Effects of a commercial pentabrominated diphenyl ether mixture on cholinergic parameters in captive mink

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Abstract

Polybrominated diphenyl ethers (PBDEs) are a class of brominated flame retardants that are recognized as global environmental contaminants and a potential health risk. They have been shown to elicit neurodevelopmental toxicity through disruption of the cholinergic neurotransmitter system in rodent models, but the effects of environmentally relevant exposures in wildlife species are unknown. The objective of this study was to assess the effects of the commercial pentabrominated diphenyl ether mixture DE-71 on cholinergic parameters in captive mink (Mustela vison) following dietary exposure of adult females and in utero, lactational and dietary exposure of their offspring. Adult females were fed diets containing 0, 0.1, 0.5 or 2.5 μg/g DE-71 from four weeks prior to breeding through weaning of their kits at six weeks of age. A portion of the weaned kits were maintained on their respective diets through 27 weeks of age. Cholinergic neurochemical biomarkers, including muscarinic acetylcholine receptor (mAChR) and nicotinic acetylcholine receptor (nAChR) binding, cholinesterase (ChE) activity and acetylcholine (ACh) concentration, were assayed in the cerebral cortex, and ChE activity measured in the plasma. Results indicated no significant effects of DE-71 on cholinergic parameters in the cerebral cortex, but a 3-fold increase in ChE activity in the plasma of adult females in the 2.5 μg/g DE-71 group. There were also no direct effects of DE-71 on mAChR or nAChR binding or ChE activity in the enzyme and receptor fractions from the whole brain of untreated mink following in vitro exposure to 0-23.6 nM DE-71. This study demonstrated that environmentally relevant exposures to DE-71 did not affect key parameters of the cholinergic neurotransmitter system in the brain of captive mink.
Les polybromodiphényléthers (PBDEs) sont une classe de produits ignifugeants bromés qui sont identifiés comme des contaminants environnementaux globaux posant un risque potentiel pour la santé. Il a été démontré qu'ils pouvaient engendrer une toxicité au niveau neurodeveloppemental du à un dérèglement du système des neurotransmetteurs cholinergiques dans des modèles de rongeur, mais les effets d’exposition sur la faune, à des niveaux présents dans l’environnement, ne sont pas connus. L'objectif de cette étude était d'évaluer les effets du mélange commercial de pentabromodiphényléther DE-71 sur des paramètres cholinergiques dans le vison (Mustela vison) captif, suite à l'exposition diététique des femelles adultes ainsi qu'à l'exposition de leurs progéniture dans l'utérus, par la lactation et par leurs nourriture. Des femelles adultes ont reçu des régimes contenant 0, 0.1, 0.5 ou 2.5 μg/g DE-71 à partir de quatre semaines avant l'accouplement jusqu'au sevrage de leurs petits à six semaines d'âge. Une partie des petits sevrés ont été maintenus sur leurs régimes respectifs jusqu'à 27 semaines d'âge. Des biomarqueurs neurochimiques cholinergiques, y compris le récepteur muscarinique d'acétylcholine (mAChR), le récepteur nicotinique d'acétylcholine (nAChR), la cholinestérase (ChE) et l'acétylcholine (ACh), ont été analysés dans le cortex cérébral, et ChE a aussi été mesuré dans le plasma. Les résultats n'ont indiqué aucun effet significatif pour DE-71 sur les paramètres cholinergiques dans le cortex cérébral, mais une augmentation de l'activité de ChE par un facteur de 3 a été détectée dans le plasma des femelles adultes dans le groupe du 2.5 μg/g DE-71. Il n'y avait également aucun effet direct de DE-71 sur ChE, mAChR et nAChR dans les fractions d'enzyme et de récepteur du cerveau entier du vison non traité suite à l'exposition in vitro de 0 à 23.6 nM DE-71. Cette étude a démontré que l'exposition à DE-71, à des niveaux présents dans l'environnement, n'a pas affecté les paramètres principaux du système des neurotransmetteurs cholinergiques dans le cerveau du vison captif.
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Contributions of Authors

This is a manuscript-based thesis and includes a single manuscript to be submitted for publication (Chapter 2). The manuscript is authored by myself and co-authored by my colleague, Dr. Niladri Basu, my collaborators, Dr. Steven Bursian and Pamela Martin, and my supervisor, Dr. Laurie Chan. The contributions of each of the authors of the manuscript are stated below.

The DE-71 mink feeding trial was designed and conducted by S. Bursian (Michigan State University) and P. Martin (Canadian Wildlife Service). The application to use animals in research was submitted by S. Bursian and approved by the All-University Committee on Animals Use and Care (Michigan State University). N. Basu and L. Chan (McGill University) established a collaboration with S. Bursian to measure biochemical changes in the brain of exposed mink. Blood sampling and necropsies of mink were assisted by K. Bull. All laboratory work, including the cholinergic neurochemical biomarker assays on brain tissue and blood plasma, was performed by K. Bull (McGill University). The nicotinic acetylcholine receptor (nAChR) binding assay method was modified by N. Basu from previously published methods, and has not yet been submitted for publication. Design and development of the in vitro assays were conducted by K. Bull. All data analysis and statistical analysis were carried out by K. Bull. The work of K. Bull was supervised by L. Chan and funded by grants provided to L. Chan. The manuscript was written by K. Bull and edited by the co-authors.
Contribution to Knowledge

This study is the first to assess the neurological effects of polybrominated diphenyl ethers (PBDEs) on a wildlife species. As the concentrations of PBDEs in the environment are increasing, it is critical to understand the effects of PBDEs on wildlife health and survival. While PBDEs have been shown to elicit neurodevelopmental toxicity through disruption of the cholinergic neurotransmitter system in rodent models, studies on the neurobehavioural effects of PBDEs outnumber those on the neurochemical effects. Furthermore, the use of single, high-dose neonatal exposure to individual congeners exceeds that of repeated or continual, low-dose perinatal exposure to commercial mixtures. There have also been no studies on the neurodevelopmental toxicity of PBDEs in wildlife species such as mink, nor on the direct neurochemical effects of PBDEs in vitro. In the current study, environmentally relevant conditions were used, including exposure (concentrations, durations and routes), PBDE (the commercial penta-BDE mixture DE-71) and animal model (the mink). Captive mink were blood sampled and necropsied following dietary exposure of adult females to continual, low-dose concentrations of DE-71 from prior to breeding through weaning of their offspring, and in utero, lactational and dietary exposure of their offspring from gestation through the growth phase. Cholinergic neurochemical biomarkers were assayed to measure the effects of DE-71 on muscarinic acetylcholine receptor (mAChR) and nicotinic acetylcholine receptor (nAChR) binding, cholinesterase (ChE) activity and acetylcholine (ACh) concentration in the cerebral cortex, and ChE activity in the plasma. The direct effects of DE-71 on mAChR and nAChR binding and ChE activity were also measured in the enzyme and receptor fractions from the whole brain of untreated mink following in vitro exposure. The results demonstrated that DE-71 did not affect key parameters of the cholinergic neurotransmitter system in the brain of captive mink, neither in vivo nor in vitro. However, previous studies indicated that single, high-dose neonatal exposure to individual PBDE congeners decreased mAChR and nAChR binding in the brain of rats and mice in vivo. Therefore, further
studies are needed to assess the neurochemical effects of PBDEs on the cholinergic neurotransmitter system in order to elucidate the mechanism of neurobehavioural toxicity under environmentally relevant conditions.
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AhR</td>
<td>Aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>BFR</td>
<td>Brominated flame retardant</td>
</tr>
<tr>
<td>bw</td>
<td>Body weight</td>
</tr>
<tr>
<td>ChE</td>
<td>Cholinesterase</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>CYT</td>
<td>Cytisine</td>
</tr>
<tr>
<td>d</td>
<td>Day</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EROD</td>
<td>Ethoxyresorufin-(O)-deethylase</td>
</tr>
<tr>
<td>HBr</td>
<td>Hydrogen bromide</td>
</tr>
<tr>
<td>LOAEL</td>
<td>Lowest observed adverse effect level</td>
</tr>
<tr>
<td>lw</td>
<td>Lipid weight</td>
</tr>
<tr>
<td>mAChR</td>
<td>Muscarinic acetylcholine receptor</td>
</tr>
<tr>
<td>MeO-PBDE</td>
<td>Methoxylated polybrominated diphenyl ether</td>
</tr>
<tr>
<td>MROD</td>
<td>Methoxyresorufin-(O)-demethylase</td>
</tr>
<tr>
<td>nAChR</td>
<td>Nicotinic acetylcholine receptor</td>
</tr>
<tr>
<td>OH-PBDE</td>
<td>Hydroxylated polybrominated diphenyl ether</td>
</tr>
<tr>
<td>PBDE</td>
<td>Polybrominated diphenyl ether</td>
</tr>
<tr>
<td>PCB</td>
<td>Polychlorinated biphenyl</td>
</tr>
<tr>
<td>PHDD/F</td>
<td>Polyhalogenated dibenzo-(p)-dioxin and dibenzofuran</td>
</tr>
<tr>
<td>PROD</td>
<td>Pentoxyresorufin-(O)-deethylase</td>
</tr>
<tr>
<td>QNB</td>
<td>Quinuclidinyl benzilate</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>T4</td>
<td>Thyroxine</td>
</tr>
<tr>
<td>THR</td>
<td>Thyroid hormone receptor</td>
</tr>
<tr>
<td>TTR</td>
<td>Transthyretin</td>
</tr>
<tr>
<td>UDPGT</td>
<td>Uridinephospho-glucuronyltransferase</td>
</tr>
<tr>
<td>ww</td>
<td>Wet weight</td>
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</tbody>
</table>
Introduction

Polybrominated diphenyl ethers (PBDEs) are a class of brominated flame retardants that are used in polymers (foam, plastics and textiles) to increase fire safety (International Programme on Chemical Safety, 1994). However, PBDEs are recognized as global environmental contaminants, and a potential risk to human and environmental health (European Chemicals Bureau, 2001). They are persistent, bioaccumulate, undergo long-range transport and elicit adverse effects. Of the three commercial PBDE mixtures, penta-BDE (DE-71), octa-BDE (DE-79) and deca-BDE (DE-83R), the predominant congeners in DE-71 (BDE-47, -99, -100 and -153) exhibit the highest bioaccumulation and most adverse effects (Agency for Toxic Substances and Disease Registry, 2004). These congeners have been found to bioaccumulate in the liver of captive mink (Bursian et al., 2006) and in the brain of wild otter (unpublished data, Basu et al.). They have been shown to elicit neurodevelopmental toxicity through the cholinergic neurotransmitter system in rodents (Viberg et al., 2002; Viberg et al., 2003a; Viberg et al., 2005), as well as thyroid hormone disruption in rats and captive mink (Zhou et al., 2002; Martin et al., 2004). The cholinergic neurotransmitter system is important in the cerebral cortex and hippocampus for functions including spontaneous behaviour, learning and memory. Thyroid hormones regulate neurodevelopment, including the cholinergic neurotransmitter system in the cerebral cortex and hippocampus (Porterfield, 2000), and may mediate the effects of PBDEs on neurodevelopment.

Reversible changes in biochemistry at the cellular level are known to precede irreversible adverse effects at the organism level (Manzo et al., 2001). As such, changes in neurochemistry may provide an early indication of neurotoxicity or adverse effects on neurobehaviour, including spontaneous behaviour, learning and memory. Functional neurotransmitter systems are essential for the health and survival of wildlife in the environment. The use of neurochemical biomarkers to elucidate the mechanism of neurotoxicity in wildlife is therefore important for the
assessment of neurotoxic effects of environmental contaminants in the characterization of risk. Neurochemical biomarkers of the cholinergic neurotransmitter system include acetylcholine (ACh), cholinesterase (ChE), muscarinic acetylcholine receptor (mAChR) and nicotinic acetylcholine receptor (nAChR) (Stamler et al., 2005; Basu et al., 2006b). They have been used to measure the direct (primary) effects of neurotoxins on cholinergic parameters in vitro, as well as the direct (primary) and/or mediated (secondary) effects in vivo. The neurochemical effects of PBDEs have not yet been studied in wildlife species such as mink.

