ABSTRACT

Using *In Vitro* to *In Vivo Extrapolation* (IVIVE) to Develop Toxicity Metrics for Human Health Risk Assessment of Polybrominated Diphenyl Ethers (PBDEs)

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Polybrominated diphenyl ethers (PBDEs) are flame retardants found in many industrial components, such as furniture foam, consumer electronics, plastics, and textiles and levels have increased in humans over the past few decades. PBDEs demonstrate adverse neurotoxic effects in mice, are lipophilic, and bioaccumulate in fish. Consequently, PBDE biomagnification may occur, which can be transferred to humans in amounts that may present adverse health effects. This investigation uses *in vitro* models to calculate bioassay-based reference doses in order to develop a human health risk assessment based on the consumption of PBDE-contaminated fish, using *in vitro* to *in vivo* extrapolation (IVIVE). The toxicity effects of ten PBDE congeners were examined and compared among those developed using traditional *in vivo* mice studies and *in vitro* models in this study using rat (Clone-9), HEPG2 and zebrafish liver cells to determine the feasibility of using alternative approaches to develop toxicity metrics to evaluate human health risk.

Using In Vitro to In Vivo Extrapolation (IVIVE) to Develop Toxicity Metrics for Human Health Risk Assessment of Polybrominated Diphenyl Ethers (PBDE)

by

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CHAPTER ONE

Introduction

Fires kill more than 3,000 people annually, while 20,000 more are injured and approximately $11 billion dollars account for in property damages in the United States alone (Karter, 2002; Birnbaum et. al, 2004). Over the past 25 years, the number of fire incidences has dropped partially due to fire prevention policies that require flame retardant chemicals to be present in many industrial products (Birnbaum et al., 2004; Dawson, 2002). Flame retardants are chemicals that have the ability to slow fire ignition and the rate at which a fire develops. As a result, flame retardants increase the escape time in the event of a fire and reduces injury (BSEF 2000; Birnbaum et al., 2004), thus saving lives (Costa et al., 2008; Birnbaum et al., 2004). Of the 175 different types of flame retardants, the brominated group currently has the largest market because of its low cost and high performance efficiency (Birnbaum et. al, 2004). More specifically and in contrast to the long banned polychlorinated biphenyls (PCBs) (Costa et al., 2008), polybrominated diphenyl ethers (PBDEs) have been increasingly used as additives, or blended with polymers, and are not chemically bound to the products in which they have been placed; thus, PBDEs are more likely to leach out of the product and into indoor and outdoor environments (Costa et al., 2008; Athanasiadou et al., 2008; de Wit, 2008; Noyes et al., 2010; Hale et al., 2006; de Wit et al., 2006; Alaee et al., 2003; Eriksson et al., 2001). PBDEs have become ubiquitous persistent organic pollutants over the past twenty years (Costa et al, 2008; Branchi et al., 2003; Hites et al., 2004; Chen et al., 2008) due to its massive production.

The production of PBDEs began in Germany in the 1970s (US EPA, 2006) and are found in the form of three commercial mixtures, or biphenyl oxides, decabromodiphenyl ether (decaBDE), octabromodiphenyl ether (octaBDE), and pentabromodiphenyl ether (pentaBDE) (Birnbaum et. al, 2004; Birnbaum et. al, 2006; ATSDR, 2004; Huang et al., 2009; Wenning, 2002). Congeners with fewer than four bromines are rarely found in commercial mixtures (Birnbaum et al., 2006). DecaBDE is the most widely used PBDE (Costa et. al, 2008) and makes up about 82% of the products manufactured globally (ATSDR, 2004). As of 2003, according to the directory of chemical producers (SRI, 2002), the Great Lakes Chemical Corporation in El Dorado, Arkansas produced pentaBDE and octaBDE, while the Albemarle Corporation in Magnolia, Arkansas produced decaBDE (BSEF 2000; Birnbaum et. al, 2004), approximately 40 miles between each company. DecaBDE is still produced in the US and in Europe, while pentaBDE and octaBDE have recently been banned in the European Union and in several states in the US, such as California (Birnbaum et. al, 2004), and are also no longer produced in these countries (Costa et al., 2008; Costa et al., 2007; Birnbaum et al., 2004; BSEF 2003).

The total worldwide production of PBDE is approximately 67,000 metric tons per year, and the U.S. accounts for slightly more than 50% of its production (BSEF 2000; Birnbaum et al., 2004). Some forms of PBDEs are naturally produced in the environment via some algal species (Usenko et al., 2012) and marine ecosystems, thus have been structurally identified in biota from marine environments (Anathasiadou et al., 2008). Gribble et al., (1998) provided examples of a number of naturally occurring organobromine compounds that are produced by marine and terrestrial plants, marine

animals, bacteria, fungi, and also humans. For instance, marine sponges are known to produce methoxylated PBDE (Gribble et al., 1999).

There are theoretically 209 PBDE congeners (de Wit 2008; Costa et al., 2008; Branchi et al., 2003; Noyes et al., 2010; Darnerud et al., 2001) that share a common structure, which vary in the degree of substitution of one to ten bromine atoms on aromatic rings (US EPA, 2006; Branchi et al., 2003; Darnerud et al., 2001; Hardy, 2002). Congeners are distinguishable in that they differ in the number of bromine atoms as well as the position of those atoms (US EPA, 2006). PBDEs are numbered using the IUPAC numbering system (Branchi et al., 2003; Ballschmiter et al., 1993; Birnbaum et al., 2006; Darnerud et al., 2003) and are divided into ten congener groups (mono- to decabromo-diphenyl ethers) (Branchi et al., 2003). General properties indicate that all PBDEs present in commercial products have low water solubility, less than 1 μg/L, and high log octanol-water partition coefficient (*K*ow) values, greater than 5 (Birnbaum et al., 2004). Thus, high levels of PBDEs are not found in water, which indicates that very small amounts are water-soluble and therefore bind to particles that eventually settle in the sediment where it can remain for years (ATSDR, 2004). PBDEs have low vapor pressure at room temperature (Branchi et al., 2003); moreover, the lower brominated congeners have higher vapor pressures than the higher brominated compounds (Birnbaum et al., 2004; de Wit, 2002). These compounds have a high resistance to physical, chemical and biologic degradation, by a boiling point between 310°C and 425°C (Branchi et al., 2003; Darnerud et al., 2001; de Wit, 2002).Human exposure routes occur via consumption of PBDE-contaminated food, breast milk (Schecter et al., 2003), ingestion or inhalation of dust and air (Schecter et al., 2010; Lober, 2007), and dermal absorption, while some of

the pathways occur via bloodstream and body fat (ATSDR, 2004). The main route of human exposure to PBDEs has been suggested to be via food (Branchi et al., 2003; Alaee et al., 1999; Darnerud et al., 2001; Wenning, 2002). Although PBDE exposure routes are not well established, more recent data suggest that diet and inhalation are the predominant routes of exposure (Allen et al., 2007; 2008; Costa et al., 2008; Pacyniak et al., 2010; Schecter et al., 2004, 2005a; 2006). Lower brominated congeners are much more likely to enter systematic circulation through the lungs and stomach, then pass into the bloodstream. The concentrations of the lower brominated PBDEs in blood, breast milk, and body fat indicate that the majority of people are exposed to low levels of PBDEs (ATSDR, 2004). Currently, the levels of lower brominated PBDE congeners are greater in individuals living within the United States than levels reported in other regions of the world (ATSDR, 2004). Due to lipophilicity and persistence in the environment, PBDEs accumulate in lipid-rich tissues (de Wit, 2008; Frederiksen et al., 2009). Consequently, consuming food, such as fish, at high trophic levels or those containing lipid-rich oils have been found to have relatively high concentrations of PBDE as an exposure route to humans (Frederiksen et al., 2009). PBDEs tend to bind to soil particles very strongly and can remain in soil for several years (ATSDR, 2004). PBDEs are not suspected to spread below the soil surface, which makes it unlikely to enter groundwater.

When PBDEs are suspended in the air, they can be present as particles and eventually return to land or water as the dust settles and are carried out by snow and rainwater (ATSDR, 2004). Lower brominated congeners, such as tetraBDE and pentaBDE, are found throughout the environment at low levels in air, sediments, animals, and food. Highly brominated congeners, such as decaBDE, are not commonly found

throughout the environment (ATSDR, 2004). However, BDE-209 has been detected in certain foods, in breast milk, and in the placenta and has been observed to debrominate to lower brominated congeners in rats (Costa et. al, 2008).

According to the US EPA (2006), there have been published studies expressing concern about PBDE exposure (i.e., Birnbaum et al., 2004; Eriksson et al., 2002a). PBDE levels could cause adverse effects in sensitive human populations, such as young children, indigenous peoples, and fish consumers (de Wit, 2002). PBDEs have been found to affect the development of the central nervous system in mice (Viberg et al., 2003). For example, a study performed by Eriksson et al., (2001) has shown that neonatal exposure to BDE-47 and BDE-99 can cause permanent aberrations in spontaneous behavior and learning and memory in adult mice. Peer-reviewed studies have demonstrated that even a single dose administered to mice during brain development can cause permanent changes in behavior, including hyperactivity (Kuriyama et al., 2005). PBDEs have also been suggested to have endocrine disrupting effects, particularly on estrogen and thyroid hormones (Lema et al., 2008), such as an increase in thyroid hormone levels in adult male sport fish consumers shown by Turyk et al., 2008 (Schecter et al., 2006; Hallgren and Darnerud, 2002), and also toxic to the development of animals (Stoker et al., 2004).

Electronic waste may be discarded or recycled (Cui and Forssberg, 2003); moreover, uncontrolled discharges of materials with the presence of brominated flame retardants may lead to high environmental exposure to these chemicals (Athanasiadou et. al, 2008). These flame retardants have recently become an environmental concern as contaminants not only in the environment, but also in the tissues of fish, birds, marine

mammals and humans because of their high potential for accumulation (Birnbaum et al., 2004). The presence of PBDEs in human tissue is also of great concern because of potential toxicological endpoints including carcinogenicity, neurotoxicity, reproductive toxicity, and endocrine toxicity (Darnerud et al., 2001; Pacyniak et al., 2010). Athanasiadou et al (2008) states that humans are exposed to PBDEs via ingestion, (i.e., Schecter et al., 2006), concerning seafood consumption, and inhalation in which PBDEs are present in ambient air both in industrialized regions (Butt et al., 2004; ter Schure et al., 2004), and the Arctic and also in household dust (Schecter et al., 2005a; Stapleton et al., 2005; Wu et al., 2007). Moreover, PBDE levels detected in human serum and adipose tissue in Europe and Asia are usually below 5 ng/g lipid, while those in North America have been found to be as high as 200 ng/g lipid (Costa et. al, 2008). When compared to Europe and Japan, the body burden of PBDEs in North America are one to two orders of magnitude higher and furthermore PBDE levels have doubled every 4–6 years (Hites, 2004; Pacyniak, et al., 2010; Petreas et al., 2003; Schecter et al., 2005b, 2007). Fully brominated deca-BDE congeners have been known to be poorly absorbed and rapidly eliminated, thus have low potential for bioaccumulation (Branchi et al., 2003; el Dareer et al., 1987; Norris et al., 1975). However, decaBDE has been shown to be toxic, indicated by Hu et al., (2007), which revealed that BDE-209 inhibited cell proliferation in HEPG2 (human carcinoma liver cells) and also caused morphological changes, such as shrinkage and retraction from their neighbors, at concentrations ranging from 10-100 μmol/L. HEPG2 is a human hepatoblastoma cell line that has an array of signal responses to different classes of drugs (Hu et al., 2007, 2009; Knowles et al., 1980). This causes concern regarding damages and/or adverse health effects that may

occur in the human liver. The complete mechanism for PBDEs is not completely known. Moreover, some proposed mechanisms of action for PBDEs are induction of oxidative stress and alterations of thyroid hormone homeostasis (He et al., 2008; Albina et al., 2010; Lema et al., 2008). These insults/injuries could result in altered neural development and functioning (Lema et al., 2008).

The liver is a major target organ that removes many chemicals, drugs, and microbial pathogens (Sahu et al., 2008; Treinen-Molsen, 2001) from the portal blood (Pacyniak et al., 2010) and is the primary organ involved in metabolizing xenobiotic substances. The liver is also one of the first organs of exposure for food-borne contaminants, which makes hepatotoxicity one of the most serious safety concerns for food additives, contaminants, dietary supplements and microbial pathogens (Sahu et al., 2008). Hepatic uptake is a prerequisite for biotransformation and subsequent elimination of various endogenous and exogenous compounds (Pacyniak et al., 2010). Although the human liver burden of PBDEs is not clear, the presence of PBDEs in human liver is particularly alarming due to hydroxylated metabolites playing a pivotal role in PBDE-mediated toxicity (Darnerud et al., 2007; Hamers et al., 2008; Meerts et al., 2000; Pacyniak et al., 2010; Zhou et al., 2002). According to toxicity studies, the liver, thyroid gland and possibly also developing reproductive organs are particular targets of PBDEs toxicity (Darnerud et al., 2001; Kuriyama et al., 2005; Hu et al., 2009). *In vivo* studies indicated BDE-47 accumulates in the liver of mice, followed by redistribution to other tissues, including the kidney and adipose tissue (Pacyniak et al., 2010; Staskal et al., 2005). Another example involves a human Swedish study (Guvenius et al., 2001), where liver tissue samples were taken from one female (age 47) and 4 males (ages 66-83) and

analyzed for PBDEs. The mean human liver concentrations for some common BDEs was between 0.07 and 3.8 ng/g lipid weight. Thus, liver cell cultures are valuable models for *in vitro* studies of toxicology (Sahu et al., 2008) and may serve as *in vitro* assay systems for environmental contamination (Ghosh et al., 1994; Tifitt and Geisy, 1991). *In vitro* models are important alternatives to that of *in vivo* models due to time consumption and cost, either complementary or supplementary (Green et al., 2001; MacGregor et al., 2001; Sahu et al., 2008). *In vitro* models offer well-defined and reproducible experimental conditions for toxicity evaluation and are excellent systems to study mechanisms of action and structure-activity relationships at the cellular and molecular level, which makes them a very useful tool to rapidly screen for potential toxins (Sahu et al., 2008). Primary hepatocytes and hepatocyte cell lines in culture retain their metabolic activities that are characteristic of the intact liver *in vivo* (Hengstler et al., 2000, 2002; Runge et al., 2001; Sahu et al., 2008). These cell lines serve as excellent *in vitro* models when studying liver function, xenobiotic metabolism, pharmacology and toxicology (Sahu et al., 2008) as well as the toxicity of chemicals, drugs and microbes (Barsi et al., 1998; Castell et al., 1997; Li et al., 1999; Michalopoulos, 1999; Sahu et al., 2001, 2008).

