THE ADVERSE EFFECTS OF 1,2-BIS(TRIBROMOPHENOXY)ETHANE IN MINK (*MUSTELA VISON*)

By

Stephanie Smith-Edwards

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ABSTRACT

THE ADVERSE EFFECTS OF 1,2-BIS(TRIBROMOPHENOXY)ETHANE IN MINK (*MUSTELA VISON*)

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Brominated flame retardants (BFRs) have been incorporated into a variety of consumer products for several years. Demonstration of BFRs in the environment, wildlife and humans has prompted concern for these emerging contaminants. Two of the commercial polybrominated diphenyl ether (PBDE) BFRs (octa-BDE and penta-BDE) are no longer being produced because of environmental concerns. As a result, the production and use of non-PBDE BFR alternatives, such as 1,2-bis(2,4,6-tribromophenoxy) ethane (BTBPE), have increased. It was of interest to determine the sensitivity of mink, a sentinel wildlife species, to BTBPE, which has been detected in the environment. Forty adult female mink were fed one of four diets containing 0, 0.014, 0.13 or 2.3 mg BTBPE/kg feed two months prior to breeding. Females were bred to untreated males. At whelping and at 3 and 6 weeks of age, kits were counted and weighed. At 6 weeks of age, six offspring from each treatment group, as well as the adult females, were necropsied. Samples of plasma, liver, fat, lungs, and feces were processed for chemical analysis and thyroids were processed for histological assessment. Ten offspring per group were maintained on their respective treatments through 7 months of age at which time the juvenile mink were necropsied and tissues processed as described above. The results of this study indicate that exposure to BTBPE at dietary concentrations up to 2.3 mg/kg feed had no effect on the reproductive performance of mink and the survivability and growth of their offspring.

DEDICATION

To my parents and friends, my children Jayla, Julian and Stephon, and my husband Julius who has sustained me through this endeavor.

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LIST OF ABBREVIATIONS

ABS	Acrylonitrile-butadiene-styrene
Ach	Acetylcholine
ADFI	Average daily feed intake
BDE	Brominated diphenyl ether
BFRs	Brominated flame retardants
BMDL	Bench mark dose limit
BTBPE	1,2-bis(2,4,6-tribromophenoxy)ethane
ChE	Cholinesterase
CNPase	Cyclic nucleotide 3'-phosphodiesterase
DBDPE	Decabromodiphenyl ethane
DCM:HEX	Dichloromethane:n-hexane
ECNI	Electron capture negative ionization
EPA	Environmental Protection Agency
EROD	Ethoxyresorufin-O-deethylase
F_1	First filial generation
FF-680	FireMaster 680
GC-MS	Gas chromatography-mass spectrometry
HBB	Hexabromobenzene
HBCDD	Hexabromocyclododecane
HIPS	High-impact polystyrene
IS	Internal standard
K_{ow}	Concentration in octanol phase/concentration in aqueous phase
LD	Lethal Dose

LD_{50}	Lethal dose to 50% of population
Lsmeans	Least square means
mAChR	Muscarinic acetylcholine receptor
MLOD	Method limit of detection
MLOQ	Method limit of quantification
MS	Mass spectrometer
MSD	Mass spectrometer detector
MSU	Michigan State University
m/z	Mass to charge ratio
n	Number of subjects
nAChR	Nicotinic acetylcholine receptor
NOAEL	No observed adverse effect level
OECD	Organization for Economic Cooperation and Development
PBBs	Polybrominated biphenyls
PBEB	Pentabromoethylbenzene
PBDE	Polybrominated diphenyl ether
PCBs	Polychlorinated biphenyls
PHA	Phytohemagglutinin
POPs	Persistent organic pollutants
POTWs	Publicly owned wastewater treatment works
PROD	Pentaoxyresorufin-O-deethylase
SAS	Statistical Analysis Systems
SE	Standard error
SIM	Selected ion monitoring
T_3	Triiodothyronine

T_4	Thyroxine
TBBPA	Tetrabromobisphenol A
TRI	Toxic Reduction Inventory
tris-BP	Tris (2,3-dibromopropyl)phosphate
TSH	Thyroid stimulating hormone
UGT	Uridine diphosphate-glucuronosyltransferases
UDPGT	Uridine diphosphate-glucuronosyltransferases
UK	United Kingdom
US	United States
UV	Ultraviolet

CHAPTER 1

LITERATURE REVIEW

Introduction

More than 3,000 people have been killed, more than 20,000 people have been injured and an estimated $11 billion in property damage has resulted from fires in the United States (US) alone in 2001 (Birnbaum and Staskal, 2004). The US has higher standards for protection against fire than Europe and as a result, more lives have been saved, fewer fire-related injuries have occurred and less economic loss have resulted by using flame retardants (Birnbaum and Staskal, 2004). Flame retardants are used to prevent or retard the initial phase of a developing fire (Sjödin et al., 2001). The incorporation of flame retardants into materials provides for longer escape time during fires by allowing less release of heat, smoke, and toxic gases, thus saving lives (Silva et al., 2004)

Brominated flame retardants (BFRs) are the largest group of flame retardants used worldwide. They have been used widely since 1975 and their use continues to increase. An estimated 410,000 metric tons of BFRs are used in the global market each year (Covaci et al., 2011). The major commercial BFRs used are tetrabromobisphenol A (TBBPA), hexabromocyclododecane (HBCDD) and polybrominated diphenyl ethers (PBDEs; Birnbaum and Staskal, 2004). Brominated flame retardants are incorporated into electronics, clothes and furniture. The electronic industry uses these compounds in printed circuit boards, connectors, plastic covers, and cables. They are also used in consumer products such as plastic covers of televisions, microwaves and other kitchen appliances as well as in carpets, paints, wood products, textiles, and paper (Birnbaum and Staskal, 2004).

Due to their chemical structure, brominated flame retardants are lipophilic and are resistant to physical and biochemical degradation, making them a potential environmental hazard. Brominated flame retardants enter the environment through industrial waste, leaching from the product itself, or from landfills, and as a result are found in the environment far from where they are produced or used. Many BFRs are persistent and bioaccumulative and can biomagnify in the food web. As a result of those properties, increased concentrations have been found in terrestrial, freshwater, and marine ecosystems, affecting the environment, wildlife and humans. Therefore they are classified as persistent organic pollutants (POPs) (Alaee and Wenning, 2002; de Wit, 2002, and de Wit et al., 2006).

The use of some BFRs has been discontinued or restricted due to the incidence of environmental contamination and/or because of adverse health effects. Specific BFRs that have been withdrawn from the market or have been restricted in use include polybrominated biphenyls (PBBs) and tris (2,3-dibromopropyl) phosphate (tris-BP) (Birnbaum and Staskal, 2004).

One specific incidence of environmental contamination involved FireMaster FF-1, which was a commercial PBB product manufactured by Michigan Chemical Company and was used as a flame retardant in industrial and consumer products (DiCarlo et al., 1978; Fries, 1985). FireMaster FF-1 was a mixture of PBB congeners formed by substituting bromine for hydrogen on the biphenyl molecule that consists of two benzene rings. Theoretically, there are 209 possible congeners considering the five sites available on each ring for binding of bromine (Figure 1.1). The hexabromobiphenyl congeners were the predominant congeners in the commercial mixture (http://www.inchem.org/documents/ehc/ehc/ehc162.htm). In 1973, several hundred pounds of FireMasterFF-1 were accidentally mixed into animal feed that ultimately resulted in the disposal of almost 30,000 contaminated dairy cows and over two million

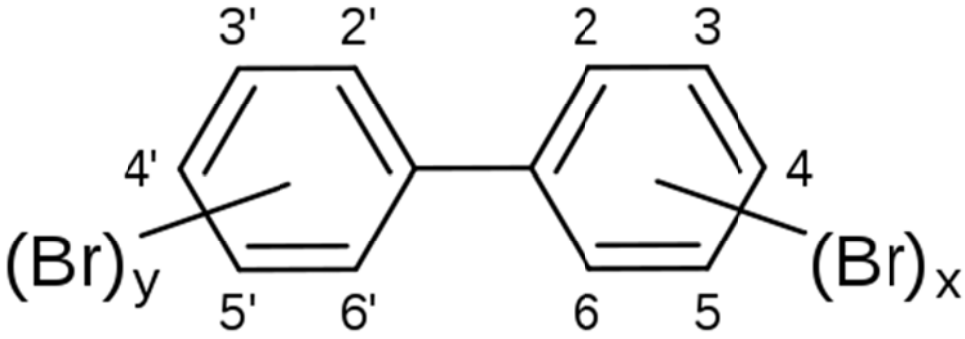

Figure 1.1. Structure of polybrominated biphenyls (PBBs)

contaminated chickens (de Wit, 2002; http://ntp.niehs.nih.gov/ntp/roc/twelfth/profiles/-PolybrominatedBiphenyls.pdf). More than 90% of the residents in Michigan had detectable quantities of PBBs stored in their body fat as a result of consumption of contaminated dairy products, eggs, and meat (Anderson et al., 1978; Miceli et al., 1985). The use of PBBs was discontinued in the US in 1976 as a result of the Michigan contamination incident (Di Carlo et al., 1978).

Tris (2,3-dibromopropyl) phosphate (Figure 1.2), was manufactured by the former Michigan Chemical Company, also the major BFR used in sleepwear between 1973 and 1977 to comply with federal regulations designed to reduce burn injuries and death. As a result, it was estimated that over 50 million children were exposed to this chemical (Blum et al., 1978). Tris (2,3-dibromopropyl)phosphate, which was shown to be mutagenic and nephrotoxic (Dybing et al., 1980; Söderlund et al., 1980), was withdrawn from the US market in 1977 (Prival et al., 1977; Blum and Ames, 1977).

The five major BFRs currently used in commerce are tetrabromobisphenol A (TBBPA), hexabromocyclododecane (HBCDD), and three commercial mixtures of polybrominated diphenyl ethers (PBDEs). The three commercial PBDE mixtures are pentabrominated diphenyl ether (pentaBDE or "penta"), octabrominated diphenyl ether (octaBDE or "octa") and decabrominated diphenyl ether (decaBDE or "deca").

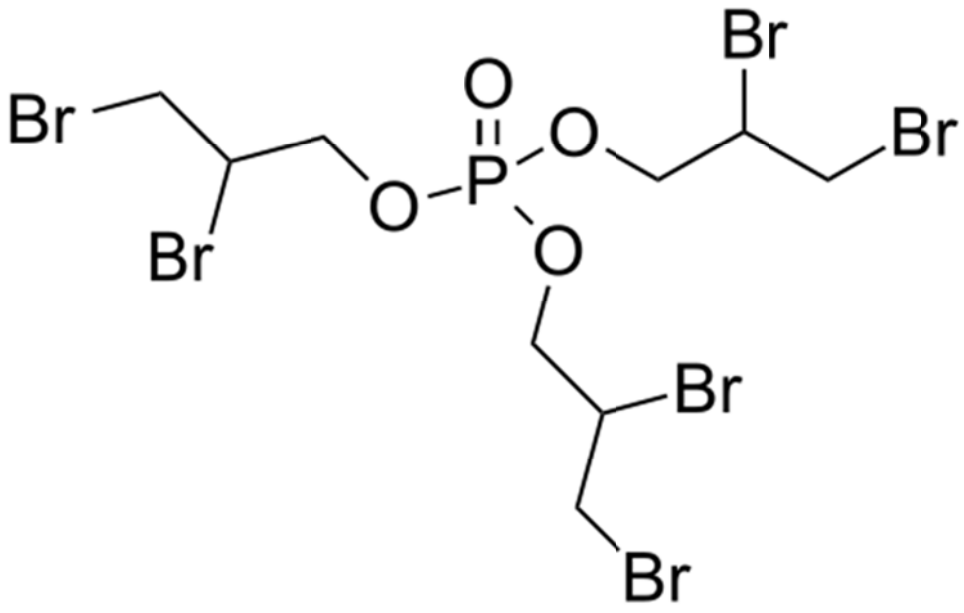

Figure 1.2. Structure of tris (2,3-dibromopropyl)phosphate (tris-BP)

Tetrabromobisphenol A

The most widely used BFR is TBBPA (Figure 1.3) with an estimated use worldwide of 150,000 metric tons (Covaci et al., 2011). Tetrabromobisphenol A is used as a reactive flame retardant in printed circuit boards, and as an additive BFR in several polymers. As a reactive BFR, TBBPA is incorporated into the product and release into the environment is minimal,

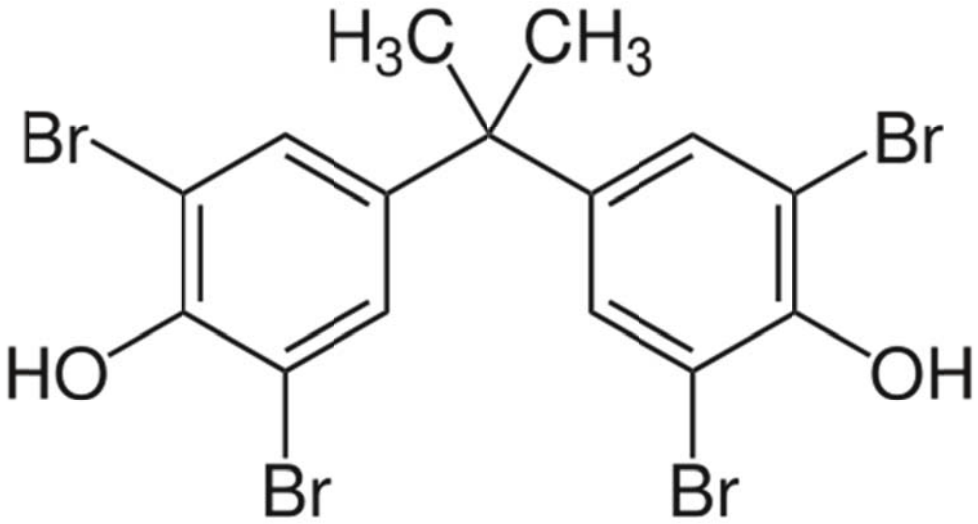

Figure 1.3. Structure of tetrabromobisphenol A (TBBPA)

whereas when TBBPA is used as an additive BFR, it can be released into the environment more readily (Birnbaum and Staskal, 2004).

Tetrabromobisphenol A and its metabolites have been detected in the atmosphere, soil and sediment, but usually not in water. Tetrabromobisphenol A was detected in the atmosphere at a concentration of 1.8 µg TBBPA/m^3 at a production site in Japan (Birnbaum and Staskal, 2004). Soil and sediment samples collected in Japan contained TBBPA at concentrations ranging from 0.5 to 140 µg/kg (dry weight) and 2 to 150 µg/kg (dry weight), respectively (Watanabe et al., 1983a, 1983b). The presence of TBBPA is limited in biota because of its relatively short half-life in water, air, and sediment. Environmental biodegradation studies of TBBPA indicate that the chemical partially breaks down under both aerobic and anaerobic conditions, with an approximate half-life of two months (Birnbaum and Staskal, 2004).

Szymanska et al. (2001) investigated the metabolism of TBBPA in rats. After a single dose of ^{14}C-TBBPA, 51 to 95% of the dose was rapidly excreted in the feces largely unchanged. After intraperitoneal (IP) administration of ^{14}C-TBBPA (250 or 1,000 mg/kg body weight), Szymanska et al. (2001) reported that the feces contained 90% TBBPA and 10% of the metabolite tribromobisphenol A. Fecal analysis suggested rapid elimination of TBBPA in the bile and possible debromination by gastrointestinal flora. The adipose tissue had the highest concentration of the compound within the first 60 minutes. At 72 hours post–injection, adipose tissue and muscle still retained a percentage of the TBBPA (3 to 6% and 11 to 14%, respectively), suggesting that TBBPA or a metabolite has the potential to bioaccumulate with repeated exposure (Szymanska et al., 2001).

Toxicity studies conducted with rodents have shown TBBPA to be relatively non-toxic. The oral LD_{50} (dose lethal to 50% of the population) is greater than 5 g/kg body weight in rats and greater than 4 g/kg body weight in mice (http://www.inchem.org/documents/ehc/ehc/ehc-172.htm). In vivo studies have not shown TBBPA to be an inhalation, dermal or ocular toxicant or teratogen (http://www.inchem.org/documents/ehc/ehc/ehc172.htm).

A 28-day repeat-dose study assessing the effects of TBBPA in rats using the Organization for Economic Cooperation and Development (OECD) guidelines was conducted by Van der Ven et al. (2008). Dietary concentrations were 0, 30, 100 or 300 mg TBBPA/kg body weight and each treatment group had 10 animals per sex. The animals were necropsied at 12 weeks of age. The effects reported were decreased plasma total thyroxine (TT_4) concentration at a benchmark dose ($BMDL_{10}$) of 48 mg/kg body weight/day and increased plasma total triiodothyronine (TT_3) concentration at a $BMDL_{10}$ of 124 mg/kg body weight/day in males.

A one-generation reproduction study assessing the effects of TBBPA in rats was conducted using the OECD guidelines (Van der Ven et al., 2008). Rats were assigned to one of eight dose groups (0, 3, 10, 30, 100, 300, 1000, 3000 mg/kg body weight/day). The design allowed a dose-response analysis and calculation of BMDL. Parental exposure started 70 days prior to mating in males and 14 days prior to mating in females. The F1 animals remained on their respective doses until necropsy, which was up to 17 weeks of age. The most sensitive effects were an increase in testis weight and an increase in pituitary weight in adult F1 males. The $BMDL_5$ and $BMDL_{10}$ based on these effects were 0.5 and 0.6 mg/kg body weight/day, respectively. The testis weight in F1 males at postnatal day 21 was also affected resulting in a $BMDL_5$ of 0.5 mg/kg body weight/day. Plasma T_4 concentration was decreased in F1 animals at a $BMDL_{10}$ of 31 and 16 mg/kg body weight/day for males and females, respectively. Plasma T_3 concentrations were increased in F1 females at a $BMDL_{10}$ of 2.3 mg/kg body weight/day (Van der Ven et al., 2008).

Saegusa et al. (2009) exposed pregnant female rats to 0, 100, 1000, or 10,000 mg TBBPA/kg feed from gestational day 10 to post-natal day 20. The dams did not have any significant toxic effects, although there was evidence of thyroid follicular cell hypertrophy in the 1000 mg TBBPA/kg feed treatment group and non-dose related increased relative thyroid gland weight in all treatment groups. The only effect in the offspring was a reduction in serum T_3 concentrations in the 100 and 1000 mg TBBPA/kg feed groups that was not related to dose. Serum T_4 and thyroid stimulating hormone (TSH) concentrations were not changed. Also, there was no evidence of impaired brain development such as hypothyroidism-related neuronal

migration or oligodendroglial development. The results suggest that TBBPA can induce developmental hypothyroidism.

Hexabromocyclododecane

Hexabromocyclododecane (Figure 1.4) is a nonaromatic, brominated cyclic alkane primarily used in thermoplastic polymers with final applications in styrene resins (Birnbaum and Staskal, 2004). It is an additive flame retardant, with total production around 22,000 metric tons per year (de Wit et al., 2010), making it a minor contributor to the BFR economy. Hexabromocyclododecane is highly lipophilic and has low water solubility (0.0034 mg/L) (Birnbaum and Staskal, 2004).

Hexabromocyclododecane has been shown to be persistent in the environment, with a half-life of three days in air and two to 25 days in water (Lyman et al., 1990). Studies have shown that HBCDD has a strong propensity to bioconcentrate, with a bioconcentration factor of approximately 18,100 in fathead minnows and fish-to-sediment ratios as great as 15 (Veith and Defoe 1979; Sellström et al., 1998).