Mink (Mustela vison) are widely distributed throughout North America and Europe (Larivière, 1999). They are commercially trapped and farmed for fur (Larivière, 1999). As a result, mink are extensively captured in the wild and studied in captivity, and can therefore be used to determine environmentally relevant exposures and establish exposure-response relationships. As a high trophic level piscivorous species (Larivière, 1999), mink are able to bioaccumulate environmental contaminants and are sensitive to those such as PCBs and PBDEs (Martin et al., 2004; Bursian et al., 2006; Martin et al., 2006). Due to the high and increasing concentrations of PBDEs in freshwater fish in North America (Hale et al., 2001; Johnson and Olson, 2001; Rayne et al., 2003; Chernyak et al., 2005), the dietary exposure of piscivorous species such as mink may pose a health risk. Therefore, the mink is a key sentinel species in environmental risk assessment (Basu et al., 2006a).

The objective of this study was to assess the effects of the commercial penta-BDE mixture DE-71 on cholinergic parameters in captive mink following dietary exposure of adult females and in utero, lactational and dietary exposure of their offspring. A one-generation DE-71 mink feeding trial was conducted, from which adult females and their offspring (6-week-old kits and 27-week-old juveniles) were blood sampled and necropsied. Cholinergic neurochemical biomarkers, including mAChR and nAChR binding, ChE activity and ACh
concentration, were assayed in the cerebral cortex, and ChE activity measured in the plasma. The direct effects of DE-71 on mAChR and nAChR binding and ChE activity were also assessed in the enzyme and receptor fractions from the whole brain of untreated mink following *in vitro* exposure.
Chapter 1: Literature Review

1.1. Flame Retardants

Fire is a major cause of property damage and loss, as well as injury and death (Gann, 1993). Smoke, gases and other by-products of fire are also a source of toxic pollutants such as polycyclic aromatic hydrocarbons (PAHs) and polyhalogenated dibenzo-\(p\)-dioxins and dibenzofurans (PHDD/Fs) (Simonson et al., 2002). Over the past several decades, increases in the use of petroleum-based and flammable polymers in residential, commercial and industrial products has led to greater fuel load in homes and buildings. Modern homes and buildings are also more energy efficient and less able to conduct heat. Combined, these factors have resulted in greater fire risk and, in turn, higher fire safety regulations (Leihbacher, 1999).

Flame retardants (FRs) are chemicals used in plastics (primarily electronics and electrical equipment, building/construction materials and transportation applications), textiles and furnishings to reduce fire risk and meet fire safety regulations (International Programme on Chemical Safety, 1997). They decrease the flammability of polymers by reducing the ignition and propagation of fire (International Programme on Chemical Safety, 1997). As a result, FRs decrease property damage and loss, injury and death, and pollution caused by fire. They provide up to 15 times more escape time (Bromine Science and Environmental Forum, 2006), which was credited for helping save lives in a recent Air France jet crash-landing at Toronto Pearson International Airport (American Fire Safety Council, 2005).

FRs are classified into four major groups: inorganic, halogenated organic, organophosphorus and nitrogen-based (Figure 1) (International Programme on Chemical Safety, 1997). Halogenated organic FRs are further classified as chlorinated and brominated. Brominated flame retardants (BFRs) are further classified as brominated monomers, reactive or additive. Brominated monomers
are used in the production of brominated polymers, reactive BFRs (such as tetrabromobisphenol A, TBBPA) are chemically bonded to polymers and additive BFRs (such as PBDE and hexabromocyclododecane, HBCD) are physically added to polymers. Reactive BFRs are freed from a polymer only when not fully polymerized, upon degradation of the polymer or through chemical reaction. As a result, reactive BFRs are released to the environment less easily than additive BFRs (Hutzinger et al., 1976).

1.2. Brominated Flame Retardants

Combustion involves the initiation and propagation of free radical reactions in the gas phase. Halogens are very effective in trapping free radicals, of which bromine has the best combination of trapping efficiency and thermal stability of the carbon-halogen bond. When heated, BFRs release HBr gas, which inhibits the free radical reactions by replacing the more reactive H and OH radicals with the less reactive Br radicals and thus inhibits combustion (International Programme on Chemical Safety, 1997).

1.3. Polybrominated Diphenyl Ethers

1.3.1. Commercial Mixtures

There are 209 possible congeners of PBDEs, which are numbered according to the International Union of Pure and Applied Chemistry (IUPAC) nomenclature based on the number (1-10) and position (2-6, 2'-6') of bromine atoms on the phenyl rings (Figure 2A). There are three commercial products of PBDEs: penta-BDE, octa-BDE and deca-BDE. They are mixtures of congeners named according to the average number of bromine atoms on the phenyl rings. In the U.S., the commercial mixtures produced by Great Lakes Chemical Corporation are DE-71 (penta-BDE), DE-79 (octa-BDE) and DE-83R (deca-BDE). The predominant congeners in DE-71 are BDE-47, -99, -100, -153 and -154 (Table 1, Figure 3) (Wellington Laboratories Inc., 2005a). The predominant congener in DE-79 is BDE-183 (37%) and in DE-83R is BDE-209 (97%) (Figure 4) (Wellington Laboratories Inc., 2005b; Wellington Laboratories Inc., 2005c).
these commercial mixtures, only DE-83R is still being produced. However, DE-71 is of greater concern for environmental and human health as the predominant congeners exhibit higher persistence, bioaccumulation, long-range transport and more adverse effects than those in DE-79 or DE-83R (International Programme on Chemical Safety, 1994; Agency for Toxic Substances and Disease Registry, 2004).

1.3.2. Uses and Applications
Penta-BDE is used primarily in flexible polyurethane (PU) foam in cushioning (e.g. mattresses, upholstered furniture, carpet underlay, bedding, tanks/pipes and others). Octa-BDE is used primarily in acrylonitrile butadiene styrene (ABS) in electronics and electrical equipment (e.g. copy machine parts, housings of televisions, computer monitors, audio and video equipment, remote controls and mobile phones). The primary application of deca-BDE is in high impact polystyrene (HIPS) in electronics and electrical equipment (e.g. housings of televisions, audio and video equipment, remote controls and mobile phones), and its secondary application is in upholstery textiles (e.g. sofas and office chairs) (Bromine Science and Environmental Forum, 2006). These polymer-based materials contain 5 to 30% PBDE by weight (International Programme on Chemical Safety, 1994).

1.3.3. Production and Use
PBDEs have been in production and use since the 1970s. The estimated total market demand for BFRs represented 25% of that for FRs in 1992 (Organization for Economic Co-operation and Development, 1994) and 39% in 1998 (TownsendTarnell Inc., U.S.) (Table 2). The global market demand increased by over 100% in a decade from 145,000 tonnes in 1990 to 310,000 tonnes in 2000 (Pettigrew, 1994; Bromine Science and Environmental Forum, 2000). In 2001, the estimated total market demand for PBDEs represented 33% of that for the major commercial BFRs (Table 3) (Bromine Science and Environmental Forum, 2006). Deca-BDE constituted 83% of PBDEs worldwide, and penta-BDE in
North America constituted 95% of penta-BDE worldwide. Production of BFRs occurs near bromine production sites, which are near a limited number of bromine sources, whereas the manufacture of products containing BFRs is more widespread (Alaee et al., 2003). The majority of market demand for BFRs in North America is in the U.S. Production of BFRs in North America is dominated by two companies in the U.S., Great Lakes Chemical Corporation and Albemarle.

1.3.4. Risk Assessments and Regulation

1.3.4.1. European Union

1.3.4.2. Asia
The Environmental Agency of Japan has not completed a risk assessment of PBDEs and there is no national regulation on marketing or use, although a voluntary industry ban on penta-BDE and octa-BDE has been in effect since 1991 and 2000, respectively (Watanabe and Sakai, 2003).

1.3.4.3. United States
As in Japan, the U.S. Environmental Protection Agency has not completed a risk assessment of PBDEs and there is no national regulation on marketing or use, although a voluntary industry ban on penta-BDE and octa-BDE has been in effect since January 2005.

1.3.4.4. Canada
Both Environment Canada and Health Canada have conducted screening assessments of PBDEs under the Canadian Environmental Protection Act (CEPA) 1999. Environment Canada released a draft report in February 2004, and Health Canada released a final report in December 2004 (Environment Canada, 2004; Health Canada, 2004). The reports propose that tetra-BDE to deca-BDE congeners or PBDEs as a group, respectively, be considered “toxic” as defined in Section 64 of CEPA 1999. The Ministers of Environment and Health have recommended that tetra-BDE to deca-BDE congeners be added to the List of Toxic Substances in Schedule 1, and the implementation of virtual elimination of tetra-BDE to hexa-BDE congeners under Subsection 65(3) (Department of the Environment and Department of Health, 2004).

1.3.5. Sources
Sources of PBDEs to the environment include production and import or export of PBDEs and/or products containing PBDEs. PBDEs are also released during the use, disposal (e.g. landfill and incineration) and recycling of products containing PBDEs. Wastewater treatment plant effluent and sewage sludge applied to
agricultural soil are sources of PBDEs. PBDEs are also released during fires, in particular major disasters such as that of the World Trade Centre in New York City (Lioy et al., 2002).

PBDEs are a source of brominated and brominated-chlorinated dibenzo-\(p\)-dioxins and dibenzofurans (PBDD/Fs, PXDD/Fs) formed during thermal processes involved in production, recycling, incineration and fires (International Programme on Chemical Safety, 1998). PBDD/Fs and PXDD/Fs are then released in the same manner as PBDEs. PBDD/Fs and PXDD/Fs have similar properties to polychlorinated dibenzo-\(p\)-dioxins and dibenzofurans (PCDD/Fs). There is also limited evidence for photochemical degradation of PBDEs to PBDD/Fs (International Programme on Chemical Safety, 1998), and both photochemical and biochemical degradation of higher brominated congeners to lower brominated congeners (International Programme on Chemical Safety, 1994).

Hydroxylated and methoxylated PBDEs (OH-PBDEs, MeO-PBDEs) are formed by natural sources (e.g. sponges and algae) and during the metabolism of PBDEs. The toxicity of OH-PBDEs (and possibly MeO-PBDEs) is higher than that of PBDEs for endpoints such as thyroid hormone disruption (Meerts et al., 2000; Legler et al., 2002).

1.3.6. Physical and Chemical Properties
The physical and chemical properties of the commercial PBDE mixtures are shown in Table 4 (Great Lakes Chemical Corporation, 2005a; Great Lakes Chemical Corporation, 2005b; Great Lakes Chemical Corporation, 2005c). PBDEs have low volatility (vapour pressure) and water solubility. They are also lipophilic (high octanol-water partition coefficient). The volatility, solubility and bioavailability (molecular weight) are higher for the lower brominated congeners. Persistence, bioavailability and lipophilicity enable bioaccumulation, while persistence and volatility or solubility enable long-range transport. As a result, the potential for bioaccumulation and long-range transport is higher for the lower
brominated congeners. However, BDE-209 (deca-BDE) has been found in peregrine falcon eggs (de Wit et al., 2006), as well as in lake sediments in the Arctic (Muir et al., 2003). The lower brominated, more volatile and soluble congeners predominate in the vapour and aqueous phases, while BDE-209 predominates in the solid phase (dust, particulates, soil, sediment and sewage sludge) (Hale et al., 2006). As distance from the source increases, concentrations decrease and the ratio of lower brominated to higher brominated congeners increases (Hale et al., 2006).

1.3.7. Routes of Exposure
Fish are an important vector in the transfer of persistent organic pollutants from the environment to high trophic level species, such as piscivorous wildlife and humans. For both piscivorous wildlife and humans, dietary intake of fish is a major route of exposure to lower brominated PBDEs, as well as OH-PBDEs and MeO-PBDEs (Asplund et al., 1999a; Bergman et al., 2002). Dietary intake of breast milk (or lactational transfer) and placental transfer are the primary routes of exposure to lower brominated PBDEs for infants and fetuses, respectively (Strandman et al., 2000; Guvenius et al., 2002).

For humans, occupational inhalation of dust and particulates is a major route of exposure to higher brominated PBDEs (Bergman et al., 2002). It has also recently been discovered that ingestion of indoor dust is a significant route of exposure to both lower and higher brominated PBDEs for children and adults (Stapleton et al., 2005; Wilford et al., 2005).

