University of Alberta

Bioaccumulation, Tissue Distribution, Excretion and Metabolism of Polybrominated Diphenyl Ethers in Farmed Mink

by

Si Zhang

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science
in
Medical Science — Laboratory Medicine and Pathology

Department of Laboratory Medicine and Pathology

Edmonton, Alberta
Spring 2008

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Your file Votre référence
ISBN: 978-0-494-45914-0
Our file Notre référence
ISBN: 978-0-494-45914-0

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Abstract

Polybrominated diphenyl ethers (PBDEs) are brominated flame retardants that have been, and still are, used in consumer products. They are now ubiquitous in the environment. This may present environmental health risks given that toxicological studies have demonstrated several adverse effects in animals.

Taking mink as a study subject, this thesis examined the tissue distribution, maternal transfer, biotransformation, and biomagnification of the technical penta-BDE mixture, DE-71. Moreover, this thesis studied in vitro biotransformation pathways using mink hepatic microsomes and rat intestinal microflora.

Most BDE congeners were accumulative in mink tissues and on a whole-body basis. Maternal transfer of PBDEs favoured lactational transfer rather than transplacental transfer. Different BDE congeners exhibited different biomagnification potential, depending on the bromine substitution pattern. The biomagnification factors were significantly higher than one for some PBDEs, but biotransformation, such as the detected hydroxylation process mediated by mink hepatic microsomes, clearly limited the biomagnification of some other PBDEs.

Acknowledgements

I would like to express my sincere thanks to Dr. Jonathan Martin for his involvement in this project; his guidance and support were invaluable.

I would also like to express my gratitude to Dr. Steven Bursian and Dr. Pamela Martin for providing mink samples. Dr. Steve Bursian also receives my sincere thanks for sharing research data and providing helpful advice on editing my first manuscript.

I would like to address my thanks to Dr. Phil Fedorak and Ms. Debbi Coy for their guidance with anaerobic techniques. Nick Warner receives my thanks for sharing his experience in the metabolism study and providing a simple and safe method for the derivatization of hydroxylated compounds.

Thank you also to my examining committee, Dr. Xing-Fang Li and Dr. Phil Fedorak, for taking the time to read and critique my work; and to Dr. Chris Le for chairing my defense.

I would also like to thank my husband, Yongjie Liu, for his encouragement and support, and my parents for encouraging me to follow my own path in life.

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List of Abbreviations

ASE	Accelerated solvent extraction
BAF	Bioaccumulation factor
BCF	Bioconcentration factor
BDE(s)	Brominated diphenyl ether(s)
BFR	Brominated flame retardant
BMF	Biomagnification factor
CNS	Central nervous system
bw	Body weight
DTT	Dithiothreitol
dw	Dry weight
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EROD	Ethoxy-resorufin-*O*-deethylase
GC	Gas chromatography
GSH	Glutathione
LOAEL	Lowest-observed-adverse-effect-level
K_{OC}	Water-organic carbon partition coefficient
K_{OW}	Water-octanol partition coefficient
lw	Lipid weight
MDL	Method detection limit
MS	Mass spectrometry

m/z	Mass-to-charge ratio
NADPH	Reduced form of nicotinamide ademine dinucleotide phosphate
NOAEL	No-observed-adverse-effect-level
OH-PBDE(s)	Hydroxylated PBDE(s)
PBDE(s)	Polybrominated diphenyl ether(s)
PCB(s)	Polybrominated biphenyl(s)
POP(s)	Persistent organic pollutant(s)
pg/L	Picogram per litre
PROD	7-pentoxy-resorufin-*O*-deethylase
PUF	Polyurethane foam
STP	Sewage treatment plant
TRH	Thyrotropin-releasing hormone
TSH	Thyroid-stimulating hormone
TTR	Transthyretin
T3	Triiodothyronine
T4	Thyroxine
UDPGA	Uridinediphosphate-glucuronic acid
UDPGT	Uridinediphosphate-glucuronosyltransferase
ww	Wet weight

Chapter 1: Introduction

1.1 Brominated flame retardants (BFRs) and their function, value, and general problems posed

Chemical flame retardants have a long history of application to prevent things from catching fire, which can be traced back to Roman times [1]. The extensive usage of these substances began after the introduction of polymeric materials which are very beneficial but more inflammable than traditional materials [1, 2].