The commercial HBCDD product contains three diastereomers, α- (10 to13%), β- (1 to 12%) and γ- (75 to 89%) HBCDD (Figure 1.5). Hexabromocyclododecane is usually found in sediments as γ-HBCDD (> 90%). However, small amounts of the α- and β-diastereomers have been found in some regions with high concentrations of HBCDD (http://www.epa.gov/opptintr/tsca8e/pubs/8ehq/2002/-oct02/8ehq_-1002_15166b.pdf). The lower level organisms contain mostly the γ-diastereomer, while the top predators contain mostly the α-diastereomer (http://www.epa.gov/opptintr/tsca8e/pubs/8ehq/-2002/oct02/8ehq_-1002_15166b.pdf).

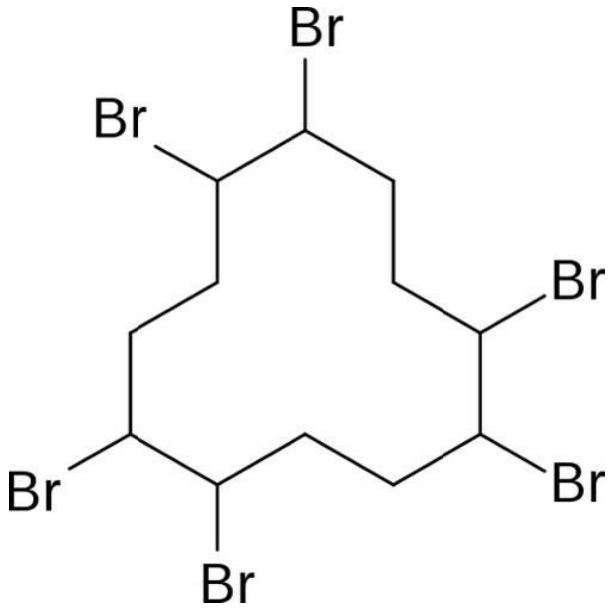

Figure 1.4. Structure of hexabromocyclododecane (HBCDD)

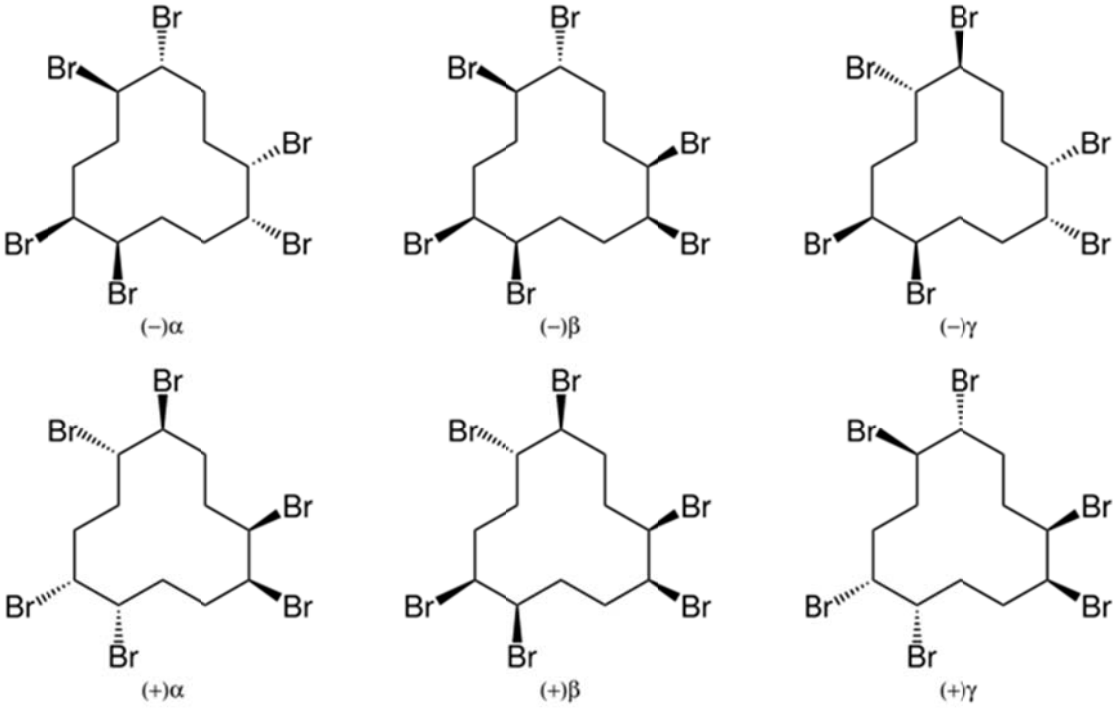

Figure 1.5. Structures of α-, (left), β-, (middle) and γ-HBCDD (right)

Laboratory studies in rats have shown HBCDD to be capable of causing adverse effects. In a HBCDD study examining endocrine effects (Van der Ven et al., 2006), rats were given oral doses of 0, 0.3, 1, 3, 10, 30, 100, and 200 mg HBCDD/kg body weight/day for 28 days. Daily dosing resulted in dose-dependent increase in liver concentrations of HBCDD, indicating absorption of the HBCDD and increased bioavailability. The marked HBCDD dose-related effects were mostly seen in the thyroid hormone axis, including decreased concentration of total T_4 ($BMDL_{10}$ of 55.5 mg/kg body weight/day), increased immunostaining for TSH in the pituitary, increased weight of the pituitary ($BMDL_{10}$ of 29 mg/kg body weight/day) and thyroid glands ($BMDL_{10}$ of 1.6 mg/kg body weight/day), and induction of T_4-UGT in the liver ($BMDL_{10}$ of 4.1 mg/kg body weight/day) resulting in increased liver weight ($BMDL_{10}$ of 22.9 mg/kg body weight/day). T_4-UGT is involved in the metabolism of T_4, thus its induction decreases plasma total T_4, which in turn leads to an increase in TSH and an increase in the weight of the thyroid gland. These HBCDD effects were only seen in females. Additional effects seen in the females were increased cholesterol ($BMDL_{10}$ of 7.4 mg/kg body weight/day) and increased tibial bone mineral density ($BMDL_{10}$ greater than 49 mg/kg body weight/day). Males had decreased splenocyte counts ($BMDL_{20}$ of 0.3–6.3 mg/kg body weight/day).

In another rat study, the developmental toxicity of HBCDD was evaluated. Hexabromocyclododecane effects were assessed in rat offspring after maternal exposure from mid-gestation through lactation at feed concentrations of 100, 1000, or 10,000 mg/kg (Saegusa et al., 2009). There was an increase in relative thyroid gland weight and incidence of thyroid follicular cell hypertrophy in dams in the 10,000 mg HBCDD/kg feed treatment group at

weaning. The offspring in the 10,000 mg HBCDD/kg feed treatment group, at this time point, had a decrease in serum T_3 concentration and an increase in serum TSH concentration. An increase in thyroid gland weight and decrease in serum T_3 concentration persisted until the adult stage in offspring exposed to 1,000 mg HBCDD/kg feed. Hexabromocyclododecane reduced density of CNPase-positive oligodendrocytes at 10,000 mg HBCDD/kg feed, which suggested impaired oligodendroglial development, probably as a result of developmental hypothyroidism. A no observed adverse effect level (NOAEL) of 100 mg HBCDD/kg feed was selected based on the developmental brain effects and changes in thyroid parameters.

Polybrominated Diphenyl Ethers

Polybrominated diphenyl ethers are another class of BFRs that are recognized as global environmental contaminants. Roughly 70,000 metric tons of PBDEs are produced annually worldwide, with half being produced in the US and Canada (Renner, 2000; Hites, 2006). The structure of a generic PBDE molecule is illustrated in Figure 1.6. Polybrominated diphenyl ethers consist of two phenyl rings with an ether linkage between the two rings. Diphenyl ether molecules contain 10 hydrogen atoms, any of which can be exchanged with bromine, resulting in 209 PBDE congeners (Alaee et al., 2003). There are three commercial mixtures of PBDEs that have been marketed as flame retardants: “penta” or pentaBDE, “octa” or octaBDE and “deca” or decaBDE (Costa and Giordano, 2007).

The commercial pentaBDE product is a mixture of 50 to 60% pentaBDEs, 24 to 38% tetraBDEs, 4 to 8 % hexaBDEs and 0 to 1% triBDEs and is sold under numerous trade names including Bromkal G1, DE 60FTM, Bromkal 70, and DE-71 (http://www.inchem.org/-documents/ehc/ehc/ehc162.htm).

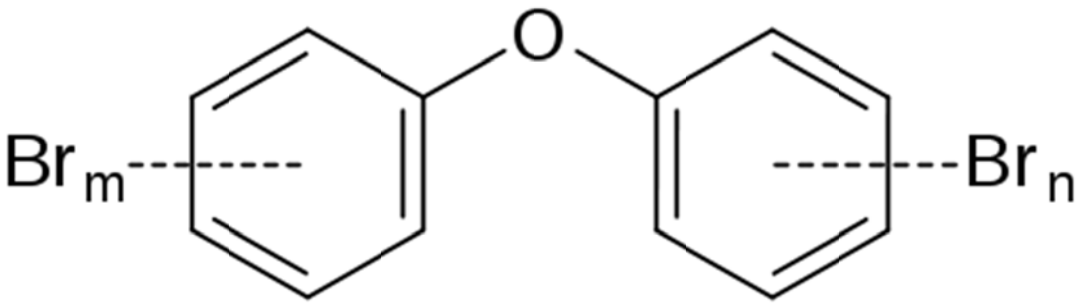

Figure 1.6. Generic structure of polybrominated diphenyl ethers (PBDEs)

PentaBDE is used as an additive flame retardant at concentrations ranging from 5 to 30% in many different polymers, resins and other substrates. The primary use of pentaBDE is in polyurethane foams, where up to 30% of the weight of the foam can be accounted for by this flame retardant (Hale et al. 2002). Penta-brominated diphenyl ether also has minor uses in phenolic resins, polyesters, and epoxy resins (Birnbaum and Staskal, 2004). Approximately 7,500 metric tons of pentaBDE are used annually with 95% being used in the US (http://www.ec.gc.ca/lcpe-cepa/09F567A7-B1EE-1FEE-73DB-8AE6C1EB7658/sar_pbde-eng.pdf).

Penta-brominated diphenyl ether may be released into the environment from various sources. Wastewater streams are an environmental source of release as a result of handling the raw material. During the curing phase of foam production, temperatures reach 160^{o}C for several hours, potentially releasing volatilized PBDE congeners (http://www.atsdr.cdc.gov/toxprofiles/tp68-c8.pdf). The estimated overall release of pentaBDE to wastewater is approximately 0.11% with approximately half of that quantity entering the

atmosphere (http://www.atsdr.cdc.gov/toxprofiles/tp68-c8.pdf). Polyurethane foam scraps, which can contain up to 30% penta, are used in products such as car seats or carpet underlay and may eventually be landfilled releasing PBDEs into the environment (http://www.atsdr.cdc.gov/toxprofiles/tp68-c8.pdf).

Penta-brominated diphenyl ether can be very persistent in the environment, binding to the organic fraction of particulate matter and the lipid fraction of biota. Components of the mixture will be expected to partition primarily to sediment (approximately 59%), followed by soils (approximately 40%), water (1.2%), and air (0.2%). The estimated half–lives of pentaBDE, with atmospheric degradation, are 600 days in aerobic sediment, 150 days in soil and 150 days in water (http://www.ec.-gc.ca/lcpe-cepa/09F567A7-B1EE-1FEE-73DB-8AE6C1EB7658/sar_pbde-eng.pdf).

The bioaccumulation of pentaBDE increases as the trophic level increases, indicating biomagnification in the food web (http://www.ec.gc.ca/lcpe-cepa/09F567A7-B1EE-1FEE-73DB-8AE6C1EB7658/sar_pbde-eng.pdf). Increasingly elevated concentrations have been detected in fish and the piscivorous avian in the food chain. Concentrations of pentaBDE at the mg/kg lipid level have been documented in marine mammals such as whales, dolphins and seals. In blubber samples collected from San Francisco Bay harbor seals, concentrations of pentaBDE rose from 88 µg/kg lipid in 1989 to 8325 µg/kg lipid in 1998 (http://www.ec.gc.ca/lcpe-cepa/09F567A7-B1EE-1FEE-73DB-8AE6C1EB7658/sar_pbde-eng.pdf).

Commercial mixtures of pentaBDE have been shown to induce both phase I (ethoxyresorufin-O-deethylase [EROD] and pentoxyresorufin-O-deethylase [PROD]), and phase II (uridinediphosphate-glucuronosyltransferase [UDPGT]) metabolic enzymes. T_4 glucuronidation by phase II UDPGT in the liver has been suggested as one of the mechanisms

contributing to circulating T_4 depletion by PBDEs and other polyhalogenated aromatic hydrocarbons (PHAHs). There are a number of studies that assessed the effects of pentBDE on thyroid hormone concentration.

There are many studies that assessed the toxicity of various commercial pentaBDE mixtures in rodents. Mice were administered a commercial pentaBDE mixture, DE-71, as either a single dose (0.8, 4, 20, 100, or 500 mg DE-71/kg body weight) or daily doses over 14 days (18, 36 or 72 mg DE-71/kg body weight/day for total doses of 250, 500, or 1000 mg DE-71/kg body weight) (Fowles et al., 1994). In the acute trial, total serum T_4 concentrations were significantly lower compared to controls at all doses except 100 mg/kg body weight. In the 14-day study, there was a significant induction of total hepatic microsomal cytochrome P450 and hepatic EROD and PROD activities at doses greater than 250 mg/kg body weight. There was also a dose-dependent decrease in the concentrations of total and free serum T_4 concentrations and an increase in serum corticosterone concentrations. An immunosuppressive effect was demonstrated by a decreased sheep erythrocyte plaque forming cell response and a decrease in thymus weight at 1000 mg DE-71/kg body weight.

In a study by Zhou et al. (2001), weanling rats were dosed with 0.3, 1.0, 3.0, 10, 30, 100, or 300 mg DE-71/kg body weight/day for four days. Hepatic EROD and PROD activities were significantly greater compared to control activities and there was a dose-related increase in uridine diphosphate glucuronyl transferase (UDPGT) activity. A dose-dependent decrease in total serum T_4 concentrations was observed.

Bromkal 70, a commercial pentaBDE mixture containing 64% pentaBDEs and 36% tetraBDEs, was administered to rats using one of three dosing regimens: a single dose of 300

mg/kg body weight, 100 mg/kg body weight/day for 4 days or 50 mg/kg body weight/day for 28 days. Relative liver weight was greater in treated animals than in controls at all doses. Additionally, there was significant induction of hepatic EROD activity in all treated animals, compared to controls at all doses (Von Meyerinck et al., 1990). The study concluded that Bromkal 70, and pentabrominated diphenyl ethers, act as mixed type inducers of liver enzymes (Von Meyerinck et al., 1990).

A 90-day feeding trial was conducted in which the commercial pentaBDE mixture DE-71 was administered to rats at doses of 2, 10 or 100 mg DE-71/kg body weight/day for 90 days. Absolute liver weight were increased by 11% in animals dosed with 10 mg DE-71/kg body weight/day and by 50 and 70% in females and males, respectively, at 100 mg DE-71/kg body weight/day. Histopathological changes in the liver included hepatocytomegaly and hepatocyte degeneration and necrosis. Slight hyperplasia of the thyroid occurred in animals dosed with 100 mg DE-71/kg body weight/day and thyroid gland weight was 30% greater compared to controls. Serum T_4 concentrations were decreased by greater than 20% at doses of 10 and 100 mg DE-71/kg body weight/day (http://www.ec.gc.ca/lcpe-cepa/09F567A7-B1EE-1FEE-73DB-8AE6C1EB7658/sar_pbde-eng.pdf).

Individual congers in commercial pentaBDE products may be neurotoxic to mammalian systems if exposure is during a critical time in development. Neonatal mice administered a single oral dose of 2,2',4,4'- tetrabromodiphenyl ether (BDE-47) at 0.7 or 10.5 mg/kg body weight or 2,2',4,4',5-pentabromodiphenyl ether (BDE-99)/kg body weight at 0.8 or 12.0 mg/kg body weight on day 10 of development had decreases in habituation, locomotion, rearing and total activity that became more pronounced with age. Adult memory and learning were also affected (Eriksson et al., 1998).

OctaBDE

Octabrominated diphenyl ether (OctaBDE) (Figure 1.7) is another commercial PBDE product that is composed of 45% heptaBDEs, 33% octaBDEs, 12% hexaBDEs, 10% nonaBDEs, 0.7% decaBDE and 0.05% pentaBDE (http://chm.pops.int/Convention/POPsReviewCommittee/-Reports/tabid/2301/Default.aspx). Trade names for OBDE include Bromkal 79-8DE, CD-79, DE-79, EB-8, FR-1208, FR-143, Tardex 80, Saytex 111 and Adine 404. Approximately 3,790 metric tons of octaBDE have been used annually with the majority being used in the US (1,500 metric tons) and Asia (1,500 metric tons) (Birnbaum and Staskal, 2004).

Approximately 70% of the octaBDE manufactured globally is added to acrylonitrile-butadiene-styrene (ABS) polymers that are then used to produce computers and business cabinets (http://www.inchem.org/documents/ehc/ehc/ehc162.htm). The polymers are also used in pipes and fittings, automotive products, business machines and appliances (http://www.ec.gc.ca/lcpe-cepa/09F567A7-B1EE-1FEE-73DB-8AE6C1EB7658/sar_pbde-eng.pdf). Octabrominated diphenyl ether is also included in high-impact polystyrene (HIPS), polybutylene, terephthalate, polyamide polymers, nylon and low-density polyethylene, polycarbonate, phenol-formaldehyde resins and unsaturated polyesters, and adhesive coatings (http://www.ec.gc.ca/lcpe-cepa/09F567A7-B1EE-1FEE-73DB-8AE6C1EB7658/sar_pbde-eng.pdf).

There are many potential routes by which octaBDE may be released into the environment. Manufacturing of plastic is one of many potential routes for release (http://www.ec.gc.ca/lcpe-cepa/09F567A7-B1EE-1FEE-73DB-8AE6C1EB7658/sar_pbde-eng.pdf). During the handling of octaBDE, there is loss of the raw material, estimated at 0.21% for powders of particle size greater than 40 µm (http://www.ec.gc.ca/lcpe-cepa/09F567A7-B1EE-1FEE-73DB-8AE6C1EB7658/sar_pbde-eng.pdf). The losses are initially to the

atmosphere, but the dust rapidly settles, creating primarily solid waste, which may be recycled or disposed of, or it may

enter the wastewater (http://www.ec.gc.ca/lcpe-cepa/09F567A7-B1EE-1FEE-73DB-8AE6C1EB7658/sar_pbde-eng.pdf). It is estimated that approximately 0.05% octaBDE is released into the atmosphere and 0.05% into wastewater during the processing of thermoplastic polymers (http://www.ec.gc.ca/lcpe-cepa/09F567A7-B1EE-1FEE-73DB-8AE6C1EB7658/sar_pbde-eng.pdf).

