 2.6%. Results from the 12 h cell culture exposure experiments are summarized below in Figure 17.

![Image](image.png)

Figure 17: 3,3’-T2 SULT activity after a 12 h exposure to BFR compounds at three dosing levels. Data are reported as percent relative to vehicle control. An asterisk indicates a significant difference from vehicle controls (p < 0.05; n = 6 from two experiments; error bars represent ± SEM).
Given the observed activity of 2,4,6-TBP at 12 hours, additional experiments were performed for exposure durations of 1 and 6 hours. Treatment of the BeWo cells with 1.0 µM and 0.5 µM of 2,4,6-TBP for 6 hours significantly decreased the 3,3'-T2 SULT activity measured in the cell homogenate by 56.8 ± 3.1% and 34.4 ± 4.2%, respectively. Treatment of the BeWo cells with 1.0 µM and 0.5 µM 2,4,6-TBP for 1 hour significantly decreased the 3,3'-T2 SULT activity measured in the cell homogenate by 62.7 ± 5.0 % and 50.5 ± 7.8 % respectively. There was no observed effect on basal 3,3'-T2 SULT activity in BeWo cells at the low dose for 1 or 6 hour exposure durations. Total data for 2,4,6-TBP at all exposure durations are shown below in Figure 18.
Cytotoxicity was assessed in all dosing concentrations for all BFR analytes for 24 hour exposure periods using the resazurin reduction assay. All values were normalized to control samples for statistical analysis and there was no observed cytotoxicity resulting from BFR exposure at any dosing concentration.

Figure 18: Basal 3,3’-T2 SULT activity in BeWo cells following exposure to 2,4,6-TBP for various exposure durations. Data are reported as percent relative to vehicle control (each data point represents \( n = 6 \) from two experiments; error bars represent ± SEM).

### 4.3.4 Cytotoxicity

Cytotoxicity was assessed in all dosing concentrations for all BFR analytes for 24 hour exposure periods using the resazurin reduction assay. All values were normalized to control samples for statistical analysis and there was no observed cytotoxicity resulting from BFR exposure at any dosing concentration.
4.3.5 Growth Media TH Analysis

THs (T4, T3, rT3, and 3,3'-T2) were measured in BeWo cell growth media following 24 hr exposures. TH concentrations were calculated as ng/mL and compared between all exposure groups and DMSO controls. There were no observed effects of treatment on any TH compounds.

Figure 19: Thyroid hormone concentrations in BeWo cell culture growth media following 24 hour exposure to 2,4,6-TBP. Thyroid hormone concentrations are expressed as the mean value (ng/mL) of three cell culture growth media samples per treatment and error bars represent ± SEM.
4.3.6 Growth Media Sulfated Metabolite Analysis

Sulfated metabolites were detected in the cell culture growth media from 3-OH BDE-47, but not 2,4,6-TBP or 6-OH BDE-47 (Figure 20). There were no observed peaks in the chromatograms from the DMSO control samples, indicating that these compounds are very likely a product of exposure to the BFRs (Figure 21).

Figure 20: Overlaid chromatogram of 3-OH BDE-47 dosing media with 3-OH BDE-47 and 3-OH BDE-47 sulfated conjugate peaks and structures shown.
mRNA expression of several SULT and TH regulating genes was measured in cells exposed to all analytes in the 24 hour experiments. Additional expression analyses were also conducted in cells exposed to 2,4,6-TBP for 1 and 12 hours. Sex-hormone binding globulin (SHBG) and SULT1C2 were poorly expressed in preliminary evaluations of BeWo cells (Ct > 38) and were excluded from future analyses. DIO3,
SULT1A1, TR-α, and TR-β were measured in all samples. DIO3 expression was highly stochastic and variable, even within the control treatment groups, and was excluded from statistical analysis. SULT1A1, TR-α, and TR-β expression was measured in all BeWo cell experimental groups, however, no significant changes in mRNA expression were observed for any BFR treatment groups. mRNA expression levels for BeWo exposure experiments are summarized below in Figures 22 - 23.

**mRNA Expression in BeWo Cells Following 24 h Exposure to 2,4,6-TBP**

![Graph showing mRNA expression ratios relative to control cells and normalized to β-actin as an internal reference gene calculated using the 2^{ΔΔCt} method after 24 h exposures to 2,4,6-TBP (n = 6 from two experiments).]

Figure 22: mRNA expression ratios relative to control cells and normalized to β-actin as an internal reference gene calculated using the 2^{ΔΔCt} method after 24 h exposures to 2,4,6-TBP (n = 6 from two experiments).
Figure 23: (A – D) mRNA expression ratios relative to control cells and normalized to β-actin as an internal reference gene calculated using the $2^{\Delta \Delta C_t}$ method (each bar represents $n = 6$ from two experiments) for experimental exposures 12 h 2,4,6-TBP, 1 h 2,4,6-TBP, 24 h BDE-99, and 24 h 3-OH BDE-47, respectively.
4.4 Discussion

This is the first study to evaluate the effects of BFR exposure on TH SULT activity, as well as the kinetic parameters involved in TH SULT enzyme disruption in the BeWo cell line. Direct enzyme inhibition was observed for 2,4,6-TBP and the OH-BDE compounds, while BDE-99, 2,4,6-TBP, and OH-BDEs also disrupted basal SULT activity following BeWo cell exposures. This study demonstrates that BFR compounds have the ability to disrupt TH SULT activity within placental cells, and adds to our growing understanding of the effects of BFR exposure on TH metabolism within the placenta.

The kinetic parameters and inhibition profiles of the BFR analytes tested in this study were evaluated using BeWo cell homogenates. 2,4,6-TBP was the most potent inhibitor of 3,3’-T2 SULT activity in the BeWo cell homogenates with an IC$_{50}$ value of 11.6 nM. The next most potent inhibitor of 3,3’-T2 SULT activity in the BeWo cell homogenates was 3-OH BDE-47 with an IC$_{50}$ value of 48.9 nM, and the least potent inhibitor was 6-OH BDE-47 with an IC$_{50}$ value of 395.5 nM. The PBDE congeners BDE-47 and BDE-99 did not exhibit any SULT inhibition in the BeWo cell homogenates. These results are consistent with a previous in vitro evaluation of BFR inhibition on TH SULT activity using pooled human liver cytosol (Butt and Stapleton 2013). This study showed no effect of the parent PBDE compounds (BDE-47 and BDE-99); however, a high degree of SULT inhibition was observed for 2,4,6-TBP, 3-OH BDE-47, and 6-OH BDE-47. Both
studies found 2,4,6-TBP to be the most potent inhibitor of TH SULT activity with IC$_{50}$ values in the low nM range. Additionally, both studies show 3-OH BDE-47 to be a more potent inhibitor of TH SULT activity than 6-OH BDE-47. However, both of these studies used different tissues sources for the SULT enzymes (liver versus placenta) and different tissue types will likely have different SULT enzyme isoform profiles and expression levels that contribute to the observed differences in enzyme inhibition.

The presence of the OH group appears to be necessary for 3,3'-T2 SULT inhibition, as the non-hydroxylated PBDE compounds showed no sign of inhibition in both studies, while the hydroxylated compounds showed a high degree of inhibition. These results are supported by previous research that showed that only OH-PCBs inhibited TH SULT activity in rat liver cytosolic fractions (low µM range), while the parent PCBs (non-hydroxylated) did not (Schuur et al. 1998c). Additional work from this research group went on to show that BDE-47 and BDE-99 were potent inhibitors of TH SULT activity only after incubation with cytochrome-P450 enriched microsomes, indicating that the hydroxylation of the parent compounds leads to a chemical structure that is able to interact with and inhibit SULT enzymes (Schuur et al. 1998b).