Particularly of interest in this study, primary rat hepatocytes have become the most frequently used and best characterized *in vitro* model for testing liver toxicity of chemicals and drugs because of its historical use by many laboratories throughout the world (Beekman et al., 2006; Sahu et al., 2008). An area of uncertainty in extrapolation of *in vitro* mechanistic studies to *in vivo* effects is the lack of understanding of tissue dose. A number of *in vitro* studies employ a range of exposure concentrations to encompass known or probable *in vivo* blood or target tissue concentration. Therefore, a

known common assumption of *in vitro* studies is that medium concentration is a predictive marker of tissue concentration. There are very few data available to support or refute this assumption (Mundy et al., 2004). More specifically, *in vitro* models have also been used to investigate toxic effects of PBDEs, such as the study of BDE-47 accumulation in neuronal cells conducted by Mundy et al., 2004. This study demonstrated that BDE-47 accumulate in neuronal cells by 100-fold and that accumulation is influenced by length of exposure, constituents of the exposure solution, and total media volume applied.

In vitro experimental systems attempt to reproduce that of a whole organism process. For instance, Pelkonen et al., (2007) used *in vitro* to *in vivo* extrapolation (IVIVE) to use data from simple systems to assess drug disposition in complex whole organisms in efforts to reproduce the whole organism process. Thus, one was able to conclude whether a compound was rapidly or slowly cleared via hepatic processes. The ultimate goal of employing the IVIVE of clearance technique is the ability to predict *in vivo* pharmacokinetics of a compound in humans (Pelkonen et al., 2007). This technique is an emerging useful tool in drug development, which was also employed in other studies (i.e., Ito and Houston, 2004; Riley et al., 2005). This method is far from perfect extrapolations, there are some factors to consider in using this type of approach, such as the use of proper scaling factors (i.e., hepatocytes to liver mass unit); the awareness of other processes within the system occurring simultaneously, but independently (i.e., renal clearance); and the assumed or measured physiological values (Pelkonen et al., 2007). Thus, the present study employs similar methods of IVIVE as Pelkonen et al., (2007), making the present study novel. Furthermore, the extrapolation outcome critically

depends on the primary experimental data and the quality of in vivo pharmacokinetics data (Nagilla et al., 2006). O'Brien et al., (2004) evaluated the utility in using *in vitro* models for testing seven classes of xenobiotics/drugs and found that human and animal hepatocytes in vitro have been related to their in vivo hepatotoxicity and the corresponding activity of their metabolizing enzymes. Even more so, this study also showed that the cytotoxic effectiveness of 16 halobenzenes towards rat hepatocytes (using higher doses and short incubation times) *in vitro* correlated with rat hepatoxic effectiveness *in vivo* (using lower doses and longer incubation times. Unfortunately, there is no "perfect" animal species that accurately predicts drug to human interactions. Therefore, animals selected for toxicity studies are primarily based on the animal models' historical use, cost, and availability of a particular strain or species instead of being based on which animal model may provide a good prediction of human metabolism of a particular drug of concern (O'Brien et al., 2004). This is the same method used for selection of cell lines for similar *in vitro* investigations like the one used in this study.

There is limited human data available, thus several studies are performed with animal models *in vivo*, such as rats or mice. For example, one study Viberg et al., (2003) found that BDE-209 can cause developmental neurotoxic effects in mice in which the effect also worsens with age. Moreover, current evidence for health effects based on data from animal models includes endocrine disruption (Darnerud and Sinjari, 1996; Fowles et al., 1994; Mundy et al., 2004; Zhou et al., 2001, 2002), developmental neurotoxicity (Branchi et al., 2002; Eriksson et al., 2002; Mundy et al., 2004), and reproductive toxicity (Mundy et al., 2004; Stoker et al., 2004).

Small fish, such as zebrafish (Ghosh et al., 1994; Hatanaka et al., 1982; Bresch et al., 1990; Bresch, 1991) and medaka, have also become popular model systems for studies of toxicology due to its rapid sexual maturity (within approximately 3 months) and are tolerant of temperature and salinity and year-round reproduction (Collodi et al., 1992; Hatanaka et al., 1982, Egami et al., 1981; Aoki et al., 1977; Bresch, 1991; Bresch et al., 1990, Babich and Borenfreund, 1991; Streisinger et al., 1981; Powers, 1989). The zebrafish is a popular economical model for studies of vertebrate development and toxicology (Collodi et al., 1992; Schultz et al., 1982a; Babich and Borenfreund, 1991) because of the established morphological, biochemical, and physiological information at all stages of early development in both juveniles and adults of males and females. Therefore, zebrafish an ideal organism for toxicology research when identifying adverse effects of chemical exposure (Hill et al., 2005). Adverse effects of chemical exposure on the brain, notochord, heart, jaw, trunk segmentation and size measurements can be quantitatively assessed by using little magnification. Moreover, the zebrafish genome is considered by some researchers to be more complex than humans due to two more pairs of chromosomes in zebrafish than the twenty-three pairs of human chromosomes (Hill et al., 2005). Zebrafish are also a unique cancer model in that it can develop almost any type of cancer (Fetisma et al., 2008; Kent et al., 2002) and many of its tumors are histologically similar to human tumors with regard to general cancer characteristics, such as genomic instability, invasiveness, transplantability, cancer stem cells existence apply to the zebrafish tumors as well (Fetisma et al, 2008; Langenau et al., 2007). Zebrafish embryos and larvae are a convenient and effective model for comparison of physiological processes and organ-systems similar to those in humans. In fact, there are several studies

in which zebrafish respond biologically to chemicals (ex. drugs and environmental toxicants) similarly to mammals (Sukardi et al., 2011). The aforementioned reasons make zebrafish a good choice for the type of investigations conducted in this study. However, there are currently few fish liver cell lines derived and exploited for studies as such even though fish has become a popular experimental model for toxicology research (Ghosh et al., 1994; Tillitt et al., 1991). Ghosh et al., (1994) characterized the zebrafish cell line and demonstrated that the cells exhibit properties in culture that are associated with differentiated liver cell function *in vivo*. Although *in vitro* cell-based models from zebrafish lack relevant whole-organism physiology to further validate findings, they are well suited for high-throughput screening (Sukardi et al., 2011). One of the disadvantages of using this model is the lack of characterization of the cell line and the lack of a full understanding of the absorption, distribution, metabolism, and excretion (ADME) profile of many chemicals using this model.

PBDE levels found in humans and wildlife continue to increase, which cause concern regarding potential ecological health risks (Lema et al., 2007). For example, Gilron et al., 2007 indicated that elevated levels of PBDEs in farmed and wild salmon have had significant impacts on public opinion and consumer behavior, influencing the sales of farmed salmon in North America and Europe. Thus, there is an essential need for the assessment of contaminants in fatty fish (an important source of omega-3 fatty acids) in balancing risks and benefits (Gilron et al., 2007). Pérez-Fuentetaja et al., 2010 states that PBDEs tend to accumulate in fatty tissues (Burreau et al., 1999) and are currently found in the tissue of fish in higher trophic levels (Luross et al., 2002; Montory et al., 2010). Thus, in excess, PBDEs may cause toxic responses. Human health risk assessors,

risk managers and public health professionals are often needed to analyze, interpret, and/or communicate existing data regarding levels of PBDEs in farmed and wild salmon (Gilron et al., 2007).

Potential Significance

There are citizens of the United States who consume fish as a part of their everyday diet. Some people fish for recreational purposes, but many who fish for survival. The overall knowledge of PBDEs is very limited and hinders environmental authorities from carrying out adequate risk assessments (de Wit, 2002). The development of a human health risk assessment will seek to determine the risks involved and to what extent adverse effects may occur when exposed to PBDEs through the consumption of fish. Knowledge of this type aims to prevent what otherwise could be a harmful, toxic or long-term human health effect from occurring. This research will provide a critical first attempt in predicting adverse health effects in humans from the consumption of fish contaminated with PBDEs. This risk assessment will establish preliminary protective measures for human health as well as opening the opportunity for society to be environmentally conscious. As a society, we must create a healthy balance between the chemicals we feel are necessary for a certain quality of life and that which we do not need in order to protect human health and the environment. Future studies may be expanded to narrow the scope of the dose at which the PBDE concentration becomes more toxic within the assays presented to reduce uncertainty.

Specific Aims

Recent studies have shown the bioaccumulation and potential concerns of PBDE contamination (Birnbaum et. al, 2004; Frederiksen et. al, 2009). We hypothesize that an *in vitro* bioassay-based reference dose (RfD) can be developed that will be similar to those developed using the traditional *in vivo* mice-based studies. These bioassay-based reference doses can then be used to complete a preliminary baseline human health risk assessment for PBDE exposure from ingestion of contaminated fish. Specifically, the objectives of this study are as follows: 1) evaluate cytotoxicity in Clone-9 (Sprague-Dawley rats), zebrafish (*Danio rerio*), and HEPG2 liver cells; 2) develop bioassay-based RfDs from the 3 models systems: Clone-9 (rat liver cells), zebrafish liver cells, and human carcinoma liver cells (HEPG2); 3) compare assay sensitivity using RfD values across 4 model systems: *in vitro* (rat, zebrafish, and human liver cells) and *in vivo* (Naval Medical Research Institute (NMRI) or C57B1) mice; 4) compare the experimental assay RfD values to the RfD values established by EPA for PBDEs; 5) conduct a human health risk assessment based upon the experimental RfDs.

The following PBDE congeners, each at five concentrations ranging from 1.25 to 20 mg/L (nominal concentrations), are examined in these experiments: BDE-28, -47, -99, -100, -153, -183, -209, 3-OH-BDE-47, 5-OH-BDE-47, and 6-OH-BDE-47. The chosen concentrations were based on a similar study found in a literature review and for the most accurate representation of a reasonable maximum exposure (RME) scenario for a baseline human health risk assessment. These specific congeners were chosen based on their occurrence in human and environmental samples, their use in commercial products, and produced metabolites. These compounds provide a significant, structural range for

the class of PBDE compounds, which includes 209 different congeners. The hydroxylated compounds were also chosen due to human and environmental findings as well as their toxic potential. Current studies have found that hydroxylated-BDE compounds are more toxic than their parent compounds (Usenko et al., 2012; Dingemans et al., 2008; Meerts et al., 2001; Van Boxtel et al., 2008). Valters et al., 2005 showed that hydroxylated PBDEs are structurally similar to the thyroid hormone, thryoxine (T4), and are found in the blood and have been shown to compete with thyroid hormones as they bind to thyroid hormone transport proteins. In a study conducted by Usenko et al., (2012), 6-OH-BDE-47 disrupted the gene transcription of a normal thyroid hormone during the developmental stages of zebrafish. In rat liver microsomes, several PBDE congeners were biotransformed to metabolites (Valters et al., 2005). Recent studies found that some hydroxylated PBDEs show evidence of greater neurotoxic, thyroid system disrupting and estrogenic potency than the related PBDE congeners (Legler, 2008; Dingeman et al., 2010; Wang et al., 2011). Moreover, it was demonstrated by Orn and Klasson-Wehler, 1998 that BDE-47 is biotransformed to hydroxylated PBDEs in rats and mice. Known for its persistence and abundance in the environment and in environmental samples (Meeker et al., 2009), BDE-47 and its hydroxylated metabolites has been of high priority. Based on the comparison of data collected from two *in vivo* bioassay results, three *in vitro* bioassay results, a bioassay-based reference dose can be estimated for the prediction of risk associated with human consumption of contaminated fish.

CHAPTER TWO

Methods and Materials

All test chemicals (BDE-28, -47, -99, -100, -153, -183, -209, 3-OH-BDE-47, 5-OH-BDE-47, 6-OH-BDE-47) used in this study were obtained from Accustandard, Inc (New Haven, CT). Ham's Nutrient Mixture F-12, Leibovitz-15 media (L-15), Dulbecco's Modified Eagle's Medium, Dulbecco's phosphate-buffered saline, and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich Chemical Company (Saint Louis, MO). Trypsin-EDTA solution, penicillin-streptomycin, and fetal bovine serum were obtained from Atlanta Biologicals (Lawrenceville, GA). Tissue culture flasks and Costar multi-well plates were obtained from BD Falcon (Bedford, MA) and Sigma, respectively. The 96-well plates used in this study were low-binding tissue culture plates with covers to assist in preventing chemical absorption and/or slowing the rate of evaporation. Alternatively, glass-lined plates would have been ideal for this study; however, these are expensive and were not a possibility for the number of chemical experiments that were run in this study. Consequently, other researchers in our lab (Usenko et al., 2011) have analytically quantified these concentrations when using 48-well plates in similar studies; there was an initial decrease of nominal concentration in the first 24 hrs followed by the concentrations remaining constant afterwards. We measured cytotoxicity via cellular viability in rat liver cells (Clone-9), zebrafish liver cells and HEPG2 liver cells *in vitro*. Cell viability was chosen as a general endpoint rather than more specific endpoint, such as neurotoxicity or reproductive toxicity, because current literature does not provide a complete mechanism of action for this class

of compounds. Choosing a more general measure of cytotoxicity allowed the consideration of any alteration that the exposures made on the cells. If the mechanism of action for PBDEs had been fully elucidated, and, for example, I was doing a more specific risk assessment (i.e., reproductive toxicity risk assessment), then it would have been more appropriate to choose a more specific measure of the relevant toxic endpoint. Test solutions for *in vitro* studies were dissolved in DMSO and delivered in basal medium at 0.5% DMSO. Solvent controls were included for each cell line to ensure the solvents did not cause a statistically significant decrease in cell viability and proliferation (refer to Appendix C).

Clone-9 Rat Liver Cells

According to the American Type Culturing Collection (ATCC; Manassas, VA), rat liver clone-9 cells were originally isolated from the normal rat liver of a 4-week old Sprague-Dawley male rat, which has been used by other investigators as an *in vitro* model for testing the hepatotoxic potential of chemicals and drugs (Sahu et al., 2008). Approximately one million Clone-9 rat liver cells were purchased (ATCC) and stored in liquid nitrogen upon arrival. The cells were subcultured before being used for experimental purposes to ensure proper health and growth. Complete growth medium consisted of Ham's F-12 medium and 10% fetal bovine serum. Before retrieving the frozen vial, medium was warmed for approximately 30 minutes at 37°Celsius. The neck of a T-75 flask was opened and flamed for sterilization, and then filled with 18 mL of the warmed culture medium.