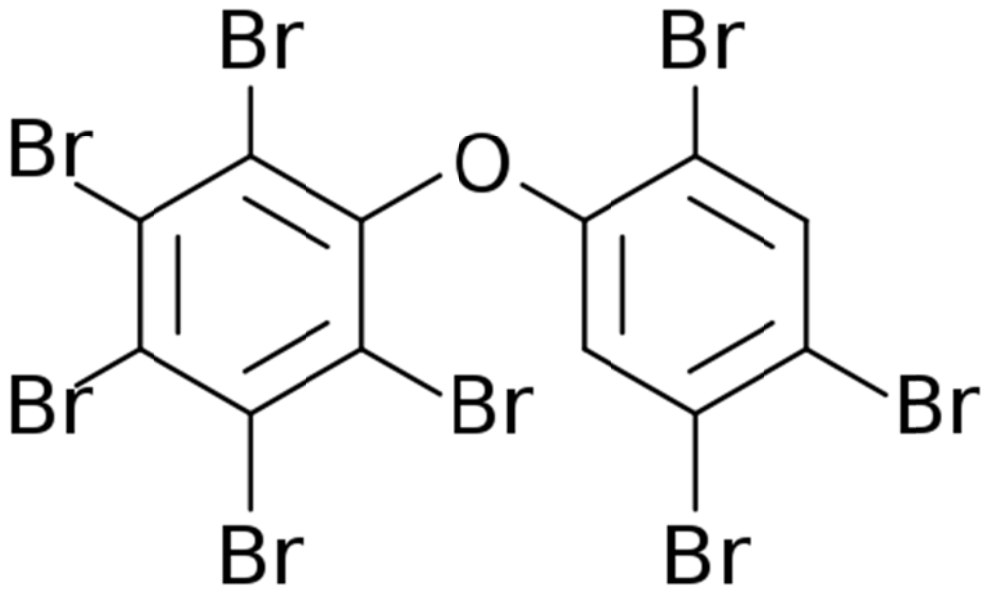

Figure 1.7. Structure of Octabrominated diphenyl ether (OctaBDE)

Congeners comprising the commercial octaBDE mixture have been detected in air samples from urban, rural, and remote sites in the US ranging from approximately 0.2 to 0.9 pg/m^3 (Strandberg et al., 2001). In the west, central, and eastern basins of Lake Ontario, there are measurable total PBDE (mono through heptaBDE congeners) concentrations of 0.0039,

0.0065 and 0.0053 ng/L, respectively (http://www.ec.gc.ca/lcpe-cepa/09F567A7-B1EE-1FEE-73DB-8AE6C1EB7658/sar_pbde-eng.pdf). Concentrations of octaBDE in United Kingdom (UK) sediments ranged from less than 0.44 to 3030 µg/kg dry weight (Allchin et al., 1999; http://www.ec.gc.ca/lcpe-cepa/09F567A7-B1EE-1FEE-73DB-8AE6C1EB7658/sar_pbde-eng.pdf). Octabrominated diphenyl ether was detected in sediment samples in Japan in 1987 and 1988 at concentrations of 8 to 21 µg/kg and 15 to 22 µg/kg, respectively (http://chm.pops.int/Convention/POPsReview-Committee/Reports/tabid/2301/Default.aspx). Concentrations of octaBDE components (hexaBDE and heptaBDE congeners) detected in lake trout collected from lakes Superior, Huron, and Ontario ranged from 11 to 53 µg/kg lipid (Alaee et al., 1999). Sampling of wild bird eggs in western and northern Canada between 1983 and 2000 determined that concentrations of total hexa- and heptaBDE congeners ranged from 0.148 to 52.9 µg/kg wet weight in great blue heron (*Ardea herodias*) eggs, 0.03 to 0.68 µg/kg wet weight in northern fulmer (*Fulmarus glacialis*) eggs and 0.009 to 0.499 µg/kg wet weight in thick billed murre (*Uria lomvia*) eggs. The presence of these congeners in arctic bird eggs on Canada's west coast and in the Canadian arctic suggests long-range transport of the hexa- and heptaBDE (http://www.ec.gc.ca/lcpe-cepa/09F567A7-B1EE-1FEE-73DB-8AE6C1EB7658/sar_pbde-eng.pdf).

There are a number of rodent studies that have assessed the toxicity of the commercial octaBDE mixture. The data available for rodents indicate a very low level of acute toxicity. An acute oral LD_{50} of 28,000 mg/kg body weight for octaBDE in rats was reported (http://www.inchem.org/-documents/ehc/ehc/ehc162.htm). In a study by Zhou et al. (2001), weanling rats were administered DE-79 at doses of 0.3, 1.0, 3.0, 10, 30, 60 or 100 mg DE-79/kg body weight/day for a four-day period. Hepatic EROD and PROD activities were significantly

increased in the highest dose group, with 20-fold induction for EROD activity and 26-fold induction for PROD activity. Additionally, there was a dose-dependent decrease in total serum T_4 concentrations, with a maximum decrease of 70% in the animals dosed with 100 mg DE-79/kg body weight/day.

In another study conducted by Great Lakes Chemical Corporation (http://www.inchem.-org/documents/ehc/ehc/ehc162.htm), rats were administered DE-79 for 28 days or 13 weeks. The doses used in the 28-day study were 0, 100, or 1000 mg DE-79/kg feed. At the end of the 28-day study, absolute and relative mean liver weight were significantly greater in the 100 and 1000 mg/kg feed treatments. The doses used in the 13-week study were 0, 100, 1,000, or 10,000 mg/kg feed. At the termination of the 13-week feeding study, statistically significant increases in absolute and relative liver weight were evident at all feed concentrations. Liver cells were enlarged, with finely granular appearing cytoplasm and increased vacuolation. Hepatocyte necrosis at the 1,000 and 10,000 mg DE-79/kg feed concentrations was also detected.

A rat fetal toxicity study was performed using octaBDE preparations of DE-79 and Saytex 111. Maternal doses of 0, 2.5, 10, 15, 25 or 50 mg/kg body weight/day were administered by gavage from days 6 to 15 of gestation. Adult females dosed with 50 mg/kg body weight/day had reduced body weight gain. An increase in mortality and fetal reabsorptions, delayed skeletal ossification and reduced fetal body weight occurred at doses of 10, 15 and 25 mg/kg body weight/day (http://www.inchem.org/documents/ehc/ehc/ehc162.htm).

Viberg et al. (2003a) assessed the neurotoxic effects of BDE-153, a congener found in commercial octaBDE mixtures, in mice. Neonates were administered a single dose of 0.45, 0.9, or 9.0 mg BDE-153/kg body weight on postnatal day 10, a time associated with rapid brain development in mice. At 2, 4, and 6 months post-dosing, treated mice had significantly altered

motor behavior. The incidence of abnormal behavioral responses was dose-related and the condition worsened with age. Adult mice also had aberrant behavior with impaired spatial learning ability and memory function.

DecaBDE

The commercial decaBDE (Figure 1.8) mixture is composed of 97-98% decaBDE and small quantities of other PBDEs (mainly nonaBDEs at 0.3-3.0%) (http://www.inchem.org/-documents/ehc/ehc/ehc162.htm). Some trade names for DBDE are 102 (E), Adine 505, AFR 1021, Bromkal 81 and Saytex 102. DecaBDE is the second most used BFR globally with approximately 56,400 metric tons being used annually (de Wit et al., 2010). The majority of its use is in the Americas (24,500 metric tons) and Asia (23,000 metric tons). DecaBDE is used in a variety of polymer applications at a rate of approximately 10 to 30% of the total product weight (Kierkegaard et al., 2009). DecaBDE is also used in terephthalates to make moldings, connectors and electrical equipment (http://www.oekorecherche.de/english/berichte/volltext/Flame%-20Retardants.pdf).

There is potential for decaBDE to be released into the environment during the manufacturing process, throughout the service life of the materials containing decaBDE and during disposal (http://www.ec.gc.ca/lcpe-cepa/09F567A7-B1EE-1FEE-73DB-8AE6C1EB7658/sar_pbde-eng.pdf).

The US EPA's Toxic Reduction Inventory (TRI) indicated that most US decaBDE manufacturer emissions were directed to land and publicly owned wastewater treatment works (POTWs). The 2001 TRI estimated the emissions from the textile industry were by far the largest decaBDE discharge to US surface waters and POTWs. Publicly owned wastewater treatment works consequently affect sewage sludge burdens and effluent receiving streams. About 98% of the

estimated air emissions were attributed to fugitive dust releases from facilities belonging to the two major US decaBDE manufactures (Hale et al., 2006).

Once decaBDE is released into the environment, it can be found in sediment and sewage sludge. Sediments taken near a manufacturing site in Sweden had decaBDE concentrations of 68-390 µg/kg dry weight and decaBDE concentrations in river sediments collected from an urban area in Japan were 33-390 µg/kg dry weight (Sellström et al., 1998). Decabrominated diphenyl ether has been found at higher concentrations in samples from riverine, estuarine and marine sediments, in comparison to other brominated diphenyl ethers, such as hexaBDE, pentaBDE and tetraBDE. The decaBDE concentrations ranged from less than 25 to 11,600 µg/kg dry weight, whereas concentrations of the latter compounds were in the range of less than the limit of detection of 70 µg/kg dry weight (Darnerud et al., 2001).

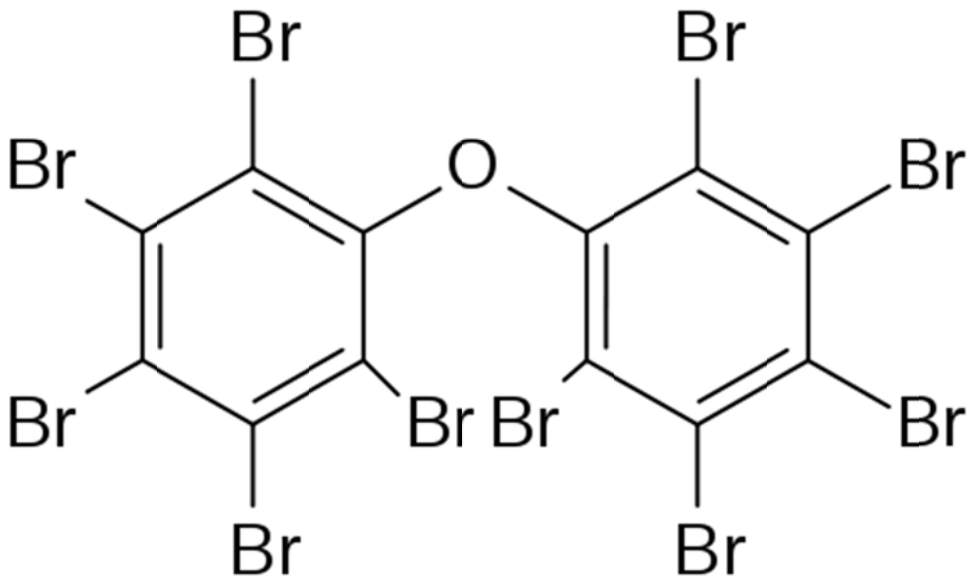

Figure 1.8. Structure of decabrominated diphenyl ether (DecaBDE)

Decabromodiphenyl ether has also been identified in air and water samples. DecaBDE was identified in 10 samples of air from a manufacturing site at atmospheric concentrations that were below the analytical detection limit of 25 μg/m^3, while aquatic concentrations of decaBDE were reported to be in the range of 0.2 to 2.5 μg/L (http://www.inchem.org/documents/ehc/-ehc/ehc162.htm#PartNumber:1). The atmospheric half-lives of tetraBDE to decaBDE ranged from 7.14 to 317.53 days (http://www.ec.gc.ca/lcpe-cepa/09F567A7-B1EE-1FEE-73DB-8AE6C1EB7658/sar_pbde-eng.pdf).

DecaBDE may be debrominated in the natural environment to lower brominated PBDEs by sunlight and UV light based on laboratory studies (Watanabe and Sakai, 2003). It is also possible that decaBDE can undergo anaerobic biodegradation and dehalogenation (http://www.atsdr.cdc.gov/ToxProfiles/tp68-c8.pdf; http://www.ec.gc.ca/lcpe-cepa/09F567A7-B1EE-1FEE-73DB-8AE6C1EB7658/sar_pbde-eng.pdf). DecaBDE can absorb light up to 325 nm, which indicates its susceptibility to photodegradation at environmental wavelengths (http://www.atsdr.cdc.gov/ToxProfiles/tp68-c8.pdf).

Although it was first believed that bioaccumulation of decaBDE in aquatic organisms was minimal due in part to the large molecular size of the decaBDE constituents (Opperhuizen et al. 1985; http://www.inchem.org/documents/ehc/ehc/ehc162.htm), recent research has shown that decaBDE can bioaccumulate in aquatic organisms. Juvenile rainbow trout (*Oncorhynchus mykiss*) were dosed with DOW FR-300 (77.4% decaBDE, 21.8% nonaBDEs and 0.8% octaBDEs). Several hexa-, octa- and nonaBDEs were detected in the liver and muscle of treated fish with concentrations increasing with duration of exposure. The presence of various PBDE congeners suggested metabolic debromination of decaBDE (Kierkegaard et al., 1999). It has been reported that peregrine falcon eggs had high concentrations of decaBDE, indicating that the

commercial mixture has the potential to bioaccumulate in terrestrial organisms (http://bfr2010.com/abst/2001/BFR2001del5.pdf#page=33).

In general, toxicity studies have not shown decaBDE to have deleterious effects. The decaBDE studies performed with aquatic organisms have shown the compound to be acutely nontoxic at concentrations up to and exceeding its limit of water solubility (< 0.1 µg/L) (http://www.ec.gc.ca/lcpe-cepa/09F567A7-B1EE-1FEE-73DB-8AE6C1EB7658/sar_pbde-eng.pdf). There are numerous rodent studies that have assessed the toxicity of decaBDE. Commercial decaBDE has been shown to have low oral toxicity. Male and female rats were fed a commercial decaBDE mixture (> 97% decaBDE with nonaBDE present at 0.3 to 3%) at concentrations of 100 and 1000 mg/kg feed for a period of 28 days. Corresponding approximate doses were 10 and 100 mg/kg body weight/day. The only observable change that could be attributed to treatment was increased bromine content in liver and fat samples from both treatment groups (http://www.inchem.org/documents/ehc/ehc/ehc162.htm).

A 30-day study in male rats that were fed Dow FR-300-BA (77.4% decaBDE, 21.8% nonaBDE, 0.8% octaBDE) at concentrations of 0.01, 0.1 and 1.0%, which corresponded to approximate daily doses of 8, 80 and 800 mg/kg body weight. The significant effects reported were liver enlargement and thyroid hyperplasia in the 0.1 and 1.0% groups, and renal and hepatic lesions in the high dose group (Norris et al., 1975; Darnerud et al., 2001).

In a decaBDE carcinogenicity study, rats and mice were fed diets containing commercial decaBDE (94 to 99% purity) for 103weeks. Tumor incidence was observed at 1,200 and 2,500 mg/kg body weight/day in rats. A dose related increase in liver adenomas was observed in rats and mice fed decaBDE. Hepatocellular adenomas and carcinomas were increased in mice at doses of 3,500 and 7,000 mg/kg body weight/day (Darnerud et al., 2001)

Pregnant rats were administered Dow-300-BA at doses of 10, 100, or 1000 mg/kg body weight on days 6 to 15 of gestation. Fetuses in the high dose group had evidence of subcutaneous edema and a significantly increased incidence of delayed skull ossification (Norris et al. 1975; Darnerud et al., 2001).

Viberg et al. (2003b) evaluated the potential neurotoxicity of BDE-209 in mice. A single oral dose of 2.22 or 20.1 mg BDE-209/kg body weight was administered to mice on postnatal days 3 or 19. An additional study used a single oral dose of 1.34, 13.4 or 20.1 mg BDE-209/kg body weight administered to 10-day-old mice (Viberg et al., 2003b). Mice exposed to the 20.1 mg BDE-209 dose on postnatal day 3 had disturbances in spontaneous behavior (motor behavior, locomotion, rearing, and total activity) as adults. The disruption of spontaneous behavior appeared to worsen with age. When comparing the habituation capacity (decrease in response to a stimulus after repeated exposure to the stimulus) in 2-, 4-, and 6-month-old mice, there was a decrease in locomotion, rearing and total activity in response to diminishing novelty of the test chamber. The mice exposed on postnatal day 3 showed the aberrant behavior pattern at 6 months of age only. Mice exposed on postnatal days 10 and 19 did not have disrupted habituation behavior. The results from this study indicate BDE-209 can be neurotoxic in adult mice exposed neonatally.

Polybrominated Diphenyl Ethers and Wildlife

Polybrominated diphenyl ethers are known global environmental contaminants, posing health risks to humans and wildlife (Segev et al., 2009). Lower brominated congeners (i.e. mono- to hexaBDEs) are generally more persistent (Gerecke et al., 2006; Law et al., 2006), bioaccumulative (Burreau et al., 2004, 2006) and toxic (Darnerud, 2003) than higher brominated congeners. DecaBDE is the PBDE used to the greatest extent in North America. However, the

lower brominated congeners are the most prominent PBDEs found in wildlife and humans. Higher brominated congeners can be debrominated to lower brominated congeners in mammals (Morck et al., 2003). The most abundant PBDE congeners found in biota and demonstrated to elicit the most adverse health effects are those that are components of the commercial pentaBDE mixture DE-71 (http://www.atsdr.cdc.gov/toxprofiles/index.asp). The concentrations of PBDEs in freshwater fish in North America, the greatest user of PBDEs, are high and increasing exponentially in certain species and locations (Hale et al., 2001; Johnson and Olson, 2001; Rayner et al., 2003; Chernyak et al., 2005). The dietary exposure of piscivorous wildlife species such as mink to PBDEs may pose a health risk to these species. While there are numerous studies that have assessed the effects of PBDEs in rodents, relatively few studies have focused on wildlife species.

Wildlife species can serve as sentinels of environmental hazards. Laboratory animals reared under controlled conditions are used in determining dose-response relationships and the molecular mechanisms of toxicity. The laboratory studies typically do not give enough information about integrated biologic responses to chronic exposures to real-world mixtures of environmental chemicals. Wildlife species can provide information on the types, characteristics, amounts and bioavailability of pollutants in an environment. Interactive effects of environmental chemicals and the role of the other environmental factors in the final toxicological response are provided by wildlife (Fox et al., 2001). Mammalian wildlife species have physiological systems that are similar to humans and rodents in terms of uptake, distribution, metabolism and distribution of toxicants. Humans and many wildlife species inhabit the same ecosystems and are exposed to the same food sources and pollutants (Basu et al., 2007). Because of these

characteristics, the use of wildlife species to examine environmental pollutants can be advantageous.

The mink is an excellent wildlife model to address issues related to environmental pollutants. Mink satisfy the requirements of a sentinel species, characteristics of which include: a wide spread distribution, high trophic status, ability to bioaccumulate pollutants, capable of being maintained and studied in captivity, captured in sufficient numbers, restricted home range, well known biology and sensitive to pollutants (Basu et al., 2007). The mink is one of the most widespread carnivores in North America and is generally found in forested regions, especially in areas containing wetlands (Arnold and Fritzell, 1990). Mink can be used in epidemiological studies because of their occurrence across wide geographical areas, which ensures their presence in polluted and non-polluted regions. Mink are abundant and frequently trapped, and they provide data on spatial and temporal trends in environmental pollutants (Basu et al., 2007). The life span of mink in the wild is approximately three years. However, in captivity mink can live up to eight years, permitting pollutants time to bioaccumulate to appreciable concentrations. Wild mink are opportunistic predators that consume a range of prey items available in their local habitat, including small mammals, frogs, snakes and birds (Basu et al., 2007). Fifty percent of a typical mink diet consists of fish, because mink usually forage close to aquatic habitats. The fish in the mink's diet represents the primary route by which persistent chemicals are accumulated (Chan et al., 2003; Wiener et al., 2003). The mink's high trophic status and limited home range provides relatively steady-state information on the types, bioavailability, and concentration of pollutants in specific regions. The pollutants measured in mink tissues reflect levels in the local prey base (Cumbie, 1975; Kucera, 1983; Foley et al., 1988). Mink are highly susceptible to many pollutants (Calabrese et al., 1992; Aulerich and Bursian, 1996). Fur ranchers observed that

mink consuming fish collected from the Great Lakes during the 1960s failed to reproduce (Aulerich et al., 1971). Subsequent feeding studies demonstrated that polychlorinated biphenyls (PCBs) present in the tissues of the fish collected from the region were the cause of the reproductive problems.

Mink can be raised in captivity, allowing derivation of quantitative exposure-response relationships, which makes them a particularly useful model in toxicology. Toxicity data can be compared between wild and captive animals, strengthening environmental risk characterization. Most studies performed in captivity address reproductive and lethal effects of environmental pollutants. Most PBDE toxicity studies are conducted in rodents, but the rodent may not be an adequate model to analyze higher trophic wildlife. Because mink are sensitive to other halogenated environmental contaminants such as PCBs, they were chosen by Environment Canada as a sentinel species to assess the effects of the commercial pentaBDE mixture DE-71.