Hydroxylated PCBs have also been shown to inhibit human estrogen SULT activity in vitro, with IC$_{50}$ values in the low nM range (Kester et al. 2000). For example, 4-OH 3,5,3',4'-tetraCB had a measured IC$_{50}$ value of 0.21 – 0.61 nM for estrogen sulfation. A more recent study also evaluated the structure-activity relationships between OH-PCBs
and TH SULT inhibition and found that all 15 of the OH-PCBs tested were potent inhibitors of TH SULT activity and that eight of the compounds actually acted as substrates for the sulfation reaction. This study also characterized the influence of the specific positions of the halogens and hydroxyl group that lead to varying degrees of inhibition (lowest IC$_{50}$ value) (Ekuase et al. 2011). Similarly, this current study confirms that OH-BDEs are more potent inhibitors of TH SULT activity than their parent PBDE compounds, and that the presence of the hydroxyl group allows for favorable binding with SULT enzymes. The higher potency observed for 3-OH BDE-47 versus 6-OH BDE-47 in this study, as well as in Butt et al. 2013, suggests that the meta-positioning of the hydroxyl group in 3-OH BDE-47 allows for more favorable binding with SULT enzymes than the ortho-positioning of the hydroxyl group in 6-OH BDE-47. The chemical structure of 3-OH BDE-47 is more similar to that of a TH than 6-OH BDE-47, as the hydroxyl group is substituted in the meta position as opposed to the ortho-position (in T3, the hydroxyl group is para-substituted). This difference in structure may result in the observed differences in enzyme inhibition in the BeWo cell homogenates.

The calculated IC$_{50}$ values from this study are all in the nM range, however, a direct comparison to the human serum and placenta tissue concentrations of these BFR compounds is difficult to assess due to differences in in vivo conditions. As a rough comparison, BDE-47, BDE-99, and ΣBDEs have been measured in human serum as high as 60.7 nM, 13.5 nM, and 78.8 nM, respectively (Stapleton et al. 2008b). OH-BDEs are
generally measured at much lower concentrations than their parent compounds, but 6-OH BDE-47 has been measured in human serum as high as 0.34 nM (Chen et al. 2013). Additionally, as presented in Chapter 2, the concentrations of BDE-47 and 2,4,6-TBP were found to be quite high in placenta tissues samples (range: 0.12 – 141 ng/g lipid and 2.18 – 568 ng/g lipid, respectively). These serum and tissue concentrations are difficult to compare to the dosing concentrations used in the BeWo exposure experiments, however, the concentrations measured in human tissues may be capable of eliciting an inhibitory effect on SULT enzyme activity in vivo. Regardless of the other potential indirect effects on SULT activity that may be mediated by BFRs, there exists the possibility of these BFR compounds to directly inhibit the catalytic capacity of SULTs within the placenta.

The mechanism of 3,3′-T2 SULT inhibition was investigated by calculating the Michaelis-Menten parameters of 3,3′-T2 sulfation in BeWo cell homogenates using 2,4,6-TBP and 3-OH BDE-47 as inhibitors. Exposure to both 2,4,6-TBP and 3-OH BDE-47 resulted in a statistically significant decrease in $V_{\text{max}}$ and increase in $K_m$ parameters relative to control cells. A decrease in $V_{\text{max}}$ and an increase in $K_m$ are indicative of mixed inhibition. Mixed inhibition is a type of enzyme inhibition in which the inhibitor can bind to the enzyme with or without the substrate bound, but has a greater affinity for one state over the other (Strelow et al. 2012). Mixed inhibition is a mixture of competitive enzyme inhibition, in which an inhibitor can only bind to an enzyme if the substrate has not already bound, and uncompetitive inhibition, in which the inhibitor can only bind to
an enzyme if the substrate has already bound. This type of enzyme inhibition suggests that 2,4,6-TBP and 3-OH BDE-47 are capable of binding SULT enzymes in the binding pocket of the active site and at an allosteric binding site. It is also worth mentioning that BeWo cells express a mixture of SULT isoforms and it is possible that these different enzyme isoforms have different relative affinities for the BFR compounds, as well as different types of enzyme inhibition kinetics (competitive versus uncompetitive). Therefore, it is conceivable that the mixed inhibition observed in this study using the BeWo cell homogenates is in fact, a reflection of the different inhibition kinetics (both competitive and uncompetitive) specific to each SULT isoform present in the total cell homogenate. This would lead to the observed mixed inhibition as a result of an additive effect of the different SULT isoforms present in the cell homogenates. Future research should focus on SULT enzyme kinetics and inhibition by using purified individual SULT isoforms in order to determine the protein-specific parameters for each isoform. However, the use of the BeWo cell homogenate is a strength of this research because it is more representative of the in vivo conditions within the placenta tissue and more accurately characterizes the net effect of BFR compounds on SULTs at the protein-specific level.

The inhibition kinetics of SULT isoforms has been extensively studied using various inhibitors and tissue types. Noncompetitive inhibition was observed for 4-OH BDE-90 on 3,3′-T2 sulfation in pooled human liver cytosol, for triclosan on 3-OH
benzo(a)-pyrene sulfation in human liver cytosol, and for OH-PCBs on estrogen sulfation (Butt and Stapleton 2013; Kester et al. 2000; Wang et al. 2004b). Competitive inhibition was observed for polyhalogenated aromatic hydrocarbons (PHAH-OHs) on estradiol sulfation in human liver cytosol and for 4-hydroxy-2',3,3',4',5-PCB on 3,3'-T2 sulfation in rat liver cytosol (Kester et al. 2002a; Schuur et al. 1998c). However, consistent with the findings from this study, mixed inhibition was observed for triclosan on estrogen sulfation in sheep placenta, for cigarette smoke toxicants on estrogen sulfation in primary human lung endothelial cells, and for 4’-OH-CB-165 on 4-nitrophenol sulfation in human liver cytosol (James et al. 2010; Wang et al. 2006; Yasuda et al. 2007). Clearly, there is a great deal of complexity involved in the inhibition kinetics of SULT enzymes related to the structural and electrostatic differences in the binding pockets and allosteric sites of the different SULT isoforms. This leads to different relative substrate affinities for each SULT isoform, and is further complicated by the fact that different tissue types contain different SULT isoform expression profiles. As a result, direct comparisons between different tissue types and cell culture lines may prove to be difficult. For example, the BeWo cell line is likely to express a different SULT isoform expression and activity profile than primary human placenta tissues, which exhibit developmental time point and cell-type dependent SULT enzyme expression. However, despite these complexities, the in vitro BeWo cell model provides a controlled experimental system in which to evaluate the underlying mechanisms of TH SULT
enzyme inhibition by BFRs. The results from this current study indicate that 2,4,6-TBP and 3-OH BDE-47 exhibit mixed inhibition in BeWo cell homogenates and are capable of significant enzyme disruption via direct protein interactions. These results suggest that BFRs such as 2,4,6-TBP and 3-OH BDE-47 present in the placenta tissue during pregnancy have the ability to inhibit the TH sulfation activity of SULT enzymes through direct inhibitor-enzyme interactions.