1.3.8. Concentrations and Trends
PBDEs were first detected in abiotic compartments as early as 1979 and in biota as early as 1981 (de Carlo, 1979; Anderson and Blomkist, 1981). They were first suggested as global environmental pollutants in 1987 (Jansson et al., 1987). PBDEs have been detected in all environmental compartments. Abiotic compartments include air, water, dust, particulates, soil, sediment and sewage
sludge, while biota include freshwater, marine and terrestrial ecosystems and humans. Human compartments include blood (plasma, serum), adipose tissue and breast milk. BDE-47 is the predominant congener in biota (and abiotic compartments in the vapour and aqueous phases), followed by BDE-99, -100, -153 and -154, the predominant congeners in penta-BDE. BDE-209 is the predominant congener in abiotic compartments in the solid phase.

1.3.8.1. Concentrations in the Environment
Concentrations of PBDEs in both abiotic compartments and biota are generally in the order of ppb (ng/g or μg/kg). However, in close proximity to point sources or at high trophic levels the concentrations are higher, in the order of ppm (μg/g or mg/kg).

1.3.8.1.1. Wildlife
Concentrations of PBDEs in freshwater fish are the most relevant to the exposure of piscivorous species such as mink. Concentrations in various species of freshwater fish in rivers and lakes (including the Great Lakes) in North America are generally in the order of 10 to 100 ng/g wet weight (ww) (Agency for Toxic Substances and Disease Registry, 2004). In some cases the concentrations are higher, in the order of 1,000 ng/g ww (Hale et al., 2001; Johnson and Olson, 2001). Mink consume 140 to 200 g/kg body weight/day (g/kg bw/d) of food, and their diet consists of approximately 50% fish (Aulerich et al., 1999; Sample and Glenn II, 1999). Therefore, exposure estimates in mink based on dietary intake of fish are in the order of 700 to 100,000 ng/kg bw/d. The lowest observed adverse effect level (LOAEL) of PBDEs is 1 mg/kg bw/day in rat dams based on thyroid hormone effects in fetuses and offspring following in utero and lactational exposure to DE-71 (Darnerud et al., 2001; Zhou et al., 2002). Although the lower intake in mink is three orders of magnitude lower than the LOAEL in rats, the higher intake is only one order of magnitude lower than the LOAEL. Concentrations of OH-PBDEs and MeO-PBDEs are in the order of 100 pg/g ww (Letcher et al., 2003), and the LOAEL for these compounds is currently unknown.
1.3.8.1.2. Humans

Concentrations of PBDEs in indoor air and dust, breast milk and food are the most relevant to human exposure. Concentrations in these compartments in North America are 260 to 1,518 pg/m$^3$ (mean, indoor air) (Shoeib et al., 2004; Wilford et al., 2004), 5,500 to 5,900 ng/g (mean, indoor dust) (Stapleton et al., 2005; Wilford et al., 2005), and 25 to 74 ng/g lipid weight (lw) (mean, breast milk) (Ryan et al., 2002; Schecter et al., 2003). Concentrations in food are 1725 pg/g ww (fish), 238 pg/g ww (meat) and 32 pg/g ww (dairy) (Schecter et al., 2004). In another market basket survey, concentrations in fish, meat, dairy and other were measured and used to estimate exposure based on dietary intake, but were not shown (Ryan and Patry, 2001).

Exposure estimates in North America based on inhalation of air and ingestion of food and dust are 124 to 385 ng/d (median) and 9,386 to 34,036 ng/d (maximum) in children and 53 to 226 ng/d (median) and 820 to 17,113 ng/d (maximum) in adults (Wilford et al., 2005). Estimates based on ingestion of breast milk are 1,774 ng/d (mean) and 10,056 ng/d (maximum) in infants (Schechter et al., 2003; Stapleton et al., 2005). Exposure to PBDEs on both a per day and per body weight basis are higher in children and infants than in adults. Estimates based on ingestion of breast milk in infants weighing 5 kg are therefore 355 ng/kg bw/d (mean) and 2,011 ng/kg bw/d (maximum), approximately 3,000- and 500-fold lower than the LOAEL of 1 mg/kg bw/d in rats, respectively. However, differences in the toxicokinetics and toxicodynamics of PBDEs between animals and humans are currently unknown.

1.3.8.2. Spatial Trends

Spatial trends of PBDEs are consistent with their physical and chemical properties (Section 1.3.6). Concentrations decrease with increasing latitude, and the ratio of lower brominated to higher brominated congeners increases (de Wit, 2002; de Wit et al., 2006). In addition, concentrations increase with increasing trophic level,
and the ratio of lower brominated to higher brominated congeners is higher in aquatic ecosystems than terrestrial ecosystems. Concentrations in fish are at least one order of magnitude higher in North America than in Europe (Hites, 2004). Similarly, concentrations in human blood, adipose tissue and breast milk are one to two orders of magnitude higher in the U.S. and Canada than in Europe or Japan (Figure 5) (Schecter et al., 2003). Concentrations in air and window films are up to 50 times higher indoors than outdoors (Butt et al., 2004; Wilford et al., 2004).

1.3.8.3. Temporal Trends
Temporal trends of PBDEs are consistent with their production and use (Section 1.3.3). Although the concentrations of PBDEs have been increasing exponentially since the 1970s, in some cases the concentrations of penta-BDE and octa-BDE are currently levelling off or decreasing, which may be due to the regulation of these commercial mixtures (Section 1.3.4). However, the concentrations of BDE-209 are still increasing, and the concentrations of PBDEs in the Arctic are likely to continue increasing due to the long-range transport of PBDEs released during the use, disposal and recycling of products containing PBDEs. Concentrations of polychlorinated biphenyls (PCBs), which have been banned since the late 1970s, are decreasing. Concentrations of PBDEs are generally lower than those of PCBs, but in some cases are similar to or higher than those of PCBs in abiotic compartments such as air and window films (Strandberg et al., 2001; Butt et al., 2004; ter Schure et al., 2004), and in biota such as freshwater and marine fish (Asplund et al., 1999b; Hale et al., 2001; Dodder et al., 2002).

1.3.8.3.1. Abiotic Compartments
Concentrations of PBDEs in abiotic compartments such as air, water and sewage sludge respond more rapidly to changes in environmental inputs than sediment and may be more useful for monitoring (Hale et al., 2006), although more historical concentrations are available for sediment. Concentrations of penta-BDE in sediment in Europe increased from the 1970s to 1995 and 1997 then
levelled off in the Netherlands and Germany, respectively, but increased to 1999 in Norway (Zegers et al., 2003). Concentrations of BDE-209 in lake sediments in the Canadian Arctic increased by an order of magnitude from 1984 to 1995 (Muir et al., 2003).

1.3.8.3.2. Biota
Concentrations of PBDEs have been increasing in freshwater, marine and terrestrial ecosystems. Concentrations in fish in the Great Lakes increased from 1983 to 1999 with a doubling time of 1.6 to 2.9 years (Chernyak et al., 2005), and those in the Columbia River increased 12-fold from 1992 to 2000 with a doubling time of 1.6 years (Rayne et al., 2003). Concentrations in marine mammals in the Canadian Arctic increased from 1981 to 2000 in ringed seals with a doubling time of 4.3 to 8.6 years, and 6.8-fold from 1982 to 1997 in beluga whales (Stern and Ikonomou, 2000; Ikonomou et al., 2002). Concentrations of PBDEs, including BDE-209, increased 7-fold in peregrine falcon eggs in Greenland from 1981 to 2003 (Vorkamp et al., 2005).

Concentrations of PBDEs in human breast milk in Sweden increased over 25 years from 1972 to 1997 with a doubling time of 5 years then levelled off from 1998 to 2000 (Meironyte et al., 1999; Meironyte and Noren, 2001). Similarly, concentrations in human breast milk in Japan increased over 15-fold from 1973 to 1988 then levelled off from 1993 to 2000 (Hori et al., 2002). Concentrations in human breast milk in Canada increased 15-fold over 10 years from 1992 to 2002 (Figure 5) (Ryan et al., 2002), and in the Canadian Arctic increased 3-fold from 1989-1991 to 1996-2000 (Pereg et al., 2003).

1.3.9. Toxicokinetics
Studies of the toxicokinetics of PBDEs are limited with respect to the congeners and species used. The uptake, distribution, metabolism and excretion of PBDEs vary among these congeners and species.
The possible mechanisms of metabolism of PBDEs include reductive debromination to form lower brominated PBDEs, oxidative debromination to form lower brominated hydroxylated or methoxylated PBDEs (OH-PBDEs, MeO-PBDEs), Phase I oxidation to form OH-PBDEs, Phase II conjugation to form sulfated PBDEs (SH-PBDEs), and methylation of OH-PBDEs to form methoxylated and hydroxylated PBDEs (MeO-OH-PBDEs) (Hakk and Letcher, 2003).

In mice administered a single oral dose of $^{14}$C-labeled BDE-99, the amount of radioactivity retained in the brain was between 3.7 and 5.1 per mille (%) after 24 hours and decreased to between 1.3 and 2.8% after 7 days (Eriksson et al., 2002b). In a similar study, mice were administered a single dose of $^{14}$C-labeled BDE-209 (Viberg et al., 2003b). In animals exposed on postnatal day 3 or 10, the amount of radioactivity retained in the brain was between 4.0 and 4.8% after 24 hours and increased to between 7.4 and 10.5% after 7 days. However, in animals exposed on postnatal day 19, only 0.6% was retained after both 24 hours and 7 days. In animals exposed on postnatal day 3, 10 or 19, the amount of radioactivity retained in the liver was 125.6%, 94.1% or 57.8%, respectively, after 24 hours and decreased to 47.7%, 46.3% or 3.12%, respectively, after 7 days.

In another study, adult female mice were administered a single oral or intravenous dose of $^{14}$C-labeled BDE-47 or BDE-99 (Darnerud and Risberg, 2006). Uptake was high, and retention was high in fatty tissues and some organs including the liver and (initially) the brain. Uptake in the fetus (placental transfer) was low but lactational transfer from the dam to the offspring was high (20%).

In other studies, rats and mice were administered single oral doses of $^{14}$C-labeled BDE-47, -99 and -209, as well as repeated oral doses of DE-71 and DE-79 (Orn and Klasson-Wehler, 1998; Hakk et al., 2001; Hakk et al., 2002; Huwe et al., 2002; Morck et al., 2003). The amount of PBDEs metabolised and excreted was
higher for the higher brominated congeners, and higher in mice than in rats. The metabolites were identified as debrominated, hydroxylated, methoxylated and sulfonated PBDEs. The majority of PBDEs retained in tissues were parent compounds and the concentrations were highest in adipose tissue for BDE-47 and -99, but highest in plasma and the liver and lowest in adipose tissue for BDE-209.

1.3.10. Endpoints and Mechanisms of Toxicity
PBDEs are similar in structure to non-coplanar PCBs and somewhat similar in structure to coplanar PCBs and polychlorinated dibenzo-p-dioxins (PCDDs) (Figure 2B, 2C). Certain hydroxylated PBDEs are similar in structure to the thyroid hormone thyroxine (T4), a hydroxylated polyiodinated diphenyl ether (Figure 2D). PCDDs (dioxins) and coplanar (non-ortho-substituted) PCBs are known to activate the aryl hydrocarbon receptor (AhR) (Chen and Bunce, 2004), non-coplanar (ortho-substituted) PCBs are known to elicit neurodevelopmental toxicity (Eriksson and Fredriksson, 1996) and hydroxylated PCBs are known to elicit thyroid hormone disruption (Legler et al., 2002; Meerts et al., 2002). Neurodevelopmental toxicity and thyroid hormone disruption are the most sensitive endpoints of PBDEs, although the dioxin-like toxicity of PBDEs is low as they are non-coplanar in structure.

1.3.10.1. Aryl Hydrocarbon Receptor and Hepatic Effects
The aryl hydrocarbon receptor (AhR) is a transcription factor activated by planar halogenated aromatic compounds (HACs) such as PCDDs and coplanar PCBs. AhR activation leads to binding of the dioxin response element (DRE) and induction of cytochrome P450 1A1 (CYP1A1)-dependent ethoxyresorufin-O-deethylase (EROD) activity and cytochrome P450 1A2 (CYP1A2)-dependent methoxyresorufin-O-demethylase (MROD) activity (Meerts et al., 1998). Dioxin-like effects include body weight loss, hepatotoxicity, dermal toxicity (chloracne), immunotoxicity, teratogenicity and carcinogenicity (International Programme on Chemical Safety, 1998). The constitutive androstane receptor (CaR) is activated by non-coplanar compounds such as non-coplanar PCBs. CaR activation leads to
induction of CYP2B1-dependent pentoxyresorufin-O-deethylase (PROD) activity (Meerts et al., 1998).