Initially, Martin et al. (2007) conducted a sub-chronic study that assessed the immunotoxicity of DE-71 in ranch mink. Forty 20-week-old male mink were randomly assigned to four treatment groups, with 10 animals per treatment group. Mink were provided feed containing 0, 1.0, 10, or 100 mg DE-71/kg feed for nine weeks. Within the first week, mink on the 100 mg DE-71/kg feed diet rejected the food and were then switched to a diet containing 5 mg DE-71/kg feed for the duration of the trial. Hepatic concentrations of ∑PBDEs (lipid corrected) increased with increasing dietary DE-71 exposure. Predominant congeners detected in the livers of the mink included BDEs 47, 153, 99, 85 and 100. The percent contribution of BDE 47 to ∑PBDEs ranged from 39 to 44 %. Mink exposed to DE-71 did not differ significantly in the phytohemagglutinin (PHA) skin response, relative to control mink, and the PHA-induced response was not associated with individual liver PBDE congener concentrations. Primary and

secondary antibody responses of mink in the 5.0 and 10 mg DE-71/kg feed groups exceeded that of control mink, suggesting an alteration, perhaps an up-regulation, in one or more aspects of the antibody response. There was also an increase in the spleen somatic index and greater germinal center development in spleens of the DE-71 treated mink. Hepatic microsomal EROD activity was positively associated with ∑PBDE concentrations and EROD induction correlated with the liver somatic index, reflecting the significantly enlarged livers in treated mink. Mean hematocrit decreased significantly in mink exposed to 10 mg DE-71/kg feed over the course of the trial. A significant decline in mean hematocrit with increasing ∑PBDEs in liver indicated a contaminant-induced effect. The percentage of neutrophils increased and the percentage of lymphocytes decreased significantly in the two highest treatment groups. Results from this study provided evidence for the vulnerability of mink to the immune effects of bioaccumulative PBDE congeners.

A chronic trial was then conducted to assess the accumulation, disposition and metabolism of DE-71 and its effects on reproductive performance of female mink and survivability, growth and neurodevelopment of their offspring (Bull et al., 2007; Zhang et al., 2008, 2009). Mink were fed diets containing 0, 0.1, 0.5, or 2.5 mg DE-71/kg feed beginning seven weeks prior to breeding through weaning of kits at six weeks post-parturition. Portions of the offspring were continued on their respective diets until 33 weeks of age. These dietary concentrations bracketed environmentally relevant concentrations.

Zhang et al. (2008) evaluated the accumulation, disposition and metabolism of DE-71 in the adults and offspring from the above study. Similar lipid-normalized concentrations of PBDEs were detected in most tissues of adult mink with the exception of the brain. Six-week-old kits had a greater proportion of PBDEs in the brain compared to adults, presumably because

of incomplete development of the blood-brain barrier. Lesser brominated congeners were transferred from the mother to the kit and the bulk of the body burden in kits at weaning resulted from lactational rather than transplacental transfer. Lipid-normalized, whole body biomagnification factors ranged from 0.5 to 5.2 for the major congeners and were greatest for BDEs 47 and 153. Hydroxylated PBDEs accounted for 28 to 32% of the excreted fraction, indicating that metabolism was an important elimination pathway.

Zhang et al. (2009) presented data from the chronic study related to effects of DE-71 on reproductive performance of adult female mink, survivability and growth of their offspring, histological and biochemical effects in the liver and thyroid, and concentrations of circulating thyroid hormones in adult, kit and juvenile mink. The dietary concentration of 2.5 mg DE-71/kg feed resulted in complete reproductive failure, while reproduction was unaffected at the lesser dietary concentrations. Juvenile mink at 33 weeks of age had disrupted thyroid hormone homeostasis as evidenced by a significant decrease of T_3 in males and females exposed to 0.5 mg DE-71/kg feed, despite a compensatory increase in total T_4 in females, but not males. Additionally, thyroid follicular epithelium cell height was increased in the 0.5 mg DE-71/kg feed males and females. Ethoxyresorufin-O-deethylase activity was significantly increased in all offspring at 33 weeks of age, but this increase was attribute to polybrominated dioxin, polybrominated furan and/or polybrominated biphenyl impurities in DE-71.

Finally, Bull et al. (2007) assessed the effect of DE-71 on cholinergic parameters in adult female mink and their offspring exposed to 0, 0.1, 0.5 or 2.5 mg DE-71/kg feed. Cholinergic parameters, including muscarinic acetylcholine receptor (mAChR) and nicotinic acetylcholine receptor (nAChR) binding, cholinesterase (ChE) activity and acetylcholine (ACh) concentration, were assessed in the cerebral cortex and ChE activity was measured in the plasma. Despite a

dose-related increase in brain PBDE concentrations, DE-71 had no significant effect on cortical cholinergic parameters.

Concern about the environmental impact and deleterious health effects of some commercial PDBE mixtures has led to discontinuation or restriction of their use. The commercial pentaBDE mixture, whose use was concentrated in North America, and octaBDE were both phased out by the European Union in 2004 and by Canada in 2006. Twelve states in the US have banned at least two commercial PBDEs, and four states (Washington, Maine, Vermont, and Oregon) have banned all three of the commercial PBDE mixtures. In the US, the producer of pentaBDE- and octaBDE voluntarily ceased production in 2004 (Costa and Giordano, 2007).

DecaBDE is still the most widely used and cost effective PBDE in the polymer industry, but alternative BFRs have been introduced as replacements for the PBDEs (Ward et al, 2008). Alternatives to PBDEs include hexabromocyclododecane (HBCDD), decabromodiphenyl ethane (DBDPE), hexabromobenzene (HBB), pentabromoethylbenzene (PBEB) and 1, 2-bis (2,4,6-tribromophenoxy) ethane (BTBPE).

1,2-Bis (2,4,6-tribromophenoxy) Ethane

1,2-Bis (2,4,6-tribromophenoxy) ethane (Figure 1.9) has been introduced as a replacement for the commercial octaBDE mixture (Renner, 2004). It is manufactured in the US by Great Lakes Chemical Corporation and is marketed commercially as FireMaster 680 or FF-680. The total annual world production was approximately16,710 metric tons in 2001 (Verreault et al., 2007). 1,2-Bis (2,4,6-tribromophenoxy) ethane is used in applications such as the production of plastic materials that require high manufacturing temperatures and light stability such as acrylonitrile-butadinene-polystyrene and high impact polystyrene (Hakk et al., 2004). The chemical structure of BTBPE is similar to that of PBBs. 1,2-Bis (2,4,6-tribromophenoxy)

ethane is very hydrophobic (K_{ow} of 3.14) and, like many BFRs, is expected to be persistent and to bioaccumulate in the environment (Hakk et al., 2004).

1,2-Bis (2,4,6-tribromophenoxy) ethane was first detected in the environment in the late 1970s (Qiu et al., 2007). Release of BTBPE into the environment may be through the manufacturing process, as well as through various waste streams such as household dust (Karlsson et al., 2007) and dust collected from electronic recycling centers (Pettersson-Julanderet al., 2004). 1,2-Bis (2,4,6-tribromophenoxy) ethane, in recent years, has also been detected in

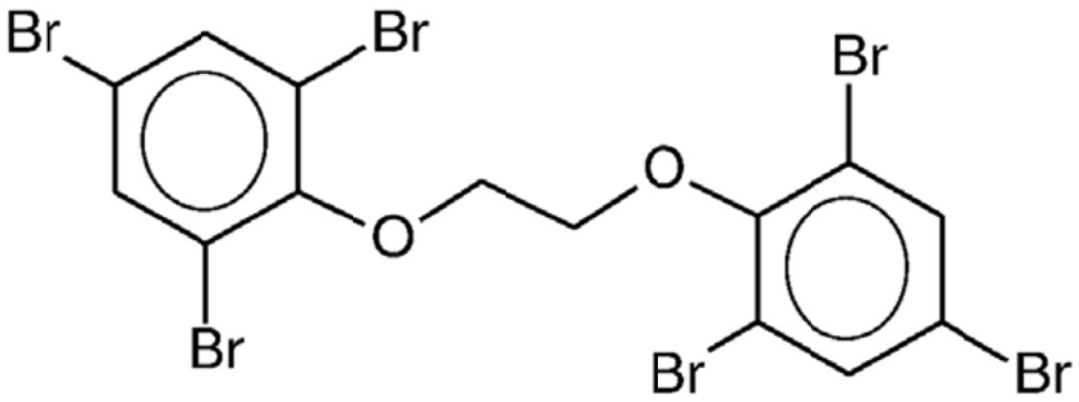

Figure 1.9. Structure of 1,2-bis (2,4,6-tribromophenoxy) ethane (BTBPE)

ambient air and sediment samples from various sites across the US (Hoh et al., 2005a; Qiu et al., 2007). Hoh et al. (2005b) found concentrations of BTBPE in the atmosphere that were greatest near its manufacturing source in El Dorado, Arkansas. This observation suggests that atmospheric transport and deposition is a significant source of BTBPE in the Great Lakes.

Sediment cores collected from Lake Ontario began to have increasing concentrations of BTBPE in the early 1980s. The maximum concentration detected was 6.7 µg/kg dry weight (Xinghua et al., 2007). This concentration was similar to the concentration detected in Lake Michigan sediment cores (7.2 µg/kg dry weight) (Xinghua et al., 2007). However, these concentrations are low compared to concentrations detected in sediment collected near a manufacturing site in Arkansas (470 µg/kg dry weight) (http://nepis.epa.gov/Exe/ZyPURL.cgi?Dockey=-40001GYV.txt). In Southern China, BTBPE concentrations found in dust collected from an electronic waste area were in the range of 14.6 to 232 µg/kg dry weight with a mean value of 107 µg/kg dry weight (Shi et al., 2009). The Shi et al. (2009) study demonstrates that concentrations of BTBPE are increasing in the environment with its increased usage. Because BTBPE is being used to replace the octaBDE, the concentrations will likely increase in the future (Xinghua et al., 2007).

A study that examined the exposure of rainbow trout to BTBPE, demonstrated the great potential for uptake of BTBPE in the aquatic food web. However, it was also demonstrated that BTBPE was rapidly degraded and depurated in the rainbow trout (Tomy et al., 2007). Quantifiable concentrations of BTBPE were reported in herring gull eggs collected from seven colonies on the Laurentian Great Lakes from the mid 1990's to 2006. The BTBPE concentrations found in Lake Superior, Lake Michigan, Channel-Shelter Island (Lake Huron), Chantry Island (Lake Huron), the Detroit River, the Niagara River and Lake Ontario were 0.11, less than 0.06, 0.08, 0.10, 0.20, 0.11, and 0.09 µg/kg wet weight, respectively (Gauthier et al., 2007, 2008). The concentrations of BTBPE found in gull eggs may reflect exposure of adult birds through soil/sediments and atmosphere, or possible dietary exposure in the form of fish such as alewife (*Alosa pseudoharengus*) and rainbow smelt (*Osmerus mordax*) present in the Great Lakes

aquatic food web (Gauthier and Letcher, 2009). Biotransformation of BTBPE may be greater in herring gulls relative to fish because gulls may possess a greater metabolic capability (Gauthier and Letcher, 2009). In gull egg pools, a number of lower brominated and unidentified compounds were detected, which could have been BTBPE debromination products (Gauthier et al., 2007). Marine mammal monitoring studies have detected BTBPE in blubber from Canadian Arctic beluga whales (*Delphinapterus leucas*) collected from several sites from 2002 to 2005 (de Wit et al., 2010). The sample concentrations ranged from 0.1 to 2.5 µg/kg lipid weight. 1,2-Bis (2,4,6-tribromophenoxy) ethane was also found in 10% of ringed seal (*Phoca hispida*) blubber samples collected in five locations in the Canadian Arctic in 2006. The concentrations ranged from less than 0.01 to 0.29 µg/kg lipid weight (Covaci et al., 2011).

During preparation of dosing solutions for pharmacokinetic studies, it was observed that BTBPE was virtually insoluble in all common vehicles used for oral dose preparations (Hakk et al., 2004). It was concluded from the solubility studies that mammalian absorption of BTBPE via ingestion would be minimal. Rats were fed 100 to 1000 mg FF-680/kg feed for 28 days. There was some accumulation of the compound in fat, liver, and muscle during the treatment period, but the compound disappeared from the tissues after cessation of dosing (Nomeir et al. 1993). In another study, rats were administered a single oral dose of ^{14}C-labeled FF-680. Eighty percent of the dose was excreted in the feces and 5% in the urine within 96 hours of administration. At 10 days following dosing, the greatest concentration was in the fat (0.38 mg/kg), whereas the maximum concentration in other tissues was 0.05 mg/kg. A maximum concentration of 0.58 mg/L occurred in the blood at 24 hours post-dosing, decreasing to 0.15 mg/L by 96 hours post-dosing. It was concluded from these studies that FF-680 was absorbed poorly through the rat gastrointestinal tract and it accumulated in the fat (Nomeir et al. 1993).

Nomeir et al. (1993) investigated the extent of absorption and disposition of ^{14}C-labeled FF-680 following dietary administration. On day one, rats were provided dietary concentrations of 0.05, 0.5, and 5% FF-680 and on days two through eleven they were provided 0.05% FF-680. The rational for the concentrations chosen were based on an LD_{50} for FF-680 that exceeded 10 g/kg of body weight. FF-680 was poorly absorbed through the gastrointestinal tract after the first 24 hours based on the lack of the radioactivity in the major tissues, excretion of less than 1% of the dose in the urine, and excretion of more than 99% of the dose in the feces. After 11 days, the excretion profile was similar to the day-one profile. The parent compound accounted for 94% of the radioactivity excreted in the feces. The gastrointestinal tract had the greatest level of radioactivity, followed by adipose tissue. The adipose tissue had detectable amounts of FF-680 in the one and ten day feed trial. The concentration in adipose tissue was 0.38 ± 10 nmole/g tissue for the 0.05 dose and 1.76 ± 3.45 10 nmole/g tissue for the 5.0 dose of the one-day trial. In the ten-day feed trial, the concentration determined in adipose tissue was 3.19 ± 0.58 nmole/g tissue. This study indicated that FF-680 accumulated in adipose tissue, but the potential for systemic exposure was minimal.

Hakk et al. (2004) conducted a study that examined the metabolism, tissue disposition, and excretion of BTBPE in male Sprague-Dawley rats. The radiolabeled BTBPE (2.0 mg/kg body weight) was administered by stomach tube to seven conventional and six bile duct-cannulated rats. It initially appeared that the intestinal absorption of BTBPE was low, with cumulative fecal excretion of BTBPE approximating 100% and 94% for conventional and bile duct-cannulated rats, respectively. Approximately 82% of the extractable fecal ^{14}C was the parent BTBPE compound. Additionally, cumulative biliary excretion of BTBPE was only

0.22% of the dose, indicating that hepatic metabolism of BTBPE was minimal. The study by Hakk et al. (2004) demonstrated that 39% of the fecal ^{14}C collected 24 hours after dosing with ^{14}C-BTBPE was extractable into solvents ranging from anisole to water. This value increased to 44% and 83% at 24 to 48 hours and 48 to 72 hours post-dosing, respectively. The biotransformation of BTBPE, as determined by the metabolites detected in the feces, included a series of oxidations, debrominations and ether cleavages with less than 4% of the 2.0 mg/kg body weight dose undergoing these reactions (Hakk et al., 2004). It was also concluded that biliary and urinary excretion of BTBPE was minimal following oral exposure, resulting in minimal tissue accumulation.

Acute toxicity studies indicated an oral LD_{50} for FF-680 exceeding 10 g/kg body weight for rats and dogs. The dermal LD_{50} of FF-680 exceeded 2 g/kg body weight for rats and 10 g/kg body weight for rabbits (Nomeir et al., 1993). No compound-related effects were seen in rats fed diets containing up to 10% FF-680 for 14 days. A 28-day dermal toxicity study indicated that rabbits dosed daily with up to 5 g/kg body weight had no clinical signs indicative of toxicity. Rats that inhaled FF-680 at 5 or 20 mg/liter for 21 days had no gross pathological changes. However, unspecified histopathological lesions were observed in the lungs. FF-680 was non-mutagenic in the Ames Salmonella/microsome test system (Nomeir et al., 1993).

Environmental persistence, metabolism/elimination and toxicity data suggest that BTBPE is not a major concern. However, because mink are sensitive to DE-71, a kinetic study and reproductive study were conducted in mink to test the hypothesis that BTBPE would not be absorbed to a great extent in mink and as a result, BTBPE would not have adverse effects on reproduction of adults and growth and survivability of offspring.

CHAPTER 2

THE PHARMOKINETICS OF 1,2 BIS (2,4,6-TRIBROMOPHENOXY) ETHANE IN MINK

Introduction

Brominated flame retardants (BFRs) are the largest group of flame retardants used worldwide because of their low cost and high performance efficiency (Birnbaum and Staskal, 2004). As of 2008, an estimated 410,000 metric tons of BFRs were used annually in the global market, which was an increase from 311,000 metric tons in 2005 (Covaci et al., 2011). Brominated flame retardants are incorporated into electronics, clothes, and furniture to prevent or to slow combustion, thus allowing for longer escape time and saving lives. The extensive use of BFRs for a variety of purposes has resulted in increased concentrations of BFRs in the environment, in wildlife, and in humans. The increase in worldwide environmental contamination by BFRs and their presence in biota, even in remote locations, have prompted concern about the potential health effects of these chemicals (deWit, 2002; Law et al., 2006; Burreau et al., 2006; Voorspoels et al., 2007).

The polybrominated diphenyl ethers (PBDEs) constitute a major class of BFRs used in industry. An estimated 70,000 metric tons of PBDEs are produced annually worldwide with half being used in the US and Canada (Renner, 2000; Hites, 2006). Polybrominated diphenyl ethers are an important class of BFRs from an environmental and health standpoint because they have been found in the environment and they persist in abiotic compartments, wildlife, and humans (Pijnenburg et al., 1995; de Boer et al., 1998, 2000; de Wit, 2002; Ikonomou et al., 2002). Two of the commercial PBDEs, octaBDE (octa) and pentaBDE (penta), are no longer produced in North America, the European Union, and China because of their presence in the

environment and concern about potential health effects in humans and wildlife (Birnbaum and Staskal, 2004; Renner, 2004; Zhou, 2006). In the US, the manufacturer of pentaBDE- and octaBDE voluntarily ceased production of these two PBDE mixtures in 2004 (Hites, 2006) and production throughout North America was phased out by 2005, effectively eliminating their use on this continent (http://www.ec.gc.ca/lcpe-cepa/09F567A7-B1EE-1FEE-73DB-8AE6C1EB-7658/sar_pbde-eng.pdf). Penta-brominated diphenyl ether, which was used primarily in North America, and octaBDE were banned by the European Union in 2004, and by several states in the US (California, Maine and Hawaii in 2006 and Washington in 2008).

A number of alternative BFRs have been developed to replace PBDEs. One alternative is 1,2-bis (2,4,6-tribromophenoxy) ethane (BTBPE; Figure 1.9), which is marketed under the trade name of FireMaster 680 as a replacement for the commercial octa mixture. Worldwide production and usage of BTBPE was estimated to be 16,710 metric tons in 2001 (Covaci et al., 2011).