It has been shown that some chemicals are able to bind SULT enzymes so well that they themselves can act as substrates and become sulfated, implicating the role of direct competitive inhibition (Grimm et al. 2013). For example, urinary concentrations of sulfated conjugates of TBBPA have been measured in humans and rats following oral administration (Hakk et al. 2000; Schauer et al. 2006). Such results are supported in this present study, as the detection of a sulfated metabolite of 3-OH BDE-47 was observed in cell culture dosing media following exposure. Interestingly, sulfation of 6-OH BDE-47 and 2,4,6-TBP was not observed, presumably due to the ortho positioning of the hydroxyl group in 6-OH BDE-47 which may create less favorable binding with SULT enzymes due to steric hindrance from bulky halogen atoms (blocking access to the hydroxyl group necessary for SULT binding and conjugation) and possibly due to the unfavorable molecular weight/size of 2,4,6-TBP for catalytic site interactions. The presence of sulfated conjugates in the cell culture growth media following exposure indicate that 3-OH BDE-47 is capable of binding SULT enzyme binding pockets and
favorably interact with the active site in such a way as to allow for sulfation of the hydroxyl moiety. The chromatograms of the sulfated metabolite of 3-OH BDE-47 present in the cell culture media are shown above in Figures 20 - 21.

After investigating the protein-level interactions and calculating the kinetic parameters of BFRs using the BeWo cell homogenates, a series of experiments were conducted to further examine the cumulative biological effects of BFR exposure on basal TH SULT activity in dosed BeWo cell cultures. The BeWo cell culture exposure experiments represent a more complete in vitro experimental model than the previously described BeWo cell homogenate experiments. This is because cells are grown within an exposure media, allowing for the BeWo cells to respond to BFR compounds in a manner similar to real-life in vivo exposures (e.g. leading to changes in gene regulation). For these experiments, the following endpoints were analyzed following BFR exposure: basal 3,3’-T2 SULT activity, TH concentrations in the cell culture growth media, and mRNA expression levels of TH-related genes.

Cell culture exposure experiments were conducted with 2,4,6-TBP, BDE-47, BDE-99, 3-OH BDE-47, and 6-OH BDE-47. Following the 24 hour exposure, basal 3,3’-T2 SULT activity was significantly reduced in all high and medium dose exposures for all BFR analytes (except BDE-47), as well as in the low dose for 2,4,6-TBP. These results indicate that BFRs are capable of disrupting basal 3,3’-T2 SULT activity in BeWo cells following a 24 hour exposure period at doses of 1 µM and 0.5 µM concentrations. 2,4,6-
TBP showed the highest degree of potency by significantly reducing basal 3,3'-T2 SULT activity at all doses, as well as by reducing basal 3,3'-T2 SULT activity by over 80% in the high dose. Additionally, BDE-99 showed significant effects at the high and medium doses following the 24 hour exposure; however, BDE-99 showed no inhibitory effect in the BeWo cell homogenate experiments. This indicates that BDE-99 must act through a mechanism of action other than direct enzyme inhibition. One possibility is that BDE-99 was metabolized to form OH-BDE metabolites, which then mediated the observed reduction in basal SULT activity. To investigate this, the growth media was extracted and analyzed for OH-BDEs following the 24 hour exposure. However, there were no OH-BDEs detected in the BDE-99 dosing media, indicating that oxidative metabolism and formation of OH-BDEs was likely not occurring in the BeWo cells and that the observed effects on basal 3,3'-T2 SULT activity were not mediated by OH-BDEs. Therefore, BDE-99 may elicit its action via indirect mechanisms such as effects on SULT protein post-translational processes and expression, SULT enzyme turnover rates via proteasomal degradation pathways, or possibly by disrupting some other pathway upstream of SULT enzyme regulation. For example, BDE-99 and 5'-OH-BDE-99 were shown to decrease DIO2 activity in cultured human astrocytes via a proteasomal degradation pathway (Roberts et al. 2015). Additionally, ubiquitination and proteasomal degradation has been verified as a mechanism of enzyme recycling for the SULT1A1*2 polymorphic variant, effectively reducing the cellular half-life of this protein (Nagar et
Therefore, it is plausible that SULT enzymes may be regulated by ubiquitination in a similar manner following BDE-99 exposure.

Next, following the 12 hour exposure, basal 3,3'-T2 SULT activity was significantly reduced in the high dose for 2,4,6-TBP and 3-OH BDE-47, as well as in the medium dose for 2,4,6-TBP. There was no observed effect on basal 3,3'-T2 SULT activity following 12 hour exposure to BDE-99 or 6-OH BDE-47. This lack of effect on basal SULT activity in the 12 hour exposures versus the 24 hour exposures indicates that exposure duration is an important factor in mediating the effects of BFRs on basal 3,3'-T2 SULT activity. For instance, it may be that the cellular machinery (ex. transport proteins, metabolizing enzymes, etc.) requires more time to transport these BFR compounds into cells and the nucleus. One factor that might contribute to these differences in binding and transport, are the physicochemical properties of the compounds such as solubility and molecular weight. For example, 2,4,6-TBP is significantly smaller and less lipophilic than OH-BDE compounds and may have a greater capacity for cellular transport into and within cells, contributing to its higher potency. Another possible explanation may be that these compounds impact SULT enzymes by upregulating proteasomal degradation pathways and lowering the cellular half-lives of SULT enzymes. As a result, the pool of SULT enzymes present in the BeWo cells may not be sufficiently degraded after a 12 hour exposure period, and require a 24 hour exposure period in order to elicit an effect on basal SULT activity. However, it appears that 2,4,6-TBP is equally potent
following a 12 hour exposure at the high and medium dose as it is in the 24 hour exposure. 2,4,6-TBP was also tested at 6 hour and 1 hour exposure durations and found to significantly reduce basal 3,3’-T2 SULT activity in the medium and high doses at both time points. The ability of 2,4,6-TBP to disrupt basal SULT activity at all exposure durations ranging from 1 hour to 24 hours indicates a rapid mechanism of action. The mechanisms by which 2,4,6-TBP, as well as the other BFRs tested in the study, impact SULT activity in BeWo cells following exposure remain to be determined. Disruption and reduction of basal TH SULT activity in the placenta may impact the tissue concentrations of THs, as well as other biomolecules that are metabolized by SULTs such as estrogen, fatty acids, and other lipophilic environmental contaminants. Therefore, the effects of SULT enzyme inhibition may have biological consequences for other signaling pathways in addition to THs.

THs (T4, T3, and rT3) were measured in cell culture exposure media collected after the exposure duration. It was hypothesized that different TH profiles would be observed for samples with significant effects on basal 3,3’-T2 SULT activity. For example, samples with significant reductions in basal 3,3’-T2 SULT activity would be expected to have higher media concentrations of T4 and T3 as a result of decreased metabolism and clearance from the cells. However, there was no significant effect of exposure on media TH concentrations observed by treatment. These results may be reflective of the maintenance of TH levels in BeWo cells during BFR exposure due to the
compensatory and self-regulating action of TH transport proteins, TH binding proteins, and/or TH metabolizing enzymes such as DI or UDPGTs. So while BFR exposure may have impacted TH concentrations through SULT inhibition in cells, there were sufficient regulatory mechanisms that maintained TH concentrations within a narrow concentration window, and detection of changes in TH concentrations in the growth media were effectively masked.