Next, the frozen vial was placed into water (usually collected from the waterbath at 37°C) until thawed. Immediately, the cell contents were drawn from the vial and

transferred to a T-75 flask. The flask neck was flamed again before closing, and then observed under the microscope before placed into the incubator at 5% CO_2 and 37°C until ready for separation. Cells were grown over a period of approximately 3-4 days until 80-85% confluent (doubling time is approximately 24 hours), during which medium was replaced every other day as long as needed. Once confluent, the hood was prepared with phosphate-buffered saline (PBS-Free), trypsin and medium (all warmed 30 minutes prior to use in the waterbath at 37°Celsius, then opened and flamed in hood). Media was removed from the T-75 flask of cells and replaced with PBS-Free (20 mL) and placed into the incubator at 37°C for about 4-6 minutes. PBS-Free was removed and replaced with trypsin (3 mL) and placed into incubator for about 3-4 minutes. The flask was lightly agitated until cells detach from the bottom of the flask. Immediately, medium (7 mL) was added to the flask and rinse a few times until most of the bottom portion of the flask has been covered with medium and cells have been suspended in medium. Cells were then placed into a 15 mL centrifuge tube, where a 20 µL sample was taken and placed into a 20 mL cell counter vial of isotone solution (ready for counting). The 15 mL centrifuge tube was placed in the centrifuge at 3000 rpm for about 3 minutes to spin down cells into a pellet clump. Next, the liquid was removed from the undisturbed pellet, and replaced with fresh medium (amount varies with cell count). The 15 mL tube was vortexed until cells became freely suspended without clumps. A sterile basin was used to mix 600 µL of cell solution and 9.4 mL of media to plate cells in a 96-well plate. This calculation plated 6,000 cells per well at 100 µL each, assuming that there are 1,000 cells per 1 mL of medium. The 96-well plate was placed in the incubator for 24 hours at 5% CO_2 and 37°C. The plate was observed after 24 hours to ensure cell attachment and an

even distribution throughout the plate. The plate was set up with one control column (medium and cells only), one solvent control column (medium, 0.5% DMSO, and cells only), and the remaining columns were dosed with the proper treatments of PBDE with five concentrations ranging from 1.25 to 20 mg/L using a 0.5 dilution factor. After 72 hours, cell viability was measured by using the Janus Green assay. For each concentration, two columns of 8 wells were used for each exposure (N = 16 wells for all exposures for each experiment). Each experiment was run three separate times and the mean and standard error were calculated for each treatment (N=48).

HEPG2 - Human Carcinoma Liver Cells

HEPG2 cells have been used in various toxicological studies. Although HEPG2 cells may not respond exactly the same as normal human liver cells for some endpoints, the chosen endpoint (viability) for this study was accurately represented in HEPG2 cells because many of the phenotypical characteristics of normal human liver cells are conserved in this cell line. Approximately one million human carcinoma liver cells were purchased from ATCC, where they were originally isolated from the cancerous liver of a 15-year old adolescent male, and then stored in liquid nitrogen upon arrival. Cells were cultured at 37°C and 5% CO_2 and used complete growth medium containing eagle's minimum essential medium with 10% fetal bovine serum. After two subcultures, the cells became approximately 80-85% confluent in approximately 5 days in a T-75 flask, the medium (18 mL) was aspirated from the flask, and then rinsed and incubated at 37°C for 3 minutes with 20 mL of PBS-Free. PBS-Free was aspirated, and then trypsin (3 mL) was added and incubated at 37°C for 5 to 7 minutes. The flask was not agitated, but instead gently shaken to disturb cells from bottom of flask. Growth medium was added

to the flask to neutralize the effect of trypsin. An aliquot was taken to count cells, which were seated at approximately 9,000 cells/well in a 96-well plate. The population doubling time was approximately once every 48 hours, where it reached 80-85% confluent in 72 hrs. For each concentration, two columns of 8 wells were used for exposure (N = 16 wells for all exposures for each experiment). Each experiment was run three separate times and the mean and standard error were calculated for each treatment (N=48). Cells were incubated for 24 hours to ensure attachment and even distribution, and then dosed with the varying concentrations of PBDEs. After 72 hours of incubation, cell viability was measured using the Janus Green B assay.

Zebrafish Liver Cells

Zebrafish liver cells follow a process similar to that of the rat liver cells. Zebrafish liver cells were originally isolated from the normal liver of an adult zebrafish. Approximately two million zebrafish liver cells were purchased from ATCC, and then stored in liquid nitrogen upon arrival. Culture medium contained 87% Leibovitz's L-15 media, 10% Heat-Inactivated Fetal Bovine Serum, 2% Zebrafish Embryo Extract and 1% Penicillin-Streptomycin. Zebrafish embryo extract was formulated by euthanizing 3-day old embryos with tricaine (200 embryos per 1 mL extract obtained from stocks at Baylor University). After 4-5 minutes or until embryos became paralyzed, the tricaine was removed and the embryos were rinsed with 0.5% bleach for 2 minutes. After the bleach was removed by pipeting, the embryos are rinsed with PBS-FREE for 2 minutes. The PBS-FREE was removed as much as possible and embryos were homogenized well. One mL of L-15 media was added to the homogenized liquid and vortexed, and then stored at -20°C. After subculturing and cells grew to approximately 80-85% confluent in about 6-

7 days in a T-75 flask, the medium was removed from the flask, replaced with trypsin (3 mL) and allowed to sit at room temperature (or may be incubated at 28°C) until the cells detached (about 10-15 minutes). The flask was not shaken while cells were detaching to prevent clumping. Fresh growth medium (7 mL) was added to the flask and a 20 µl aliquot was taken for cell count. The cells were centrifuged at 1000 rpm for 10 minutes. Trypsin-based medium was aspirated and the pellet was resuspended in fresh growth medium. Cells were seeded in a 96-well plate at approximately 15,000 cells per well and incubated at 28°C for 24 hours (to ensure attachment and even distribution). There were two replicates of eight wells for each concentration (N = 16 wells for all exposures for each experiment). Each experiment was run three separate times and the mean and standard error were calculated for each treatment (N=48). The population doubling time was not exactly known, but was slow. However, when seeding at this density, the well became confluent at approximately 80-85% at the end of 72 hours. The plate was dosed with the varying concentrations of PBDE, and then cell viability was measured using Janus Green B assay.

Determination of Cell Viability

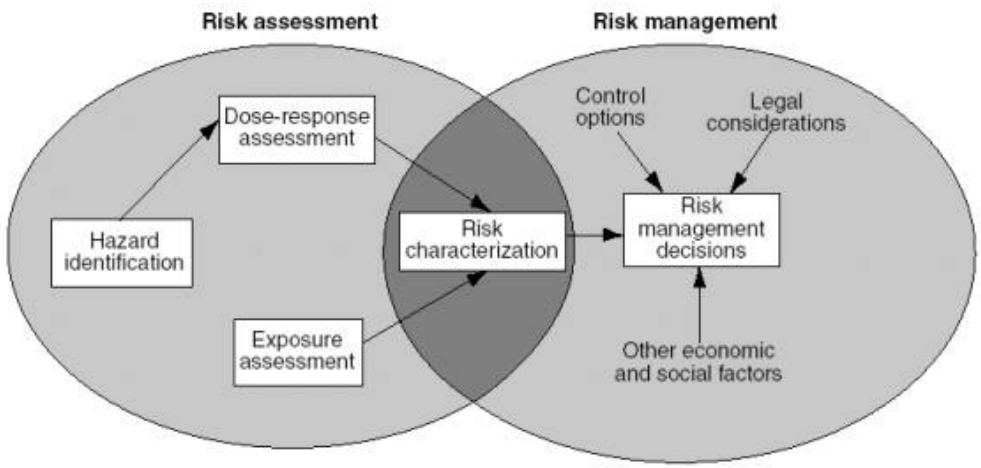
Janus Green B is a more sensitive and rapid alternative to cell counting under a microscope. It is a dye used to rapidly quantitate cell growth, proliferation and viability on surviving and damaged cells by intensively staining the cell's nuclei and mitochondria and lightly staining the cell's cytoplasm (Rieck et al., 1993). Mitochondria become decolorized when all of the oxygen is removed (Bensely et al., 1911, 1938) upon adding cyanide. Mitochondria regain their blue color when cyanide is washed away (Lewis, 1923; Lewis et al., 1924). Thus, it is suggested that Janus green B indicator depends

upon the enzyme activities of the cell. This supravital dye (similar to trypan blue) allows viable, healthy cells to exclude the dye, while dead or dying cells absorb the dye through the ruptured cell membrane. Absorbance is measured photometrically by a multi-well scanning spectrophotometer to measure optical density at 654 nm.

Janus Green B is used to measure cell viability in Clone-9, HEPG2 and zebrafish liver cells. 96-well plates containing the particular cells with varying PBDE congeners and concentrations are incubated at 37°C, 37°C, and 28°C, respectively, for 72 hours. Medium was aspirated followed by two PBS-Free rinses at 100 µl per well. Any remaining PBS-Free was removed from wells. 100 µl of Janus green dye is added to the wells for a minimum of 60 seconds and aspirated. All wells were washed twice again with PBS-Free, while any remaining dye was also washed away after the second rinse. One hundred microliters of absolute ethanol and 100 µl of nanopure were added to the wells. The plate was immediately placed on a spectrophotometer at an optical density reading of 654 nm to measure cell viability.

Risk Assessment

The process of a human health risk assessment (HHRA) consists of four basic steps, which can be observed in the US EPA's diagram below (Figure 1). The first step is hazard identification, which qualitatively describes the likelihood that a chemical agent can produce adverse health effects under certain environmental exposure conditions (Asante-Duah, 2002). This step identifies contaminants, toxicological properties, pathways and routes of exposure. Therefore, this study identified the hazard of concern, where its properties, background information, and characteristics were previously described in the introduction.



Source: EPA Office of Research and Development.

Figure 1. Risk Assessment Paradigm.

The second step evaluates toxicity assessment, which quantitatively estimates the relations between magnitudes of exposure and the degree and/or probability of occurrence of a particular health effect. Toxicity assessments compiles quantitatively and qualitatively toxicology and evaluate health effects and uncertainty (Asante-Duah, 2002). Within this assessment, the RfD is derived by using NOAEL (no-observed-adverse-effect level) or LOAEL (lowest-observed-adverse-effect level) values. The NOAEL is the highest experimentally-determined dose at which tested organisms have shown no statistically or biologically significant indication that the toxicant presents toxic effects, whereas the LOAEL is the lowest experimentally-determined dose at which adverse effects are noted in tested organisms. For these experiments, the NOAEL or LOAEL values from the class of PBDE congeners are used conservatively to determine an RfD among each assay. One of the main routes of human exposure to PBDEs is through ingestion. We developed an oral RfD based upon the consumption of locally caught fish. This scenario was selected based upon discoveries of alarming environmental

concentrations of PBDEs, lipophilic compound with the potential to bioaccumulate in fish (Anderson et al., 2008). The oral RfD equation used is as follows:

RfD equation:

$$ORfD = \frac{NOAEL}{UF \times MF}$$

Where,

ORfD - the reference dose

NOAEL -the no-observable-adverse-effect-level

UF - the uncertainty factor

MF - the modifying factor

The oral reference dose (ORfD), based on the same concept as acceptable daily intake, is the maximum amount of a chemical (mg/kg/body weight/day) that a human body can absorb without experiencing chronic health effects (Asante-Duah, 2002). The RfD is an equation that also includes uncertainty and modifying factors, which are factors that bring a more sound scientific judgment regarding the RfD value by multiplying by a 10-fold factor to account for interspecies and intraspecies differences and errors. One use of an UF include a 10-fold factor when extrapolating from less than chronic results on experimental animals when there are no useful long-term human data. This factor accounts for uncertainty involved in extrapolating from acute or subchronic NOAELs to chronic NOAELs, referenced as “10S”. Another 10-fold factor is used when extrapolating from valid experimental results in studies using prolonged exposure to average healthy humans, which accounts for the variation in sensitivity among members of the human population due to heterogeneity, referenced as “10H”. There is also a 10-fold UF that can be applied when extrapolating from valid results of long-term studies on

experimental animals when there are inadequate or no results of human exposures available, which accounts for the uncertainty involved in extrapolating from animal to humans, referenced as “10A”.

The UF referenced as “10L” is applied when deriving an RfD from a LOAEL, instead of a NOAEL, which accounts for the uncertainty involved in extrapolating from LOAELs to NOAELs. Lastly, a 10-fold UF can be applied when extrapolating from valid results in experimental animals when the data are considered “incomplete”, which accounts for the inability of any single animal study to adequately address all possible outcomes in humans, referenced as “10D”. Additional uncertainty factors used in this study were employed to experimental assays based upon the order(s) of magnitude difference of the rat (*in vivo*) assay from literature. Modifying factors are additional uncertainty factors based on professional judgment and are added into an equation to account for additional uncertainty that is not previously accounted for with standard uncertainty factors. Essentially, the RfD is used as a measure of exposure for non-carcinogens that is considered to be without adverse effects (Asante-Duah, 2002). Therefore, the derivation of the RfD potentially present a basis for the employment of a human health risk assessment using the Visual SmartRisk Software, 3.0, provided by Baylor University.

The third step includes exposure assessment, which determines the extent of human exposure. The exposure assessment analyzes contaminant release scenarios and potential receptors, while estimating expected exposure levels and calculating intake, or doses (Asante-Duah, 2002). The last step includes risk characterization, which integrates the findings of the first three components to describe the nature and magnitude of health

risk associated with environmental exposure to a chemical substance or a mixture of substances. Risk characterization estimate potential for non/carcinogenic risks and evaluate uncertainties, while summarizing risk information (Asante-Duah, 2002). Risk is calculated using the following two equations (US EPA, RAGS 2001):

For carcinogens: Risk = Intake x Cancer Slope Factor

Where,

Intake can be defined as the amount of inhaled, ingested or dermally-absorbed material during a specified time period (mg/kg-day). Specifically, this study is only concerned with materials that are orally ingested. A cancer slope factor is a plausible upper-bound estimate of the probability of a response per unit intake of a chemical over a lifetime. This is represented by the slope of the dose-response curve in the low-dose region. In other words, the cancer slope factor is used to estimate an upper-bound probability for an individual to develop cancer due to a lifetime exposure to a particular level of a carcinogen (Asante-Duah, 2002).

For non-carcinogens: Hazard Quotient = Intake ÷ ORfD

Where,

Hazard Quotient is the ratio of the average daily dose of the chemical to the ORfD for that specific chemical. Thus, a value less than 1.0 indicates that risk exposure is likely insignificant, however a value greater than 1.0 indicates a potentially significant risk (Asante-Duah, 2002).

Statistical Analysis

Data were analyzed using a one-way analysis of variance (ANOVA) followed by Dunnett's test for comparison to control (in this study, solvent control). Dunnett's test

was used because it compares all other columns to the solvent control column. Statistical significant differences were determined at $\alpha = 0.05$. The level of significance was set at $p < 0.05$. All computations analyzed for *in vitro* testing used Sigmaplot and JMP 8.0 software (SAS Institute, Inc). The ANOVA confirmed assumptions tested were based upon the data being normally distributed; each sample was random and independent; and that the populations had equal standard deviations (or variances). In cases where the normality assumptions of the ANOVA were not met, a nonparametric method, the Kruskal-Wallis method, was employed.

CHAPTER THREE

Results

For all liver cells, the control included liver cells and complete growth medium only, while the solvent control consisted of liver cells, complete growth medium and 0.5% DMSO. Cells tested with various percentages of DMSO did not show a statisically significant response at concentrations of 0.5% and lower (data not shown). Accordingly, the concentration of DMSO did not exceed 0.5% in any experiment. PBDE compounds were dissolved in DMSO with complete growth medium. The solvent control was used to compare the observed effects of all treatments levels. There is no statistically significant difference between the control and solvent control in any test. In addition all experiements were run with two controls (control and solvent control) to demonstrate no toxicity was observed using the appropriate concentration of solvent (DMSO at 0.5%).