To understand how flame retardants work, it is necessary to understand the combustion process. There is an important process involved in the ignition of solid materials: pyrolysis. During heating of solid materials, high energy free radicals ($H^{\cdot}$ and $^{\cdot}OH$) present in the gas phase react with and decompose the carbon bond of solid materials (i.e. fuel). Short-chain and small flammable molecules are then released into the gas phase and start to burn, thus providing more heat and, in turn, more flammable gases to burn [3]. When the relatively small fire provides enough heat to ignite all flammable materials simultaneously in a region, flashover occurs [1, 3].

Organohalogen compounds can act as electron accepters to form non-flammable gases with free radicals emitted during the initial stages of pyrolysis. This reduces both the generation of heat and the production of flammable gases induced by free radical chain reactions [1, 4, 5]. As a result, flashover is delayed, thus providing people extra time to escape or to put out the fire. Effective organohalogen flame retardants must have

a strong ability to capture free radicals and a suitable decomposing temperature that is slightly lower than the host polymer [4, 5]. With increasing radius of halogen elements, the ability of capturing free radicals increases, but halogenated compounds become less stable under slightly elevated temperature [5]. To achieve the best balance, BFRs are very suitable and have become the most cost-effective flame retardants [3, 5].

The practice of requiring flame retardants in flammable materials saves lives and reduces fire damage and associated costs [3]. However, the benefits of BFRs must be balanced with the associated risks. The risks of using BFRs were not noticed until BFRs were found in remote areas of the globe, thus providing a clue that they had become ubiquitous in our environment [6]. Subsequent studies on the environmental concentrations in various regions of the world did not catch much attention until BFRs were reported to be doubling their concentrations in breast milk every five years in 1998, whereas polychlorinated biphenyls (PCBs) were reported to be simultaneously declining [7]. Later on, further evidence emerged relating to the increasing trend of BFRs in the environment [8, 9]. This led to studies on the environmental fate, persistence, and toxicity of BFRs. Initially toxicity studies related to their acute toxic effects from high doses, and later to sub-chronic and chronic toxicity with environmentally relevant concentrations [9]. In general, results indicated that some BFRs were similar in many ways to persistent organic pollutants (POPs) — chemicals that are slow to degrade in the environment, become widely distributed geographically due to long-range transport, and accumulate in fatty tissues of living organisms through bioconcentration and food chain accumulation and which can be toxic at relatively low concentrations [10, 11].

BFRs can be classified based on the way in which they are incorporated into their host polymers: either reactive or additive. Reactive BFRs such as tetrabromobisphenol A are incorporated with host polymers by covalent bonds, whereas additive BFRs, such as the polybrominated diphenyl ethers (PBDEs) and hexabromocylododecane (HBCD) are simply dissolved in host polymers. Consequently, the latter can leach into the environment more easily [4, 5, 12] and thus may pose a higher risk of exposure to humans and wildlife. The present thesis focuses on PBDEs, a family of additive BFRs which were historically and still today, produced and applied in the greatest quantities compared to all other additive BFRs [1].

1.2 Overview of PBDEs

1.2.1 What are PBDEs and their physicochemical properties

1.2.1.1 PBDEs and their structures

PBDEs are a family of structurally related BFRs. From a chemical architecture point of view, the structure of PBDEs (Figure 1-1) includes an ether bond connecting two phenyl rings, and 1-10 bromine atoms may be covalently bound to the 10 available positions of the two phenyl rings. Depending on the degree of bromination and the position of the bromine atoms on the phenyl rings, there are 209 possible PBDE congeners. These congeners are numbered following the same rules established for PCBs (Table 1-1).