The mRNA expression of TH responsive and regulating genes were also measured in the cell culture BFR exposure experiments. There was no observed effect of treatment for any of the genes investigated. However, for DIO3, there was highly variable and stochastic gene expression observed at all treatment levels, including the DMSO control groups. This is in contrast to the consistent results observed for TR-α, TR-β, and SULT1A1, which show non-significant deviation from control group mRNA expression values. It is unclear why the DIO3 mRNA expression was so variable in the BeWo cells, however, this variability excluded DIO3 from additional analysis. There was no observed effect of treatment on the mRNA expression profiles of TR-α, TR-β, and SULT1A1. These results are interesting because there was a significant decrease in basal 3,3′-T2 SULT activity in BeWo cells following these BFR exposures. Our initial hypothesis was that BFR exposure would cause the mRNA expression of SULT enzymes to be reduced and lead to the observed reduction in basal activity. However, this hypothesis is not supported by the mRNA expression data, as SULT1A1 gene expression
did not vary with BFR treatment. From these data we can conclude that SULT1A1 mRNA expression is not affected by BFR exposure in BeWo cells; however, the full mechanism behind the decrease in basal 3,3′-T2 SULT activity remains to be determined. It is likely that direct enzyme inhibition contributes to the decrease in basal 3,3′-T2 SULT activity following exposure, but the relative contributions of other indirect pathways is not known at this time. There are many other possible mechanisms of action that could contribute to the observed reduction in basal 3,3′-T2 SULT activity. For example, there are many post-translational processes/modifications that could affect the amount of SULT1A1 protein that becomes synthesized (alterations in protein expression levels), post-translational ubiquitination that leads to enhanced proteosomal degradation, and epigenetic modifications that can lead to gene suppression (Nagar et al. 2006). These potential mechanisms of action warrant further investigation in order to determine the mechanism of action underlying the observed biological effect. One additional consideration is that only the mRNA expression of one SULT isoform, SULT1A1, was measured in this current study. While SULT1A1 is expected to be the dominant SULT isoform expressed in BeWo cells, it may be that other isoforms were impacted following BFR exposure and contributed to the reduction in basal 3,3′-T2 SULT activity. However, the mRNA expression of SULT1C2 was also investigated in this current study and was found to be poorly expressed or not detected despite previous reports of high expression.
(< 30 cycles) of SULT1C2 in BeWo cells (Tamura et al. 2001). Future studies should examine the expression profiles of the other SULT isoforms in BeWo cells.

Inhibition of SULT enzymes in the human placenta has the potential to elicit downstream biological effects. First, as previously discussed, inhibition of TH SULT activity may potentially lead to disruption of TH tissue concentrations, which could have downstream effects on TH-mediated pathways. However, no effect on TH concentrations were observed in BeWo cells following BFR exposure in this current study. However, SULT enzymes play a broad role in the biotransformation and metabolism of many other endogenous compounds including bile acids, neurotransmitters, peptides, hormones, and lipids, as well as xenobiotics such as drugs, chemical carcinogens, and environmental contaminants. Not only is sulfation an important Phase II metabolic pathway in adults, but sulfation is the primary metabolic pathway within the placenta and developing fetus (Coughtrie 2002). As a result, disruption of TH SULT activity within the placenta is likely to impact a wide range of metabolic pathways outside of TH homeostasis. For example, reductions in basal SULT activity may lead to lower rates of sulfation of environmental contaminants, effectively increasing tissue concentrations of these chemicals due to lack of clearance and further perpetuating SULT enzyme inhibition within the placenta. Another potential outcome for SULT inhibition in the placenta, is the disruption of other sulfation pathways for endogenous compounds. Examples of other endogenous substrates that utilize sulfation
include estradiol, testosterone, dopamine, cholesterol, and cortisol (Pacifici 2005). Notably, estradiol signaling is very important in mediating gestational development within the placenta during pregnancy, as well as in modulating cellular communication between the fetus, placenta, and mother (Albrecht and Pepe 1999; Challis JRG et al. 2000). Disruption of estradiol sulfation may lead to a perturbation of estradiol homeostasis and increased expression of estradiol responsive genes, resulting in estrogenic effects within the placenta and/or fetus. While TH sulfation was the focus of this current study, the integrated role of SULT enzymes in multiple signaling and metabolic pathways suggests that inhibition of the activity of this important class of enzymes may have broader implications within the placenta. Additionally, the TH disrupting capacity of other environmental contaminants such as PCBs, dioxins, and PFOAs, should be investigated in the BeWo cell model. Many of these chemicals have been shown to exhibit varying degrees of potency and relative effects on the HPT axis (ex. TR agonists vs. TR antagonists), and their individual effects, as well as mixture effects should be evaluated within an in vitro placental cell culture model.

Overall, the ability of BFRs, especially the hydroxylated compounds, to disrupt TH SULT activity in BeWo cells at environmentally relevant concentrations warrants further investigation into the mechanisms of action underlying these effects, as well as the biologically relevant downstream effects of exposure.
5. Discussion

As part of this research, THs were measured in human placenta tissue for the first time, and the results indicate that PBDEs and 2,4,6-TBP bioaccumulate within the human placenta and are associated with TH levels. BFRs and THs were also associated with endogenous DIO3 and TH SULT activity in a sex-specific manner, suggesting that there are differences in BFR bioaccumulation and toxicity based on infant sex.

Additionally, research using a human choriocarcinoma placenta cell line (BeWo) showed that BFRs are capable of disrupting basal 3,3'-T2 SULT activity following various exposure durations (1, 6, 12, and 24 h). 2,4,6-TBP was shown to be the most potent inhibitor of SULT activity in the BeWo cell model. One novel finding was that BDE-99 was capable of disrupting SULT activity despite showing no inhibitory effect in samples using cell homogenates, indicating that BDE-99 must act through a mechanism of action other than direct protein inhibition. There was no observed biotransformation of BDE-99 in BeWo cells following exposure, indicating the OH-BDE metabolites were not mediating the inhibitory effects. Following BFR exposure, BeWo cells showed no change in mRNA expression of various TH-related genes, including SULT1A1, indicating that BFRs mediate their effect on SULT activity via a mechanism of action other than downregulation of mRNA expression. Overall, BFRs are capable of disrupting TH homeostasis in the human placenta and future work should focus on the mechanisms of
action of TH SULT disruption, as well as the downstream biological effects of BFR exposure within the placenta.

5.1 Challenges Associated with Epidemiological Studies Examining Thyroid Hormone Endpoints

Research presented in Chapter 2 and Chapter 3 provides the first measurements of THs and BFR in a cohort of human placenta tissue samples. In addition, significant associations were observed between BFR and TH levels, in addition to associations between BFRs and DIO and SULT activities in placental tissues. While this research provides important insight into the potential effects of BFRs on TH metabolism and tissue concentrations, it is possible that our observations were confounded by factors commonly encountered using real-life human samples (e.g. interindividual differences). As a result, Aim 3 (Chapter 4) examined the effects of BFRs on TH SULT activity using a highly controlled in vitro cell culture model system, in order to better target and isolate the potential mechanisms of action underlying these observed associations.

There exists a great deal of inconsistencies between human epidemiological studies that have investigated associations between serum concentrations of THs and PBDEs. This is in contrast to the reproducible and consistent results observed in animal studies, wherein PBDEs consistently induce hypothyroxinemia in exposed animals. Additionally, other environmental toxicants, such as lead, show a strong consensus between the human epidemiological studies related to human lead exposure and neurotoxic endpoints, as well as agreement between these human data and animal
exposure studies related to lead exposure and neurotoxicity (Rice 1996). Clearly, TH biology in humans is a challenging epidemiological focus, and a majority of the inconsistencies observed in human epidemiological research related to THs and PBDEs may be attributed to differences in study designs, as well as environmental and biological factors. These variables are discussed in detail below.