1,2-Bis (2,4,6-tribromophenoxy) ethane has been found in the environment, being detected in ambient air and sediment samples from various sites across the US (Hoh et al., 2005a; Qiu et al., 2007). Additionally, BTBPE has been found in the eggs of northern fulmars from the Faroe Islands and in glaucous gulls (*Larus hyperboreus*) from Svalbard and herring gulls (*Larus argentatus*) from across the Great Lakes (Karlsson et al., 2006; Verreault et al., 2007; Gauthier and Letcher, 2009). The concentrations found in the herring gull eggs in the Great Lakes region ranged from less than 0.06 to 0.20 µg/kg wet weight (Gauthier et al., 2007, 2008). Marine mammal monitoring studies have detected BTBPE in blubber from Canadian Arctic beluga whales collected from several sites from 2002 to 2005 (de Wit et al., 2010). The sample concentrations ranged from 0.1 to 2.5 µg/kg lipid weight (de Wit et al., 2010). 1,2-Bis

(2,4,6-tribromophenoxy) ethane was also found in 10% of ringed seal blubber samples collected from five locations in the Canadian Arctic in 2006. The concentrations ranged from less than 0.01 to 0.29 μg/kg lipid weight (Covaci et al., 2011). With increased usage of BTBPE, there is a potential for environmental concentrations of this BFR to increase (Xinghua et al., 2007).

Because BTBPE has been detected in the environment, there are concerns about its potential health effects in wildlife species. Although toxicity studies of BTBPE have been conducted using rodent models (Nomeir et al., 1993; Hakk et al., 2004), the rodent may not be an adequate model for higher trophic wildlife. A recent study that assessed the kinetics of DE-71, which is a commercial pentaBDE mixture, in mink demonstrated that there were major differences in the biotransformation capabilities of mink and rodents (Zhang et al., 2008). Additionally, Zhang et al. (2008, 2009) reported that mink were more sensitive to DE-71 than rodents based on reproductive endpoints and thyroid homeostasis. Because of its sensitivity to a related BFR, it was of interest to determine the effects of BTBPE in mink, which is regarded as a sentinel wildlife species for potential environmental contaminants (Basu et al., 2007). The first study conducted was a pharmacokinetic study that assessed the distribution, metabolism and excretion of BTBPE. Kinetic studies in rodents suggest that BTBPE is absorbed to a limited extent and that the majority of the compounded is excreted unchanged in the feces (Nomeir et al., 1993; Hakk et al., 2004). Thus the hypothesis of this study is that 1,2-bis(tribromophenoxy)-ethane will appear in feces unchanged and will not be absorbed, or metabolized to a great extent.

Materials and Methods

Chemical. 1,2-Bis (2,4,6-tribromophenoxy) ethane (purity greater than 97%, as indicated by the manufacturer) was purchased from Wellington Laboratories (Guelph, Ontario, Canada).

Diet preparation. Because of the insolubility of BTBPE, the powder was added directly to other dry ingredients of the standard Michigan State University Experimental Fur Farm diet (Table 2.1). An appropriate quantity of BTBPE was added to a small container containing vitamin premix, trace mineral mix and biotin, which was then tumbled for 30 minutes. Diets were mixed in two batches (453 kg of each diet for batch 1 and 138 to 231 kg for batch 2). The dry ingredients were then added to a 550 kg capacity paddle mixer containing water, oil and phosphoric acid and mixed for one minute. The remaining ingredients of the mink diet were added to the mixer and the feed continued to mix for an additional 20 minutes. Samples of feed were collected as the feed was being dispensed into labeled plastic containers for subsequent freezing. Feed samples were used for proximate analysis and for verification of BTBPE concentration in the feed (National Wildlife Research Centre, Ottawa, Ontario, Canada).

Study design. Forty first-year, virgin female, natural dark mink, housed at the MSU Experimental Fur Farm, were randomly assigned to one of two treatment groups (20 animals per group). The two groups consisted of a control group that was fed the standard experimental fur farm diet and a treatment group that was fed the standard diet containing BTBPE at a targeted concentration of 2.0 mg BTBPE/kg feed (actual concentration of 2.3 mg BTBPE/kg feed). Animals were started on their respective treatment diets on January 15, 2009 and exposure continued for 21 days. Feed and water were available *ad libitum*. Dietary intake of BTBPE was estimated by determining feed consumption for two consecutive days each week. Fecal and urine samples were taken on days 0, 7, 14 and 21. Five animals from both the control and treatment group were euthanized and necropsied at day 7, 14 and 21. Prior to euthanasia, animals were anesthetized with an intramuscular injection of ketamine HCl (30 mg/kg body weight) and blood samples were taken by cardiac puncture. Blood was collected in a Vacutainer tube containing

sodium heparin. The blood was gently mixed for two minutes and centrifuged for five minutes at room temperature. The isolated plasma was then stored at -80°C until shipment to the National Wildlife Research Centre for analysis of T_3 and T_4. After blood collection, animals were euthanized with CO_2, weighed and necropsied. The brain, liver and lung were removed and weighed. Samples of brain, liver, lung and abdominal fat were frozen at -80°C for subsequent contaminant analysis. The remaining 10 animals were fed the control diet for an additional 21 days. Urine and fecal samples were taken on day 42. Animals were then anesthetized, sampled for blood, weighed, euthanized and necropsied on day 42 as described above.

Chemical analyses. Diet samples were analyzed for BTBPE using a gas chromatography-mass spectrometry (GC-MS) (electron capture negative ionization [ECNI])-based method (MET-ORGRES-NEW BFR/PBDE; Revision #2, April, 2010) with minor modifications. 1,2-Bis (2,4,6-tribromophenoxy) ethane and 13C12-BTBPE standard solutions (purity greater than 97%, as indicated by the manufacturer) were purchased from Wellington Laboratories (Guelph, Ontario, Canada). Solvents used for extraction and clean up were OmniSolve quality (EMD Chemicals, Gibbstown, NJ, USA) and suitable for gas chromatography and residue analysis. Diatomaceous earth was heated at 600°C overnight in a muffle furnace prior to use. Silica was activated at 120°C overnight in an oven and then treated with sulfuric acid (equal weight). The mixture was homogenized for more than 24 hours prior to use.

Table 2.1 Composition of standard mink diet

Ingredients	% of diet
Mink cereal[1]	16.65
Chicken[2]	21.55
Spray-dried liver[3]	6.86
Spray-dried egg[3]	6.86
Spray-dried blood cells[4]	1.47
Soybean oil[5]	2.45
Water	35.26
Fish meal[5]	6.86
Vitamin premix[6]	0.42
Trace mineral premix[7]	1.42
Phosphoric acid, 85%[8]	0.98
d-biotin[9]	0.03
Larvacide[10] (mL/kg feed)	1.32 mL/kg diet
1,2-Bis (tribromophenoxy) ethane (BTBPE)[11]	0, 0.014, 0.13 or 2.3 mg/kg diet

[1]GnF-20, National Fur Foods, Division of Milk Specialties Co., New Holstein, WI.
[2]Fresh whole ground chicken-hens, Michigan State University Poultry Research and Teaching Center, East Lansing, MI.

Table 2.1 (con't)

[3]VanElderen, Inc., Martin, MI.

[4]AP301G, APC, Ankeny, IA.

[5]North American Nutrition, Lewisburg, OH.

[6]Vitamin A, 916,652 IU/kg; vitamin D_3, 91,674 IU/kg; vitamin E, 11,000 IU/kg; vitamin K activity, 2200 mg/kg; menadione, 733 mg/kg; vitamin B12, 5.5 mg/kg; riboflavin, 733 mg/kg; d-pantothenic acid, 2935 mg/kg; niacin, 4400 mg/kg; thiamine,

[7]Calcium, 13.40%; copper, 2000 mg/kg; iodine, 30 mg/kg; iron, 2.0 %; manganese, 2000 mg/kg; selenium, 60 mg/kg; zinc, 2.0 %; Akey, Louisburg, OH.

[8]Astaris, St. Louis, MO.

[9]Biotin 100 (222.2 mg/kg), ADM, Des Moines, IA.

[10]Lavadex, active ingredient: cyromazine (N-cyclopropyl-1,2,5-triazine-2,4,5-triamine, 2%), Novatis Animal Health, Greensboro, NC.

[11]Kinetic trial: 0 or 2.3 mg/kg diet; reproduction trial: 0, 0.014, 0.13 or 2.3 mg/kg diet. Great Lakes FF-680, Great Lakes Chemical, West Lafayette, IN.

The approximate size of the samples selected for analysis was 2.0 g, excluding fat and feces, which were 0.2 and 0.5 g, respectively. These samples were weighed and ground with diatomaceous earth and then spiked with internal standard (IS – see below). The samples were then extracted with 50:50 dichloromethane: n-hexane (DCM: HEX) solution. A 10 % portion of the extract was used for gravimetric lipid determination. For the remaining 90% of the extract, sulfuric acid-impregnated silica (50%) was used to clean up the sample of biogenic material. Acid silica (5.0 g) cartridges were conditioned with successive 4 × 5 mL of 25% DCM:HEX. After the sample was loaded on the cartridge, the analytes were eluted with 4 × 5 mL of 25% DCM:HEX (v/v). The eluent was then concentrated and solvent exchanged to isooctane in preparation for analysis (GC-MS [ECNI]).

The IS used was mass-labeled $_{13}$C12-BTBPE, which was validated previously as representative for quantification of native BTBPE. A series of calibration standard solutions was prepared, which contained six concentrations of the target compound with a constant concentration of IS (100 µg13C12-BTBPE/kg). These standard solutions were analyzed by GC/MS (ECNI) and the results indicated that there was a highly and linearly correlated concentration versus ECNI response ($r^2 > 0.996$) over a concentration range of approximately 1.0 µg/kg to 500 µg/kg.

Samples were analyzed on an Agilent gas chromatograph 6890 equipped with a 5973 quadrupole mass spectrometer (MS) detector (Agilent Technologies, Mississauga, Ontario, Canada). The injector temperature was set to 240°C. The oven temperature was programmed as follows: 100°C for 2 minutes, 25°C/minute to 325°C, held for 2 minutes. A volume of 1 µL was injected with pulsed-splitless injection mode (injection pulse at 25.0 psi until 1.00 minute; purge

flow to split vent of 96.4 mL/minute to 0.80 min; gas save flow of 20 mL/minute at 2.0 minutes). The analytical column was a 15 m × 0.25 mm i.d. DB-5 HT capillary column with a film thickness of 0.10 μm. The ECNI source temperature was 250°C, the quadrupole temperature was 150°C and the transfer line temperature was 280°C. Methane was used as the reagent gas.

Selected ion monitoring (SIM) was used for quantification of the target compounds based on the most selective mass fragment under the above mass spectrometry detection operating conditions. 1,2-Bis (2,4,6-tribromophenoxy) ethane was monitored with ions of 251 and 330 (m/z) for di- and tri-bromophenoxy anions, respectively. Ion selection for the internal standard was 257 and 336 (m/z) (for di- and tri-bromophenoxy anions) for $_{13}$C12-BTBPE. Quantification for these samples was achieved via Agilent ChemStation Advanced Data Analysis software (Agilent Technologies, Mississauga, Ontario, Canada), based on the internal standard quantification approach.

The average recovery of the internal standard varied, depending on the sample matrix under study. Generally, recoveries of $_{13}$C12-BTBPE internal standard were 80 ± 32%. An internal standard method was used for quantification, where the concentrations of target compounds were inherently recovery-corrected by internal standard.

A method blank was included in each batch of samples (n of 10 to 12) to monitor any potential contamination resulting from the entire analysis process. Blank samples did not contain any detectable (below the method limit of detection [MLOD]) BTBPE, and thus background subtraction was not necessary. A pork liver homogenate was spiked with target compounds (25 ng BTBPE) as a method control (for liver, lung, brain and diet) and olive oil (for fat) was analyzed with each batch of samples to test the recovery and reproducibility. An acceptable

coefficient of variation of less than 5 % was obtained. To monitor instrumental reproducibility and response stability, a set of external and internal standards was injected for every eight to 10 sample analyses, with isooctane solvent injected prior to and after analysis of standards.

The method limit of quantification (MLOQ) and MLOD were defined as the minimum amount of analyte that produced a peak with a calculated signal to noise ratio of 10 and 3, respectively. Depending on the tissue or sample matrix, the BTBPE MLOQ for the method ranged from 0.16 µg/kg wet weight to 1.36 µg/kg wet weight and the MLOD ranged from 0.05 µg/kg wet weight to 0.46 µg/kg wet weight.

Results

The target dietary concentration of BTBPE in the present study was 2.0 mg/kg feed and the analyzed concentration was 2.3 mg/kg feed. The concentration of BTBPE in the control diet was below the MLOD.

Relative liver, lung and brain weight of mink fed 2.3 mg BTBPE/kg feed for up to 21 days and then placed on clean feed for an additional 21 days were not significantly different compared to control weight (Table 2.2).

1,2-Bis (2,4,6-tribromophenoxy) ethane was detected predominately in the feces with lesser concentrations in adipose tissue of mink fed diets containing 2.3 mg BTBPE/kg feed for 21 days. Minimal concentrations of BTBPE were detected in the liver, brain, lung and urine (Table 2.3). The concentration of BTBPE in the feces was consistent over the 21-day exposure period while the concentration in adipose tissue appeared to reach steady state after the second week of exposure. When animals were placed on the control feed, the concentration of BTBPE in the feces decreased to below the MLOD by day 42, while the concentration in the adipose tissue decreased by almost 70% at day 42 compared to the concentration at day 21.

Table 2.2 Mean relative weight of liver, lung, and brain of mink exposed to 2.3 mg 1,2 bis (2,4,6-tribromophenoxy) ethane (BTBPE)/kg feed

		Relative organ weight (percent of body weight)[2]							
		Body Weight (g)[1]		Liver		Lung		Brain	
Day	n	Control	BTBPE	Control	BTBPE	Control	BTBPE	Control	BTBPE
7	5	1324.4 (64.5)	1215.0 (64.5)	3.42 (2.59 – 4.24)	3.49 (2.79 - 4.19)	0.60 (0.53 – 0.67)	0.64 (0.57 - 0.70)	0.60 (0.48 – 0.72)	0.68 (0.63- 0.73)
14	5	1173.6 (64.5)	1251.2 (64.5)	3.55 (3.12 -3.99)	3.53 (3.11 – 3.94)	0.65 (0.52 –0.78)	0.54 (0.48 – 0.60)	0.71 (0.62 – 0.80)	0.71 (0.61 – 0.82)
21	5	1302.0 (64.5)	1321.8 (64.5)	3.59 (3.09 -4.09)	3.33 (2.98 – 3.69)	0.56 (0.49 – 0.63)	0.52 (0.45 – 0.60)	0.66 (0.56 – 0.77)	0.65 (0.52 -0.77)
42	5	1248.6 (64.5)	1249.2 (64.5)	3.34 (3.17 – 3.50)	3.19 (2.72 – 3.66)	0.69 (0.60 – 0.79)	0.61 (0.52 – 0.69)	0.69 (0.61 – 0.76)	0.62 (0.53 -0.71)

[1]Data presented as mean body weight with standard error in parentheses.

[2]Data presented as mean with 95% confidence limits in parentheses.

Table 2.3 Mean concentration (µg/kg, wet weight) of 1,2 bis (2,4,6-tribromophenoxy) ethane (BTBPE) in various tissues of mink fed diets containing 2.3 mg BTBPE/kg feed for 21 days and then placed on clean feed for 21 days[1]

Day	n	Treatment	Liver	Lung	Brain	Fat	Feces	Urine
7	5	Control	not analyzed[2]	not analyzed	not analyzed	<MLOD	<MLOD	not analyzed
		BTBPE	2.8 ± 2.3	1.7 ± 0.5	<MLOD	12 ± 6.6	1784 ± 511	0.025[3]
14	5	Control	not analyzed	not analyzed	not analyzed	<MLOD	<MLOD	not analyzed
		BTBPE	1.1 ± 1.3	1.6 ± 1.1	<MLOD	38 ± 7.5	1156 ± 448	0.025 ± 0.0
21	5	Control	not analyzed	not analyzed	not analyzed	<MLOD	<MLOD	not analyzed
		BTBPE	0.058 ± 0.040	not analyzed	not analyzed	36 ± 28	1296 ± 972	0.025 ± 0.0
42	5	Control	not analyzed	not analyzed	not analyzed	0.10[3]	<MLOD	not analyzed
		BTBPE	not analyzed	not analyzed	not analyzed	12 ± 3.9	0.2 ± 0.0	not analyzed
MLOQ			0.21	0.13	0.20	0.61	1.4	0.16
MLOD			0.070	0.050	0.070	0.20	0.46	0.050

[1]Data presented as mean ± Standard Deviation.

[2]Because of the low BTBPE concentrations observed in tissues of the 2.3 mg BTBPE/kg feed treatment, the control tissues were not expected to be elevated, and therefore were not analyzed. Similarly, when concentrations were low at an early sampling period it was expected that concentrations would be equally low or lower at subsequent sampling periods.

[3]Sample size of one.

Discussion

Exposure of mink to BTBPE for 21 days had no significant effect on relative organ weight (Table 2.2). The lack of a significant effect of BTBPE on liver weight differs from the hepatic effects caused by exposure to commercial PBDE mixture. Rats fed diets containing 100 or 1000 mg octaBDE/kg feed for 28 days or up to 13 weeks, had significantly greater mean absolute and relative liver weight compared to the control group. Histologically, enlarged centrilobular and midzonal hepatocytes containing eosinophilic bodies were reported (http://www.inchem.org/documents-/ehc/ehc/ehc162.htm). Rats dosed with 2, 10 or 100 mg DE-71/kg body weight/day for up to 90 days, followed by a 168-day recovery period had absolute liver weights that were 11% heavier in the 10 mg/kg body weight/day dose group and 50 to 70% heavier in females and males exposed to100 mg/kg body weight/day, respectively, compared to controls (http://www.inchem.org/documents/ehc/ehc/ehc162.htm). Mink fed diets containing 0, 1.0, 5.0 or 10 mg DE-71/kg feed for eight weeks had increased liver weight compared to controls that were correlated with increased hepatic ethoxyresorufin O-deethylase (EROD) activities in the 5.0 and 10 µg DE-71/kg feed groups (Martin et al., 2007). The lack of an effect of BTBPE on relative liver weight in the present study could be due to the relatively low dose that was used, the relatively short exposure period or limited absorption of the chemical.

Results of the present study indicate that mink eliminated the majority of ingested BTBPE as the parent compound in the feces and that only a small quantity of the chemical accumulated in the adipose tissue (2.8% of the quantity that was detected in the feces). Tissue concentrations decreased rapidly once the animals were placed on clean feed. These results are comparable to results reported in rodent studies. In a study conducted by Nomeir et al. (1993), it was concluded that FF-680 was poorly absorbed in the gut of rats, but it accumulated in the fat.

Lipophilic tissues contained the most BTBPE in rat studies by Nomeir et al. (1993) and Hakk et al. (2004). In the Nomeir et al. (1993) study, BTBPE was fed at 0.05, 0.5 or 5.0 % of diet for one day. Twenty-four hours after the start of administration of BTBPE, there was no radioactivity detectable in any of the tissues analyzed, except adipose tissue, skin, and thymus in which low concentrations of radioactivity were detected in some animals. The concentrations detected in adipose tissue was 0.38 ± 10 nmole/g tissue for the 0.05 % dietary concentration, and 1.76 ± 3.45 10 nmole/g tissue for the 5.0 % dietary concentration of the one-day trial. Radioactivity was detected in urine at 0.26 to 0.73 % of the dose and between 84.7 and 99.5% of the estimated dose was recovered in the feces 96 hours after termination of the one-day experiment. These results indicate poor absorption.

In a second study, Nomeir et al. (1993) fed BTBPE to a group of rats at 0.05% of diet for 10 days. After 10 days, the concentration of FF-680 was greatest in adipose tissue (0.06% of the total administered), followed by kidney, skin, and thymus. Nomeir et al. (1993) concluded that the potential for systemic exposure by ingestion was minimal in rats. Similar observations were made in the present mink study with BTBPE, in that the majority of the compound was excreted in the feces. In a trial by Hakk et al. (2004), a single oral dose of 2.0 mg BTBPE/kg body weight in 0.5 mL peanut oil was administered to rats. Greater than 94% of BTBPE dose was excreted in the feces. Less than 4% of the administered BTBPE was subject to oxidation and oxidative debromination and ether cleavage and these metabolites were detected in the feces. There was minimal tissue retention of BTBPE, but the highest concentrations were found in lipophilic tissues such as adipose tissue, adrenal glands, and thymus.