Clone-9, Rat Liver Cell Viability (in vitro)

After Clone-9 cells were exposed to parent and hydroxylated PBDEs for 72 hours, cell viability was evaluated. The results of the cell viability assay for the parent PBDE congeners for Clone-9 cells is found in Figure 2. BDE-28 shows statistically significant decreases in percentage cell viability in a dose-dependent manner. BDE-99 and -153 also show significant decreases in percentage cell viability in a dose-dependent manner with the exception of the lowest concentration (1.25 mg/L), where we observed no decrease in cell viability. Additionally, we observed statistically significant decreases in cell viability with BDE-209 in each of the concentrations tested with the exception of the

highest concentration (10 mg/L). There were no measures of BDE-209 at 20 mg/L, since we did not obtain a stock solution at a high enough concentration to produce a 20 mg/L solution.

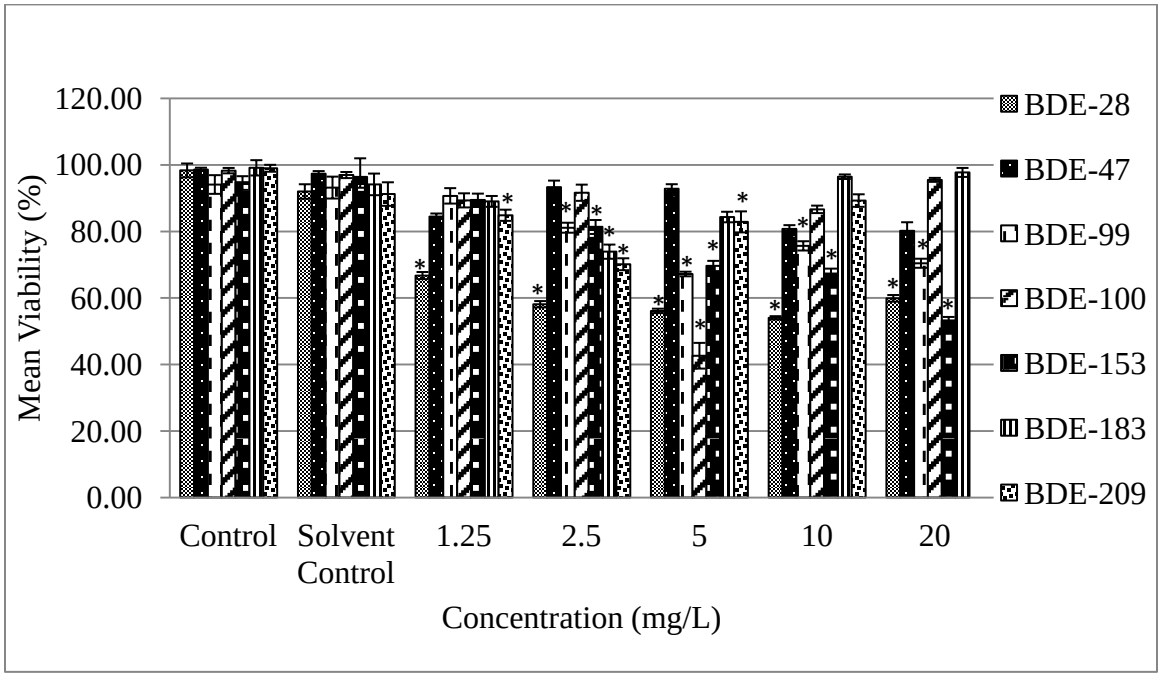


Figure 2. Mean percent viability of Clone-9 cells after exposure to parent polybrominated diphenyl ether (PBDE) congeners for 72 h in 96-well plate. All values are expressed as mean ± S.E.M. (n = 48). Asterisk (*) indicates statistically significant difference compared to solvent control ($p < 0.05$, Dunnett's test).

We also measured cell viability for Clone-9 cells after exposure to hydroxylated PBDE compounds (Figure 3). Hydroxylated compounds have recently been found in human serum, cord blood and breastmilk (Lacorte and Ikonomou, 2009). We observed some interesting changes in cell viability for 3-OH-BDE-47. While we observed a dose-dependent change in viability, the decreases in cell viability were not significantly different in treated wells when compared to the solvent control. The hydroxylated congener, 5-OH-BDE-47, demonstrated a statistically signficant decrease in percentage

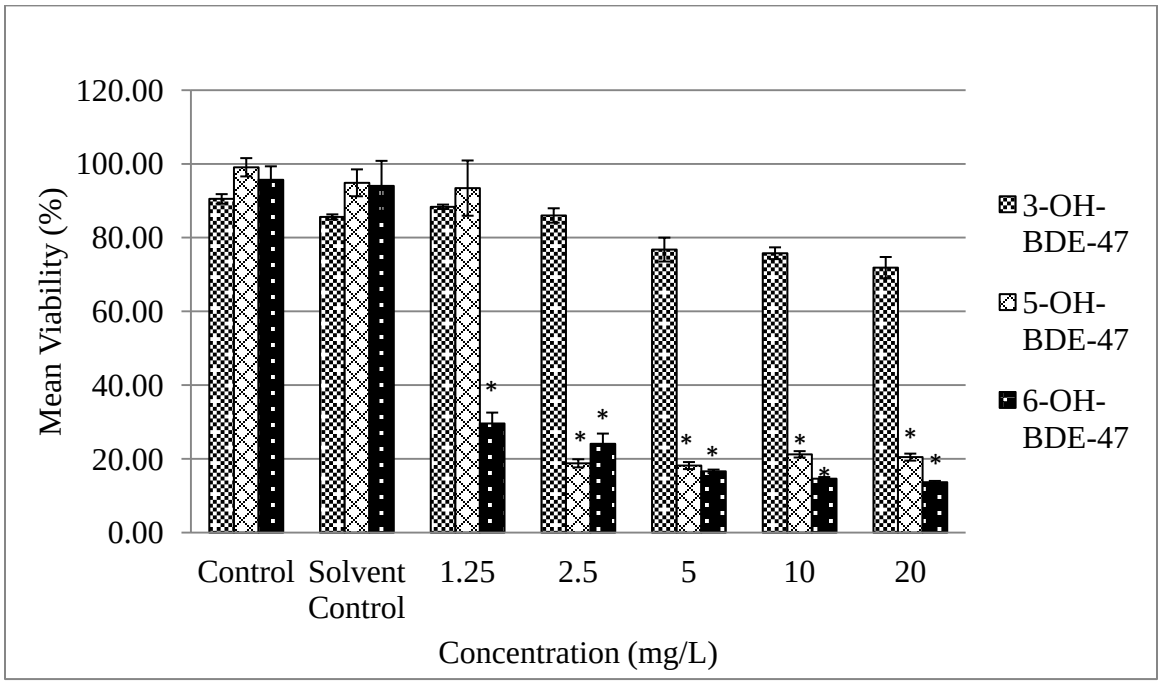


Figure 3. Mean percent viability of Clone-9 cells after exposure to hydroxylated polybrominated diphenyl ether (PBDE) congeners for 72 h in 96-well plate. All values are expressed as mean ± S.E.M. (n = 48). Asterisk (*) indicates statistically significant difference compared to solvent control ($p < 0.05$, Dunnett's test).

cell viability beginning at 2.5 mg/L. Further, we observed singificant cytotoxicity with exposures to 6-OH-BDE-47 in Clone-9 cells. A statistically significant decrease in percentage cell viability occurred in a dose-dependent manner from 1.25 to 20 mg/L when exposed to 6-OH-BDE-47. Furthermore, there was an approximate 70% decrease in cell viability at the lowest concentration (1.25 mg/L) tested, while there was an approximate 80% decrease at the highest exposure level (20 mg/L). This response is similar to the response seen by other researchers in our lab using other models (i.e., Usenko et al., 2011).

HEPG2 Human Liver Cell Viability (in vitro)

The percentage viability for HEPG2 cells using parent PBDE congeners are shown in Figure 4. BDE-28, -47, and -99 exhibits statistically significant decreases in percentage cell viability in a dose-dependent manner. There was an interesting trend with BDE-183 that showed statistically significant decreases in percentage cell viability in all concentrations tested. With BDE-209 cell viability began to significantly decrease statistically among concentrations ranging from 1.25 to 5 mg/L with the exception of 10 mg/L. There were no measures of BDE-209 at 20 mg/L, since we did not obtain a stock solution at a high enough concentration to produce a 20 mg/L solution.

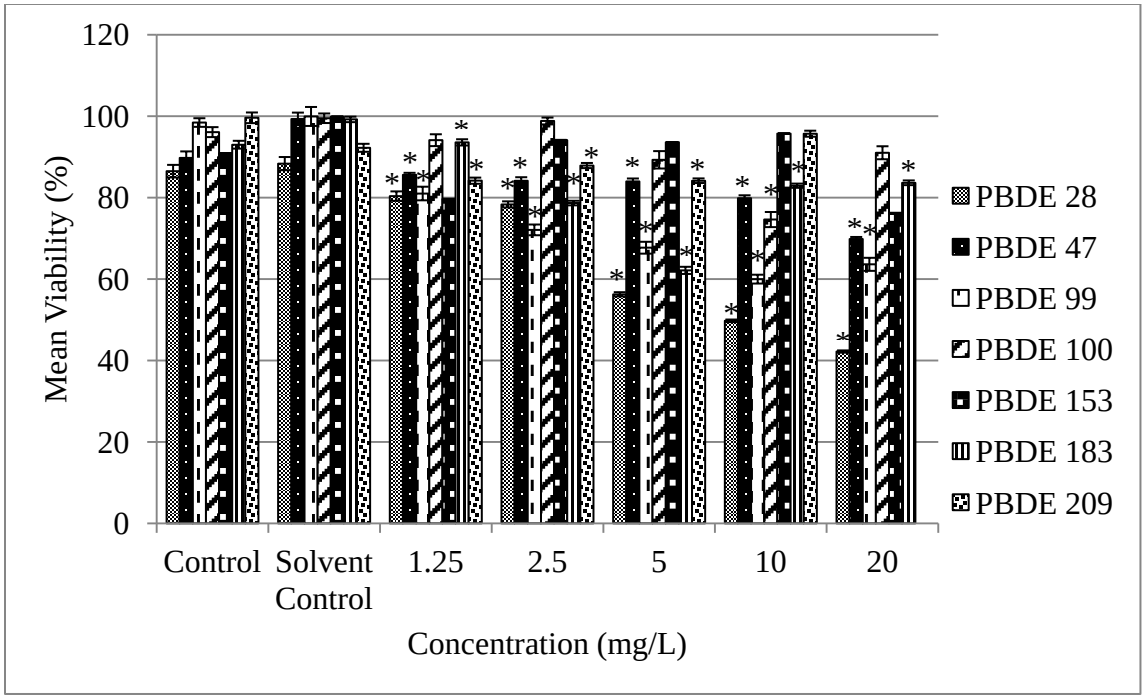


Figure 4. Mean percent viability of HEPG2 cells after exposure to parent polybrominated diphenyl ether (PBDE) congeners for 72 h in 96-well plate. All values are expressed as mean ± S.E.M. (n = 48). Asterisk (*) indicates statistically significant difference compared to solvent control ($p < 0.05$, Dunnett's test).

Figure 5 illustrates the percentage viability for HEPG2 cells exposed to hydroxylated PBDE congeners, where 3-OH-BDE-47 does not show significant decreases in percentage cell viability in any of the concentrations tested. However, there was a significant decrease in cell viability beginning at higher concentrations (10 and 20 mg/L) with exposure to 5-OH-BDE-47. The only hydroxylated compound tested that demonstrates a decrease in cell viability across all the concentrations tested in this study is 6-OH-BDE-47.

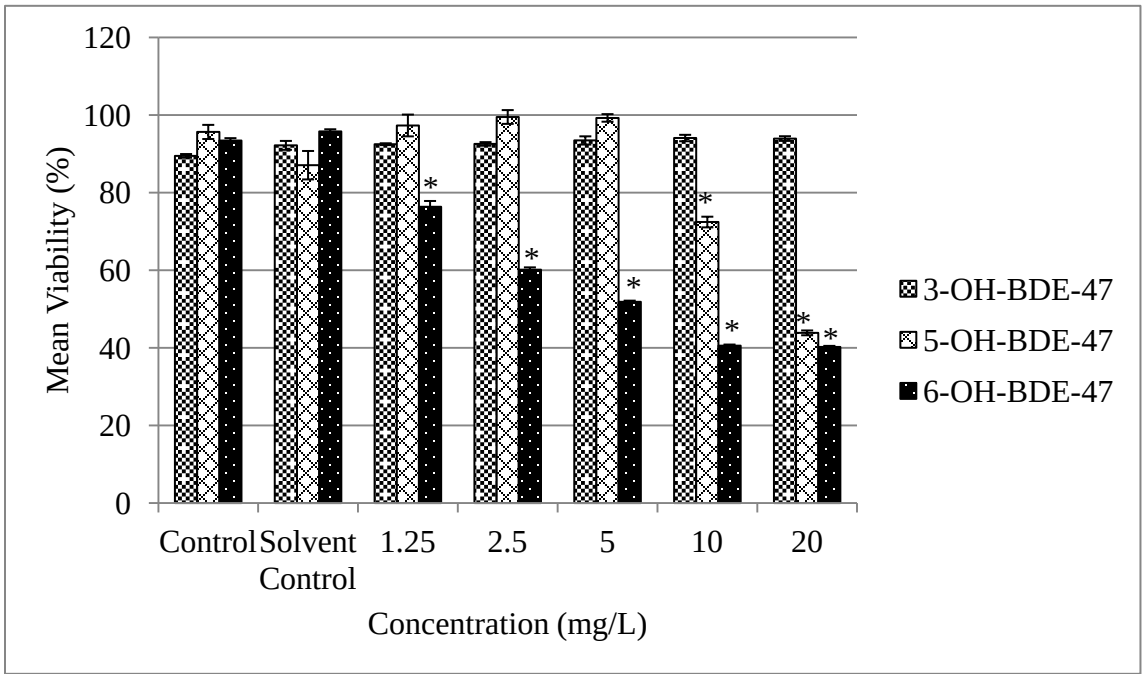


Figure 5. Mean percent viability of HEPG2 cells after exposure to hydroxylated polybrominated diphenyl ether (PBDE) congeners for 72 h in 96-well plate. All values are expressed as mean ± S.E.M. (n = 48). Asterisk (*) indicates statistically significant difference compared to solvent control ($p < 0.05$, Dunnett's test).