First, the determination of maternal iodine and selenium status is an often overlooked or excluded variable from these studies. These variables are often not measured because many research studies perform TH and PBDE analyses as a secondary measure, using existing biobanks for serum samples and/or cohort studies that were designed for other research purposes. During pregnancy, there is an increased demand for and consumption of iodide due to the increase in maternal T4 production. It is estimated that T4 production during pregnancy is 50% higher than normal and that this increased demand requires an additional 50-100 µg of iodide per day from dietary sources in order to maintain these higher TH production rates (Glinoer et al. 1990). In addition to the increased demand for iodide needed for the synthesis of THs during pregnancy, the placenta facilitates the transfer of maternal iodine to the fetal circulatory compartment beginning approximately at 10-12 weeks gestation and increasing until birth. Increased production and secretion of hCG by the placenta facilitates the stimulation of the sodium-iodide symporter (NIS) in the cytotrophoblast cell layer, allowing for the transport of maternal iodine to the fetus (Bidart et al. 2000). In addition,
as pregnancy progresses, placental vascularization increases and tissue oxygen concentrations rise from approximately 1% $O_2$ in early pregnancy, to 8% $O_2$ at week 16, leading to a complementary increase in the expression of NIS (Li et al. 2011). This mechanism provides the availability of iodine within the fetal compartment during pregnancy, which allows the fetal thyroid gland to synthesize its own THs. The estimated daily transfer of maternal iodine to the fetal compartment is approximately 50-75 µg at its peak during the end of gestation. Overall, there is an approximate doubling in the required daily requirement of dietary iodide in pregnant women (250 – 300 µg/day) from these combined physiological demands. Pregnancy represents the most vulnerable period of life for iodide insufficiency, and it is estimated that approximately 57% of pregnant US women have low urinary iodine concentrations, indicating insufficient intake (Caldwell et al. 2013). The effects of maternal and fetal iodine insufficiency have been well documented and include events such as interruptions in pregnancy, impaired fetal neurodevelopment, lower IQ scores, and in the most severe of cases, cretinism (Bougma et al. 2013; Zimmermann 2012). Due to the critical importance of dietary iodide and maternal iodine status during pregnancy, these factors need to be measured in a standardized manner in human epidemiological studies examining effects on the TH system. This is especially important for pregnancy cohorts from developing parts of the world, as well as individuals from lower socioeconomic standing, in which access to dietary iodide via iodized salt and/or natural
food sources may be limited or nonexistent. However, iodine has a short half-life and excretion rates have large inter-individual variability (Perrine et al. 2014). As a result, single urinary iodine measurements are insufficient in estimating the iodine status of individuals, and even the use of 10 serial urine samples has been shown to only be capable of estimating an individual’s iodine status with 20% precision (König et al. 2011). Race-ethnicity differences in iodine status have also been reported, and should be accounted for when conducting human epidemiological research on TH status. For example, according to data from the National Health and Nutrition Examination Survey (NHANES) for the 2003 - 2006 period, estimated urinary iodine concentrations were 34% lower in non-Hispanic blacks versus non-Hispanic whites while controlling for sociodemographic factors (Pfeiffer et al. 2013). Overall though, evaluating maternal iodine status is important in interpreting the larger suite of TH endpoints measured in such studies.

Next, a related dietary micronutrient, selenium, requires equal attention in pregnancy studies due to its critical role in the synthesis of DI enzymes. Selenium is used to create the rare amino acid, selenocysteine, which is an important amino acid residue in the active site of DI enzymes and necessary for normal catalytic function. Selenium has a narrow therapeutic index and approximately 60-75 µg of selenium are required per day from dietary sources in order to maintain suitable selenoenzyme activity (Lacka and Szelig 2015). There are many dietary sources of selenium such as
Brazil nuts, seafood, meats, and grains (Chun et al. 2010). However, the selenium content of plant-based foods depends largely on the soil conditions and geographic location of agriculture, and Midwestern and Western states have significantly higher concentrations of selenium than the South and Northeast (Niskar et al. 2003). As a result, geographic location as well as diet should be acknowledged when assessing the selenium status of a study population. Due to the important role that selenium plays in DI enzyme synthesis and activity, which in turn impacts thyroid gland function and TH synthesis and metabolism, selenium status is closely linked with various thyroid related endpoints. Low serum selenium concentrations have been associated with a higher prevalence of pathological thyroid conditions such as hypothyroidism, subclinical hypothyroidism, autoimmune thyroiditis, and enlarged thyroid glands (Wu et al. 2015). Additionally, according to data from the NHANES for the 2011 - 2012 period, serum Se levels were significantly lower in adolescents than adults, in low income (<$20,000) individuals, and non-Hispanic blacks, while non-Hispanic Asians had the highest serum levels of Se (Jain and Choi 2015). For these reasons outlined above, measurement of maternal serum selenium concentrations throughout pregnancy, as well as the use of dietary questionnaires should be included in pregnancy cohort studies as a confounding variable.

Another source of inconsistency among research studies is the normalization of serum and tissue levels based on lipid content. Measurements of PBDEs and other
lipophilic environmental contaminants (log $K_{ow} > 4$) are often reported on a lipid normalized basis because it is assumed these compounds will be primarily associated with blood lipid concentrations. However, in this current study, as well as in Abdelouhab et al. 2013, PBDEs were not correlated with total lipid concentrations in the placenta tissue or maternal blood. In contrast, other studies have shown significant correlations between the lipid-normalized concentrations of PBDEs in human serum, adipose tissue, and liver samples, suggesting that the assumption that PBDEs equilibrate within body lipids is valid (Covaci et al. 2008; Hirai et al. 2012). These discrepancies question the validity and justification of lipid normalization for environmental contaminants. Yet another complicating factor in adjusting for lipid content is the method by which lipids are quantified. Most studies using serum measurements do not measure total lipids directly in the serum, but estimate lipid content using the Phillips formula (Phillips et al. 1989). This method of estimation may be a source of additional variability in lipid measurements and may not be suitable for use in pregnant women, whose lipid profiles differ from those of the non-pregnant adult population (Sattar et al. 1999). However, the use of direct blood lipid measurements can be problematic since maternal lipid profiles change throughout pregnancy as well as postprandially, meaning that epidemiological studies should try to collect fasting samples at equivalent time points in all participating individuals (Phillips et al. 1989). In this current study, gravimetric analysis of placenta tissues was used to quantify tissue total lipid content.
and normalize BFR concentrations. The mean lipid measurements in placenta tissue from this study agree well with previously reported values that were obtained using different lipid measurement techniques (Chen et al. 2014b; Main et al. 2007). However, some studies have shown that the distribution of PBDEs and other environmental contaminants can vary between different lipoproteins that have different polarities, suggesting that normalizing BFR concentrations to total lipid content may not be the most accurate method (Mohammed et al. 1990; Sandermann 2003). Fine-scale lipid determination has been performed using Matrix-Assisted Laser Desorption/Ionization (MALDI)-TOF-TOF MS/MS to quantify the main classes of lipids in blood plasma and placenta tissues, and it has been shown that these tissue lipid profiles differ between preeclamptic and normal pregnancy tissues (Korkes et al. 2014). The use of this method to characterize placenta tissue lipid profiles may provide greater insight into the bioaccumulation patterns of BFRs and allow for more accurate lipid normalization by specific lipid type within each tissue sample. Regardless of the complicating factors described above, the validity of lipid adjustment for PBDEs and other lipophilic environmental contaminants still remains under question.