Non-ortho-substituted PCB congeners are coplanar in structure, while ortho-substituted PCB congeners are non-coplanar. However, all PBDE congeners are non-coplanar in structure due to the ether linkage. Individual PBDE congeners have been shown to be either weak AhR agonists or AhR antagonists in primary rat hepatocytes in vitro (Chen and Bunce, 2003), although DE-71 and DE-79 (but not DE-83R) induced EROD and PROD activity in rat liver in vivo (Zhou et al., 2001). EROD activity induction may be due to dioxin-like contaminants present in the commercial mixtures and not the individual congeners, while PROD activity induction may be due to the PBDEs.

In addition to induction of liver enzymes, DE-71 and DE-79 (but not DE-83R) increased liver-to-body weight ratio in rats (Zhou et al., 2001; Zhou et al., 2002).

1.3.10.2. Thyroid Effects
There are several possible mechanisms of thyroid hormone disruption (Brouwer et al., 1998). Effects on the thyroid gland include inhibition of the synthesis and release of thyroid hormones. Effects on thyroid hormone metabolism and excretion include activation of Phase I oxidation and Phase II conjugation enzymes and inhibition of the deiodinase enzyme. Effects on thyroid hormone transport and activity include inhibition of the binding of thyroid hormone to transport proteins and thyroid hormone receptor (THR).

Although there is currently no direct evidence for inhibition of the synthesis and release of thyroid hormones by PBDEs, morphological and histological effects on the thyroid gland have been observed in rats and mice (International Programme on Chemical Safety, 1994).
BDE-47, DE-71 and DE-79 (but not DE-83R) have been found to induce Phase I oxidation enzymes (EROD, MROD, PROD) and Phase II conjugation enzymes (uridinephospho-glucuronyltransferase, UDPGT) in rat liver in vivo. A decrease in the thyroid hormone thyroxine (T4) and binding of T4 to the transport protein transthyretin (TTR) was also observed (Zhou et al., 2001; Hallgren and Darnerud, 2002). Similar effects were observed for DE-71 in rat dams, fetuses and offspring, and were greater in offspring (during development) (Zhou et al., 2002).

Synthesized 6-OH-BDE-47 (but not BDE-47) and OH-PBDE metabolites from rat liver microsomes (but not parent congeners) bound competitively to human TTR with high affinity in vitro (Meerts et al., 2000; Legler et al., 2002). Synthesized OH-PBDE analogs of T4 bound competitively to THR-alpha and THR-beta with low affinity in vitro (Marsh et al., 1998).

Binding of OH-PBDEs to TTR may result in transport of PBDEs across the placenta and the blood-brain barrier, with effects on thyroid hormone in both mother and fetus and effects on neurodevelopment in the fetus, as was observed for OH-PCBs in rats (Meerts et al., 2002).

1.3.10.3. Neurodevelopmental Effects

The possible mechanisms for the neurodevelopmental toxicity of PBDEs include the disruption of thyroid hormone and impairment of neurotransmission through intracellular signalling and neurotransmitter systems (intercellular signalling) (McDonald, 2002). Effects of PBDEs on neurobehaviour include spontaneous behaviour (locomotion, rearing, total activity), learning and memory, while effects on neurochemistry include disruption of the phospholipase A2 (PLA2) and protein kinase C (PKC) pathways and the cholinergic neurotransmitter system.

Maternal, fetal and neonatal thyroid hormones regulate neurodevelopment, including the cholinergic and dopaminergic neurotransmitter systems in the cerebral cortex and hippocampus (Porterfield, 2000). The effects of PBDEs on neurodevelopment may be mediated in part by the effects on thyroid hormone.
DE-71 has been found to induce UDPGT and decrease T4 in rat dams, fetuses and offspring (Section 1.3.10.2) (Zhou et al., 2002). Similar effects on liver enzymes and thyroid hormones were observed in rat offspring, but no effects on neurobehaviour were observed (Taylor et al., 2002).

PBDEs have been shown to elicit adverse effects on neurobehaviour. Neonatal exposure to BDE-47 disrupted spontaneous behaviour in adult mice (Eriksson et al., 1998). Neonatal exposure to BDE-99 disrupted spontaneous behaviour in adult mice and rats, and impaired learning and memory in adult mice (Eriksson et al., 1998; Eriksson et al., 2002b; Viberg et al., 2002; Viberg et al., 2004; Viberg et al., 2005). Perinatal exposure to BDE-99 also disrupted sensory and motor behaviour in mice and rats (Branchi et al., 2002; Kuriyama et al., 2005). Neonatal exposure to BDE-153, -183, -203 and -206 disrupted spontaneous behaviour and impaired learning and memory in adult mice (Viberg et al., 2003a; Viberg et al., 2006), while neonatal exposure to BDE-209 disrupted spontaneous behaviour in adult mice (Viberg et al., 2003b). However, neonatal exposure to DE-71 disrupted learning in rats (Dufault et al., 2005), while perinatal exposure to DE-71 did not disrupt sensory or motor behaviour in rats (Taylor et al., 2002; MacPhail et al., 2003; Taylor et al., 2003).

The effects of PBDEs on neurobehaviour may be mediated in part by changes in neurochemistry. BDE-47, -99 and -153, as well as DE-71 and DE-79 have been found to disrupt intracellular signalling through the phospholipase A2 (PLA2) and protein kinase C (PKC) pathways in rat neurons in vitro (Kodavanti and Derr-Yellin, 2002; Kodavanti and Ward, 2005; Kodavanti et al., 2005). Individual PBDE congeners have also been shown to disrupt the cholinergic neurotransmitter system (intercellular signalling). In addition to effects on spontaneous behaviour, learning and memory, neonatal exposure to BDE-99 altered the effects of adult exposure to nicotine (a nicotinic acetylcholine receptor, nAChR, agonist) on spontaneous behaviour and decreased muscarinic acetylcholine receptor (mAChR) density in the hippocampus in adult rats, while neonatal exposure to
BDE-153 decreased nAChR density in the hippocampus in adult mice (Viberg et al., 2002; Viberg et al., 2003a; Viberg et al., 2005).

The cholinergic neurotransmitter system is important in the cerebral cortex and hippocampus for functions including spontaneous behaviour, learning and memory (Figure 6). The neurotransmitter acetylcholine (ACh) is synthesized from choline (Ch) and acetyl coenzyme A (AcCoA) by the enzyme choline acetyltransferase (ChAT) in the presynaptic neuron. ACh is taken up into vesicles by the ACh transporter (AChT) and, following an action potential, ACh is released into the synapse by fusion of the vesicles with the presynaptic membrane. ACh binds the muscarinic and nicotinic acetylcholine receptors (mAChR, nAChR) activating intracellular signalling pathways and is rapidly metabolized to choline and acetate by the enzyme cholinesterase (ChE) on the postsynaptic neuron. Ch is taken up into the presynaptic neuron by the choline transporter (ChT). ChE is also synthesized in the liver and secreted into blood (plasma, serum) via very low density lipoproteins (VLDL) (Kutty and Payne, 1994).

1.4. Neurochemical Biomarkers
Reversible changes in biochemistry at the cellular level are known to precede irreversible adverse effects at the organism level (Manzo et al., 2001). As such, changes in neurochemistry may provide an early indication of neurotoxicity or adverse effects on neurobehaviour, including spontaneous behaviour, learning and memory. Changes in neurochemistry are also easier to measure in wildlife than changes in neurobehaviour due to limited accessibility to live animals, and are easier to measure in wildlife than in humans due to limited accessibility to viable tissues. The use of neurochemical biomarkers to elucidate the mechanism of neurotoxicity in wildlife is therefore important for the assessment of neurotoxic effects of environmental contaminants in the characterization of risk.
Neurochemical biomarkers of the cholinergic neurotransmitter system include acetylcholine (ACh), cholinesterase (ChE), muscarinic acetylcholine receptor (mAChR) and nicotinic acetylcholine receptor (nAChR) (Stamler et al., 2005; Basu et al., 2006b). They have been used to measure the direct (primary) effects of neurotoxins on cholinergic parameters in vitro, as well as the direct (primary) and/or mediated (secondary) effects in vivo.

1.5. Mink
Mink (Mustela vison) are widely distributed throughout North America and Europe (LariviÈre, 1999). They are commercially trapped and farmed for fur (LariviÈre, 1999). As a result, mink are extensively captured in the wild and studied in captivity, and can therefore be used to determine environmentally relevant exposures and establish exposure-response relationships. As a high trophic level piscivorous species (LariviÈre, 1999), mink are able to bioaccumulate environmental contaminants and are sensitive to those such as PCBs and PBDEs (Martin et al., 2004; Bursian et al., 2006; Martin et al., 2006). Therefore, the mink is a key sentinel species in environmental risk assessment (Basu et al., 2006a).

Although concentrations of PBDEs in wild mink have not yet been reported, a recent study determined the concentration of total BDEs in the brain of wild otter collected from Nova Scotia to be 0.10 to 0.69 ng/g ww (range) and 0.33 ng/g ww (mean; 44% BDE-99, 33% BDE-100 and 23% BDE-153) (unpublished data, Basu et al.). The bioaccumulation of PBDEs in mink brain is likely to be similar to that in otter brain, as they are closely related species. The concentrations of PBDEs shown to decrease mAChR density in rats and nAChR density in mice are 16 mg/kg bw BDE-99 and 9 mg/kg bw BDE-153, respectively, or approximately 80 ng/g ww BDE-99 and 45 ng/g ww BDE-153 (approximately 5% bioaccumulation) in the brain, 1000- and 300-fold higher than the mean concentrations measured in wild otter brain (Eriksson et al., 2002b; Viberg et al., 2003a; Viberg et al., 2003b; Viberg et al., 2005). Furthermore, the dietary
exposure to PBDEs in wild mink is estimated to be as high as only one order of magnitude lower than the dietary intake of DE-71 known to elicit thyroid hormone disruption in rats (1 mg/kg bw/d in dams, effects in fetuses and offspring following in utero and lactational exposure) (Zhou et al., 2002). Moreover, a preliminary study found that dietary exposure to DE-71 decreased T4 in captive mink (5 μg/g in the feed for 70 days) (Martin et al., 2004). In addition, PBDEs have been found to bioaccumulate in the liver of captive mink following consumption of wild carp collected from the Saginaw River (Michigan, USA) (Bursian et al., 2006). Therefore, mink may be at risk of neurodevelopmental toxicity as a primary direct effect of PBDEs and/or as a secondary effect mediated by thyroid hormone disruption. However, the neurological effects of PBDEs have not yet been studied in wildlife species such as mink.

1.6. Rationale
The predominant congeners in DE-71 (penta-BDE) exhibit higher persistence, bioaccumulation, long-range transport and more adverse effects than those in DE-79 (octa-BDE) or DE-83R (deca-BDE). They have been found to bioaccumulate in the liver of captive mink and the brain of wild otter, and to elicit neurodevelopmental toxicity through the cholinergic neurotransmitter system in rodents and thyroid hormone disruption in rats and captive mink. Functional neurotransmitter systems are essential for the health and survival of wildlife in the environment. The cholinergic neurotransmitter system is important in the cerebral cortex for functions including spontaneous behaviour, learning and memory. Thyroid hormones regulate neurodevelopment, including the cholinergic neurotransmitter system in the cerebral cortex, and may mediate the effects of PBDEs on neurodevelopment. Studies on the neurobehavioural effects of PBDEs outnumber those on the neurochemical effects, and the use of single-dose neonatal exposure to individual congeners exceeds that of repeated-dose perinatal exposure to commercial mixtures. Wildlife species and humans are exposed to common ecological factors, thus wildlife species are more relevant to
environmental and human health than laboratory animals. The neurochemical effects of PBDEs have not yet been studied in mink.