Zebrafish Liver Cells

The zebrafish liver cell viability assay for parent PBDEs are shown in Figure 6. There are statistically significant differences among concentrations that occur at 5, 10, and 20 mg/L for BDE-28 when compared to the solvent control. We observed a

statistically significant cell viability decrease at the highest concentration, 10 mg/L, for BDE-47. There were no measures of BDE-47 and BDE-209 at 20 mg/L, since we did not obtain a stock solution at a high enough concentration to produce a 20 mg/L solution. BDE-99 showed a decrease in cell viability in a dose-dependent manner beginning at 2.5 mg/L. Exposures to BDE-153 resulted in a statistically significant decrease in cell viability among concentrations ranging from 1.25 to 20 mg/L.

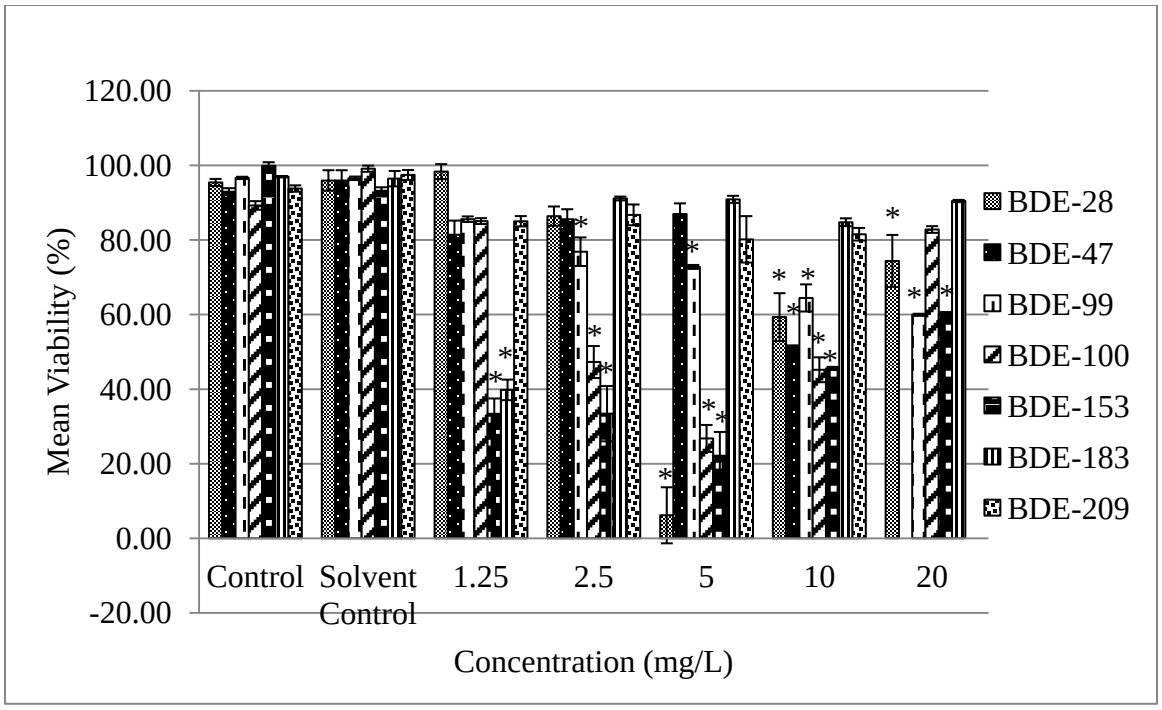


Figure 6. Mean percent viability of zebrafish liver cells after exposed to parent polybrominated diphenyl ether (PBDE) congeners for 72 h in 96-well plate. All values are expressed as mean ± S.E.M. (n = 48). Asterisk (*) indicates statistically significant difference compared to solvent control ($p < 0.05$, Dunnett's test).

The only statistically significant difference in reduced cell viability was observed at the highest concentration, 20 mg/L, for 3-OH-BDE-47 in Figure 7. From 1.25 to 5 mg/L, we observed a statistically significant decrease in cell viability when cells were

exposed to 5-OH-BDE-47. However, 6-OH-BDE-47 demonstrated a statistically significant reduction in cell viability from 2.5 to 20 mg/L.

As noted previously, the comparison of these models with others in this study was important in gaining an idea of which model would most closely represent a response in the standard mice model that is used by US EPA.

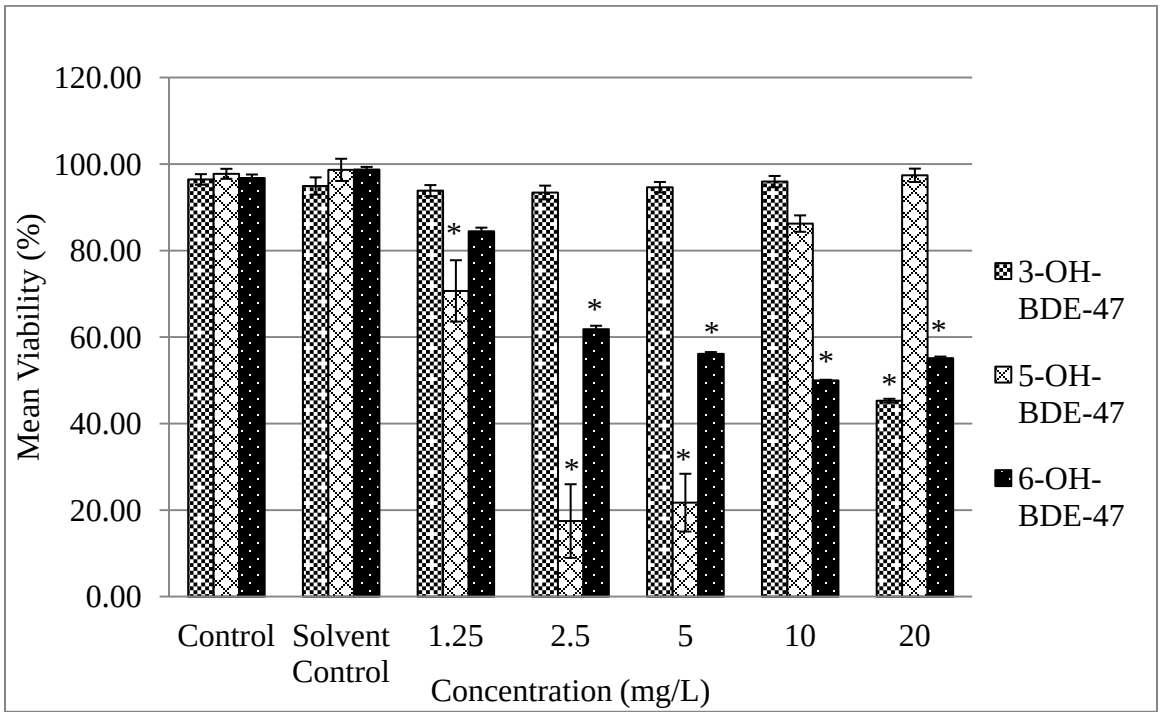


Figure 7. Mean percent viability of zebrafish liver cells after exposure to hydroxylated polybrominated diphenyl ether (PBDE) congeners for 72 h in 96-well plate. All values are expressed as mean ± S.E.M. (n = 48). Asterisk (*) indicates statistically significant difference compared to solvent control ($p < 0.05$, Dunnett's test).

Derivation of the NOAEL from the NOAEC

We previously mentioned that the NOAEL or LOAEL (mg/kg-day) is used when deriving the RfD, where the NOAEL is the highest (or lowest, LOAEL) experimentally-determined dose at which organisms does not show (or show – LOAEL) statistically or

biologically significant indication of adverse effects occurring. However, the experiments conducted within this study used NOAEC, no-observed-adverse-effect-concentration, or LOAEC, lowest-observed-adverse-effect-concentration, (mg/L) values due to the deliverance of PBDE compounds provided at certain concentrations within the appropriate medium for each assay. Asante-Duah, 2002 defines the NOAEC and LOAEC as "the highest or lowest concentration in an exposure medium in a study that is, or is *not*, associated with an adverse effect on the test organisms." Thus, Table 1 displays the NOAEC (mg/L) values for each PBDE compound in each model system tested in this study. In cases where there were no NOAEC values observed, we used LOAEC values (denoted by an asterisk).

Table 1. The no-observable-adverse-effect-concentration (NOAEC) values (mg/L) for each polybrominated diphenyl ether (PBDE) congener from experimental bioassays in this study. Asterisk (*) denotes use of the lowest-observable-adverse-effect-concentration (LOAEC) values when NOAEC values were not identified.

Congener	Clone-9 *in vitro* NOAEC (mg/L)	HEPG2 *in vitro* NOAEC (mg/L)	ZFL *in vitro* NOAEC (mg/L)
BDE-28	1.25*	1.25*	2.5
BDE-47	20	1.25*	5
BDE-99	1.25	1.25*	5
BDE-100	2.5	5	1.25
BDE-153	1.25	20	1.25*
BDE-183	1.25	1.25*	1.25*
BDE-209	1.25*	1.25*	20
3-OH-47	20	20	10
5-OH-47	1.25	5	1.25*
6-OH-47	1.25*	1.25*	1.25

When calculating the RfD, the aforementioned uncertainty factors were applied based upon the characteristics of the congeners and techniques used in this study. There is a possibility of up to five, 10-fold uncertainty factors that could be used in calculating a

RfD. In addition we used a number of scaling factors (Table 2) when converting the NOAEC (mg/L) to the NOAEL (mg/kg-day) in order to appropriately derive the RfD (mg/kg-day). This process also used the extrapolation of data from an *in vitro* model to an *in vivo* model (IVIVE), which is the modifying factor that takes into consideration the additional uncertainty.

Table 2. The derivation and definition of abbreviated scaling factor parameters used when converting no-observable-adverse-effect-concentration (NOAEC) in mg/L to no-observable-adverse-effect-level (NOAEL) in mg/kg-day values.

Parameter	Definition (units)	Calculation
CM	Convert total # of cells into mass unit (kg)	# of cells per treatment (N=16) at 72 hrs; ÷ by 10^6 to convert to (mg); ÷ by 10^6 to convert to (kg)
LV	Estimation of the % liver of hepatocyte cells that is liver (%)	(80%) x (1.5 kg)
BW_{LV}	Estimation of the % of body weight that is liver (%)	(CM x LV) ÷ (70 kg)
NOAEC or LOAEC	No-observed-adverse-effect-conservation or Lowest-observed-adverse-effect-conservation	(NOAEC or LOAEC x volume); then ÷ by BW_{LV} ; and ÷ by 3 d
RfD	Reference Dose (mg/kg-day)	NOAEL ÷ UF x MF

Actual scaling factor values are noted in Appendix B. These extrapolations followed a similar method of calculations conducted by Pelkonen et al., 2007. Pelkonen et al., 2007 suggested IVIVE techniques by using simple system data and applying it to complex whole organisms when assessing drug disposition.

For each cell line, the number of cells per well were calculated and seeded based upon the population doubling time of 24 hours over a 72-hour period. One of the scaling factors applied first involved converting (CM) the overall total number of cells at the end of the 72-hour period to mass (milligram). Using the estimation, one million hepatocyte cells per mg in a human liver (Pelkonen et al., 2007), the calculated CM is divided by one million, and then converted to kilogram. Next, since 80% of human liver cells (LV) are hepatocyte cells, we multiplied the standard human liver weight (1.5 kg) by 80%. The next step was to determine the percentage of body weight (BW_{LV}) that is the liver based on the CM. Thus, the CM is multiplied by LV, and then divided by the standard adult human body weight (70 kg). The observed NOAEC or LOAEC value (mg of chemical per 1 L of culture media) of each congener was individually multiplied by the volume of solution used per well (0.0001 L). The last step divides the mg value by the kg value (BW_{LV}) in order to completely change the NOAEC (mg/L) to a NOAEL (mg/kg). The resulting NOAEL value is divided once more by the duration (days) of PBDE exposure (3 days for cells). These calculations result in NOAEL values that can then be used to calculate a RfD for use in risk assessment. To arrive at the final RfD, the final NOAEL value is divided by a denominator that multiplies the uncertainty factors by the modifying factors, giving rise to the RfD (mg/kg-day).

All scaling factors as well as the methods used in this study were established with the consultation and guidance from the US EPA. The RfD is then used in calculating the overall amount of non-carcinogenic risk involved when consuming PBDE-contaminated fish.

Reference Dose

Overall, we determined that the HEPG2 model most closely represents results that would be produced using the traditional mouse models. Consequently, we only focused on one specific model (HEPG2) and observed the order of magnitude differences that occur between the two models (HEPG2 and mice). After consultation with researchers at US EPA, we decided that in order to appropriately take into consideration the uncertainty associated with the IVIVE used in this study, there was a need to include an additional modifying factor. An additional modifying factor ranging from 10 to 1,000 was incorporated into the RfD calculation to account for additional uncertainties from extrapolations, confidence in the estimation for the model data, and lack of additional studies on the PBDE congeners included in this study. Table 3 shows the compared RfDs calculated for the HEPG2 assay performed in this investigation to the four EPA-derived RfDs from mouse studies. Using the chosen HEPG2 model to estimate RfDs for the six additional PBDE congeners evaluated in this study allowed for a baseline human health risk assessment to be run for consumption of contaminated fish.

Human Health Risk Assessment

In order to estimate the potential non-cancer critical health effects of risk involved for an individual ingesting PBDE-contaminated fish, we used Visual SmartRisk Software, 3.0 (Pioneer Technologies, Olympia, Washington) to derive and quantify an equation that fits our specific exposure scenario. Upon an extensive literature review of PBDE concentrations in fish, we used the highest measured concentrations of the individual PBDE congeners in fish to represent a conservative “Reasonable Maximum Exposure”, or RME (Schecter et al., 2006 and Rice et al., 2002). Fish fillet data was the

Table 3. Reference dose comparisons for the traditional mouse model derived reference doses as well as the reference doses derived by *in vitro* to *in vivo* extrapolation (IVIVE) using HEPG2 cells.

	PBDE Congeners									
Model	BDE-28	BDE-47	BDE-99	BDE-100	BDE-153	BDE-183	BDE-209	3-OH-BDE-47	5-OH-BDE-47	6-OH-BDE-47
Mouse	N/A	2.33E-04	1.33E-04	N/A	1.50E-04	N/A	7.40E-03	N/A	N/A	N/A
HEPG2	8.44E-04	8.44E-04	1.35E-04	3.38E-03	1.35E-04	8.44E-04	8.40E-03	1.35E-03	3.38E-03	8.44E-04

most appropriate choice for this study, however there was limited data available for the congeners we tested. Consequently, we used whole wet weight fish concentrations (Appendix A, the exposure point concentrations), which presented the most complete set of data available for these congeners. Since we found no currently reported measurements of 5-OH- and 6-OH-BDE-47 in fish tissue, we used the same concentration as that reported for 3-OH-BDE-47 in fish tissues as an estimate for the concentrations of 5-OH- and 6-OH-BDE-47. However, 5-OH- and 6-OH-BDE-47 have been found to be the most abundant hydroxylated congeners in human serum (Zota et al., 2011). All parent PBDE fish tissue concentrations were based upon U.S. whole fish wet weight (mg/kg wet weight). Further, the fish consumption of some populations (i.e., Vietnamese-America and Latino) along the Texas Gulf Coast includes the whole fish (especially in soups and stew) and further is consumed daily at a 3 to 12 percent higher consumption rate than average populations (NRDC, 2010).