Results of the present mink kinetic study indicate that BTBPE is not absorbed to a great extent and is rapidly eliminated, largely unchanged, via the feces. These data suggest that, at the feed concentration assessed, it is unlikely that BTBPE will have adverse effects in mink.

CHAPTER 3

THE EFFECTS OF 1,2 BIS (2,4,6-TRIBROMOPHENOXY) ETHANE ON REPRODUCTION OF ADULT FEMALE MINK AND SURVIVAL AND GROWTH OF THEIR OFFSPRING

Introduction

Brominated flame retardants (BFRs) are currently the largest group of flame retardants marketed, because of their low cost and high performance efficiency (Birnbaum and Staskal, 2004). Flame retardants are used to prevent or retard the initial phase of a developing fire (Sjödin et al., 2001). The electronic industry uses BFRs in printed circuit boards, connectors, plastic covers, and cables. They are also used in kitchen appliances, clothing, furniture, carpets, paints, wood products, textiles, and paper (Birnbaum and Staskal, 2004; Gill et al., 2004).

Brominated flame retardants are lipophilic chemicals that are resistant to physical and biochemical degradation, making them a potential environmental hazard. They are found in the environment far from where they are produced or used. Increased concentrations have been found in terrestrial, freshwater, and marine ecosystems thus affecting the environment, wildlife, and humans (Alaee and Wenning, 2002; de Wit, 2002, and de Wit et al., 2006). The use of some BFRs has been discontinued or restricted due to environmental contamination and/or because of adverse health effects (Birnbaum and Staskal, 2004).

Polybrominated diphenyl ethers are a class of BFRs that are recognized as global environmental contaminants. An estimated 70,000 metric tons of PBDEs are produced annually worldwide with half being used in the US and Canada (Renner, 2000; Hites, 2006). There are three commercial mixtures of PBDEs that have been marketed: pentabrominated diphenyl ether (pentaBDE or “penta”), octabrominated diphenyl ether (octaBDE or “octa”) and decabrominated

diphenyl ether (decaBDE or “deca ") (Costa and Giordano 2007). The primary use of pentaBDE is in polyurethane foam, where up to 30% of the weight of the foam can be accounted for by this flame retardant (Hale et al., 2002). Penta-brominated diphenyl ether has been shown to bioaccumulate and increase as the trophic level increases indicating biomagnification in the food web (http://www.pops.int/-documents/meetings/cop_1/chemlisting/pentaBDEfinal.pdf). The most abundant PBDE congeners found in biota and those congeners demonstrated to elicit the most adverse health effects are components of the commercial pentaBDE mixture DE-71 (http://www.atsdr.cdc.-gov/ToxProfiles/tp68). The major use for octaBDE is as an additive to acrylonitrile-butadiene-styrene (ABS) polymers, which are then used to produce computers and business cabinets (http://www.inchem.org/documents/ehc/ehc/ehc162.htm). The congeners comprising the commercial octaBDE mixture have been detected in air samples from remote sites in the US, Lake Ontario basins, and sediments in the UK (Allchin et al., 1999; Strandberg et al., 2001; http://www.ec.gc.ca/lcpe-cepa/09F567A7-B1EE-1FEE-73DB-8AE6C1EB7658/sar_pbde-eng.pdf). There have been hexa, hepta and octaBDE congeners found in bird eggs on Canada’s west coast and in the Canadian Arctic, suggesting long-range transport of these congeners (http://www.ec.gc.ca/lcpe-cepa/09F567A7-B1EE-1FEE-73DB-8AE6C1EB7658/sar_pbde-eng.pdf). Decabromodiphenyl ether is the second most-used BFR globally with approximately 56,400 metric tons used annually (de Wit et al., 2010). It has been reported that peregrine falcon (*Falco peregrinas*) eggs had high concentrations of decaBDE, indicating that the commercial mixture has the potential to bioaccumulate in terrestrial organisms (http://bfr2010.com/abst/-2001/BFR2001_del5.pdf#page=33).

The mink is an excellent wildlife model to address issues related to environmental pollutants and is considered to be a sentinel species (Basu et al., 2007). Mink are one of the most

widespread carnivores in North America (Arnold and Fritzell, 1990). They are opportunistic predators that consume a range of prey items available in their local habitat, including frogs, snakes, birds, and small mammals (Wise et al., 1981). Approximately 50% of a typical mink diet consists of fish, because mink usually forage close to aquatic habitats. The fish in the mink's diet represents the primary route by which persistent chemicals are accumulated (Chan et al., 2003; Wiener et al., 2003). The concentrations of PBDEs in freshwater fish in North America are high and increasing exponentially in certain species and locations (Hale et al., 2001; Johnson and Olson, 2001; Rayner et al., 2003; Chernyak et al., 2005). The dietary exposure of piscivorous wildlife species, such as mink, to PBDEs may pose a health risk. While there are numerous studies that have assessed the effects of PBDEs in rodents, relatively few studies have focused on wildlife species.

Because mink are sensitive to other halogenated environmental contaminants such as polychlorinated biphenyls (PCBs), they were chosen by Environment Canada as a sentinel species to assess the effects of the commercial pentaBDE mixture DE-71. Zhang et al. (2008, 2009) examined the effects DE-71 in mink. In the first report (Zhang et al., 2008), the accumulation, disposition and metabolism of DE-71 was assessed in adult female mink and their offspring exposed to feed concentrations of 0, 0.1, 0.5, or 2.5 mg DE-71/kg. Polybrominated diphenyl ether congeners were detected in most tissues of the adults. Six-week-old kits had a greater proportion of PBDEs in the brain compared to adults, presumably because of incomplete development of the blood-brain barrier. Lesser brominated congeners were transferred from the dam to the kit with the bulk of the body burden in kits at weaning resulting from lactational rather than transplacental transfer. Hydroxylated PBDEs accounted for 28 to 32% of the excreted fraction, indicating that metabolism was an important elimination pathway. In the

second report (Zhang et al., 2009), the effects of the same dietary concentrations on reproductive performance of adult female mink, survivability and growth of their offspring and thyroid homeostasis in adult, kit and juvenile mink were assessed. The dietary concentration of 2.5 mg DE-71/kg resulted in complete reproductive failure, while reproduction was unaffected at the lesser dietary concentrations. Juvenile mink had disrupted thyroid hormone homeostasis as evidenced by a significant decrease of plasma T_3 in males and females exposed to 0.5 mg DE-71/kg feed, despite a compensatory increase in plasma total T_4 in female juveniles. Additionally, thyroid follicular epithelium cell height was increased in juvenile males and females fed 0.5 mg DE-71/kg feed.

The environmental impact and deleterious health effects of some commercial PDBE mixtures has led to discontinuation or restriction of PBDE usage. In the US, the producer of pentaBDE- and octaBDE voluntarily ceased production in 2004 (Costa and Giordano, 2007). The commercial pentaBDE and octaBDE mixtures were both phased out in the European Union in 2004 and in Canada in 2006. DecaBDE is still the most widely used and cost effective PBDE in the polymer industry, but alternative BFRs have been introduced as replacements for the PBDEs (Ward et al., 2008). One alternative is 1, 2-bis (2,4,6-tribromophenoxy) ethane (BTBPE), which has been introduced as a replacement for the commercial octaBDE mixture (Renner, 2004).

The annual world production of BTBPE, which is sold under the trade name of FireMaster 680 (FF-680), was approximately 16,710 metric tons in 2001 (Verreault et al., 2007). It is used in the production of plastic materials that require high manufacturing temperatures and light stability, such as acrylonitrile-butadinene-polystyrene and high impact polystyrene (Hakk et al., 2004). 1, 2-Bis (2,4,6-tribromophenoxy) ethane may be released into the environment through the manufacturing process, as well as through various waste streams. Other sources

include household dust (Karlsson et al., 2007) and dust collected from electronic recycling centers (Pettersson-Julander et al., 2004).

In recent years, BTBPE has been detected in ambient air and sediment samples from various sites across the US (Hoh et al., 2005a; Qiu et al., 2007). 1, 2-Bis (2,4,6-tribromophenoxy) ethane was detected in quantifiable concentrations in herring gull eggs collected from seven colonies on the Laurentian Great Lakes from the mid-1990s to 2006 (Gauthier et al., 2007, 2008). The concentrations of BTBPE found in gull eggs ranged from less than 0.06 to 0.20 μg/kg wet weight, reflecting exposure of adult birds through soil/sediments and atmosphere, or possible dietary exposure in the form of fish such as alewife and rainbow smelt present in the Great Lakes aquatic food web (Gauthier and Letcher, 2009). Marine mammal monitoring studies detected BTBPE in blubber from beluga whales collected from several sites in the Canadian Arctic between 2002 and 2005 (de Wit et al., 2010). Concentration ranged from 0.1 to 2.5 μg/kg lipid weight. In the same study, BTBPE was also found in 10% of the ringed seal blubber samples collected in five locations in the Canadian Arctic in 2006 at concentrations ranging from less than 0.01 to 0.29 μg/kg lipid weight (Covaci et al., 2011).

Studies have demonstrated that concentrations of BTBPE are increasing in the environment as a result of its increased usage in industry (Shi et al., 2009). Because BTBPE is being used to replace the commercial octaBDE mixture, concentrations will likely increase in the future (Xinghua et al., 2007). The environmental persistence, metabolism/elimination and toxicity data suggest that BTBPE is not a major concern. However, BTBPE has been detected in wildlife and there are concerns about its potential health effects in these species. Rodent studies demonstrated that BTBPE is absorbed to a limited extent and that the majority of the compound is excreted in the feces unchanged (Nomeir et al., 1993; Hakk et al., 2004). The DE-71 study

with mink demonstrated that there are major differences in the biotransformation capabilities of mink and rodents (Zhang et al., 2008). Additionally, Zhang et al. (2008, 2009) reported that mink were more sensitive to DE-71 than rodents, based on reproductive endpoints and thyroid homeostasis. Because of its sensitivity to a related BFR, it was of interest to determine the effects of BTBPE in mink. Therefore, our hypothesis for this study was that BTBPE would not affect mink reproduction or kit survivability. Furthermore, BTBPE would not affect thyroid homeostasis.

Materials and Methods

Chemicals. 1, 2-Bis (2,4,6-tribromophenoxy) ethane (purity greater than 97%as indicated by the manufacturer) was purchased from Wellington Laboratories (Guelph, ON, Canada).

Diet preparation. Because of the insolubility of BTBPE, the powder was added directly to other dry ingredients of the standard Michigan State University Experimental Fur Farm diet (Table 2.1). An appropriate quantity of BTBPE was added to a small container containing vitamin premix, trace mineral mix and biotin, which was then tumbled for 30 minutes. These dry ingredients were then added to a 200 kg capacity paddle mixer containing water, oil and phosphoric acid and mixed for 1 minute. The remaining ingredients of the mink feed were added to the mixer and the complete feed continued to mix for an additional 20 minutes. Samples of feed were collected as it was being dispensed into labeled plastic containers for subsequent freezing. Feed samples were used for proximate analysis (Litchfield Analytical Services, Litchfield, MI, USA) and for verification of dietary BTBPE concentrations (National Wildlife Research Centre, Ottawa, Ontario, Canada).

Study design. Forty first-year, virgin female, natural dark mink were randomly assigned to one of four treatment groups, which were fed the standard Michigan State University

Experimental Fur Farm diet containing BTBPE at concentrations of 0.0, 0.014, 0.13 or 2.3 mg/kg feed. Animals were housed individually in wire mesh breeder cages (76 cm L x 46 cm W x 38 cm H) suspended above the ground in an open-sided mink shed. A wooden nest box (38 cm L x 25 cm W x 29 cm H), bedded with aspen shavings or excelsior (wood wool), was attached to the outside of each cage. Females were started on the BTBPE diets on January 1, 2009, which was two months prior to breeding. Animals (including kits) were continued on these diets until weaning of kits at six weeks post-parturition. Feed and water were available ad libitum. Dietary intake of BTBPE was estimated by determining feed consumption weekly, from January 23 until February 10, 2009. Body weight of adult females was recorded monthly. The females were bred to untreated males from March 1 until March 21, 2009. Each female was given an opportunity to mate every fourth day until a presumed successful mating occurred as determined by posture during mating and post-coital appearance of the female's vaginal area. A mated female was given an opportunity to breed with a different male the day following a successful mating and on the eighth and ninth days after a successful mating (a common commercial mink breeding practice). After the first week in April, nest boxes were checked daily for evidence of whelping. Whelping occurred from April 15 to May 15, 2009. Live and stillborn kits were counted at birth and gender determined. Live kits and their dams were weighed at whelping and at three and six weeks post-whelping. Reproductive parameters measured included number of females bred, number of females whelping, litter size, and survivability of kits from birth to six weeks of age. Growth parameters assessed were kit body weight at birth, three and six weeks of age.

Offspring were weaned at six weeks of age, at which time all adult females and six offspring per treatment group were necropsied. Adults were weighed, anesthetized with ketamine (30 mg/kg body weight, intramuscular injection; Fort Dodge Animal Health, Fort

Dodge, Iowa, USA) and blood was drawn via cardiac puncture. Whole blood samples were collected in syringes coated with heparin and transferred to heparinized Vacutainer® tubes (BD, Franklin Lakes, NJ, US). The blood was gently mixed for 2 minutes and then centrifuged at room temperature for 5 minutes at 400 x g. The isolated plasma was stored at -80°C until shipment to the National Wildlife Research Centre for analysis of T_3 and T_4 concentrations. Adults were euthanized with CO_2 and necropsied. The liver, kidneys, spleen, heart, brain, thyroid gland and adrenal glands were removed and weighed. Samples of liver were frozen at -80°C for subsequent contaminant analysis. The thyroid gland was preserved in 10% neutral buffered formalin for subsequent histological assessment. The six kits from each treatment group were euthanized and necropsied as described above.

Ten kits from each treatment group (five females and five males) were continued on their respective diets until approximately 27 weeks of age during which time animals were weighed monthly. The juveniles were then anesthetized, sampled for blood, euthanized and necropsied as described above for the adult females. In addition, abdominal fat samples were collected and frozen at -80°C for subsequent contaminant analysis.

BTBPE analysis. Determination of BTBPE in liver and fat samples followed the method of MET-ORGRES-NEW BFR/PBDE (Revision #2, April. 2010) developed and optimized by Organics Research Group of the National Wildlife Centre (Ottawa, Ontario, Canada) with minor modifications.

1, 2-Bis (2,4,6-tribromophenoxy) ethane and $_{13}$C12-BTBPE standard solutions (purity greater than 97%, as indicated by the manufacturer) were purchased from Wellington

Laboratories (Guelph, Ontario, Canada). Solvents used for extraction and clean up were OmniSolve quality and suitable for gas chromatography and residue analysis. Diatomaceous earth was heated at 600°C overnight in a muffle furnace prior to use. Silica was activated at 120°C overnight in an oven and then treated with sulfuric acid (equal weight). The mixture was homogenized for more than 24 hours prior to use.

Samples were weighed (fat, 0.2 g; liver, 2.0 g; feed, 3.0 g), ground with diatomaceous earth and spiked with internal standard. Samples were then extracted with 50:50 dichloromethane: n-hexane (DCM: HEX) using an accelerated solvent extractor. A 10 % portion of the extract was used for gravimetric lipid determination. The remaining 90% of the extract was cleaned of biogenic material using sulfuric acid impregnated silica (50%). Acid silica (5.0 g) cartridges were prewashed with successive 4 × 5 mL of 25% DCM in hexane, and the samples were eluted with 4 × 5 mL of 25 % DCM in hexane (v/v). The eluent was then concentrated and solvent exchanged to isooctane in preparation for gas chromatography-mass spectrometry electron capture negative ion analysis (GC-MS [ECNI]) using an Agilent 6890gas chromatograph equipped with a 5973 quadrupole mass spectrometer (MS) detector (Agilent, Mississauga, Ontario, Canada).

The internal standard (IS) used in this procedure was mass-labeled $_{13}$C12-BTBPE, which was validated previously as representative for quantification of native BTBPE. A series of calibration standard solutions was prepared, which contained six concentration levels of the target compound with a constant level of IS (100 ppb $_{13}$C12-BTBPE). These standard solutions were analyzed by GC-MS (ECNI) and the results showed that there was a highly and linearly

correlated concentration versus ECNI response ($r^2 > 0.996$) over a concentration range of approximately 1.0 µg/kg to 500 µg/kg.

The GC operation parameters were as follows: injector temperature, 240°C; oven temperature/time, 100°C for 2 minutes, 25°C/minute to 325°C, held for 2 minutes; a volume of 1 µL was injected with pulsed-splitless injection mode (injection pulse at 25.0 psi until 1.00 minute; purge flow to split vent of 96.4 mL/minute to 0.80 minutes; gas save flow of 20 mL/minute at 2.0 minutes); analytical column: 15 m × 0.25 mm i.d. DB-5 HT capillary column with a film thickness of 0.10 µm.

The MS operation parameters were as follows: electron capture negative ionization (ECNI) source temperature, 250°C; quadrupole temperature, 150°C; transfer line temperature, 280°C. Methane was used as the reagent gas.

Selected ion monitoring (SIM) was used for quantification of the present target compounds based on the most selective mass fragment under the above mass spectrometer detector (MSD) operating conditions. 1, 2-Bis (2,4,6-tribromophenoxy) ethane was monitored with ions of 251 and 330 (m/z) for di- and tribromophenoxy anions, respectively. Ion selection for the internal standard $_{13}$C12-BTBPEwas 257 and 336 (m/z) for di- and tribromophenoxy anions. Quantification of these samples was achieved via Agilent ChemStation Advanced Data Analysis software, based on the internal standard quantification approach.

The average recovery of the internal standard was 99 ± 11% for fat, 79 ± 21% for feed, and 68 ± 15% for liver. An internal standard method was used for quantification, where the concentrations of target compounds were inherently recovery-corrected by internal standard.

A method blank was included in each batch of samples (n =8) to monitor any potential contamination resulting for the entire analysis process. Blank samples did not contain any detectable BTBPE, thus background subtraction was not necessary. A pork liver homogenate, which was spiked with target compounds (25 ng BTBPE) as a method control (for liver and feed) and olive oil (for fat) were analyzed with each batch of samples (n = 8 to 10) to test the recovery and reproducibility. An acceptable coefficient of variation of less than 5 % was obtained. To monitor instrumental reproducibility and response stability, a set of external and internal standards was injected for every 8 to 10 sample analyses, with isooctane solvent injected prior to and after.

The method limit of quantification and detection (MLOD) were defined as the minimum amount of analyte that produced a peak with a signal to noise ratio of 10 and 3, respectively. The MLOQ for the method ranged from 0.12 µg/kg wet weight to 0.72 µg/kg wet weight for BTBPE and the MLOD ranged from 0.04 µg/kg wet weight to 0.24 µg/kg wet weight depending on the matrix (feed, liver, adipose tissue).

Thyroid Histology. Both thyroid lobes were embedded into single paraffin block using a Tissue Tek VIP 5 tissue processor (Sakura, Torrance, CA, USA). Sections (5 µm) were affixed to glass microscope slides and stained with hematoxylin and eosin. The thyroid was examined for proliferative follicular lesions using a Nikon Eclipse 50i compound microscope (Mississauga, Ontario, Canada). Photomicrographs were taken with an Olympus Qcolor 3 digital camera (Olympus, Richmond Hill, Ontario, Canada) and assessed using ImagePro Express software (version 5.1; Media Cybernetics, Bethesda, MD, USA). A representative thyroid section was used to quantify epithelial cell activity, and cell heights were measured at four cardinal points on all colloid-containing follicles along the axis.