The objective of this study was to assess the effects of the commercial penta-BDE mixture DE-71 on the cholinergic neurotransmitter system in captive mink (Mustela vison) following dietary exposure of adult females and in utero, lactational and dietary exposure of their offspring. A one-generation DE-71 mink feeding trial was conducted, from which adult females and their offspring (kits and juveniles) were blood sampled and necropsied. Cholinergic neurochemical biomarkers including mAChR and nAChR binding, ChE activity and ACh concentration were assayed in the cerebral cortex, and ChE activity measured in the plasma. The direct effects of DE-71 on mAChR and nAChR binding and ChE activity were also assessed in the whole brain of untreated mink following incubation with DE-71 in vitro to determine if the effects of DE-71 on these cholinergic parameters in vivo may be direct or mediated.

1.7. Hypotheses
The null hypotheses tested in the current study were as follows:

Hₐ: There are no effects of in utero, lactational and/or dietary exposure to DE-71 on cholinergic parameters in the cerebral cortex or plasma of captive mink.

Hₐ: There are no direct effects of in vitro exposure to DE-71 on cholinergic parameters in the enzyme and receptor fractions from the whole brain of untreated captive mink.
Figure 1: Classification of flame retardants.
Figure 2: Structure of (A) PBDE, (B) PBDE vs. PCDD (dioxin), (C) PBDE vs. PCB and (D) a hydroxylated PBDE vs. a thyroid hormone (thyroxine, T4).
Table 1: Concentrations of the predominant PBDE congeners in the commercial penta-BDE mixture DE-71 in percent.
(Wellington Laboratories Inc., 2005a)

<table>
<thead>
<tr>
<th>Congener</th>
<th>Structure</th>
<th>Concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDE-47</td>
<td>2,2',4,4'-tetra-BDE</td>
<td>32.4</td>
</tr>
<tr>
<td>BDE-99</td>
<td>2,2',4,4',5-penta-BDE</td>
<td>43.9</td>
</tr>
<tr>
<td>BDE-100</td>
<td>2,2',4,4',6-penta-BDE</td>
<td>8.92</td>
</tr>
<tr>
<td>BDE-153</td>
<td>2,2',4,4',5,5'-hexa-BDE</td>
<td>3.84</td>
</tr>
<tr>
<td>BDE-154</td>
<td>2,2',4,4',5,6'-hexa-BDE</td>
<td>3.30</td>
</tr>
</tbody>
</table>
Figure 3: High-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) chromatogram of commercial PBDE mixture DE-71 (penta-BDE).

(Wellington Laboratories Inc., 2005a)
Figure 4: High-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) chromatograms of commercial PBDE mixtures DE-79 (octa-BDE) and DE-83R (deca-BDE).

(Wellington Laboratories Inc., 2005b; Wellington Laboratories Inc., 2005c)
Table 2: Estimated worldwide demand for flame retardant chemicals in 1992 and 1998 in tonnes and percent.

(Organization for Economic Co-operation and Development, 1994; TownsendTarnell Inc., U.S.)

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromine</td>
<td>150,000</td>
<td>25</td>
<td>39</td>
</tr>
<tr>
<td>Chlorine</td>
<td>60,000</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>100,000</td>
<td>16</td>
<td>24</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>30,000</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Antimony</td>
<td>50,000</td>
<td>8</td>
<td>27</td>
</tr>
<tr>
<td>Aluminum</td>
<td>170,000</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>50,000</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>
Table 3: Estimated total market demand for the major commercial brominated flame retardants (BFRs) by region in 2001 in metric tons.
(Bromine Science and Environmental Forum, 2006)

<table>
<thead>
<tr>
<th>BFR</th>
<th>Americas</th>
<th>Europe</th>
<th>Asia</th>
<th>Rest of the World</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBBPA</td>
<td>18,000</td>
<td>11,600</td>
<td>89,400</td>
<td>600</td>
<td>119,600</td>
</tr>
<tr>
<td>HBCD</td>
<td>2,800</td>
<td>9,500</td>
<td>3,900</td>
<td>500</td>
<td>16,700</td>
</tr>
<tr>
<td>Deca-BDE</td>
<td>24,500</td>
<td>7,600</td>
<td>23,000</td>
<td>1,050</td>
<td>56,150</td>
</tr>
<tr>
<td>Octa-BDE</td>
<td>1,500</td>
<td>610</td>
<td>1,500</td>
<td>180</td>
<td>3,790</td>
</tr>
<tr>
<td>Penta-BDE</td>
<td>7,100</td>
<td>150</td>
<td>150</td>
<td>100</td>
<td>7,500</td>
</tr>
<tr>
<td>Total</td>
<td>53,900</td>
<td>29,460</td>
<td>117,950</td>
<td>2,430</td>
<td>203,740</td>
</tr>
</tbody>
</table>
Table 4: Physical and chemical properties of the commercial PBDE mixtures.
(Great Lakes Chemical Corporation, 2005a; Great Lakes Chemical Corporation, 2005b; Great Lakes Chemical Corporation, 2005c)

<table>
<thead>
<tr>
<th>Property</th>
<th>Penta-BDE</th>
<th>Octa-BDE</th>
<th>Deca-BDE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vapour pressure (at 21°C)</td>
<td>$4.69 \times 10^{-5}$ Pa</td>
<td>$6.59 \times 10^{-6}$ Pa</td>
<td>$4.63 \times 10^{-6}$ Pa</td>
</tr>
<tr>
<td>Water solubility (at 25°C)</td>
<td>13.3 µg/L</td>
<td>&lt;1.0 µg/L</td>
<td>0.1 µg/L</td>
</tr>
<tr>
<td>Log octanol-water partition coefficient</td>
<td>6.57</td>
<td>6.29</td>
<td>6.27</td>
</tr>
</tbody>
</table>
Figure 3. Median concentrations (ng/g lipid) of BDE-47, BDE-99, and BDE-153 in human milk from different countries. Data from Ryan et al. (2002) and Ryan and Patry (2001) for Canada, from Schroeter-Kermani et al. (2000) for Germany, from Noren and Merionyte (2000) for Sweden, and from Strandman et al. (2000) for Finland.

Figure 5: Spatial and temporal trends of PBDEs in human breast milk.

(Schecter et al., 2003)
Figure 6: The cholinergic pathway of neurotransmission.

1) Biosynthesis of neurotransmitter (acetylcholine, ACh), 2) Storage of ACh in vesicles, 3) Release of ACh into synapse, 4) Binding of ACh to receptors, 5) Metabolism of ACh, 6) Uptake of choline (Ch).
Chapter 2: Effects of a commercial pentabrominated diphenyl ether mixture on cholinergic parameters in captive mink

Manuscript Title:
Effects of a commercial pentabrominated diphenyl ether mixture on cholinergic parameters in captive mink

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2.1. Abstract
Polybrominated diphenyl ethers (PBDEs) are a class of brominated flame retardants that are recognized as global environmental contaminants and a potential health risk. They have been shown to elicit neurodevelopmental toxicity through disruption of the cholinergic neurotransmitter system in rodent models, but the effects of environmentally relevant exposures in wildlife species are unknown. The objective of this study was to assess the effects of the commercial pentabrominated diphenyl ether mixture DE-71 on cholinergic parameters in captive mink (*Mustela vison*) following dietary exposure of adult females and *in utero*, lactational and dietary exposure of their offspring. Adult females were fed diets containing 0, 0.1, 0.5 or 2.5 µg/g DE-71 from four weeks prior to breeding through weaning of their kits at six weeks of age. A portion of the weaned kits were maintained on their respective diets through 27 weeks of age. Cholinergic neurochemical biomarkers, including muscarinic acetylcholine receptor (mAChR) and nicotinic acetylcholine receptor (nAChR) binding, cholinesterase (ChE) activity and acetylcholine (ACh) concentration, were assayed in the cerebral cortex, and ChE activity measured in the plasma. Results indicated no significant effects of DE-71 on cholinergic parameters in the cerebral cortex, but a 3-fold increase in ChE activity in the plasma of adult females in the 2.5 µg/g DE-71 group. There were also no direct effects of DE-71 on mAChR or nAChR binding or ChE activity in the enzyme and receptor fractions from the whole brain of untreated mink following *in vitro* exposure to 0-23.6 nM DE-71. This study demonstrated that environmentally relevant exposures to DE-71 did not affect key parameters of the cholinergic neurotransmitter system in the brain of captive mink.

*Keywords:* Polybrominated diphenyl ether (PBDE), Brominated flame retardant (BFR), Cholinergic neurotransmitter system, Development, Mink, Wildlife
2.2. Introduction

Polybrominated diphenyl ethers (PBDEs) are a class of brominated flame retardants that are used in polymers (foam, plastics and textiles) to increase fire safety (International Programme on Chemical Safety, 1994). However, PBDEs are recognized as global environmental contaminants, and a potential risk to human and wildlife health (European Chemicals Bureau, 2001). They are persistent, bioaccumulate, undergo long-range transport and elicit adverse effects. Of the three commercial PBDE mixtures, penta-BDE (DE-71), octa-BDE (DE-79) and deca-BDE (DE-83R), the predominant congeners in DE-71 (BDE-47, -99, -100 and -153) exhibit the highest bioaccumulation and most adverse effects (Agency for Toxic Substances and Disease Registry, 2004). These congeners have been found to bioaccumulate in the liver of captive mink following consumption of wild carp collected from the Saginaw River (Michigan, USA) (Bursian et al., 2006), as well as in the brain of wild otter collected from Nova Scotia (unpublished data, Basu et al.). They also have been shown to elicit adverse effects in captive mink following consumption of diets containing DE-71 (Martin et al., 2004). As the concentrations of PBDEs in freshwater fish in North America are high and increasing (Hale et al., 2001; Johnson and Olson, 2001; Rayne et al., 2003; Chernyak et al., 2005), the dietary exposure of humans and piscivorous wildlife species such as mink may pose a health risk.

Individual PBDE congeners have been shown to elicit neurodevelopmental toxicity through disruption of the cholinergic neurotransmitter system in rodent models (Viberg et al., 2002; Viberg et al., 2003a; Viberg et al., 2005). The effects of PBDEs on neurobehaviour may be mediated in part by changes in neurochemistry. In addition to effects on spontaneous behaviour, learning and memory, neonatal exposure to BDE-99 alters the effects of adult exposure to nicotine (a nicotinic acetylcholine receptor, nAChR, agonist) on spontaneous behaviour and decreases muscarinic acetylcholine receptor (mAChR) density in the hippocampus of adult rats, while neonatal exposure to BDE-153 decreases...
nAChR density in the hippocampus of adult mice (Viberg et al., 2002; Viberg et al., 2003a; Viberg et al., 2005).

Neurochemical biomarkers of the cholinergic neurotransmitter system include acetylcholine (ACh), cholinesterase (ChE), mAChR and nAChR (Stamler et al., 2005; Basu et al., 2006b). They have been used to measure the direct (primary) effects of neurotoxins on cholinergic parameters in vitro, as well as the direct (primary) and/or mediated (secondary) effects on cholinergic parameters in vivo. However, there have been no studies on the neurochemical effects of PBDEs in wildlife species such as mink, nor on the direct effects of PBDEs on cholinergic parameters in vitro. Mink are commercially trapped and farmed for fur (Larivière, 1999) and as a result are extensively captured in the wild and studied in captivity. Mink can therefore be used to determine environmentally relevant exposures and establish exposure-response relationships, and is a key sentinel species in environmental risk assessment (Basu et al., 2006a).

The objective of this study was to assess the effects of the commercial penta-BDE mixture DE-71 on cholinergic parameters in the cerebral cortex and plasma of captive mink (Mustela vison) following dietary exposure of adult females and in utero, lactational and dietary exposure of their offspring. The direct effects of DE-71 on mAChR and nAChR binding and ChE activity were also assessed in the receptor and enzyme fractions from the whole brain of untreated mink following in vitro exposure.