The components in the hazard index equation (Table 4) were taken from the US EPA default values for human health risk assessment. The values are based on US EPA Exposure Factors Handbook (1997), peer-reviewed articles, the risk assessment guidance for superfund (RAGS), as well as assumptions that were based upon scientific reasoning according to the exposure scenario in this study. The following equations were used to estimate the hazard index in consuming PBDE-contaminated fish (primarily BDE-47, -99, -153, and -209).

$$\text{ORfD} = \frac{\text{NOAEL or LOAEL}}{\text{UF x MF}}$$

$$\text{Ingestion of Seafood } (ING_{sf})_i = \frac{(EPC_i \text{ x CR x EF x ED x CF x FC})}{(\text{BW x AT})}$$

Hazard Quotient = $(ING_{sf})_i \div ORfD_i$

Hazard Index: = $\sum HQ$

Where,

ING_{sf} = ingestion exposure to contaminated seafood

ORfD = Oral Reference Dose (mg/kg-day)

Table 4. Parameter abbreviations defined with their standard default values. These parameters are calculated in the seafood ingestion equation (ING_{sf}). Note: Ad – adults; ch – child.

Parameter	Definition (units)	Default Value
EPC_i	Exposure Point Concentration in fish per congener (mg/kg)	See Appendix
CR	Consumption Rate (g/day)	78.3 (Ad); 52.25 (ch)
EF	Exposure Frequency (days/yr)	104
ED	Exposure Duration (years)	30 (Ad); 7 (ch)
CF	Conversion Factor (kg/g)	0.0001
FC	Fraction Ingested from Contaminated Source (unitless)	1
BW	Body Weight (kg)	70 (Ad); 30 (ch)
AT	Average Time (days)	10,950 (Ad); 2,555 (ch)

The ING_{sf}, otherwise known as intake (mg/kg-day) for this study, was calculated using the parameters described in Table 4. As previously noted, the EPC_i values were found in literature (Appendix A) for each individual compound. The CR of 78.3 g/day for male and female adults (ages 18 and up) and 52.25 g/day for all children (ages 3-17 years) has been grouped and averaged based upon the values provided in the EPA Exposure Factors Handbook (1997) as well as the EPA Child Exposure Factor Handbook (2008). The EF for both adults and children are 104 days/yr because EPA assumes that recreational fish consumers ingest about 2 fish meals (approximately 8 ounces (224grams)) per week (95^{th} percentile) at 52 weeks per year on average (US EPA OSWER, 1991; US EPA, 2000; 2002). This consumption rate for some populations will

results in a higher estimation of risk considering that some fish consumers will consume fish meals every day of the week. For adults it is estimated that 30 years is used as the standard ED, while 7 years is the estimated exposure for children, since we assumed that the median age for children to be 10 years; thus, the age difference between 17 and 10 is equal to 7 (US EPA OSWER, 1991). The FC is an assumed unitless value that represents the fraction of the source that is contaminated. Conservative estimations assume the whole source (i.e., fish) is contaminated. The standard adult BW is 70 kg, while it is normally standardized at 15 kg for children; however, based upon the chosen median age of 10, we assumed an average BW of a child at this age to be about 30 kg (US EPA OSWER, 1991). The AT is calculated by multiplying the ED by 365 days; thus, the AT for adults was 10,950 days, while the AT for children was 2,555 days. The ORfD is the calculated oral reference dose value (mg/kg-day) that the human body can be exposed to without experiencing chronic health effects. Finally, the hazard quotient is the ration of toxicant exposure (estimated or measured; i.e., intake) to a reference value corresponding to a threshold of toxicity (i.e., RfD). Further, the hazard index is the sum of the hazard quotients for each substance in the assessment that affect the same target organ or organ system. In this study the hazard index indicates the potential non-cancer health effects of the combined PBDE congeners exposed to the human receptor from ingestion of PBDE-contaminated fish.

As previously mentioned, our primary objective was to determine a bioassay model that would best represent that of the traditional mouse model for deriving RfD values. This ultimately allows us to estimate RfD values for other PBDE congeners that have no toxicity data available for them. There was only one order of magnitude

difference between the hazard index values of the HEPG2-Four Congeners and the mice model-derived RfDs (only the four EPA compounds: BDE-47, -99, -153, -209) for both adults and children (Table 5). The HEPG2-All Congeners model provided a hazard index value that was one order of magnitude off that of the mice for adults, while it was the same order of magnitude as that of the mice for children. These results indicate that using the HEPG2 model to estimate a RfD for these compounds can provide a means to determine a preliminary baseline risk estimate from exposures. Further, it could be used as a screening tool to focus future studies based on estimated RfDs and the associated risk.

Table 5. Non-carcinogenic risk assessment results for PBDE exposures via ingestion of contaminated fish. Three risk assessments were conducted using different sets of references doses: US EPA reference doses (four congeners), the HEPG2 model estimated reference doses (four congeners), and the HEPG2 model estimated reference doses for all congeners in this study. Risk assessments concluded that risk was not exceeding acceptable limits (i.e., HI $\geq$ 1).

Non-Carcinogenic Risk Assessment Results		
Assay	Adult	Child
US EPA RfDs-Four Congeners	1.34E-02	2.09E-02
HEPG2-Four Congeners	5.62E-03	8.76E-03
HEPG2-All Congeners	7.32E-03	1.14E-02

CHAPTER FOUR

Discussion

This study utilized four models (Clone 9, HEPG2, and Zebrafish Liver cells) to investigate IVIVE. In light of new initiatives like "Tox 21", aimed at reducing cost and time involved in traditional toxicity testing, this study is novel and timely. According to US EPA, Tox 21 is the consolidation of several federal resources and expertise from EPA, National Institutes of Environmental Health Sciences/National Toxicology Program, National Institutes of Health and the Food and Drug Administration to use technology to screen thousands of chemicals for potential toxicity; use screening data to predict the potential toxicity of chemicals; and develop a cost-effective approach for prioritizing the thousands of chemicals that need toxicity testing.

The present study used a series of models to address these issues by determining if IVIVE techniques can be used to make predictions for toxicity factors that are otherwise non-existent. Essentially, we exposed the aforementioned models to a class of environmentally relevant PBDE compounds and observed how they affected liver cell viability. Through lab experiments, we obtained bioassay-based NOAEC, or LOAEC, values (mg/L) and applied scaling factors via IVIVE techniques to obtain NOAEL, or LOAEL, values (mg/kg-day) to be used to derive RfDs. Traditionally, the RfD is calculated by determining the most sensitive and significant NOAEL or LOAEL for non-cancer effects and dividing that by the product of a series of uncertainty factors and a modifying factor. This NOAEL is determined from animal studies, traditionally mouse studies that are collected and evaluated for scientific merit, completeness, and observed

effect. This technique takes a great deal of time for several reasons: Animal studies are lengthy and are costly; many studies are incomplete in terms of number of animals, sex ratios, chronic versus acute exposures, and dosing ranges. There is a need for collecting data that can be used in a preliminary sense to not only estimate risk but focus future toxicity testing.

After comparing RfD values among all of the experimental models in this study, we determined that the HEPG2 model was the best model to represent the mouse model due to its similarities in comparison to the four EPA-derived RfD values (BDE-47, -99, -153 and -209). The identification of this specific model (HEPG2) allowed us to apply the IVIVE (modifying) factor to obtain a RfD value either close to or the same order of magnitude as that of that mice-derived RfDs. As such, we successfully applied IVIVE factors, which gave rise to RfD values the same order of magnitude as that of the mouse. This allowed us to apply the same IVIVE factors to the other PBDE congeners in our study, which had no previously-derived RfD values. Overall, we were able to use IVIVE methods to derive RfDs for compounds without RfDs through the use of an *in vitro* bioassay model.

In summary, our experiments included the use of three *in vitro* models (Clone-9, HEPG2, and Zebrafish liver cells). A decrease in *in vitro* liver cell viability was the endpoint evaluated after exposures to PBDE compounds in this study. After a 72-hour exposure to the PBDE compounds, we found that BDE-28, -99, 5-OH-BDE-47 and 6-OH-BDE-47 have consistently decreased cell viability to some extent in all *in vitro* models. Further, 6-OH-BDE-47 was observed to be the most toxic hydroxylated compound when tested in all *in vitro* models. Other PBDE compounds were either found

to significantly decrease cell viability to some extent or have no effect on cells at the given concentrations in their respective models. This may be due to the robustness of a particular cell line or the variability in response to different compounds within the same cell line as they approach senescence. Another possibility is that we reached a solubility limit factor for certain PBDE compounds.

Clone-9 and HEPG2 (in vitro)

Clone-9 cells showed a significant decrease in cell viability with the lowest brominated congener (BDE-28) in a dose-dependent manner. This assay also revealed that there were no significant differences in cell viability when exposed to BDE-47. In contrast to the Clone-9 cells, the HEPG2 cell viability study determined that BDE-47 caused a statistically significant decrease in cell viability in a dose-dependent manner. There were statistically significant decreases in Clone-9 and HEPG2 cell viability among concentrations ranging from 1.25 to 5 mg/L, when exposed to BDE-209. As mentioned previously, BDE-209 inhibited cell growth in HEPG2 cells when exposed to BDE-209 at lower concentrations than this study (Hu et al, 2007). Morck et al., 2003 found that an oral exposure of male rats to BDE-209 was not namely distributed to lipid-rich tissues, such as adipose tissues and lungs, but rather discovered at higher concentrations in plasma and blood-rich tissues, such as the liver and heart. Also, 6-OH-BDE-47 was shown to be the most toxic among the hydroxylated compounds tested by significantly decreasing percentage cell viability in a dose-dependent manner for Clone-9 and HEPG2 cells. One study found 6-OH-BDE-47 to decrease cell viability at 20 µM in rat pheochromocytoma (PC12) cells (Dingemans et al., 2010).

HEPG2 cells exhibit a decrease in percentage cell viability in a dose-dependent manner with BDE-28, -47, -99, and 6-OH-BDE-47. Previous studies have shown that lower brominated congeners are more bioavailable and therefore can be more toxic. Similarly, biological activity is significantly different depending on the position of the hydroxyl group on the parent compound (Usenko et al., 2011, Usenko et al., 2012). This also indicates that cytotoxicity does vary among PBDE congeners possibly due to structure-activity relationships, bioavailability as well as the species of cells tested. The HEPG2 assay in the present study is consistent with Hu et al., (2007), although the latter study demonstrates a decrease in cell viability at lower concentrations ranging from 10-100 μmol/L. In the present study quantitative analysis was not evaluated until 72 hours of exposure to 6-OH-BDE-47 in the *in vitro* studies. We observed the cells under the microscope at each 24-hour interval. Under observation, there was an obvious decrease in cell viability as each 24-hour period progressed when exposed to 6-OH-BDE-47 among all treated wells, where some or most cells became detached from the bottom of the culture flask and retraction and shrinkage occurred of the attached cells.

Zebrafish Liver Cells

Zebrafish liver cells expressed statistically significant decreases in cell viability at concentrations 5, 10 and 20 mg/L for BDE-28, when compared to the solvent control. This indicates that BDE-28 begins to be toxic to these cells at or around 5 mg/L in this study. BDE-99 presented toxic effects in a dose-dependent manner cells beginning at 2.5 mg/L and continuing through to 20 mg/L. We observed an interesting trend with BDE-153 as it demonstrated a statistically significant decrease in cell viability at all tested concentrations. Although cell viability decreased from 1.25 to 5 mg/L, it began to

increase at concentrations thereafter. This suggests that solubility is possibly a major factor. The zebrafish liver cells were the only *in vitro* model in which exposure to 3-OH-BDE-47 caused a significant decrease in cell viability, which occurred at 20 mg/L. The zebrafish model may be more sensitive than other *in vitro* models to 3-OH-BDE-47 and may represent a normal human liver cell more accurately than the HEPG2 model. Recent studies have shown that zebrafish liver tumors are very similar to human liver tumors in both histology and transcriptome level, highlighting the potential of using zebrafish liver cells to model human hepatocellular carcinomas (Lam et al., 2006; 2006; Mizgireuv, et al., 2006) . Cell viability was statistically different when compared to the solvent control for concentrations 1.25 to 5 mg/L for the 5-OH-BDE-47 exposures. There was a dose-dependent decrease in zebrafish cell viability when exposed to 6-OH-BDE-47. Overall, this study suggests that 6-OH-BDE-47 may be acutely toxic in terms of cell viability among the variety of species tested.

It has been demonstrated by Van Boxtel et al., 2008 that 6-OH-BDE-47 is also acutely toxic to developing zebrafish (embryos exposed from 3 to 72 hours). This study reported abnormal development in a dose-dependent manner at concentrations ranging from 25-50 nM, while exposure to 100 nM of 6-OH-BDE-47 caused acute toxicity by indication of developmental arrest. The developmental effects resulted in defects such as pericardial edema and yolk sac deformations. Concentrations higher than 25 nM of 6-OH-BDE-47 also resulted in mortality. Van Boxtel et al., 2008 also studied the toxic effects of 6-OH-BDE-47 in adult zebrafish with concentrations ranging from 100 nM to 1 µM for up to 96 hours. Exposure to concentrations of 500 nM and 1 µM of 6-OH-BDE-47 resulted in immediate changes in the behavior of the adult zebrafish, such as gasping

for air at the surface (within 45 minutes) and suffocation (within 12 minutes). Further, Van Boxtel et al., (2008) pooled liver samples for analysis and found that liver concentrations of 6-OH-BDE-47 showed considerable accumulation on a short-term basis (96 hours). Overall, it appears from literature, as well as the data collected in this study, that the hydroxyl group added to a PBDE congener backbone significantly changes the biological activity of that compound. The effects of the position of the hydroxyl group on the specific PBDE congener backbone is an area that should be further investigated to make more accurate predictions of toxicity metrics for these compounds.

Traditional Mouse Assays

USEPA has derived RfD values for only four of the 209 possible congeners of PBDEs, which includes BDE-47, -99, -153, and -209. Table 6 displays a summary of the RfD values derived by the US EPA after determining the NOAELs from several studies. The specific studies were chosen by EPA based on their rubric of scientific integrity and completeness of the study. Further, the USEPA selected these PBDE compounds based on occurrence in humans and the environment and derived RfD values using the traditional method (animals studies) of obtaining a NOAEL value.

Firstly, BDE-47 (CASRN 5436-43-1) has been found in human maternal milk, adipose tissue, liver and blood, which indicates that it is absorbed from the environment and distributed to tissues. Animal data available indicates that the nervous system is a sensitive target. The only suitable animal study used to derive an oral RfD for BDE-47 evaluation was performed by Eriksson et al., (2001) (US EPA, 2008). In this neurobehavioral study, adult male mice were administered single oral doses of 0, 0.7, or 10.5 mg/kg of BDE-47. Effects were evaluated at 2 and 4 months of age, which included

Table 6. EPA Environmental Protection Agency (EPA)-derived reference dose values based upon the no-observable-adverse-effect-level from various studies.