The methods used to measure and quantify THs vary between different studies. Free THs are often measured using immunoassays, however, such measurement assays have shown to be skewed and biased by the concentrations of TH-bound transport proteins present in the media. A quantitative evaluation of immunoassay performance
was conducted using varying levels of FT4 and protein-bound T4 (PBT4), and found that the presence of PBT4 positively biased the FT4 measurements and that different immunoassays varied widely in their measurements (Nelson et al. 1994). Other studies use equilibrium dialysis to measure free TH concentrations and this method produces more consistent and accurate results (Thienpont et al. 2013). In this current study, THs were measured in placenta tissue samples for the first time by LC-MS/MS. This method offers high selectivity and measures individual analytes (T4, T3, rT3, and 3,3’-T2) providing precise measurements; however, only it can only measure total TH concentration values and cannot distinguish the free (unbound) TH fraction. While the use of an LC-MS/MS based method for TH quantification was suitable for this current study, the evaluation of free and total THs concentrations within other biological matrices may require the distinction between free and bound fractions. Regardless of the quantification method, the use of tissue-specific measurements over serum measurements offers many advantages. For example, while serum concentrations of THs are important for maintaining homeostasis via feedback mechanisms within the HPT axis, the actual tissue and intra-cellular concentrations of THs are the most important determinant of TH action. The tissue-specific measurements made in this study are representative of not only the TH concentrations capable of mediating TH action within the placenta, but also the available pool of THs available for transport to the fetal
compartment. As a result, the biological relevance of TH concentration measurements within the placenta tissue is higher than of TH concentrations in maternal serum.

The endogenous activity of SULT enzymes was evaluated in Chapter 2 using human placenta tissue subsamples. This data set provides a unique evaluation of the tissue-specific enzyme activity rates of the placenta; however, there are some limitations associated with these measurements. Notably, there are genetic polymorphisms in human SULT enzymes that create significant differences in enzyme activity rates, substrate affinities, and enzyme half-lives. This phenomenon has been studied extensively in the SULT1A1 gene, which is the dominant SULT isoform expressed in humans, as well as within the placenta tissue. There are three genetic polymorphisms in the human SULT1A1 gene that result in three allozymes: SULT1A1*1, SULT1A1*2, and SULT1A1*3 (Raftogianis et al. 1997). Additionally, there is variability in SULT1A1 allele frequencies based on racial background. For example, SULT1A1*1 is the dominant allele in Caucasians (66%), followed by SULT1A1*2 (33%) and almost no detection of SULT1A1*3. In Asians, SULT1A1*1 is the dominant allele (91%), followed by SULT1A1*2 (8%) and almost no detection of SULT1A1*3. Finally, in Blacks, SULT1A1*1, SULT1A1*2, and SULT1A1*3 show a mixed frequency (48%, 29%, and 23%, respectively) (Carlini et al. 2001). Carlini et al. also observed significant differences in the allele frequencies of all three SULT1A2 allozymes by ethnicity. These inter-population differences in SULT isoforms may contribute to the differences observed in drug
metabolism and disease susceptibility between different ethnic populations. For example, within the pregnancy cohort of this current study, 68% of participants were non-Hispanic black women and the remaining fractions were Hispanic and non-Hispanic white women. The genetic polymorphism of each mother is not known for this pregnancy cohort, however, it can be expected that some of the variability of the measured SULT enzyme activity rates from this study can be attributed to differences in SULT allozyme expression. These genetic differences further complicate the interpretation of these data, as the ability to detect subtle differences in endogenous enzyme activity related to BFR exposure may be clouded by these underlying genetic differences in basal SULT activity. The functional differences in the SULT1A1 allozymes have been characterized using purified SULT1A1 proteins in a cell culture system and several different substrates (Nagar et al. 2006). This study observed significant differences in the catalytic activity of these different proteins towards different classes of substrates ($V_{\text{max}} 1^* > 3^* > 2^*$). Additionally, it was shown that the SULT1A1*2 allozyme exhibited a cellular half-life 6-fold lower than that of SULT1A1*1 and SULT1A1*3. This difference in half-lives appears to be mediated by a ubiquitin-proteasome-dependent mechanism in a similar manner to what has been observed for SULT1A3 allozymes and S-methyltransferase variants (Wang et al. 2003, 2004a). Therefore, these differences between SULT polymorphic variants should to be evaluated and taken into account when conducting human epidemiological research. Differences in thyroid cancer
presentation and survival are also associated with race and socioeconomic status, with black patients and those with low socioeconomic status having worse outcomes for thyroid cancer and disease (Harari et al. 2013). Given these significant racial-ethnic differences observed in SULT1A1 expression and activity, as well as the other racial differences related to TH endpoints, such as the higher prevalence of Graves disease in black and Asian/Pacific Islanders compared with whites, but higher rates of Hashimoto thyroiditis in whites than blacks, one strength of Chapter 2 and 3 was the relatively homogenous nature of the pregnancy cohort from the intentional oversampling of African-American individuals (Hollowell et al. 2002; McLeod and Cooper 2012; McLeod et al. 2014). This leads to a more similar study population with potentially fewer confounding variables; however, it may also reduce the ability to extrapolate results back to the general US population given that only 12.3% of the US population is non-Hispanic black and 17% are Hispanic. Overall, the pregnancy cohort used in this current study represents a unique, understudied, and potentially vulnerable subpopulation in the US, and future research should continue to examine differences in TH biology related to race and ethnicity.

Production and metabolism of THs is regulated by the HPT axis, as well as within specific peripheral tissue types. In euthyroid individuals, serum TH parameters show substantial inter-individual variability, while the intra-individual variability is within a narrow range (Browning et al. 1986, 1988). This suggests that individuals have a
unique HPT axis setpoint that is largely determined by genetic factors. This factor provides one of the main challenges in conducting human epidemiological research on TH biology, in that each individual’s setpoint of TH homeostasis needs to be evaluated in order to determine if there is a perturbation of that individual’s baseline setpoint. This is an enormously difficult prospect for study design and further complicated by the complexities of TH homeostasis during pregnancy. Despite the challenges involved, future research will need to address these complexities in TH homeostasis at the individual level in order to understand the potential effects of environmental contaminant exposure.

Lastly, another variable that could affect associations between PBDEs and TH endpoints is the influence of PBDE metabolites. This current study did not quantify OH-BDEs within the cohort of placenta tissue samples. As previously discussed, OH-BDEs have a significantly higher capacity for disrupting TH-related processes such as DI and SULT enzyme inhibition, TH transporter binding, and TR binding. This enhanced endocrine disruption is a result of the greater structural similarities between OH-BDEs and THs. Specifically, the presence and positioning of the OH group confers these TH disrupting effects for OH-BDE metabolites. As a result, the correlation analyses performed in Aim 2 of this study may not fully capture the chemical(s) mediating the effects on DIO3 and/or SULT disruption because only PBDE parent compound concentrations were considered. However, since many OH-BDE metabolites are
correlated with their parent compounds (Stapleton et al. 2011), it is possible that the significant associations observed for BDE-99 and BDE-209 may be driven by the concentrations of their metabolites, rather than the parent compounds themselves. Future work should measure OH-BDEs in placenta tissue, as these compounds are most likely to drive TH disruption.