2.3. Materials and Methods

2.3.1. Chemicals

10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) was purchased from Molecular Probes (Eugene, OR). Atropine and liquid scintillation cocktail were obtained from ICN Biomedicals (Aurora, OH). Bio-Rad protein assay dye reagent concentrate was purchased from Bio-Rad Laboratories (Hercules, CA). $[^3]H$-Cytisine HCl ($[^3]H$-CYT; 30.4 Ci/mmol) and $[^3]H$-Quinuclidinyl benzilate
([^\^]H)-QNB; 42 Ci/mmol) were purchased from NEN/PerkinElmer (Boston, MA). Dimethyl sulfoxide (DMSO) was obtained from Fisher Scientific (Fair Lawn, NJ). Heparin was obtained from USB Corporation (Cleveland, OH). Pentabromodiphenyl ether (DE-71) was a gift from Great Lakes Chemical Corporation (West Lafayette, IN). Acetylcholine (ACh), acetylcholinesterase (AChE), 1,5-bis(4-allyldimethyl-ammoniumphenyl)pentan-3-one dibromide (BW284c51), bovine serum albumin, choline oxidase, horseradish peroxidase, hydrogen peroxide, (-)-nicotine hydrogen tartrate, resorufin, tetraisopropy1 pyrophosphoramide (iso-OMPA), Triton X-100 and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO) and were of analytical grade or higher.

2.3.2. Experimental Design
Mink were housed, bred, weaned, anesthetized, blood sampled, euthanized and necropsied at the Michigan State University (MSU) Experimental Fur Farm (East Lansing, MI) as previously described (Bursian et al., 2006). The MSU Experimental Fur Farm ranch diet was used as the base of the four treatment diets, which contained 0, 0.1, 0.5 or 2.5 μg/g DE-71. Samples of each treatment diet were frozen for subsequent analysis of PBDEs (Martin et al., 2006; personal communication).

Forty first-year virgin, natural dark, female mink from the MSU Experimental Fur Farm herd were randomly assigned to the four treatment groups (10 mink/group). Animals were started on their respective treatment diets beginning 2 February 2004. Feed and water were available ad libitum. Dietary intake of DE-71 was estimated from feed consumption and body weight of females measured weekly from 4 February 2004 to 26 February 2004 and 30 January 2004 to 26 February 2004, respectively.

The females were mated to untreated males between 1 March 2004 and 20 March 2004. Whelping began on 19 April 2004 and ended on 8 May 2004. Nest boxes
were checked daily, and live and stillborn kits were counted at birth and their
gender determined. Reproductive parameters were measured, including breeding
success, whelping success, litter size and survivability of kits from birth to six
weeks of age (unpublished data, Bursian et al.).

Kits were completely weaned by six weeks of age. All adult females were
anesthetized, blood sampled, euthanized and necropsied on 2, 3 and 10 June 2004,
and six kits from each treatment group at approximately six week of age were
necropsied on 9 June 2004. Ten kits from each treatment group were maintained
on their respective treatment diets. At approximately 27 weeks of age, all
juveniles from each treatment group were necropsied on 8, 9 December 2004.
Whenever possible, offspring were chosen such that no more than one kit or
juvenile from each litter and treatment group was present, and all kits or juveniles
from each treatment group were within 7-10 days of age. Samples of brain and
plasma were collected for subsequent analysis of cholinergic parameters.

2.3.3. Sample Preparation

2.3.3.1. Brain Tissue

Whole brains were immediately frozen in liquid nitrogen and stored at -80°C.
Tissues were prepared as described by Stamler et al. (2005) with minor
modifications. Dissected cerebral cortex or whole brains were homogenized
(Tissue Tearor, Model 398, BioSpec Products, Bartlesville, OK) for 30 s in ice-
cold Na/K buffer (50 mM NaH₂PO₄, 5 mM KCl, 120 mM NaCl, pH 7.4).
Membrane fractions were prepared by centrifugation of the homogenate at 32,000
x g for 15 min at 4°C. The resulting pellet was washed twice under the same
conditions and resuspended in ice-cold NaK buffer. Enzyme fractions were
prepared by sonication (Sonic Dismembrator, Model 60, Fisher Scientific,
Pittsburgh, PA) of the homogenate for 10 s in ice-cold Na/K buffer and 0.1%
Triton X-100, followed by centrifugation at 12,000 x g for 10 min at room
temperature to obtain the supernatant. The membrane and enzyme preparations
were immediately frozen in liquid nitrogen and stored at -80°C. The
concentration of protein was determined using the Bradford assay (Bradford, 1976) with bovine serum albumin as the standard.

2.3.3.2. Blood
Whole blood samples were collected in syringes containing heparin, transferred to heparinized Vacutainer tubes (BD, Franklin Lakes, NJ) and gently mixed for at least two min. Plasma was isolated by centrifugation of whole blood at 900 x g for 10 min at room temperature and stored at 4°C.

2.3.4. In vivo Study
2.3.4.1. mAChR Binding Assay
mAChR binding was measured as described by Stamler et al. (2005) with minor modifications. Cerebral cortex membrane fractions (20 μg protein) were added in triplicate to a 96-well, 0.22-μm GF/B glass filter system (Millipore, Boston, MA). Samples were pre-incubated with Na/K buffer (total binding) or 100 μM atropine (a mAChR antagonist) (non-specific binding) for 30 min at room temperature, then incubated with 1 nM [³H]-QNB for 60 min at room temperature with gentle agitation. It has been shown that specific binding at 1 nM [³H]-QNB is indicative of mAChR density in mink brain tissues (Stamler et al., 2005). The incubation was terminated by rapid vacuum filtration and the filters were washed twice with ice-cold Na/K buffer. Filters were extracted and allowed to soak overnight in liquid scintillation cocktail. Radioactivity retained by the filters was quantified by a liquid scintillation counter (LS 3801, Beckman Instruments, Irvine, CA) with approximately 60% counting efficiency. Non-specific binding was approximately 3% of total binding. Intra- and inter-assay variation were generally less than 10% and 3% relative standard deviation (RSD), respectively.

2.3.4.2. nAChR Binding Assay
nAChR binding was measured as previously described (Trauth et al., 1999) with the following modifications. Cerebral cortex membrane fractions (25 μg protein) were added in triplicate to a 96-well, 0.22-μm GF/B glass filter system (Millipore,
Samples were pre-incubated with Tris buffer (50 mM Tris, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, pH 7.4) (total binding) or 100 µM (-)-nicotine hydrogen tartrate (a nAChR agonist) (non-specific binding) for 30 min at room temperature, then incubated with 1 nM [³H]-CYT for 1-2 min at room temperature with gentle agitation followed by 75 min at 4°C without agitation. The incubation was terminated by rapid vacuum filtration and the filters were washed three times with ice-cold Tris buffer. Filters were extracted and allowed to dissolve overnight in liquid scintillation cocktail. Radioactivity retained by the filters was quantified as described above. Non-specific binding was approximately 35% of total binding. Intra- and inter-assay variation were generally less than 10% and 6% RSD, respectively.

2.3.4.3. ChE Activity Assay

ChE activity was measured as described by Stamler et al. (2005) with minor modifications. Cerebral cortex enzyme fractions (0.5 µg protein) or plasma (20 nL plasma) and resorufin standard solutions (0-2.5 µM resorufin) were added to a 96-well microplate and incubated with Na/K reaction buffer (20 µM Amplex Red, 1 U/mL horseradish peroxidase, 0.1 U/mL choline oxidase and 50 µM ACh). ChE hydrolyzes ACh to form the coproduct choline. The oxidation of choline by choline oxidase to form the coproduct H₂O₂ is coupled to the oxidation of Amplex Red by horseradish peroxidase to form the fluorescent product resorufin. Fluorescence was measured every 5 min between 30 and 90 min at 540/590 nm (excitation/emission) by a microplate fluorometer (Wallac Victor2, PerkinElmer, Boston, MA) at room temperature. Intra- and inter-assay variation were generally less than 5% and 10% RSD, respectively.

2.3.4.4. ACh Concentration Assay

ACh concentration was measured as described by Basu et al. (2006b) with minor modifications. Cerebral cortex enzyme fractions (20 µg protein) and ACh standard solutions (0-2.5 µM ACh) were added to a 96-well microplate and incubated with Na/K reaction buffer (50 µM Amplex Red, 1 U/mL horseradish
peroxidase, 0.1 U/mL choline oxidase and 0.5 U/mL AChE). Fluorescence was measured every 5 min between 45 and 60 min as described above. Intra- and inter-assay variation were generally less than 5% and 10% RSD, respectively.

2.3.5. *In vitro Study*

The direct effects of DE-71 on mAChR binding, nAChR binding and ChE activity *in vitro* were assessed using modified versions of the assays described above.

2.3.5.1. *mAChR and nAChR Binding Assays*

Whole brain membrane fractions were pre-incubated with buffer (total binding) or antagonist/agonist (non-specific binding) for 15 min at room temperature, then with DE-71 (0-23.6 nM DE-71 in 1% DMSO) or 1% DMSO, respectively, for 15 min at room temperature. The water solubility of DE-71 is 13.3 µg/L or 23.6 nM at 25°C (Great Lakes Chemical Corporation, 2005a). The assays were performed three separate times. To assess the effects of DMSO on mAChR binding and nAChR binding *in vitro*, samples were pre-incubated with buffer or antagonist/agonist, then with 0, 0.1% or 1% DMSO.

2.3.5.2. *ChE Activity Assay*

Whole brain enzyme fractions were incubated with Na/K reaction buffer and either DE-71 (0-23.6 nM DE-71 in 0.1% DMSO) or 100 µM BW284c51 (an acetylcholinesterase, AChE, inhibitor) or 1 mM iso-OMPA (a butyrylcholinesterase, BChE, inhibitor). The assay was performed three separate times. To assess the effect of DMSO on ChE activity *in vitro*, samples were incubated with Na/K reaction buffer and 0-1% DMSO.

2.3.6. *Statistical Analysis*

Statistical analyses were performed using SAS Release 8.02 (SAS Institute, Cary, NC). SAS PROC GLM was used to model a one-way ANOVA for the effect of treatment on the cholinergic parameters assayed for adult females and *in vitro* studies, and to model a two-way ANOVA for the effects of treatment and gender.
on the cholinergic parameters assayed for kits and juveniles. In most cases only one female and/or one male offspring from each litter were present in each treatment group. In the cases where more than one female and/or more than one male offspring from a given litter were present in a given treatment group, the average of the females and/or the average of the males were taken to avoid over-inflation of statistical power. Although each treatment group contained two to seven litters, many litters contained only one offspring or offspring of only one gender, and litter was excluded from the analyses to avoid confounding with gender. A p-value of \( p < 0.05 \) was considered statistically significant in all analyses. When a significant difference was detected, the Tukey multiple comparison test was performed.

2.4. Results

2.4.1. Dietary Intake of DE-71
The dietary intake of DE-71 in adult females prior to breeding was estimated to be approximately 0, 0.01, 0.05 and 0.25 mg/kg bw/d for the 0, 0.1, 0.5 and 2.5 \( \mu g/g \) DE-71 groups, respectively (unpublished data, Bursian et al.).

2.4.2. Treatment Groups
In the highest treatment group (2.5 \( \mu g/g \) DE-71), no adult females whelped, although all bred and the majority had implantation sites (unpublished data, Bursian et al.). There were consequently no offspring (kits or juveniles) in the 2.5 \( \mu g/g \) DE-71 group.

2.4.3. In vivo Study
There were no significant effects of in vivo exposure to DE-71 on mAChR binding, nAChR binding, ChE activity or ACh concentration in the cerebral cortex of adult females, 6-week-old kits and 27-week-old juveniles (Tables 5-7, Figure 7).
ChE activity in the cerebral cortex of 6-week-old kits in the 0.5 μg/g DE-71 group was significantly lower (17%) than that in the 0.1 μg/g DE-71 group (p=0.047) but was not significantly lower than that in the control group (0 μg/g DE-71) (p=0.111) (Figure 7).

ChE activity in the plasma of adult females in the highest treatment group (2.5 μg/g DE-71) was 3-fold higher than that in all other treatment groups (p<0.0001) (Figure 8). There was no significant effect of DE-71 on ChE activity in the plasma of 6-week-old kits or 27-week-old juveniles (Figure 8), although no offspring were whelped in the 2.5 μg/g DE-71 group. There were no correlations between ChE activity in plasma and cerebral cortex, but there were significant positive correlations between ChE activity in plasma and both liver weight (r=0.46, p=0.0029) and liver-to-body weight ratio (r=0.62, p<0.0001) in adult females.