Congener	Based upon NOAEL values from	RfD Value	Reference
BDE-47	Eriksson et al., 2001	2.3 E-04 mg/kg-day	EPA, 2008; IRIS CAS No. 5436-43-1
BDE-99	Viberg et al., 2004a	1.3 E-04 mg/kg-day	EPA, 2008; IRIS CAS No. 60348-60-9
BDE-153	Viberg et al., 2003a	1.5 E-04 mg/kg-day	EPA, 2008; IRIS CAS No. 68631-49-2
BDE-209	Viberg et al., 2003a	0.007 mg/kg-day	EPA, 2008; IRIS CAS No. 1163-19-5

three different measures of spontaneous behavior (locomotion, rearing, and total activity). Significant changes took place in the high-dose group versus controls at both 2 and 4 months of age. Habituation capability also worsened with age with increasing doses of BDE-47. This suggests that the capability of the mice to habituate to a new environment was adversely affected by BDE-47. Based on habituation rations, the NOAEL was found to be 0.7 mg/kg (US EPA, 2008). UFs applied to the NOAEL included: interspecies variability - extrapolating from animals to humans (factor of 10); intrahuman variability - susceptibility to human subpopulations (factor of 10); extrapolating from single dose to lifetime exposure duration (a factor of 3); database deficiencies (a factor of 10). As a result, a total UF of 3,000 was applied to the NOAEL of 0.7 mg/kg using Eriksson et al., (2001) study, which derived an RfD value of 2.3 E-04 mg/kg-day (US EPA, 2008). Overall, BDE-47 is lipophilic, therefore there is a strong potential for accumulation in lipid-rich tissues. It also has a long half-life, resulting in exposure that lasts much longer than acute exposure time.

Secondly, BDE-99 (CASRN 60348-60-9) has also been found in adipose tissues, blood, liver, and maternal milk, indicating that it is, like BDE-47, absorbed from the

environment and distributed to tissues. However, there is animal data available, which indicates that the nervous system is a sensitive target (US EPA, 2008). US EPA chose a study conducted by Viberg et al., 2004a for deriving a RfD for BDE-99 in which neurobehavioral effects were observed. Male and female mice were administered single oral doses (0, 0.4, 0.8, 4.0, 8.0, or 16 mg/kg) of BDE-99 on the 10^{th} PND (postnatal day). The 10^{th} PND is a period of maximum vulnerability during mouse brain development (Eriksson et al., 2002). Adverse effects occurred at 0.8 mg/kg in ages 2-, 5-, and 8-month old mice, which included hypoactive spontaneous motor behavior in the beginning of the test period, hyperactive behavior at the end of test period, and a decrease in habituation capability. All of these effects became more pronounced as age increased. Behavioral changes in mice did not occur as such when BDE-99 was exposed to ages greater than two months old (Branchi et al., 2005). As a result, concerns of neurodevelopmental effects arise since fetuses and infants are exposed to BDE-99 via maternal/cord blood and human breast milk.

The NOAEL of BDE-99 occurred at 0.4 mg/kg based on behavioral changes. Similar UFs were applied to the NOAEL, interspecies variability- extrapolating from animals to humans (factor of 10); intrahuman variability - susceptibility to human subpoplulations (factor of 10); extrapolating from single dose to lifetime exposure duration (a factor of 3); database deficiencies (a factor of 10). Consequently, a total UF of 3,000 was applied to the NOAEL of 0.4 mg/kg using Viberg et al., 2004a study, which yields an RfD value of 1.3 E-04 mg/kg-day. BDE-99 is lipophilic and possesses an extended half-life, resulting in systemic exposure which lasts much longer than that of the exposure duration.

Another congener for which there is mouse data available is BDE-153 (CASRN 68631-49-2). Like the previously mentioned congeners it has also been found in adipose tissues, blood, liver and maternal milk. The highest levels of BDE-153 are found in adipose tissues, followed by muscle, liver, and skin (US EPA, 2008). The animal study used to derive the RfD for BDE-153 was conducted by Viberg et al., 2003a. Male mice were administered single oral doses (0, 0.45, 0.9, or 9.0 mg/kg) of BDE-153 on the 10^{th} PND. Adverse effects began at 0.9 and 9.0 mg/kg, which included hypoactive spontaneous motor behavior in the beginning of testing, hyperactive behavior at the end of testing (while becoming more pronounced with increasing age), and effects on learning and memory capability. The NOAEL of 0.45 mg/kg was based on the effects of spontaneous behavior, learning, and memory in mice. UFs applied to the NOAEL consisted of the following: interspecies variability - extrapolating animal data to humans (a factor of 10); intrahuman variability - susceptibility of human subpopulation (a factor of 10); extrapolating a single dose to lifetime exposure duration (a factor of 3); and accountability for a deficient database (a factor of 10). A total UF of 3,000 was applied to the NOAEL of 0.45 mg/kg using the Viberg et al., 2003a study, which yields an RfD of 1.5 E-04 mg/kg-day. BDE-153 is highly lipophilic, thus acute exposure will result in exposure that lasts longer than first day of dosing.

BDE-209 (CASRN 1163-19-5) has been found in human serum and maternal milk. The highest levels of BDE-209 are found in the liver, muscle and skin. Contrary to lower brominated congeners, high levels of BDE-209 were not found to be in the adipose tissues (US EPA, 2008). A study conducted by Viberg et al., 2003 was the study used to derive an RfD for BDE-209. In this study, adult NMRI (Pregnant Naval Medical

Research Institute) male mice were exposed to a single oral dose of BDE-209 as neonates PND 3, 10, or 19. Adult mice at 2, 4 and 6 months of age were evaluated after being exposed to from doses ranging from 2.22 to 20.1 mg/kg of BDE-209 on PND 3. Based on locomotion, rearing, and total activity, habituation ratios were significant from control groups. Adult mice that were exposed neonatally up to 20.1 mg/kg on either the 10^{th} or 19^{th} PND did not show significant changes in spontaneous activity after 2, 4, or 6 months. This further indicates that there is a critical window for the induction of behavioral disturbances and that adult mice exposed neonatally to the high dose of BDE-209 demonstrated to be persistent and worsened with age. Based upon the effects of locomotion, rearing and total activity, the NOAEL of 2.22 mg/kg was identified. UFs applied to the NOAEL consisted of the following: interspecies variability – extrapolating animal data to humans (a factor of 10); intrahuman variability – susceptibility of human subpopulation (a factor of 10); extrapolating from a single dose to lifetime exposure duration (a factor of 3). Therefore, a total UF of 300 was applied to the NOAEL of 2.22 mg/kg using the Viberg et al., 2003a study, which yields an RfD of 0.007 mg/kg-day. Toxicokinetic studies show that BDE-209 can be absorbed via oral route to a limited extent, however, does not accumulate in tissues and undergoes clearance (US EPA, 2008). When exposed acutely, BDE-209 is highly lipophilic and has a long half-life, which results in exposure that is also lasts much longer than acute exposure time. There are studies suggesting that BDE-209 has carcinogenic potential (US EPA, 2008; NTP, 1986), however in this study we only considered the non-carcinogenic effects.

Risk Assessment Analysis

The RfDs for the consumption of fish contaminated with PBDEs were calculated in collaboration with US EPA researchers, and are based on the NOAEL values, the uncertainty factors, and the aforementioned scaling factors. For a non-cancer effect, the RfDs were later used to determine the overall hazard index. Since EPA has only developed RfD values for four of the PBDE congeners, the current study focused on comparing those same RfDs to the experimental bioassay-derived RfDs in this investigation. According to EPA's RfDs from the mice assays, the maximum amount of BDE-47 that the human body can absorb without experiencing chronic health effects is 2.33E-04 mg/kg-day, while the maximum amount for BDE-153 is 1.50E-04 mg/kg-day. BDE-99 has the lowest RfD value of the four congeners at 1.33E-04 mg/kg-day. However, BDE-209 has the highest RfD value of 7.40E-03 mg/kg-day, meaning that an individual can be exposed to more of this congener without experiencing an effect. The RfD value for BDE-209 is higher than those of the lower brominated congeners, which is expected because of its low absorption, low uptake, and excretion (predominately in fecal matter) (US EPA, 2008). Due to the presence in fecal matter and in blood, it is evident that decaBDE is metabolized, forms phenolic metabolites, and debrominates, resulting in a variety of lower brominated congeners (US EPA, 2008).

The ING_{sf} and RfD values of each PBDE compound were calculated to produce individualized hazard quotients, which were summed together to form a hazard index value. The hazard index represents the potential non-cancer health effects as a result of exposure to all chemicals present. The acceptable non-carcinogenic risk level is a hazard index that is less than or equal to unity (1.0). Thus, a hazard index value less than or

equal to 1.0 is an acceptable benchmark, whereas a value greater than 1.0 results in the likelihood of the impacts of adverse non-carcinogenic health impacts (Asante-Duah, 2002). Table 5 illustrates the non-carcinogenic risk estimates that compare US EPAs four established PBDE RfDs, the HEPG2 model estimations for the same four compounds and the HEPG2 model using all of the investigated compounds in this study. The risk assessment using the EPA-established RfDs yielded the highest risk values for adults in comparison to the other assessment estimates. This suggests that EPAs risk estimates were less conservative for the combined four PBDE congeners when compared to our study; nevertheless, both adult and child values obtained a hazard index value less than 1.0.

We found that risk estimates for adults and children were also less than 1.0 for the HEPG2 model using EPAs four compounds, which indicates that the risk of exposure to these four compounds combined is below the acceptable non-carcinogenic risk level. Further, these risk estimates express the most conservative values in this study and suggests that the human population and sensitive subpopulation can be exposed to concentration levels currently found in fish for the four-combined compounds without observable adverse effects occurring from exposure. Finally, the non-carcinogenic risk estimates using the HEPG2 model that combined all PBDE compounds demonstrated increased risk estimations (not above unity). This indicates that there is an additive effect in the potential for adverse effects to occur in adults from exposure to multiple PBDEs through fish ingestion. The default assumption for exposures to multiple compounds is that their effects are additive. This may be inaccurate when considering potential mixture interactions that are otherwise neglected. The potential for greater than additive effects

(synergism), or even less than additive (antagonism), will change the true risk from exposures to mixtures of compounds. Overall, the HEPG2 model is a good *in vitro* alternative for the mouse model, since it expresses hazard index values closest to that of the mouse when using IVIVE.

Our study is consistent with the four steps of the human health risk assessment (HHRA) paradigm. We found from literature that the production of commercial PBDEs were of rising concern due to its ubiquitous presence in the humans, wildlife, and the environment and its potential adverse effects in humans. We researched toxicological properties as well as potential pathways and routes of PBDE exposure. We used four bioassay models to evaluate several PBDE concentrations: three *in vitro* models and one *in vivo* model. Further, we constructed an exposure scenario for the ingestion of contaminated fish for both adults and children. In assessing toxicity, we discovered the lack of toxicity data available for the majority of PBDE compounds. Since RfD values are established from NOAEL or LOAEL values (mg/kg-day), we used a number of scaling factors (IVIVE) and modifying factors in addition to the standard EPA uncertainty factors to convert from NOAEC or LOAEC values (mg/L) to NOAELs or LOAELs. During this step, we further compared all of the RfD values among each assay to the four PBDE RfDs established by US EPA and identified a model (HEPG2) that most closely represented that of the US EPA mouse model. Therefore, the HEPG2 model specifically became the model of interest in this study. The final step of the HHRA is risk characterization, where we calculated hazard index values based on calculated RfDs and intake values in this study to describe levels of risk (hazard index < 1.0) for the consumption of PBDE-contaminated fish. As a result, we found that all risk assessment

estimates were below the acceptable level of non-carcinogenic risk for human populations and sensitive populations. These results indicate that populations exposed to the concentrations of PBDEs in fish evaluated in this study would have risk levels below the acceptable limits.

Uncertainties

Risk assessment is a complex process that involves scientific judgment and decision making that has uncertainly involved. Uncertainty occurs because risk assessment is a complex process, which requires the integration of the release of pollutants into the environment as well as the fate and transport of pollutants in a variety of different and variable environments, by processes that are often poorly understood or too complex to quantify accurately. In addition, other parameters that also contribute a significant amount of uncertainty include the potential for adverse health effects to occur in humans, whether extrapolated from animal studies or in this case using IVIVE; and the probability of adverse effects in a human population that is highly variable genetically, as well as in age, activity level, and lifestyle. Variable uncertainty and model uncertainty are also generally recognized by risk assessors as major sources of uncertainty. Random, or sample, errors are common sources of variable uncertainty, which are especially critical for small sample sizes. It is more difficult to recognize non-random, or systematic, errors that are due to sampling, experimental design, or choice of assumptions. In this study, the sample size is not considered small in terms in *in vitro* toxicology; however, the concentration range is limited and should be extended (as highlighted in the future work).

One major source of uncertainty, and one of the motivating factors for this study, is the uncertainty involved with the toxicity factor estimates. Due to the lack of toxicity information for the majority of PBDE congeners, there must be estimates made, which incorporate uncertainty into the risk assessment. Methods that estimate toxicity factors are a necessity to complete a risk assessment, thus illustrating the need for methods and or studies to estimate those factors. Another source of uncertainty in the risk assessment for this study includes using IVIVE techniques to estimate toxicity factors. However, with the appropriate assumptions and realistic expectations of the extrapolations, this method can provide a means to screen many chemicals. Further, it can be refined through iterations that involve more precise data and additional techniques (i.e., *in silico* modeling and animal studies) to reduce uncertainty and provide more accurate estimates. In addition, using the NOAEL to estimate toxicity factors introduces additional uncertainty, although this approach is the traditionally used method and is part of the USEPA Risk Assessment Guidance for Superfund. Alternative methods could be employed to reduce this uncertainty, such as the use of the Benchmark Dose method to develop reference doses for use in risk assessments.

CHAPTER FIVE

Conclusions and Future Direction

The possibility of using IVIVE methods to determine a toxicity metric for preliminary human health risk assessment is demonstrated in this study. We found that the HEPG2 *in vitro* model most accurately represented results one might find when using a traditional mouse model. We further demonstrated the use of the developed RfDs in a risk assessment scenario for the ingestion of fish. Based on our findings, a risk estimate was calculated using congeners that currently do not have developed toxicity metrics. This provides preliminary insight into potential risk from exposures to these compounds. *In vitro* methods offer an approach that reduces time, costs, and animal lives to obtain relevant toxicity data for use in human health risk assessments. There is a need to further investigate additional *in vitro* and *in vivo* models (i.e., zebrafish) to determine if there is a model that is even more effective than the HEPG2 model in determining toxicity metrics for risk assessment. Moreover, this study currently only provides a means to be a high throughput screening and prioritization rather than a complete alternative to in vivo testing. With more development these types of methods may be a start to at least partially replace or reduce the amount of *in vivo* testing. In addition, the concentration ranges used in future studies should be aimed at elucidating the true NOAEL values by testing additional concentrations in a more focused range based on these initial experiments.