Thyroid hormone analysis. The T_3 and T_4 enzyme-immunoassay protocols were a modification of previously published protocols (Graham et al., 2001). The optimum concentrations and dilutions of antibodies and biotinylated T_3 and T_4 were determined by checkerboard titration. In brief, Nunc microtiter plates (Thermo Fisher Scientific, Rochester, NY, USA) were coated with T_3 or T_4 antibody (Sigma-Aldrich, Oakville, Ontario, Canada) dissolved in coating buffer (0.015 mol/L Na_2CO_3, 0.035 mol/L $NaHCO_3$; pH 9.6) and incubated overnight at room temperature. Coated plates were washed with 0.04% Tween 20 (Sigma-Aldrich, Oakville, Ontario, Canada), 100 µl of assay buffer was added to each well and plates were incubated at room temperature for 1 hr. Then, 50 µl of diluted plasma sample and standards were dispensed into appropriate wells, followed immediately by 100 µl of biotinylated T_3 or T_4. Plates were incubated overnight at room temperature. After incubation, plates were washed and 200 µl of streptavidin-peroxidase conjugate (1 µL in 22 mL assay buffer; Roche Molecular Biochemicals, Indianapolis, IN, USA) were added to each well. After incubation (45 minutes, room temperature), plates were washed and 200 µl of substrate solution (0.5 µl of 0.016 mol/L tetramethylbenzidine in dimethyl sulfoxide and 100 µl 0.175 mol/L H_2O_2 diluted in 24 µl 0.01 mol/L $C_2H_3O_2Na$; pH 5.0) was added to each well. After incubation (45 minutes, room temperature), the enzyme reaction was stopped with 50 µl of stop solution (3 mol/L H_2SO_4). The optical density was measured at 450 nm. Triiodothyronine and T_4 were used as standards and serial dilutions of plasma gave displacement curves parallel to that of the standard curve.

Statistical analysis. Reproductive data were analyzed as a completely randomized design using the mixed procedure of SAS (version 9.2; Statistical Analysis Systems, Cary, NC, USA). The body weights for adult females and, juvenile females and males were analyzed as repeated measures. Differences between treatments for adult female and kit body weight and number of live kits at birth, three and six weeks of age were ascertained using least square means with Tukey adjustment. Kit survivability was expressed as a percent and therefore transformed for analysis using arcsine transformation. The means reported reflect the back-transformed data. Since standard error (SE) is not readily back-transformed, indication of variance is expressed as 95% confidence intervals around the mean. Organ weight at necropsy were expressed as percent of body weight and therefore transformed as indicated above. Similar analysis was used for kit growth except the model also contained the effect of sex and treatment by sex interaction. For body weight, fat and liver BTBPE concentrations, and T_3 and T_4 concentrations, least square means utilizing Tukey adjustment were used to ascertain differences between treatments. Differences between treatments were considered to be significant at $p < 0.05$.

Results

Dietary BTBPE concentrations. The targeted dietary concentrations of BTBPE were 0.0, 0.02, 0.2, and 2.0 mg/kg feed, wet weight. The analyzed dietary concentrations were 0.014, 0.13 and 2.3 mg/kg feed, wet weight. The MLOQ and MLOD were 0.12 and 0.04 µg/kg wet weight respectively. Table 3.1 presents the analyzed dietary concentrations of BTBPE.

Effects of BTBPE on adult female feed consumption. The daily estimated feed intake was not significantly different between the control and 2.3 mg BTBPE/kg feed treatment groups (the only groups assessed) from January 23 to February 10, 2009 (Table 3.2). The data represents pre-breeding feed consumption of adult females.

Table 3.1 Dietary concentrations of 1,2 bis (2,4,6-tribromophenoxy) ethane (BTBPE)

Targeted dietary BTBPE concentration (mg/kg feed, wet weight)	Analyzed dietary concentration (mg/kg feed, wet weight)
0.0	< MLOD[1]
0.02	0.014
0.2	0.13
2.0	2.3

[1]Concentration below the method limit of detection (MLOD).

Table 3.2 Average daily feed intake (ADFI) of adult female mink fed 0.0 or 2.3 mg 1, 2 bis (2, 4, 6-tribromophenoxy) ethane (BTBPE)/kg feed from January 23 to February 10, 2009

Dietary BTBPE concentration (mg/kg feed, wet weight)	ADFI(g/d)
0.0	139
2.3	138
SE	1.6
p-value	0.6549

Reproduction, offspring survivability, body weight, and organ weight. There were no significant differences in the number of females whelping, the total number of kits whelped or the number of live kits whelped per litter. Kit survivability through six weeks of age was not adversely affected by exposure to in utero and lactational exposure to BTBPE (Table 3.3).

There were no statistically significant treatment-related differences in adult pre-breeding body weights (Table 3.4), maternal and kit body weights at whelping and at three and six weeks of age (Table 3.5), and juvenile female body weights through 27 weeks of age (Table 3.7). Body weights of 27-week-old juvenile males exposed to BTBPE were significantly less compared to controls (Tables 3.6).

At weaning, relative liver weight of adult control animals was significantly greater compared to relative liver weight of adult females in the 2.3 mg BTBPE/kg feed group (p = 0.0319). There were no other significant differences in relative organ weight of adult females (Table 3.8).

Relative liver weight of six-week-old female kits was significantly greater in the 0.014 mg BTBPE/kg feed group compared to relative liver weight of animals in the 2.3 mg BTBPE/kg feed group (p = 0.0198). There were no other significant changes in relative organ weight of six-week-old females (Table 3.9), nor were there significant differences in relative organ weight of six-week-old male kits (Table 3.10).

Relative spleen weight of juvenile females was less in the 0.014 mg BTBPE/kg feed group in comparison to relative spleen weight of juvenile females in the 0.13 mg BTBPE/kg feed group (p = 0.0526). There were no other significant effects for relative weight of other organs for juvenile females (Table 3.11). In juvenile males, relative weight of the adrenal glands in the 0.13 mg BTBPE/kg feed group was significantly less compared to the control group

Table 3.3 Effect of 1,2 bis (2,4,6-tribromophenoxy) ethane (BTBPE) on mink reproduction

Dietary BTBPE concentration (mg/kg feed, wet weight)	# Females whelping/# females bred	Total kits whelped[1]	Live kits whelped[1]	% Survivability at birth	% Survivability at 3 weeks of age[2]	% Survivability at 6 weeks of age[2]
0	8/10	7.9	7.4	93.7	87.4	86.1
		(0.78)	(0.76)	(83.7 -104)	(68.0 – 107)	(67.1 – 105)
0.014	8/10	7.0	5.4	80.7	88.3	88.3
		(0.78)	(0.76)	(62.0 - 99.3)	(74.0 -103)	(74.0 – 103)
0.13	10/10	7.7	6.8	88.9	93.7	92.7
		(0.70)	(0.68)	(74.7 -103)	(85.9 – 101)	(83.1 – 102)
2.3	10/10	8.9	7.6	86.0	81.8	81.8
		(0.74)	(0.72)	(73.0 - 98.9)	(68.2 - 95.3)	(68.2 – 95.3)
p-value		0.3774	0.1814	0.5610	0.3696	0.4215

[1]Data presented as mean with the standard error in parenthesis.

[2]Data presented as mean with the 95% confidence interval in parenthesis.

Table 3.4 Effect of 1,2 bis (2, 4, 6-tribromophenoxy) ethane (BTBPE) on adult female mink pre-breeding weight[1]

		Weight (g)		
Dietary BTBPE concentration (mg/kg feed, wet weight)	n	Initial	30 d on trial	60 d on trial
0.0	10	1271	1378	1306
		(52.4)	(51.6)	(55.9)
0.014	10	1285	1398	1303
		(52.4)	(51.6)	(55.9)
0.13	9	1278	1377	1295
		(55.2)	(54.4)	(58.9)
2.3	10	1276	1364	1295
		(52.4)	(51.6)	(55.9)
p-value treatment	0.9966			
p-value time	< 0.0001			
p-value treatment*time	0.6557			

[1]Data are least square means (lsmeans) with the standard error in parenthesis.

Table 3.5 Effect of 1,2 bis (2, 4, 6-tribromophenoxy) ethane (BTBPE) on adult female and kit body weights (g) at whelping and at three and six weeks post-whelping[1]

Targeted dietary BTBPE concentration (mg/kg feed, wet weight)	n	Adult females at whelping	Adult females at 3 weeks post-whelping	Adult females at 6 weeks post-whelping	Kits at whelping	Kits at 3 weeks of age	Kits at 6 weeks of age
0.0	10	1255	1079	966	9.5	118	311
		(53)	(46)	(44)	(0.6)	(8)	(23)
0.014	10	1300	1136	1013	9.5	118	308
		(53)	(46)	(44)	(0.6)	(8)	(23)
0.13	9	1294	1135	985	9.5	126	340
		(48)	(41)	(39)	(0.5)	(7)	(21)
2.3	10	1290	1111	1018	9.7	122	311
		(50)	(43)	(41)	(0.6)	(8)	(22)
p-value		0.9294	0.7854	0.8138	0.9886	0.8245	0.6702

[1]Data presented as least square means with the standard error in parenthesis.

Table 3.6 Effect of 1,2 bis (2,4,6-tribromophenoxy) ethane (BTBPE) on body weight[1] (g) of juvenile males

Dietary BTBPE concentration (mg/kg feed, wet weight)	n	Age				
		10 weeks	14 weeks	19 weeks	23 weeks	27 weeks
0.0	10	1137	1670	2098	2283	2369[a]
		(65.8)	(60.4)	(60.4)	(68.9)	(56.7)
0.014	10	974	1490	1841	1988	1961[b]
		(65.8)	(60.4)	(60.4)	(68.9)	(56.7)
0.13	10	1103	1655	2029	2153	2143[b]
		(65.8)	(60.4)	(60.4)	(68.9)	(56.7)
2.3	10	966	1544	1945	2154	2087[b]
		(65.8)	(60.4)	(60.4)	(68.9)	(56.7)
p-value treatment		< 0.0166				
p-value time		< 0.0001				
p-value treatment*time		0.2281				

[1]Data are presented as least square means with the standard error in parenthesis.

Table 3.7 Effect of 1,2 bis (2,4,6-tribromophenoxy) ethane (BTBPE) on body weights[1] (g) of juvenile females

Dietary BTBPE concentration (mg/kg feed, wet weight)	n	Age				
		10 weeks	14 weeks	19 weeks	23 weeks	27 weeks
0.0	10	797	1145	1358	1426	1447
		(45.2)	(75.8)	(116.7)	(128.3)	(145.5)
0.014	10	713	1057	1265	1384	1405
		(45.2)	(75.8)	(116.7)	(128.3)	(145.5)
0.13	10	722	1141	1389	1456	1459
		(45.2)	(75.8)	(116.7)	(128.3)	(145.5)
2.3	10	743	1036	1189	1250	1217
		(45.2)	(75.8)	(116.7)	(128.3)	(145.5)
p-value treatment		0.2580				
p-value time		< 0.0001				
p-value treatment*time		0.0996				

[1]Data are presented as least square means. Standard error for all lsmeans was 66.4

Table 3.8 Effects of dietary 1,2 bis (2, 4, 6-tribromophenoxy) ethane (BTBPE) on adult female mink relative organ weights[1] (% of body weight)

Targeted dietary BTBPE concentration (mg/kg feed, wet weight)	n	Body weight at necropsy (g)	Liver	Thyroid gland	Heart	Spleen	Adrenal glands	Kidneys	Brain
0.0	10	842	4.1[a]	0.0080	0.82	0.40	0.013	0.87	0.98
		(40)	(3.6-4.5)	(0.0070-0.0090)	(0.77-0.88)	(0.30-0.50)	(0.0090-0.017)	(0.78-0.95)	(0.87-1.1)
0.014	10	864	3.7[ab]	0.0070	0.78	0.33	0.011	0.80	0.96
		(38)	(3.2-4.2)	(0.0050-0.0080)	(0.69-0.85)	(0.24-0.41)	(0.0090-0.013)	(0.71-0.89)	(0.85-1.1)
0.13	10	852	3.6[ab]	0.0080	0.77	0.34	0.011	0.80	0.95
		(38)	(3.2-4.0)	(0.0060-0.0090)	(0.72-0.82)	(0.29-0.39)	(0.010-0.012)	(0.69-0.91)	(0.87-1.0)
2.3	10	871	3.3[b]	0.0070	0.80	0.36	0.010	0.78	0.94
		(38)	(2.9-3.6)	(0.0060-0.0090)	(0.74-0.86)	(0.29-0.44)	(0.0080-0.011)	(0.72-0.83)	(0.84-1.0)
p-value		0.9512	0.0526	0.4064	0.4603	0.4057	0.2253	0.4338	0.9204

[1]Data are presented as means with standard error (body weight) or 95% confidence interval (relative organ weight) beneath in parenthesis.

[ab]Values with different superscripts within the same column are significantly different ($p < 0.05$).

Table 3.9 Effects of dietary 1,2 bis (2,4,6-tribromophenoxy) ethane (BTBPE) on female kit relative organ weights[1] (% of body weight) at 6 weeks of age

Dietary BTBPE concentration (mg/kg feed, wet weight)	n	Body weight (g)	Liver	Thyroid gland	Heart	Spleen	Adrenal glands	Kidneys
0.0	5	393	5.0[ab]	0.0090	0.65	0.47	0.014	0.87
		(34)	(4.1-5.9)	(0.0080-0.010)	(0.53-0.78)	(0.39-0.54)	(0.010-0.018)	(0.79-0.95)
0.014	5	359	5.5[a]	0.0090	0.60	0.53	0.011	0.93
		(34)	(5.0-6.1)	(0.0060-0.012)	(0.50-0.70)	(0.41-0.64)	(0.008-0.014)	(0.84-1.03)
0.13	5	468	4.7[ab]	0.0080	0.64	0.55	0.012	0.87
		(34)	(4.8-5.0)	(0.0060-0.010)	(0.56-0.71)	(0.42-0.68)	(0.0090-0.014)	(0.75-0.98)
2.3	5	413	4.5[b]	0.0080	0.62	0.47	0.011	0.86
		(34)	(4.2-4.9)	(0.0050-0.011)	(0.60-0.64)	(0.33-0.60)	(0.0080-0.013)	(0.83-0.90)
p-value		0.1795	0.0198	0.8577	0.6530	0.3962	0.2870	0.3520

[1]Data are presented as means with standard error (body weight) or 95% confidence interval (relative organ weight) beneath in parenthesis.

[ab]Values with different superscripts within the same column are significantly different ($p < 0.05$).

Table 3.10 Effects of dietary 1,2 bis (2,4,6-tribromophenoxy) ethane (BTBPE) on male kit relative organ weights[1] (% of body weight) at 6 weeks of age

Dietary BTBPE concentration (mg/kg feed, wet weight)	n	Body weight (g)	Liver	Thyroid	Heart	Spleen	Adrenal glands	Kidneys
0.0	5	457 (59)	5.5 (5.2-5.8)	0.0090 (0.0060-0.011)	0.60 (0.54-0.66)	0.45 (0.42-0.48)	0.016 (0.012-0.021)	1.0 (0.91-1.1)
0.014	5	404 (59)	5.4 (4.4-6.4)	0.0080 (0.0060-0.010)	0.59 (0.49-0.70)	0.49 (0.43-0.55)	0.014 (0.012-0.016)	1.0 (0.94-1.1)
0.13	5	494 (59)	5.1 (4.4-5.9)	0.0080 (0.0060-0.011)	0.62 (0.50-0.73)	0.55 (0.34-0.76)	0.014 (0.011-0.018)	0.91 (0.78-1.0)
2.3	5	488 (59)	5.1 (3.5-6.7)	0.0080 (0.0040-0.011)	0.65 (0.57-0.73)	0.54 (0.39-0.69)	0.0161 (0.002-0.030)	1.0 (0.78-1.2)
p-value		0.7005	0.8236	0.7572	0.6538	0.4134	0.9084	0.3932

[1]Data are presented as means with standard error (body weight) or 95% confidence interval (relative organ weight) beneath in parenthesis.

Table 3.11 Effects of dietary 1,2 bis (2,4,6-tribromophenoxy) ethane (BTBPE) on juvenile female relative organ weights[1] (% of body weight) at 27 weeks of age

Dietary TBPE concentration (mg/kg feed, wet weight)	n	Body weight (g)	Liver	Thyroid gland	Heart	Spleen	Adrenal glands	Kidneys	Brain	Reproductive tract
0.0	5	1447 (86)	3.2 (2.6-3.8)	0.004 (0.003-0.005)	0.50 (0.42-0.58)	0.21[ab] (0.17-0.26)	0.005 (0.003-0.007)	0.52 (0.46-0.58)	0.62 (0.47-0.77)	0.07 (0.01-0.12)
0.014	5	1405 (86)	3.4 (2.9-3.9)	0.004 (0.003-0.006)	0.54 (0.47-0.62)	0.20[a] (0.18-0.21)	0.006 (0.004-0.008)	0.53 (0.49-0.58)	0.61 (0.51-0.71)	0.07 (0.03-0.11)
0.013	5	1459 (86)	3.7 (3.1-4.2)	0.004 (0.003-0.006)	0.58 (0.42-0.75)	0.25[b] (0.22-0.29)	0.006 (0.004-0.007)	0.54 (0.44-0.64)	0.58 (0.50-0.66)	0.10 (0.05-0.15)
2.3	5	1217 (86)	3.2 (2.9-3.5)	0.004 (0.003-0.006)	0.62 (0.53-0.71)	0.22[ab] (0.18-0.27)	0.007 (0.005-0.010)	0.56 (0.51-0.60)	0.74 (0.62-0.86)	0.07 (0.04-0.10)
p-value		0.2080	0.2778	0.9130	0.1882	0.0526	0.2096	0.7898	0.0776	0.3569

[1]Data are presented as means with standard error (body weight) or 95% confidence interval (relative organ weight) beneath in parenthesis.

[ab]Values with different superscripts within the same column are significantly different ($p < 0.05$).

(p = 0.0320). The relative brain weight of juvenile males in the 0.13 mg BTBPE/kg feed group was significantly less than relative brain weight of animals in the 0.014 (p = 0.0127) and 2.3 mg BTBPE/kg feed groups (p = 0.0105) and relative brain weight of male juveniles in the 2.3 mg BTBPE/kg feed group was significantly greater (p = 0.0022) compared to controls (Table 3.12).

Thyroid histology and hormone concentrations of juveniles. The thyroid follicles were round to ovoid, and were comprised of a single layer of cuboidal to columnar epithelial cells surrounding a colloid-filled lumen. Colloid vacuolation was absent to moderate, with no apparent difference among treatment groups (Figure 3.1A). In most of the mink examined, diffuse follicular cell hyperplasia was evident in one or both lobes of the thyroid; incidence and severity did not vary among treatment groups. Hyperplastic areas exhibited microfollicular architecture as well as larger irregular-shaped follicles with papillary in-folding and relatively sparse, light-staining colloid. These areas were poorly demarcated, non-encapsulated, and lacked cellular atypia (Figure 3.1B). Cystic hyperplasia was seen in a single individual in the 0.014 mg BTBPE/kg feed group. This lesion formed a large discrete nodule comprised of low cuboidal epithelial cells with papillary projections surrounding a distended colloid-filled lumen (Figure 3.1C). There was no evidence of neoplasia in any groups. Mean epithelial cell height was significantly greater in the mink exposed to 0.13 mg BTBPE/kg feed compared to controls (p = 0.043).