2.4.4. In vitro Study

The effects of *in vitro* exposure to DMSO, the solvent carrier of DE-71, on cholinergic parameters were assessed. Receptor binding decreased to 99.7% and 94.2% of the control (0% DMSO) at 0.1% DMSO for mAChR and nAChR, respectively. Receptor binding decreased even further to 89.6% and 90.5% of the control at 1% DMSO for mAChR and nAChR, respectively. ChE activity decreased in a negative exponential manner from 85.2% of the control at 0.1% DMSO to 35.6% of the control at 1% DMSO (Appendix A). To maximize the receptor binding or enzyme activity and the solubility of DE-71, 1% DMSO was used for subsequent *in vitro* receptor binding assays and 0.1% DMSO was used for subsequent *in vitro* enzyme activity assays.

There were no significant direct effects of *in vitro* exposure to DE-71 on mAChR binding, nAChR binding or ChE activity in the receptor and enzyme fractions from the whole brain of untreated mink (Table 8). mAChR binding at 0.745 nM
DE-71 was significantly higher (5%) than that in the control (0 ng/L DE-71) (p<0.05) but not from any other concentration of DE-71.

The ChE activity in the whole brain of untreated mink was between 90-95% AChE and 5-10% BChE.

2.5. Discussion

In a preliminary 70-day DE-71 mink feeding trial, there was a decrease in feed consumption and body weight at dietary concentrations of 5 μg DE-71/g feed and 10 μg DE-71/g feed (unpublished data, Bursian et al.). As a result, a maximum dietary concentration of 2.5 μg DE-71/g feed was selected for the current 1-generation DE-71 mink feeding trial.

There were no significant effects of in vivo exposure to DE-71 on mAChR binding, nAChR binding, ChE activity or ACh concentration in the cerebral cortex of adult females, 6-week-old kits and 27-week-old juveniles. The mAChR binding, ChE activity and ACh concentration measured in the cerebral cortex and ChE activity measured in the plasma were similar to those measured in untreated captive mink in a previous study (Basu et al., 2006b). However, the results of the current study do not corroborate those of Viberg et al. (2003a, 2005), in which neonatal exposure to BDE-99 decreased mAChR density in the hippocampus of adult rats and neonatal exposure to BDE-153 decreased nAChR density in the hippocampus of adult mice. Neonatal exposure to BDE-99 and -153 also disrupted spontaneous behaviour (locomotion, rearing and total activity) and impaired learning and memory in adult mice and rats (Eriksson et al., 1998; Eriksson et al., 2002b; Viberg et al., 2002; Viberg et al., 2003a; Viberg et al., 2004; Viberg et al., 2005), while neonatal exposure to BDE-99 altered the effects of adult exposure to nicotine (a nAChR agonist) on spontaneous behaviour (Viberg et al., 2002). However, perinatal exposure to BDE-99 disrupted sensory and motor behaviour in mice (Branchi et al., 2002; Kuriyama et al., 2005) and neonatal exposure to DE-71 disrupted learning in rats (Dufault et al., 2005), while
perinatal exposure to DE-71 did not disrupt sensory or motor behaviour in rats (Taylor et al., 2002; MacPhail et al., 2003; Taylor et al., 2003). There are several important differences between the current study and those of Viberg et al. (2003a, 2005) (Table 9), which may account for the differences in the results.

The individual congeners BDE-99 and -153 used by Viberg et al. (2003a, 2005) are the most (43.9%) and fourth-most (3.84%) predominant congeners in the DE-71 commercial penta-BDE mixture used in the current study, respectively (Wellington Laboratories Inc., 2005a). However, DE-71 contains other predominant congeners such as BDE-47 (second-highest, 32.4%) and -100 (third-highest, 8.92%), which are also environmentally relevant. They have been found to bioaccumulate in the liver of captive mink following consumption of wild carp collected from the Saginaw River (Michigan, USA) (Bursian et al., 2006), as well as in the brain of wild otter collected from Nova Scotia (unpublished data, Basu et al.). Commercial mixtures may also contain contaminants not present in individual congeners.

The animal model used in the current study was the mink, while that used by Viberg et al. (2003a, 2005) was the rodent (rats and mice). Although the neurobehavioural effects of exposure to PBDEs have not been studied in mink, there are similarities in the neurobehavioural effects of exposure to non-coplanar PCBs between mink and mice (Aulerich et al., 1971; Eriksson and Fredriksson, 1996). There are also similarities in the neurobehavioural effects of exposure to non-coplanar PCBs and PBDEs in rodents (Eriksson and Fredriksson, 1996; Eriksson et al., 2002a), but differences in the neurochemical effects. While BDE-99 decreased hippocampal mAChR density in rats and BDE-153 decreased hippocampal nAChR density in mice, PCB-52 and -153 did not significantly affect cortical or hippocampal cholinergic receptor density in mice (Eriksson and Fredriksson, 1996; Eriksson et al., 2002a; Viberg et al., 2003a; Viberg et al., 2005). PCB-153 increased cortical mAChR density but did not affect hippocampal mAChR density in rats (Coccini et al., 2006). Differences in the
toxicokinetics and toxicodynamics of PBDEs between mink and rodents, and therefore the uncertainty factors, have not yet been determined and may not be based on those of PCBs. However, as a wildlife species, the mink is a more environmentally relevant animal model than the rodent (Basu et al., 2006a).

The radioligand used for mAChR binding in both the current study and that of Viberg et al. (2005) was [3H]-QNB, which binds all five mAChR subtypes (M1-M5). The M1, M2 and M4 subtypes predominate in both the cerebral cortex and hippocampus (Volpicelli and Levey, 2004). However, the radioligand used for nAChR binding in the current study was [3H]-cytisine, which binds the α4β2 nAChR subtype, while that used in the study of Viberg et al. (2003a) was [3H]-α-bungarotoxin, which binds the α7 nAChR subtype. The α4β2 subtype predominates in the cerebral cortex, while the α7 subtype predominates in the hippocampus (Alkondon and Albuquerque, 2004). Moreover, differences in the distribution of the mAChR and nAChR subtypes in the cerebral cortex and hippocampus between mink and rodents have not yet been determined.

The exposures used in the current study were continual, low-concentration dietary doses of DE-71 in adult females from prior to breeding (approximately 0.01 to 0.25 mg/kg bw/d; unpublished data, Bursian et al.) through weaning of their offspring, as well as in utero, lactational and dietary doses in their offspring from gestation through the growth phase. Furthermore, no offspring were whelped in the highest treatment group (2.5 μg/g DE-71) (unpublished data, Bursian et al.), and the next highest treatment group was 5-fold lower (0.5 μg/g DE-71). However, in the studies of Viberg et al. (2003a, 2005), neonatal rats and mice were administered single, high-concentration oral doses of BDE-99 and -153 (0.8 to 16 mg/kg bw and 0.45 to 9 mg/kg bw, respectively). Neurochemical changes were only observed at the highest doses (16 and 9 mg/kg bw, respectively). Mink consume 140 to 200 g/kg bw/d of food, and their diet consists of approximately 50% fish (Aulerich et al., 1999; Sample and Glenn II, 1999). The concentrations of PBDEs in freshwater fish in North America are in the order of 0.01 to 1 μg/g
ww (Hale et al., 2001; Johnson and Olson, 2001), and directly comparable to the range of dietary concentrations used in the current study (0.1 to 2.5 µg DE-71/g feed). Therefore, exposure estimates in mink based on dietary intake of fish are in the order of 0.7 to 100 µg/kg bw/d, also comparable to the range of dietary intake in the current study (approximately 10 to 250 µg/kg bw/d; unpublished data, Bursian et al.). As such, the concentrations and durations of exposure used in the current study are environmentally relevant.

The age at which the animals were sacrificed following developmental exposure to PBDEs was higher in the studies of Viberg et al. (2003a, 2005) than in the current study. Neurochemical changes following developmental exposure to DE-71 were not observed in juvenile mink (27 weeks of age), although decreases in mAChR and nAChR density following developmental exposure to BDE-99 and -153 were observed in adult rats (2 months of age) and mice (6 months of age), respectively (Viberg et al., 2003a; Viberg et al., 2005). Furthermore, the neurobehavioural effects following developmental exposure to BDE-47, -99, -153 and -209 observed in adult mice worsen with age (Eriksson et al., 1998; Viberg et al., 2003a; Viberg et al., 2003b). Therefore, there may be a latency period in the onset and progression of the neurodevelopmental toxicity of PBDEs.

There were no significant effects of in vivo exposure to DE-71 on ChE activity in the plasma of 6-week-old kits and 27-week-old juveniles. However, there was a 3-fold increase in ChE activity in the plasma of adult females in the highest treatment group (2.5 µg/g DE-71) from that in all other treatment groups. As ChE in the plasma is synthesized in the liver and secreted via very low density lipoproteins (VLDL) (Kutty and Payne, 1994), the increase in ChE activity in the plasma may indicate effects of DE-71 on liver function rather than on neurochemistry. This is supported by significant positive correlations between ChE activity in the plasma and both liver weight and liver-to-body weight ratio in adult females, as well as a lack of correlations between ChE activity in the plasma and the cerebral cortex. The results of the current study corroborate those of
Zhou et al. (2001, 2002), in which an increase in liver weight and liver-to-body weight ratio was also observed in rats exposed to DE-71. The physiological and ecological significance of the increase in ChE activity in the plasma is not yet understood.

There were no effects of *in vitro* exposure to DE-71 on mAChR binding, nAChR binding or ChE activity in the receptor and enzyme fractions from the whole brain of untreated mink, indicating that DE-71 did not directly affect these cholinergic parameters. The results of the *in vitro* study support those of the *in vivo* study. The effects of *in vivo* exposure to BDE-99 and -153 on the cholinergic neurotransmitter system observed by Viberg et al. (2002, 2003a, 2005) in rodents are therefore likely not direct but rather mediated in part by other mechanisms such as thyroid hormone disruption. Thyroid hormones regulate neurodevelopment, including the cholinergic neurotransmitter system in the cerebral cortex and hippocampus (Porterfield, 2000). In other studies, a significant decrease in T4 was observed in rats exposed to 1 mg/kg bw/d DE-71 *in utero* and during lactation (Zhou et al., 2002), and in captive mink exposed to 5 µg/g DE-71 in the feed for 70 days (Martin et al., 2004).

In conclusion, this study demonstrated that environmentally relevant exposures to DE-71 did not affect key parameters of the cholinergic neurotransmitter system in the brain of captive mink, neither *in vivo* nor *in vitro*. The lack of effects of DE-71 on cholinergic parameters in mink did not corroborate the effects of BDE-99 and -153 on cholinergic receptors observed by Viberg et al. (2003a, 2005) in rodents, although there are several important differences between the current study and those of Viberg *et al.* (2003a, 2005). Moreover, the lack of neurobehavioural effects of DE-71 observed in rats (Taylor *et al.*, 2002; MacPhail *et al.*, 2003; Taylor *et al.*, 2003) did not corroborate the neurobehavioural effects of DE-71 and BDE-99 observed in rats and mice, respectively (Branchi *et al.*, 2002; Dufault *et al.*, 2005; Kuriyama *et al.*, 2005). Further studies are needed to determine the differences across species and brain regions in the effects of PBDEs.
on the cholinergic neurotransmitter system and the mechanism of
neurodevelopmental toxicity at environmentally relevant doses (continual or
repeated, low-concentration) and durations (in utero, lactation, growth period,
adult).

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(H.M.C.) and ArcticNet (H.M.C.). The application to use animals in research
(05/03-069-00) was approved by the Michigan State University All-University
Committee on Animals Use and Care.