There are various shortcomings of the traditional RfD approach, namely, the use of the NOAEL. Some of those weaknesses include: (1) the NOAEL does not incorporate

information on the slope of the dose-response curve or the variability in the data; (2) the NOAEL is likely to be higher with smaller sample sizes or an inadequate study, requiring a longer and more robust animal study; (3) the NOAEL is limited to one of the experimental doses; and (4) the number and spacing of doses in a study influence the dose chosen for the NOAEL and we have noted this as a future research need for this study. The true risk of a NOAEL can vary from zero to over 20% depending on the end point, spacing of doses, and numbers of animals used (Leisenring and Ryan, 1992). Methods to determine a more accurate RfD might include investigations using a benchmark dose method rather than the traditional NOAEL method. Further, these risk assessments do not take into account mixture interactions. To determine the overall risk more accurately, we need to look into the mixture interactions and effects of these compounds when they are in mixtures with the same class of compounds and more realistically other classes of compounds commonly found in the environment (i.e., PCBs, Dioxins, Pesticides, and pharmaceuticals and personal care products (PPCPs)).

APPENDICES

APPENDIX A

Table A.1. The highest, peer-reviewed reported PBDE concentrations (mg/kg wet weight) in United States fish per congener to our knowledge. Concentrations are based upon whole fish wet weight, except those denoted by asterisk (which are based upon non-US fish fillets). Please note that to our interest, 3-OH-BDE-47 was the only measured hydroxylated PBDE congener found in fish, thus we assumed that 5-OH-BDE-47 and 6-OH-BDE-47 obtained the same concentrations for the purpose of this study.

PBDE Congener	Fish Conc. (mg/kg wet wt)	Source	Reference
BDE-28	0.000142	salmon whole wt	Schecter et al., 2006
BDE-47	0.00622	large mouth bass (whole)	Rice et al., 2002
BDE-99	0.00093	large mouth bass (whole)	Rice et al., 2002
BDE-100	0.00075	large mouth bass (whole)	Rice et al., 2002
BDE-153	0.00126	carp (whole)	Rice et al., 2002
BDE-183	0.00411	carp (whole)	Rice et al., 2002
BDE-209	0.001269	catfish whole wt	Schecter et al., 2006
3-OH-BDE-47	0.00002	Marine - yellow croaker fillet (Not US; Hong Kong)	Wang et al., 2011
5-OH-BDE-47	0.00002	Marine - yellow croaker fillet fillet (Not US; Hong Kong) assumed	Wang et al., 2011
6-OH-BDE-47	0.00002	Marine - yellow croaker fillet fillet (Not US; Hong Kong) assumed	Wang et al., 2011

APPENDIX B

Table B.1. Scaling factor values used to calculate reference doses as no-observable-adverse-effect-concentration (NOAEC) or lowest-observable-adverse-effect-concentration (LOAEC) values are converted to no-observable-adverse-effect-level (NOAEL) or lowest-observable-adverse-effect-level (LOAEL) values for all PBDE congeners tested.

HEPG2: (9K c/w at72 hrs = 18K)	BDE-28	BDE-47	BDE-99	BDE-100	BDE-153
cells / treatment: (9K x 16 wells)	2.88E+05	2.88E+05	2.88E+05	2.88E+05	2.88E+05
mg cells/treatment:	2.88E-01	2.88E-01	2.88E-01	2.88E-01	2.88E-01
kg cells/ treatment:	2.88E-07	2.88E-07	2.88E-07	2.88E-07	2.88E-07
80% cells of Liver are hepatocyte (kg)	1.20E+00	1.20E+00	1.20E+00	1.20E+00	1.20E+00
% of BW that is liver:	4.94E-09	4.94E-09	4.94E-09	4.94E-09	4.94E-09
mg of chemical in treatment:	1.25E-04	1.25E-04	1.25E-04	5.00E-04	2.00E-03
NOAEL (mg/kg-day)	8.44E+03	8.44E+03	8.44E+03	3.38E+04	1.35E+05
RfD (mg/kg-day)	8.44E-04	8.44E-04	8.44E-04	3.38E-03	1.35E-04

HEPG2: (9K c/w at 72 hrs = 18K)	BDE-183	BDE-209	3-OH-BDE-47	5-OH-BDE-47	6-OH-BDE-47
cells / treatment: (9K x 16 wells)	2.88E+05	2.88E+05	2.88E+05	2.88E+05	2.88E+05
mg cells/treatment:	2.88E-01	2.88E-01	2.88E-01	2.88E-01	2.88E-01
kg cells/ treatment:	2.88E-07	2.88E-07	2.88E-07	2.88E-07	2.88E-07
80% cells of Liver are hepatocyte (kg)	1.20E+00	1.20E+00	1.20E+00	1.20E+00	1.20E+00
% of BW that is liver:	4.94E-09	4.94E-09	4.94E-09	4.94E-09	4.94E-09
mg of chemical in treatment:	1.25E-04	1.25E-04	2.00E-03	5.00E-04	1.25E-04
NOAEL (mg/kg-day)	8.44E+03	8.44E+03	1.35E+05	3.38E+04	8.44E+03
RfD (mg/kg-day)	8.44E-04	8.44E-03	1.35E-03	3.38E-03	8.44E-04

Table B.1. continued.

Clone 9 cells: 6K c/w at 72 hrs = 48K	BDE-28	BDE-47	BDE-99	BDE-100	BDE-153
cells / treatment: (48K x 16 wells)	7.68E+05	7.68E+05	7.68E+05	7.68E+05	7.68E+05
mg cells/treatment:	7.68E-01	7.68E-01	7.68E-01	7.68E-01	7.68E-01
kg cells/ treatment:	7.68E-07	7.68E-07	7.68E-07	7.68E-07	7.68E-07
80% cells of Liver are hepatocyte(kg):	1.20E+00	1.20E+00	1.20E+00	1.20E+00	1.20E+00
% of BW that is liver:	1.32E-08	1.32E-08	1.32E-08	1.32E-08	1.32E-08
mg of chemical in treatment:	1.25E-04	2.00E-03	1.25E-04	2.50E-04	1.25E-04
NOAEL (mg/kg-day)	3.16E+03	5.06E+04	3.16E+03	6.33E+03	3.16E+03
RfD (mg/kg-day)	3.16E-02	5.06E+00	3.16E-01	6.33E-01	3.16E-01

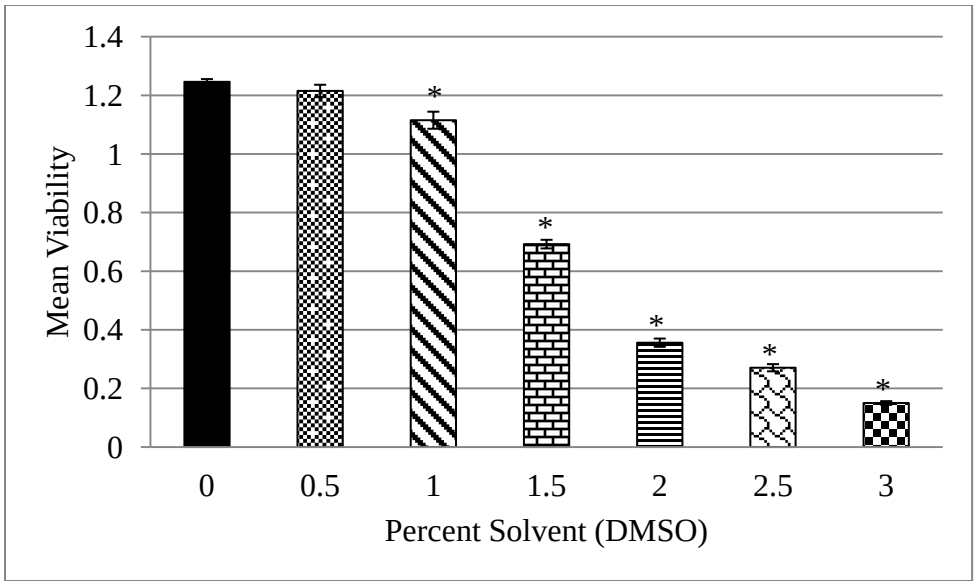
Clone 9 cells: (6K c/w at 72 hrs = 48K)	BDE-183	BDE-209	3-OH-BDE-47	5-OH-BDE-47	6-OH-BDE-47
cells / treatment: (48K x 16 wells)	7.68E+05	7.68E+05	7.68E+05	7.68E+05	7.68E+05
mg cells/treatment:	7.68E-01	7.68E-01	7.68E-01	7.68E-01	7.68E-01
kg cells/ treatment:	7.68E-07	7.68E-07	7.68E-07	7.68E-07	7.68E-07
80% cells of Liver are hepatocyte(kg):	1.20E+00	1.20E+00	1.20E+00	1.20E+00	1.20E+00
% of BW that is liver:	1.32E-08	1.32E-08	1.32E-08	1.32E-08	1.32E-08
mg of chemical in treatment:	1.25E-04	1.25E-04	2.00E-03	1.25E-04	1.25E-04
NOAEL (mg/kg-day)	3.16E+03	3.16E+03	5.06E+04	3.16E+03	3.16E+03
RfD (mg/kg-day)	3.16E-01	3.16E-02	5.06E+00	3.16E-01	3.16E-02

Table B.1. continued.

Zebrafish liver cells: (15K c/w at 72 hrs = 20K)	BDE-28	BDE-47	BDE-99	BDE-100	BDE-153
cells / treatment: (20K x 16 wells)	3.20E+05	3.20E+05	3.20E+05	3.20E+05	3.20E+05
mg cells/treatment:	3.20E-01	3.20E-01	3.20E-01	3.20E-01	3.20E-01
kg cells/ treatment:	3.20E-07	3.20E-07	3.20E-07	3.20E-07	3.20E-07
80% cells of Liver are hepatocyte(kg):	1.20E+00	1.20E+00	1.20E+00	1.20E+00	1.20E+00
% of BW that is liver:	5.49E-09	5.49E-09	5.49E-09	5.49E-09	5.49E-09
mg of chemical in treatment:	2.50E-04	5.00E-04	5.00E-04	1.25E-04	1.25E-04
NOAEL (mg/kg-day)	1.52E+04	3.04E+04	3.04E+04	7.60E+03	7.60E+03
RfD (mg/kg-day)	1.52E+00	3.04E+00	3.04E+00	7.60E-01	7.60E-02

Zebrafish liver cells: (15K c/w at 72 hrs = 20K)	BDE-183	BDE-209	3-OH-BDE-47	5-OH-BDE-47	6-OH-BDE-47
cells / treatment: (20K x 16 wells)	3.20E+05	3.20E+05	3.20E+05	3.20E+05	3.20E+05
mg cells/treatment:	3.20E-01	3.20E-01	3.20E-01	3.20E-01	3.20E-01
kg cells/ treatment:	3.20E-07	3.20E-07	3.20E-07	3.20E-07	3.20E-07
80% cells of Liver are hepatocyte(kg):	1.20E+00	1.20E+00	1.20E+00	1.20E+00	1.20E+00
% of BW that is liver:	5.49E-09	5.49E-09	5.49E-09	5.49E-09	5.49E-09
mg of chemical in treatment:	1.25E-04	2.00E-03	1.00E-03	1.25E-04	1.25E-04
NOAEL (mg/kg-day)	7.60E+03	1.22E+05	6.08E+04	7.60E+03	7.60E+03
RfD (mg/kg-day)	7.60E-02	1.22E+01	6.08E+00	7.60E-02	7.60E-01

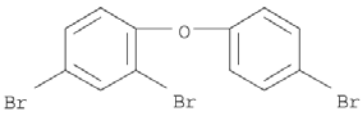
APPENDIX C



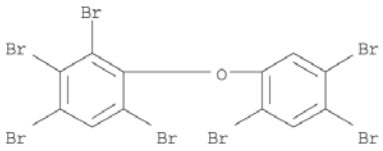
Preliminary Solvent Control study with various concentration of dimethyl sulfoxide (DMSO) and its effect on mean viability of liver cells.

APPENDIX D

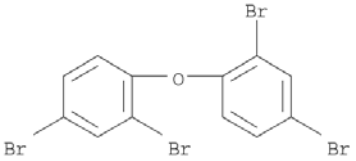
Structures of PBDE congeners used in experiments of this study.



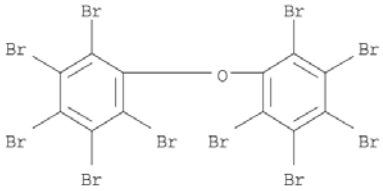
BDE-28 (2,4,4'-Tribromodiphenyl ether)



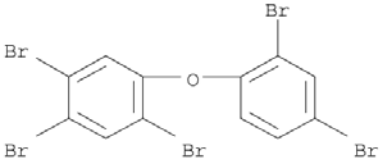
BDE-183 (2,2',3,4,4',5',6-Heptabromodiphenyl ether)



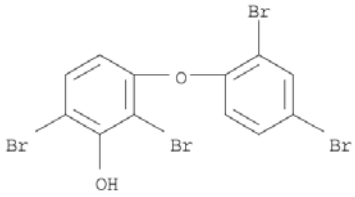
BDE-47 (2,2',4,4'-Tetrabromodiphenyl ether)



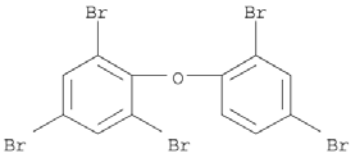
BDE-209 (2,2',3,3',4,4',5,5',6,6'-Decabromodiphenyl ether)



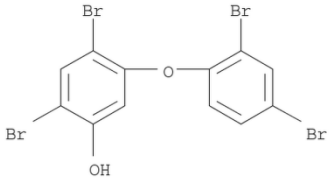
BDE-99 (2,2',4,4',5-Pentabromodiphenyl ether)



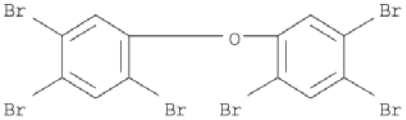
3-OH-BDE-47 (3-Hydroxy-2,2',4,4'-tetrabromodiphenyl ether)



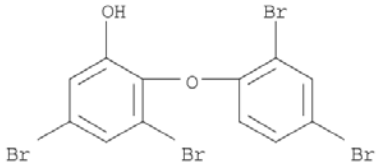
BDE-100 (2,2',4,4',6-Pentabromodiphenyl ether)



5-OH-BDE-47 (5-Hydroxy-2,2',4,4'-tetrabromodiphenyl ether)



BDE-153 (2,2',4,4',5,5'-Hexabromodiphenyl ether)



6-OH-BDE-47 (6-Hydroxy-2,2',4,4'-tetrabromodiphenyl ether)

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