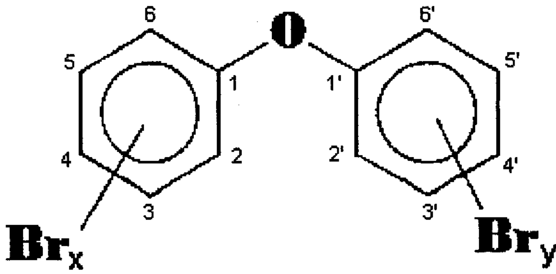


Figure 1-1: The general structure of PBDEs, where x + y = 1 to 10.

Table 1-1: Names, structures and congener numbers of selected PBDEs.

Name	**Congener number**	**Structure**
2,4,4'-tribromodiphenyl ether	BDE 28	
2,2',4,4'-tetrabromodiphenyl ether	BDE 47	
2,2',4,4',5-pentabromodiphenyl ether	BDE 99	
2,2',4,4',6-pentabromodiphenyl ether	BDE 100	
2,2',4,4',5,5'-hexabromodiphenyl ether	BDE 153	
2,2',4,4',5,6-hexabromodiphenyl ether	BDE 154	

Name	Congener number	Structure
2,2',3,4,4',5,6'-heptabromodiphenyl ether	BDE 183	
Decabromodiphenyl ether	BDE-209	

In the laboratory, pure PBDE congeners can be synthesized by coupling a diphenyliodonium salt with bromophenolate without subsequent cleanup procedures [13]. In industry, however, most PBDE products are manufactured by bromination of diphenyl ether in the presence of a Friedel-Craft catalyst (i.e. $AlBr_3$ or $FeBr_3$) in a brominated solvent such as dibromomethane; and thus usually contain many isomers and homologues in the resulting technical mixtures [4, 5, 12]. Three major technical brominated diphenyl ether mixtures were manufactured and sold commercially, termed penta-BDE, octa-BDE, and deca-BDE classified based on the average bromine substitution content (Table 1-2).

Table 1-2: Compositions of commercial PBDE mixtures.

Technical Mixtures	**Composition (Percentage of Total) [14]**							
	Tri-BDEs	Tetra-BDEs	Penta-BDEs	Hexa-BDEs	Hepta-BDEs	Octa-BDEs	Nona-BDEs	Deca-BDEs
Penta-BDE	0–1%	24–38%	50–62%	4–8%				
Octa-BDE				10–12%	43–44%	31–35%	9–11%	0–1%
Deca-BDE							0.3–3%	97–98%

1.2.1.2 Physicochemical properties

It is essential to discuss the physicochemical properties of PBDEs (Table 1-3) in order to understand their environmental partitioning and fate. The large values of log K_{ow} of PBDEs reflect their relatively poor water solubility and high lipophilicity. Therefore, PBDEs tend to partition to lipid-rich compartments such as fish from water. This is presumed to be their mechanism of bioconcentration.

Table 1-3: Water solubility and octanol-water partitioning coefficients (log K_{ow}) of PBDEs.

	Penta-BDE	**Octa-BDE**	**Deca-BDE**	**Ref.**
S_w [a](25°C) (mol/L)	3.04×10^{-8} (BDE 47) 7.74×10^{-9} (BDE 99) 7.82×10^{-11} (BDE 153)	n.a.	n.a.	[15]
log K_{ow} [a]	6.78 (BDE 47) 7.39 (BDE 99) 8.05 (BDE 153)	n.a.	n.a.	[15]
log K_{ow} [b]	6.5–7.0	8.4–8.9	10	[12] (citing Watanabe and Tatsukawa, 1990)
Vapour pressure (Pa at 25°C)	2.19×10^{-5} (BDE 47) 2.88×10^{-6} (BDE 85) 1.26×10^{-5} (BDE 99)			[16]
Melting point (°C)	92 (BDE 99) 97–98 (BDE 100)	~200	290–306	[9] (citing from other resources)
Boiling point (°C)	310–425 (for all three commercial mixtures)			[4]

[a] values are from reference [15]. [b] values are from reference [12]. n.a. – not available.