5.2 Limitations of the BeWo Cell Culture Model

The BeWo cell culture model was used as a controlled experimental system in which to examine the effects of BFRs on TH SULT activity and the mechanisms of action underlying these effects in placental cells. Insight into the kinetic parameters of TH SULT disruption was gained, however, the downstream biological effects of TH SULT disruption, as well as the specific indirect mechanisms of action underlying this disruption have yet to be determined. As a result, there are several limitations that should be considered for the use of the BeWo cell culture model. First, the use of a cancer cell line is a significant limitation of Aim 3. Although BeWo cells have been verified to be suitable models for primary human placenta cells, the full physiology of these cells has not been evaluated and there may exist differences in the uptake and metabolism of BFRs and THs. Additionally, the use of the cell model system represents an isolated and simplified version of the primary tissues and may not reflect the in vivo conditions of the human placenta during pregnancy. There exists a great deal of interactions with other tissues types such as maternal blood and fetal tissues during
pregnancy that are not accounted for when using the BeWo cell culture model. These interactions mediate growth, differentiation, protein expression, and hormone secretion/metabolism that are all absent from the BeWo cell model system. Next, BeWo cells may not express the full suite of TH-specific enzymes and transporter proteins as primary placental cells. For example, previous studies have measured SULT1A1, 1A3, 1C1, 1C2, 2A1, and 1E1 mRNA as well as DIO2 and DIO3 mRNA in BeWo cells (Hobbs et al. 2002; Tamura et al. 2001). However, SULT1C2 mRNA was not detected and DIO3 mRNA was found to be highly variable in this current study. Clearly, there exist some inconsistencies in the profiling of SULT and DI enzymes within the BeWo cell line, and there exists the possibility that the full expression of TH-related proteins are not present. Next, the BeWo cell line represents an early stage placenta since they are primarily cytotrophoblast cells. Future studies could induce differentiation within the BeWo cells to form a primarily syncytiotrophoblast cell population which would be more representative of 6+ week developed placenta. Differences in the expression patterns of DI enzymes have been shown using immunohistochemistry in first and third trimester human placenta tissues, indicating strong expression of DIO3 in syncytiotrophoblasts but not cytotrophoblasts (Chan et al. 2003). Therefore, BeWo cell differentiation may provide a cell culture model that is more representative of a mature placenta organ with a more complete biological profile for evaluating the effects of BFRs on TH metabolism. One additional modification to the BeWo cell line that could be performed in future
research, is the incubation of BeWo cells at different O\textsubscript{2} concentrations ranging from 1% to 10% O\textsubscript{2} in order to simulate the hypoxic conditions of early pregnancy. Many developmental processes within the placenta are mediated by O\textsubscript{2} concentrations, including the expression of NIS, TTR, and DIO3 (Li et al. 2011; Patel et al. 2011b; Simonides et al. 2008). In this current study, BeWo cells were grown and incubated at 21% O\textsubscript{2}, however, future work should evaluate the differences in TH metabolism and transport at lower O\textsubscript{2} concentrations. Altering BeWo cell differentiation and incubating cells at realistic O\textsubscript{2} concentrations are two experimental modifications that may increase the utility of the BeWo cell model. Finally, there is no way to evaluate the potential sex differences in TH metabolism using the BeWo cell culture model since the BeWo cell line is derived from a male donor. Given the importance of sex-specific differences observed in Chapter 3, as well as the growing body of literature supporting these effects, a model system that evaluates sex differences would be useful in future studies. Overall, the BeWo cell culture system provides clear and controlled mechanistic insight into the effect of BFRs on TH SULT activity; however, the extrapolation of these results to \textit{in vivo} conditions remains unclear.

\section*{5.3 Data Gaps and Future Research Areas}

The research presented in Chapter 4 investigated the potential mechanisms of action underlying the disruption of SULT activity in BeWo cells. While inhibition of SULT activity was observed with BFR exposure, there was no observed effect of BFR
exposure on SULT mRNA expression or TH concentrations in growth media. As a result, it appears that there are other biological mechanisms by which BFRs exert their effect on SULT activity. Future studies should continue to investigate the potential mechanisms of action of BFRs in order to increase our understanding of BFR exposure during pregnancy. Some potential mechanisms include: post-translational modifications to TH-related proteins (SULTs, etc.), effects on degradation pathways of SULT enzymes, effects on CAR and PXR expression/activation, epigenetic modifications to TH-related genes, and disruption of TH transport proteins. For example, DI enzymes are regulated at multiple levels, including reduced translational efficiency via endoplasmic reticulum control and post-translational (reversible) ubiquitination and proteasomal degradation (Arrojo E Drigo et al. 2013b, 2013a; Roberts et al. 2015). Therefore, it is plausible that SULT enzymes undergo a similar form of regulation, however, the proteasomal degradation pathways of SULT enzymes have not been widely studied (Nagar et al. 2006; Wang et al. 2004a). Future research could repeat the BFR exposure experiments in BeWo cells using a proteasomal inhibitor to assess the relative contribution of proteasomal degradation on decreases in basal SULT activity. Next, it is well known that CAR and PXR play important roles in regulating the expression of SULT enzymes. As previously discussed, the activation of these receptors has been shown for some BFR compounds and it seems probable the CAR and PXR mediated pathways play an important role in regulating basal SULT activity in response to BFR exposure in placenta
tissues. Within this current study, there was no observed effect of BFR exposure on SULT1A1 mRNA expression in BeWo cells indicating that CAR and PXR pathways were not activated, however, this may be a reflection of differences in BeWo cell biology versus primary placenta cells, and cannot be fully ruled out as a potential mechanism of action. Finally, at the level of TH transporters, including binding proteins such as TTR, as well as membrane transporters such as MCTs and OATPS, there is the potential for disruption. As previously discussed, the structural similarity of many BFRs with THs allows these compounds to competitively bind TH transporter proteins. It is also hypothesized that TH-like compounds can utilize membrane transporters such as OATPs for transport into cells (Pacyniak et al. 2010). This would disrupt normal TH cellular transport by competing with THs and potentially saturate membrane transporter function. Furthermore, OATP and other transporter protein expression is regulated by TH concentrations, and disruption of TH concentrations and/or aberrant activation via TH mimicking BFR compounds could disrupt transporter tissue expression (Zhao et al. 2015). Overall, there are many potential mechanisms of action that may be responsible for the observed reductions in basal SULT activity in BFR exposed BeWo cells.