Dietary exposure to BTBPE resulted in significant changes in plasma T_3 and T_4 concentrations in adult female mink. Mean T_3 concentration was significantly greater in animals in the 2.3 mg BTBPE/kg feed group compared to animals in the other groups and mean T_4 concentration was significantly less in adult females in the 0.014 mg BTBPE/kg feed group

Table 3.12 Effects of dietary 1,2 bis (2,4,6-tribromophenoxy) ethane (BTBPE) on juvenile male relative organ weights[1] (% of body weight) at 27 weeks of age

Dietary BTBPE concentration (mg/kg feed, wet weight)	n	Body weight (g)	Liver	Thyroid gland	Heart	Spleen	Adrenal glands	Kidneys	Brain	Testes
0.0	5	2369[a] (57)	3.2 (2.9-3.6)	0.004 (0.002-0.005)	0.60 (0.48-0.73)	0.17 (0.12-0.22)	0.004[a] (0.003-0.006)	0.56 (0.50-0.63)	0.43[ac] (0.39-0.48)	0.10 (0.06-0.14)
0.014	5	1961[b] (57)	3.2 (2.6-3.8)	0.003 (0.002-0.004)	0.55 (0.46-0.64)	0.16 (0.12-0.19)	0.005[ab] (0.005-0.006)	0.57 (0.50-0.63)	0.50[bc] (0.45-0.54)	0.09 (0.03-0.14)
0.13	5	2143[b] (57)	3.0 (2.3-3.7)	0.003 (0.003-0.003)	0.65 (0.53-0.77)	0.16 (0.14-0.19)	0.006[b] (0.005-0.006)	0.56 (0.45-0.68)	0.42[a] (0.37-0.46)	0.12 (0.08-0.16)
2.3	5	2087[b] (57)	2.8 (2.4-3.3)	0.003 (0.002-0.003)	0.58 (0.49-0.66)	0.17 (0.12-0.22)	0.005[ab] (0.005-0.006)	0.52 (0.48-0.56)	0.52[b] (0.47-0.56)	0.10 (0.04-0.17)
p-value		0.0010	0.5045	0.2497	0.3479	0.8700	0.0359	0.6015	0.0010	0.6662

[1]Data are presented as means with standard error (body weight) or 95% confidence interval (relative organ weight) in parenthesis.

[abc]Values with different superscripts within the same column are significantly different ($p < 0.05$).

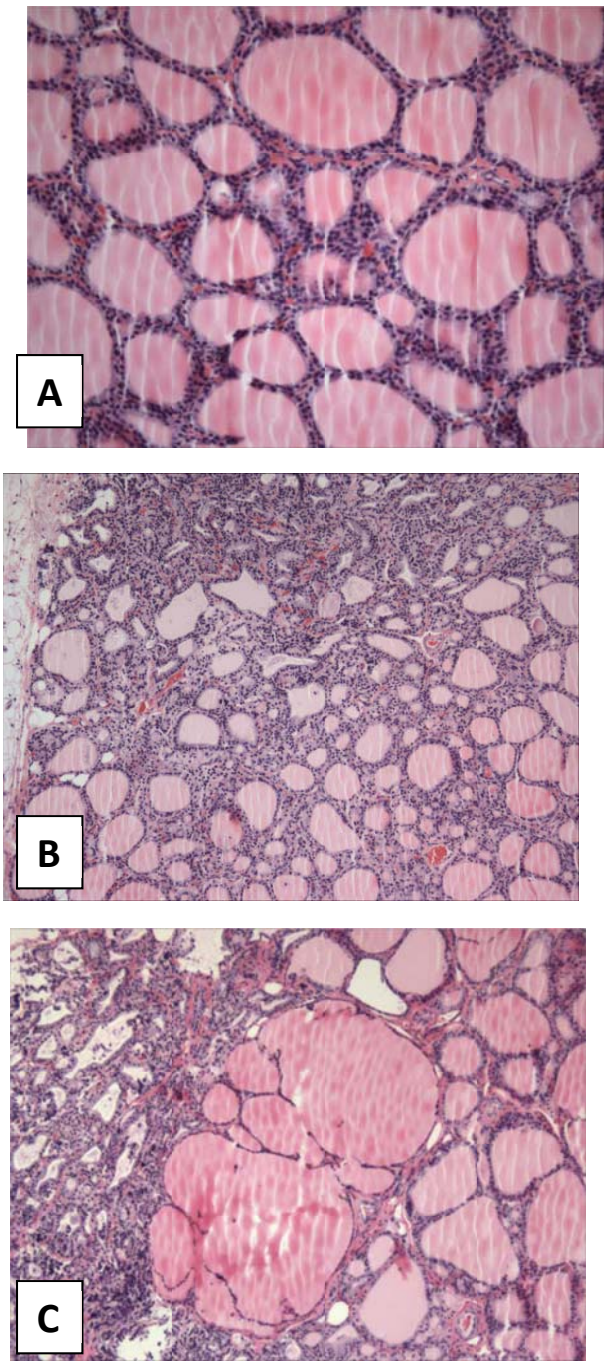

Figure 3.1 Effect of dietary BTBPE on histology of juvenile mink thyroid glands. (A) Control (200x): Ovoid follicles are comprised of cuboidal epithelium surrounding eosinophilic colloid. (B) 2.3 mg BTBPE/kg feed (100x): Diffuse follicular cell hyperplasia, showing microfollicular architecture and papillary folding of epithelium (upper left), adjacent to normal follicles. Note that this condition occurred in all treatment groups. (C) 0.014 mg BTBPE/kg feed (100x): Cystic follicular hyperplasia; with a large discrete nodule comprised of low cuboidal epithelial cells with papillary projections surrounding a distended colloid filled lumen.

compared to females in the other groups (Table 3.13).

Hepatic and adipose BTBPE concentrations. 1,2 Bis (2,4,6-tribromophenoxy) ethane accumulated to a greater extent in the adipose tissue compared to the liver, although concentrations in both tissues were from one to several orders of magnitude less than dietary concentrations. Hepatic concentrations of BTBPE in exposed adult females and kits were similar to control concentrations (data not presented). In 27–week-old juveniles, mean hepatic concentration of BTBPE in the females fed 2.3 mg BTBPE/kg feed was significantly greater compared to mean concentrations in the other groups (hepatic concentration of BTBPE was not determined for the 0.014 mg/kg feed group) (Table 3.14). 1,2Bis (2,4,6-tribromophenoxy) ethane was quantifiable in the adipose tissue of juveniles.

Juveniles exposed to 2.3 mg BTBPE/kg feed had a significantly greater mean concentration of BTBPE in their adipose tissue than did juveniles in the other treatment groups (Table 3.15). The bioaccumulation factor (ratio of the tissue concentration of BTBPE and the concentration of BTBPE in the feed) was approximately 0.1 for the 2.3 mg BTBPE/kg feed group.

Discussion

1,2bis (2,4,6-tribromophenoxy) ethane had no adverse effects on mink feed consumption (Table 3.2). Similarly, rats exposed to BTBPE at dietary concentrations of 0.05, 0.5, and 5% for one day or 10 days had feed consumption that was not different than feed consumption of control animals (Nomeir et al., 1993). In a DE-71 mink study, adult females were fed diets containing 0, 0.1, 0.5, or 2.5 mg DE-71/kg feed. Feed consumption was significantly lower in the 0.5 and 2.5 mg DE-71/kg feed treatments compared to controls (Zhang et al., 2009). In another DE-71 mink study, adult male mink were fed diets containing 0, 1, 5, or 10 mg DE-71/kg feed for nine weeks

Table 3.13 Effects of dietary 1,2 bis (2,4,6-tribromophenoxy) ethane (BTBPE) on plasma triiodothyronine (T_3) and thyroxine (T_4) concentrations in adult female mink

Dietary BTBPE concentration (mg/kg feed, wet weight)	T_3[1] (ng/ml)	T_4[1] (ng/ml)
0.0	19.8[a]	111[a]
0.014	18.9[a]	61.1[b]
0.13	18.3[a]	103[a]
2.3	44.8[b]	101[a]
SE	6.7	10
p-value	0.0196	0.0051

[1]Data are presented as lsmeans.

[ab]Values with different superscripts within the same column are significantly different ($p < 0.05$).

Table 3.14 Hepatic concentrations (µg/kg, wet weight) of 1,2 bis (2,4,6-tribromophenoxy) ethane (BTBPE) in juvenile mink[1]

Dietary BTBPE concentration (mg/kg feed, wet weight)	Male	Female	MLOQ µg/kg wet weight	MLOD µg/kg wet weight
0.0	0.035 (0.025)	0.035[a] (0.025)	0.21	0.07
0.13	0.035 (0.025)	0.035[a] (0.025)	0.21	0.07
2.3	0.077 (0.033)	0.230[b] (0.033)	0.28	0.09
p-value treatment	0.0010			
p-value sex	0.0381			
p-value treatment * sex	0.0306			

[1]Data are presented as lsmeans with standard error in parenthesis.

[ab]Values with different superscripts within the same column are significantly different ($p < 0.05$).

Table 3.15 Adipose concentrations (µg/kg, wet weight) of 1, 2 bis (2,4,6-tribromophenoxy) ethane (BTBPE) in juvenile mink

Dietary BTBPE concentration (mg/kg feed, wet weight)	Adipose concentration
0.0	0.012[a]
0.014	1.5[a]
0.13	11[a]
2.3	212[b]
Standard error	9.01
p-value	< 0.0001

[1]Data are presented as lsmeans.

[ab]Values with different superscripts within the same column are significantly different ($p < 0.05$).

(Martin et al., 2007). The animals in the 10 mg DE-71/kg feed group had a slight decline in feed consumption while feed consumption in the other groups was similar.

1,2 bis (2,4,6-tribromophenoxy) ethane also had no significant effect on adult mink reproduction as assessed by the number of females whelping, litter size and kit survivability through six weeks of age (Table 3.3). This is in contrast to the deleterious reproductive effects induced by dietary exposure of adult female mink to comparable concentrations of DE-71, a commercial pentaBDE mixture (Zhang et al., 2009). Female mink fed a diet containing 2.5 mg DE-71/kg feed experienced complete reproductive failure, while females fed diets containing 0.5 mg/kg feed or less had average whelping rates of 70 to 90%. The presence of implantation sites in the uteri of the females fed 2.5 mg DE-71/kg feed suggested that the animals were able to conceive and that DE-71 was inducing fetotoxic effects during gestation.

Body weight of adult female mink, neonatal, lactating and weanling kits, and juvenile mink generally were not adversely affected by exposure to BTBPE (Tables 3.4-3.7) with the exception of male juveniles at 27 weeks of age. At this time point, body weights of all males fed diets containing BTBPE were significantly less than control male body weights (Table 3.12). Studies conducted with BTBPE in rats did not assess body weight (Nomeir et al., 1993; Hakk et al., 2004), so comparisons cannot be made for the compound. However, studies assessing the effects of DE-71 in mink have included body weight as an endpoint. Zhang et al. (2009) reported that body weight of adult female mink fed diets containing 0, 0.1, 0.5, or 2.5 mg DE-71/kg feed were not affected at time of whelping or at six weeks post-whelping, despite the fact that feed intake was significantly lower in the 0.5 and 2.5 mg DE-71/kg feed groups. Additionally, no significant differences were reported in offspring body weight through 33 weeks of age. Martin et al. (2007) fed adult male mink diets containing DE-71 at concentrations

of 0, 1.0, 5.0, or 10 mg/kg feed for nine weeks. Mink from groups exposed to 5.0 and 10 mg DE-71/kg feed had significant reductions in body weight.

Exposure to BTBPE resulted in a few differences in relative organ weight in the present study, although the effects were not generally dose related. The only effect that could be considered treatment related was a decrease in mean relative liver weight of adult females in the 2.3 mg BTBPE/kg feed group compared to controls. Organ weight was not evaluated in the rodent BTBPE rodent study (Nomeir et al., 1993; Hakk et al., 2004). The DE-71 mink reproduction study performed by Zhang et al. (2009) did evaluate organ weight. Adult female mink were fed diets containing 0, 0.1, 0.5, or 2.5 mg DE-71/kg feed and the liver somatic index was significantly greater in the 2.5 mg DE-71/kg feed treatment group compared to other treatment groups. Additionally, there was a significant increase in liver somatic index of juvenile mink in the 0.5 mg DE-71/kg feed treatment group (there were no offspring in the 2.5 mg DE-71/kg feed group). Martin et al. (2007) also reported changes in organ weight in mink as a result of dietary exposure to DE-71. Adult male mink exposed to 10 mg DE-71/kg feed had significantly greater mean relative spleen and absolute adrenal gland weight compared to the control group. Additionally, relative liver weight of males in the 5.0 and 10 mg DE-71/kg feed groups were significantly greater compared to controls, which is opposite to the effect on liver weight reported in the present study.

The effects of BTBPE on thyroid hormone concentrations have not been reported in mammals, however, the similarity in structure between BTBPE and DE-71 suggested that BTBPE could be a disrupter of the thyroid gland such has been reported for DE-71 in mink (Zhang et al., 2009). In the latter study, plasma T_3 concentrations were decreased and plasma T_4 concentrations were increased as a result of exposure to DE-71, suggesting a disruption in the

conversion of T_4 to the metabolically active T_3. In the present study, mean plasma T_3 concentration was significantly greater in adult females in the 2.3 mg BTBPE/kg feed group compared to other treatment groups while mean plasma T_4 concentration was significantly lower in the 0.014 mg BTBP/kg feed group compared to the other treatment groups. Increased plasma T_3 is related to hyperthyroidism, the clinical signs of which can include increased appetite and decreased body weight. Because the females in the 2.3 mg BTBPE/kg feed group did not have significant weight loss or increased feed intake in comparison to females in the other treatment groups, it is assumed that the increase reported here was transitory and probably not treatment related. A decrease in plasma T_4 may indicate hypothyroidism, a clinical sign of which is an increase in body weight. Because the decrease in T_4 reported here occurred only at the lowest dose and because an increase in body weight was not reported for mink in this treatment group, it is likely that the change in T_4 was transitory and not treatment related. A lack of treatment-related histological changes in the thyroid gland of mink exposed to BTBPE support the conclusion that BTBPE, at the concentrations fed, has no effect on the thyroid gland of mink.

The potential of BTBPE to be a thyroid axis disrupter has been assessed in rainbow trout. Dietary exposure to BTBPE had no effect on thyroid gland morphology, deiodinase activity that results in conversion of T_4 to T_3, or plasma concentrations of T_3 and T_4. These results suggest that BTBPE has no effect on the thyroid gland of the rainbow trout, supporting the conclusion that BTBPE did not affect the thyroid gland of the mink.

The histological assessment of the juvenile thyroid glands in the present study indicated a significant increase in thyroid epithelial cell height in the 0.014 mg BTBPE/kg feed group

compared to controls. An increase in thyroid epithelial cell height can suggest toxic effects on the thyroid gland. A dose-related increased in thyroid cell height was reported in mink fed diets containing DE-71 (Zhang et al., 2009). Because the changes in plasma thyroid hormone concentrations and thyroid cell morphology were not dose-related, it is difficult to make conclusive statements regarding the effects of BTBPE on thyroid homeostasis.

1,2bis (2,4,6-tribromophenoxy) ethane was detected in the liver and was predominant in adipose tissue of juvenile mink, although at concentrations less than dietary concentrations. These results are similar to those reported in a BTBPE toxicokinetic study with rodents that indicated that little of the compound is retained in the animal and that adipose tissue is the preferred site of accumulation for that portion that is not eliminated (Nomeir et al., 1993). In a 10-day study, BTBPE was fed to rats at 0.05% of the diet daily. At the end of the study, the concentrations of BTBPE were highest in adipose tissue (0.06% of dose), followed by kidney, skin, and thymus, which had a minimal percentage of the dose (< 0.01%), suggesting that the potential for systemic exposure by ingestion was minimal in rats (Nomeir et al., 1993). Similarly, Hakk et al. (2004) administered a single oral dose of 2.0 mg BTBPE/kg body weight to rats and reported minimal tissue retention of BTBPE, with the greatest concentrations occurring in the lipophilic tissues such as adipose tissue, the adrenal glands and thymus.

Conclusion

The results of the present study indicated that exposure to BTBPE at dietary concentrations up to 2.3 mg/kg feed had no effect on reproductive performance of mink and survivability and growth of kits and growth of juvenile females. Body weights of juvenile males exposed to BTBPE were significantly less compared to controls at 27 weeks of age. Exposure to BTBPE had no significant effect on adult female body or organ weights, with the exception of

reduced relative liver weight in the 2.3 mg BTBPE/kg feed group. The significant differences seen in female kits, and juvenile female organ weights were not treatment related. The significant differences seen in juvenile male organ weight were not treatment related. However the significant effect seen in juvenile male body weights could be treatment related, and should be studied further.

Histological assessment of juvenile thyroid glands indicated increased thyroid epithelial height at 0.014 mg BTBPE/kg feed. 1,2 Bis (2,4,6-tribromophenoxy) ethane affected thyroid hormone homeostasis, as indicated by an increase in adult female plasma T_3 concentrations. 1,2 Bis (2,4,6-tribromophenoxy) ethane was measureable in adipose tissue, but not in the liver, suggesting that BTBPE is effectively cleared from the liver, but can accumulate in the fat. With BTBPE increased usage and the ability for it to accumulate in the environment, effects of higher doses may need to be assessed.

CHAPTER 4

CONCLUSION

1,2 bis (tribromphenoxy)-ethane (BTBPE) is considered to be an alternative flame retardant that is currently being used as a replacement for the commercial octaBDE. Assessment of environmental concentrations suggests that BTBPE has not accumulated to the same extent as the PBDEs. However since BTBPE is being marketed as a replacement for octaBDE, there is the potential that environmental concentrations will increase.

The results from the present studies were comparable to those reported for rainbow trout exposed to BTBPE. Tomy et al. (2007) determined that BTBPE was rapidly degraded and depurated in the fish. The present mink studies with BTBPE showed that the compound was rapidly eliminated at concentrations expected to occur in the environment. At higher doses of BTBPE, concentrations would be expected to increase in the adipose tissue, and potentially could cause adverse effects.

Results of the present pharmacokinetic mink study were similar to those reported in previous rodent studies (Nomeir et al., 1993), which indicated that the majority of orally administered BTBPE was excreted in feces. Lipophilic tissues of the mink, such as the adipose tissue, can accumulate BTBPE in small amounts, which is similar to the effects observed in rats (Nomeir et al., 1993; Hakk et al., 2004). Because of the minimal amount of absorption of BTBPE in the mink, few if any adverse effects would be expected as a result of prolonged exposure to this chemical, at the feed concentrations evaluated in the present studies.

The results of the present mink reproduction study confirmed few adverse effects at the feed concentrations examined. Because there were no changes in feed consumption, reproductive

parameters, body weights of adults and kits, juvenile female body weights or organ weights of adults and offspring that were considered to be treatment related, it was concluded that BTBPE demonstrated no toxicological effects. Regardless of the exposure concentration in the present studies, BTBPE was effectively cleared from the liver, and only small amounts accumulated in the adipose tissue. 1,2 bis (tribromphenoxy)-ethane affected thyroid hormone homeostasis, as indicated by an increase in adult female T_3 concentrations. The change in T_3 concentrations may suggest that exposure to BTBPE can result in subtle endocrine effects that should be studied further.

In future studies, an increase in dietary concentrations must be assessed as the concentration of BTBPE is increasing in the environment. The present reproductive BTBPE study in mink showed a significant effect on T_3 concentration. Because deiodination and conjugation are principle pathways for thyroid hormone metabolism, hepatic T_4 glucuronidation should be examined using higher dietary concentrations. Additionally, the effect of higher dietary concentrations on hepatic microsomal EROD and PROD, UDPGT activity must be assessed. Disruption of thyroid homeostasis, can cause effects on body weight and brain development.

A decrease in body weight of juvenile males was reported in the reproduction study. The decrease in body weight at the highest dietary concentration compared to the control should be investigated further. The decrease in body weight was a significant effect seen in the reproductive study, but an obvious reason for the effect could not be determined since feed consumption was determined for the juveniles. Additionally, thyroid hormone concentrations did not appear to be associated with body weight changes reported for the juvenile males.

In conclusion, both the toxicokinetic and reproductive studies of BTBPE in the mink demonstrated that the flame retardant did not induce overt toxic effects at environmentally relevant concentrations. Because environmental concentrations of BTBPE are expected to increase in time, it is advised that higher dietary concentrations be evaluated in mink.

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