There are no conflicts of interest. The sources of funding had no role in the study
design; in the collection, analysis or interpretation of data; in the writing of the
report; or in the decision to submit the paper for publication.
Table 5: Effects of in vivo exposure to DE-71 on mAChR binding in cerebral cortex of adult female mink, 6-week-old kits and 27-week-old juveniles

<table>
<thead>
<tr>
<th>Diet (μg/g DE-71)</th>
<th>Cerebral cortex mAChR binding (fmol [3H]-QNB/mg protein)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adult females</td>
<td>Six-week-old kits</td>
<td>27-week-old juveniles</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2214 ± 50 (9)</td>
<td>2023 ± 60 (4)</td>
<td>2224 ± 72 (3)</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>2280 ± 47 (10)</td>
<td>2045 ± 49 (6)</td>
<td>2279 ± 37 (10)</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>2267 ± 47 (10)</td>
<td>2184 ± 49 (6)</td>
<td>2223 ± 37 (10)</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>2153 ± 47 (10)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Adult females were fed diets containing 0, 0.1, 0.5 or 2.5 μg/g DE-71 from four weeks prior to breeding through weaning of their kits at six weeks of age. A portion of the weaned kits were maintained on their respective diets through 27 weeks of age. Mink were necropsied and mAChR binding was measured in the receptor fraction. Statistical analyses were performed using one-way ANOVA for adult females (treatment) and two-way ANOVA for kits and juveniles (treatment and gender). Values represent least squares mean ± SEM (n). All p-values > 0.05.
Table 6: Effects of *in vivo* exposure to DE-71 on nAChR binding in cerebral cortex of adult female mink, 6-week-old kits and 27-week-old juveniles

<table>
<thead>
<tr>
<th>Diet (µg/g DE-71)</th>
<th>Cerebral cortex nAChR binding (fmol [³H]-CYT/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adult females</td>
</tr>
<tr>
<td>0</td>
<td>63.3 ± 1.9 (9)</td>
</tr>
<tr>
<td>0.1</td>
<td>67.0 ± 1.8 (10)</td>
</tr>
<tr>
<td>0.5</td>
<td>66.8 ± 1.8 (10)</td>
</tr>
<tr>
<td>2.5</td>
<td>61.0 ± 1.8 (10)</td>
</tr>
</tbody>
</table>

* Adult females were fed diets containing 0, 0.1, 0.5 or 2.5 µg/g DE-71 from four weeks prior to breeding through weaning of their kits at six weeks of age. A portion of the weaned kits were maintained on their respective diets through 27 weeks of age. Mink were necropsied and nAChR binding was measured in the receptor fraction. Statistical analyses were performed using one-way ANOVA for adult females (treatment) and two-way ANOVA for kits and juveniles (treatment and gender). Values represent least squares mean ± SEM (n). All p-values > 0.05.
Table 7: Effects of in vivo exposure to DE-71 on ACh concentration in cerebral cortex of adult female mink, 6-week-old kits and 27-week-old juveniles

<table>
<thead>
<tr>
<th>Diet (μg/g DE-71)</th>
<th>Cerebral cortex ACh concentration (nmol ACh/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adult females</td>
</tr>
<tr>
<td>0</td>
<td>8.52 ± 0.64 (9)</td>
</tr>
<tr>
<td>0.1</td>
<td>8.66 ± 0.61 (10)</td>
</tr>
<tr>
<td>0.5</td>
<td>8.51 ± 0.61 (10)</td>
</tr>
<tr>
<td>2.5</td>
<td>8.29 ± 0.61 (10)</td>
</tr>
</tbody>
</table>

* Adult females were fed diets containing 0, 0.1, 0.5 or 2.5 μg/g DE-71 from four weeks prior to breeding through weaning of their kits at six weeks of age. A portion of the weaned kits were maintained on their respective diets through 27 weeks of age. Mink were necropsied and ACh concentration was measured in the enzyme fraction. Statistical analyses were performed using one-way ANOVA for adult females (treatment) and two-way ANOVA for kits and juveniles (treatment and gender). Values represent least squares mean ± SEM (n). All p-values > 0.05.
Figure 7: Effects of *in vivo* exposure to DE-71 on ChE activity in cerebral cortex of (A) adult female mink, (B) 6-week-old kits and (C) 27-week-old juveniles.
Adult females were fed diets containing 0, 0.1, 0.5 or 2.5 μg/g DE-71 from four weeks prior to breeding through weaning of their kits at six weeks of age. A portion of the weaned kits were maintained on their respective diets through 27 weeks of age. Mink were necropsied and ChE activity was measured in the enzyme fraction. Statistical analyses were performed using one-way ANOVA for adult females (treatment), two-way ANOVA for kits and juveniles (treatment and gender) and Tukey multiple comparison test. Values represent least squares mean ± SEM (n). Lower case letters (a/b) represent significant differences between diets (p<0.05).
Figure 8: Effects of *in vivo* exposure to DE-71 on ChE activity in plasma of (A) adult female mink, (B) 6-week-old kits and (C) 27-week-old juveniles.
Adult females were fed diets containing 0, 0.1, 0.5 or 2.5 µg/g DE-71 from four weeks prior to breeding through weaning of their kits at six weeks of age. A portion of the weaned kits were maintained on their respective diets through 27 weeks of age. Mink were blood sampled and ChE activity was measured in the plasma. Statistical analyses were performed using one-way ANOVA for adult females (treatment), two-way ANOVA for kits and juveniles (treatment and gender) and Tukey multiple comparison test. Values represent least squares mean ± SEM (n). Lower case letters (a/b) represent significant differences between diets (p<0.05).
Table 8: Direct effects of *in vitro* exposure to DE-71 on mAChR binding, nAChR binding and ChE activity in receptor and enzyme fractions from whole brain of untreated mink.

<table>
<thead>
<tr>
<th>Concentration (nM DE-71)</th>
<th>mAChR binding (% of control)</th>
<th>nAChR binding (% of control)</th>
<th>ChE activity (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>100 ± 0.00 (a)</td>
<td>100 ± 0.00</td>
<td>100 ± 0.00</td>
</tr>
<tr>
<td>0.00745</td>
<td>104 ± 2.31 (a,b)</td>
<td>105 ± 7.05</td>
<td>98.7 ± 1.17</td>
</tr>
<tr>
<td>0.0236</td>
<td>104 ± 1.53 (a,b)</td>
<td>115 ± 12.1</td>
<td>96.7 ± 2.69</td>
</tr>
<tr>
<td>0.0745</td>
<td>101 ± 1.47 (a,b)</td>
<td>112 ± 5.00</td>
<td>101 ± 2.10</td>
</tr>
<tr>
<td>0.236</td>
<td>102 ± 1.53 (a,b)</td>
<td>116 ± 2.31</td>
<td>98.3 ± 1.70</td>
</tr>
<tr>
<td>0.745</td>
<td>105 ± 0.58 (b)</td>
<td>105 ± 11.0</td>
<td>98.1 ± 0.87</td>
</tr>
<tr>
<td>2.36</td>
<td>103 ± 2.00 (a,b)</td>
<td>112 ± 12.8</td>
<td>96.6 ± 1.11</td>
</tr>
<tr>
<td>7.45</td>
<td>104 ± 1.00 (a,b)</td>
<td>110 ± 5.77</td>
<td>99.4 ± 1.89</td>
</tr>
<tr>
<td>23.6</td>
<td>103 ± 1.00 (a,b)</td>
<td>103 ± 4.16</td>
<td>97.4 ± 1.97</td>
</tr>
</tbody>
</table>
Receptor and enzyme fractions from the whole brain of untreated mink were incubated with 0-23.6 nM DE-71, and mAChR binding, nAChR binding and ChE activity were measured. Statistical analyses were performed using one-way ANOVA and Tukey multiple comparison test. Values represent mean ± SD (n=3). Letters (a/b) represent significant differences within columns (p<0.05).
Table 9: Comparison of the current study and those of Viberg et al. (2003a, 2005).

<table>
<thead>
<tr>
<th>Results</th>
<th>Current study</th>
<th>Viberg et al. studies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No effect on mACHR, nACHR</td>
<td>Decrease in mACHR, Decrease in nACHR</td>
</tr>
<tr>
<td>PBDE</td>
<td>DE-71</td>
<td>BDE-99</td>
</tr>
<tr>
<td>Animal model</td>
<td>Mink</td>
<td>Rat</td>
</tr>
<tr>
<td>Brain region</td>
<td>Cerebral cortex</td>
<td>Hippocampus</td>
</tr>
<tr>
<td>Radioligand</td>
<td>[³H]-cytisine (nACHR, α4β2 subtype)</td>
<td>[³H]-α-bungarotoxin (nACHR, α7 subtype)</td>
</tr>
<tr>
<td>Dose</td>
<td>Continual, low-concentration</td>
<td>Single, high-concentration</td>
</tr>
<tr>
<td>Exposure</td>
<td>Adult, in utero, lactation, growth period</td>
<td>Neonate</td>
</tr>
<tr>
<td>Age at sacrifice</td>
<td>Adult, 6 weeks (kit), 27 weeks (juvenile)</td>
<td>Adult</td>
</tr>
</tbody>
</table>
Final Conclusion & Summary

Polybrominated diphenyl ethers (PBDEs) are a class of brominated flame retardants that are recognized as global environmental contaminants and a potential health risk. They have been shown to elicit neurodevelopmental toxicity through disruption of the cholinergic neurotransmitter system in rodent models, but the effects of environmentally relevant exposures in wildlife species are unknown. The objective of this study was to assess the effects of the commercial pentabrominated diphenyl ether mixture DE-71 on cholinergic parameters in captive mink (Mustela vison) following dietary exposure of adult females and in utero, lactational and dietary exposure of their offspring. Adult females were fed diets containing 0, 0.1, 0.5 or 2.5 µg/g DE-71 from four weeks prior to breeding through weaning of their kits at six weeks of age. A portion of the weaned kits were maintained on their respective diets through 27 weeks of age. Cholinergic neurochemical biomarkers, including muscarinic acetylcholine receptor (mAChR) and nicotinic acetylcholine receptor (nAChR) binding, cholinesterase (ChE) activity and acetylcholine (ACh) concentration, were assayed in the cerebral cortex, and ChE activity measured in the plasma. Results indicated no significant effects of DE-71 on cholinergic parameters in the cerebral cortex, but a 3-fold increase in ChE activity in the plasma of adult females in the 2.5 µg/g DE-71 group. There were also no direct effects of DE-71 on mAChR or nAChR binding or ChE activity in the enzyme and receptor fractions from the whole brain of untreated mink following in vitro exposure to 0-23.6 nM DE-71.

There were significant effects of DE-71 on reproductive parameters (a decrease in whelping success) and liver parameters (an increase in plasma ChE activity, liver weight and liver-to-body weight ratio) of adult females in the 2.5 µg/g DE-71 group. Within this one-generation DE-71 mink feeding trial, studies on the effects of DE-71 on reproduction (Bursian et al., 2006; personal communication), as well as liver and thyroid function and histology (Martin et al., 2006; personal communication), are being conducted. In addition, the bioaccumulation of
PBDEs and PBDE metabolites in the cerebral cortex and plasma, as well as other tissues, (Martin et al., 2006; personal communication) are being measured.

In conclusion, this study demonstrated that environmentally relevant exposures to DE-71 did not affect key parameters of the cholinergic neurotransmitter system in the brain of captive mink, neither in vivo nor in vitro. The lack of effects of DE-71 on cholinergic parameters in mink did not corroborate the effects of BDE-99 and -153 on cholinergic receptors observed by Viberg et al. (2003a, 2005) in rodents, although there are several important differences between the current study and those of Viberg et al. (2003a, 2005). Moreover, the lack of neurobehavioural effects of DE-71 observed in rats (Taylor et al., 2002; MacPhail et al., 2003; Taylor et al., 2003) did not corroborate the neurobehavioural effects of DE-71 and BDE-99 observed in rats and mice, respectively (Branchi et al., 2002; Dufault et al., 2005; Kuriyama et al., 2005).

Further studies are needed to determine the differences across species and brain regions in the effects of PBDEs on the cholinergic neurotransmitter system and the mechanism of neurodevelopmental toxicity at environmentally relevant doses (continual or repeated, low-concentration) and durations (in utero, lactation, growth period, adult). The differences in bioaccumulation of PBDEs and PBDE metabolites across species and brain regions are also unknown. Future studies are also needed to determine the effects of PBDEs on other neurotransmitter systems such as the dopaminergic or GABAergic systems, as well as the interactions between neurotransmitter systems. The potentially additive or synergistic effects of mixtures of environmental contaminants, including organohalogen compounds such as PBDEs, PCBs, and perfluorinated compounds (PFCs), as well as metals such as mercury, are also not well known. These studies are important for the assessment of the neurotoxic effects of PBDEs and other environmental contaminants in the characterization of risk.
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Appendices

Appendix A: Effect of \textit{in vitro} exposure to DMSO on ChE activity in enzyme fraction from whole brain of untreated mink $^a$.

$^a$ The enzyme fraction from the whole brain of untreated mink was incubated with 0-1\% DMSO, and ChE activity was measured. Values represent mean.