This research also demonstrates for the first time that BFR concentrations and TH DIO3 and SULT activity in human placenta tissues are related to infant sex. These results add to the growing body of literature that supports sex-specific effects of environmental
contaminant exposure, as well as sex-specific differences in placental biology. The mechanisms underlying these sex-specific effects are not known at this time, however, these differences represent a significant gap in our understanding of basic toxicology and risk assessment for human health. Many epidemiological studies have documented sex-specific differences in fetal growth and fetal/neonatal morbidity and mortality (Di Renzo et al. 2007). For example, women carrying male fetuses have higher rates of gestational diabetes, fetal macrosomia, cord prolapse, and Cesarean sections. There is growing evidence that male sex is an independent risk factor for adverse pregnancy outcomes and that females have a distinct advantage over males within the perinatal period. For example, male infants are 20% more likely to experience a poorer outcome in pregnancies complicated by pre-eclampsia, preterm delivery, and IUGR (Di Renzo et al. 2007). Additional studies have demonstrated that male and female fetuses respond to intrauterine stressors differently, specifically maternal asthma, pre-eclampsia, and preterm delivery (Murphy et al. 2003; Stark et al. 2009, 2008). The results of these studies indicate that males use a “risky” strategy by continuing to grow normally (at the expense of normal placental growth) under adverse intrauterine conditions, which places them at a higher risk of becoming compromised in the event of an additional stressor. Females use an opposite and more conservative strategy by adapting to the intrauterine environment and limiting fetal growth while maintaining placental growth in order to better survive further intrauterine stressors (Eriksson et al. 2010).
to these epidemiological studies, the genetic mechanisms underlying sex differences in
the placenta have been investigated. For example, DNA microarrays have been used to
evaluate the differences in gene expression of 19 human placentas and significant
individual differences were observed between all samples, indicating that there is very
high diversity in human placenta gene expression profiles and that each individual
placenta may exhibit a unique molecular adaptation to similar maternal environments
(Sood et al. 2006). It was also shown that gene expression was highly sex-specific and
that females expressed higher levels of many genes related to immune function
pathways. DNA microarrays have also been used to evaluate differences between male
and female placentae in pregnancies complicated by asthma (Osei-Kumah et al. 2011).
There were 59 gene changes identified in female placentae and only six gene changes in
the placentae of males. Further analysis revealed that the 59 genes in females are
connected with hundreds of protein interactions and signaling networks that contribute
to the female response of reducing fetal growth and maintaining placental growth. In
contrast, the lack of genetic response in male placentae with maternal asthma is
indicative of a lack of response, which allows the male fetus to continue growing in an
adverse intrauterine environment. Yet another microarray study was conducted using
110 term placentas from the National Children’s Study Vanguard birth cohort (Li et al.
2015). Li et al. examined the expression of 654 micro RNAs (miRNAs) in relation to
placenta tissue concentrations of various environmental contaminants, including PBDEs.
This study identified significant associations between BDE-99 and BDE-209 with miRNA expression levels (no effect of infant sex) and is the first study to linking environmental contaminant exposure with placental miRNA expression profiles. A proteomics approach to examining sex differences in placental protein expression has also been used. Analysis of placental protein extracts from asthmatic and non-asthmatic women using surface enhanced laser desorption ionization-time of flight mass spectrometry (SELDI-TOF) identified sexually dimorphic differences in protein peaks, indicating sex-specific responses of placental protein expression to maternal asthma (Osei-Kumah et al. 2008). Overall, males and females institute different strategies to respond to the same adverse maternal environment. Males use a minimalist approach with minor gene, protein, and functional changes within the placenta in order to ensure continued fetal growth despite a compromised intrauterine environment. Based on this approach, males are associated with a greater risk of IUGR, preterm delivery, or infant death. On the other hand, females respond to maternal stressors in order to ensure continued placental growth and function while limiting fetal growth. This strategy increases the likelihood of fetal survival in the event of an additional intrauterine stressor by maintaining a healthy placenta with sufficient nutrient and oxygen supply (summarized below in Figure 24). While the sex differences in placental function have not been extensively studied in relation to environmental contaminant exposure, the current body of literature supporting sex differences in placental gene/protein expression related to
other adverse endpoints is suggestive of sex differences related to contaminant exposure and response. For example, the differences in BFR bioaccumulation between male and female infants in human placenta tissues from Chapter 2 (higher concentrations in males) may be explained by differences in placental morphology. There was no difference in the measured total lipid content between male and female placenta tissues, so it is possible that a difference in tissue protein content could be driving the observed effect. BDE-209 has been shown to bind carrier proteins at a higher rate than lower brominated PBDEs, so it is possible that male placentae express high concentrations of carrier proteins than females, leading to a higher tissue accumulation of BDE-209 (Hakk et al. 2002). In addition to protein differences, it could be possible that male placentae from this cohort had less vascularization than female placentae, leading to lower rates of BFR clearance. More research is necessary to elucidate the physiological basis of these sex differences in the human placenta. Nevertheless, the placenta plays an essential role in the developmental origin of health and disease, and there is growing evidence that demonstrates the sex-specific relationships between environmental factors on placental development and the risk of disease later in life. As a result, the perspective of viewing the fetus, as well as the placenta, as asexual entities needs to be replaced in order to appreciate these important sex-specific differences during pregnancy. Future toxicological research should focus on understanding sex differences in environmental toxicant exposure, especially related to gestation and early-life development when the
HPG axis is maturing and sexual dimorphism is occurring. Moreover, as an easily collectable tissue in humans, the placenta represents an ideal system for studying how sex-specific regulatory pathways respond to intrauterine stressors, and potentially contribute to later-life effects.

Figure 24: Summary of the differences in placental response to similar intrauterine environments based on infant sex. Adapted from Clifton et al., 2006.
One final consideration for future research on this topic is the elucidation of a downstream or biological effect of SULT disruption within the placenta. The research presented in Chapter 2, 3, and 4 show that BFRs affect the activity of TH SULT enzymes in human placenta tissue and cell culture. However, the downstream effects of this disruption have yet to be determined. It is hypothesized that disruption of TH homeostasis in the placenta can lead to perturbations in fetal TH concentrations (e.g. higher T3 levels), which would disrupt TH-mediated developmental processes such as neurodevelopment. However, this hypothesis was only partially supported by the human placenta tissue subsamples and not supported by the BeWo cell culture experiments. Additional work should investigate the potential downstream effects of TH SULT disruption. For example, a longitudinal pregnancy cohort would provide excellent insight into the later-life consequences of BFR exposure in placenta tissues. The investigation of behavioral and cognitive endpoints in children, paired with previously collected placental BFR and TH concentrations, as well as TH DIO3 and SULT activity would allow for a comparison of these variables and determination of a correlative relationship. Additional endpoints may include morphometric measurements of the placenta organ, TH measurements in children, iodine and selenium measurements, and measurement of endogenous DI and SULT activity in children from the cohort. This would be important for increasing our understanding of the potential later-life effects of BFR exposure in the placenta.
In addition to the consideration of a direct effect of TH SULT disruption on TH tissue concentrations and TH-mediated processes, the broad-range of integrated effects of THs needs to be recognized. THs are permissive hormones and act on nearly every cell of the body in humans. As a result, TH biology is deeply interconnected with many other biological systems and there exists a great deal of cross-talk between the HPT axis and other signaling pathways. Many of the documented associations that have been observed between environmental stressors, biological responses, and/or physiological outcomes have been discussed in detail in the previous sections. An outline of those associations is shown below in Figure 25, with the potential role of BFRs and DI/SULT disruption inserted into the larger signaling pathway network. While the details underlying the sex-specific differences in BFR bioaccumulation and endogenous DI and TH SULT activity in placental tissues, as well as downstream effects of SULT disruption within the human placenta are not known at this time, the research conducted within this dissertation contributes to our growing understanding of this complex period of human development.
Environmental Stressors
- Maternal nutritional status
- Maternal asthma
- Maternal diabetes
- Maternal obesity
- Maternal stress
- Environmental toxicants
  - BFRs*

Biological Response (within the placenta)
- Global gene expression
- Protein expression
- Immune function
- Steroid hormones
  - Glucocorticoids
    - Cortisol
  - Sex Steroids
    - Testosterone
    - Estrogen
- Growth factors
- Placenta morphometrics
- HPG Axis
- HPT Axis
  - DIO3*
  - SULT*
- Epigenetics
  - Gene imprinting

Outcome
- Infant mortality
- Birthweight
- Pre-eclampsia
- Preterm delivery
- IUGR
- Metabolic syndrome
- Neurodevelopmental
  - Cognitive
  - Behavioral

Figure 25: Overview of potential pathways of in placental responses (some sex-specific) to environmental stressors. All factors have shown associations with one another. Arrows represent cross-talk between different response pathways. * indicates the contributions from this current body of work.
5.4 Conclusion

The original hypothesis of this dissertation was that BFR concentrations would be correlated with lower levels of endogenous TH SULT activity in human placenta tissues as a result of enzyme inhibition. It was additionally thought that this inhibition would lead to changes in TH tissue concentrations. The results of Chapter 2 and Chapter 3 indicate that BFRs are associated with TH tissue concentrations and endogenous DI and SULT activity in human placenta tissues, however, the relationships are sex-specific and opposite in direction. Additional work was done to elucidate the mechanisms of action and biological effects of BFRs in a placental cell culture model (BeWo). The results of Chapter 4 indicate that BFRs are capable of inhibiting TH SULT activity at the protein level via mixed inhibition, as well as via indirect effects following incubation exposures. However, there was no observed effect on TH concentrations or TH-specific mRNA expression following cell culture exposure. Taken together, this dissertation research has highlighted the sex-specific effects of BFRs in human placenta tissue samples, as well as the potency of BFR compounds to disrupt TH SULT activity in BeWo cells. Future work should continue to investigate the sex differences in human placenta biology, the mechanisms of action of BFRs on SULT activity, and the downstream biological effects of SULT disruption, in order to increase our understanding of this important organ during a critical time in human development.
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Biography

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