According to their vapour pressure, PBDEs have relatively low volatility, and within the family of homologues the vapour pressure decreases with increasing number of bromine atoms [16, 17]. Vapour pressure is also associated with the position of

substituted bromine, such that *ortho*-position substituted PBDEs have higher vapour pressure than their *meta*- or *para*-isomers [17].

1.2.2 Application, production, and regulations of PBDEs

The only use for PBDEs is in flame retardant applications. They are added at concentrations ranging from 5 to 30% by weight into raw materials including polymers, resins, substrates, and common plastics [9, 14]. The raw materials are then incorporated into various final products such as electronic devices (e.g. TV sets, computers, appliances), building materials (e.g. insulation, laminate flooring), and textiles (e.g. carpets, automotive interiors) [14], which people come into contact with in daily life. The commercial deca-BDE has been, and still is, the most widely used BDE mixture in electric components, whereas the penta-BDE was mainly used in coating and stuffing materials, and the octa-BDE was primarily used (95%) in acrylonitrile-butadiene-styrene (i.e. ABS) plastic [2, 14].

The market demand for PBDEs (Table 1-4) was estimated to account for 33% of the global BFRs production in 1999, and based on estimates from 2001, the total demand had increased by 265 metric tons with a decrease in demand for penta- and octa-BDE and an increase in demand for deca-BDE [1, 2, 12]. From a continental perspective, the Americas consumed the largest volumes of penta-BDE, which accounted for 98% of the production volume of total penta-BDE in 1999 and for 95% in 2001, and also consumed 40% and 44% of octa- and deca-BDE, respectively, in 2001 [2].

Table 1-4: Market demand for PBDEs worldwide.

PBDE Products		Americas	Europe	Asia	Total
1990 [12]					
	Penta-BDE	n.a.	n.a.	n.a.	4,000
	Octa-BDE	n.a.	n.a.	n.a.	6,000
	Deca-BDE	n.a.	n.a.	n.a.	30,000
1999 [1]					
	Penta-BDE	8,290	210	n.a.	8,500
	Octa-BDE	1,375	450	2,000	3,825
	Deca-BDE	24,300	7,500	23,000	54,800
2000 [2]					
	Penta-BDE	7,100	150	150	7,500
	Octa-BDE	1,500	610	1,500	3,790
	Deca-BDE	24,500	7,600	23,000	56,100

n.a. not available. Unit: metric ton.

However, when PBDEs were found to exhibit similar properties as POPs, a number of countries and international organizations began to establish regulations to ban production, or certain uses, of PBDEs, particularly the penta- and octa-BDEs [5, 18, 19]. The use of penta-BDE was voluntarily phased out from the Japanese market [5], and the European Union (EU) led the way in phasing out the use of penta- and octa-BDEs in 2003, and further banned them in August 2004. As of July 1, 2006, the EU has also banned the deca-BDE for use in electronic products and is considering a full ban of the deca-BDE by 2008 [20]. In the US, several states also took action in phasing out the use of penta- and octa-BDEs since 2004 [18]. Except for Washington and Maine, no other states have enacted restrictions on the use of deca-BDE [18].

In Canada, PBDEs were added to the *List of Toxic Substances* under the *Canadian Environmental Protection Act* by the Canadian government in 2006 [20]. Commercial penta-BDEs have also been voluntarily phased out in Canada since 2005, and the regulation completely banning the manufacturing, use, sale, or import of commercial

penta- and octa-BDEs came into effect at the end of 2007 [20]. However, the use of deca-BDE was not restricted [21].

1.2.3 PBDEs in the environment

A schematic representation of the multiple pathways for PBDEs in the environment is shown in Figure 1-2.

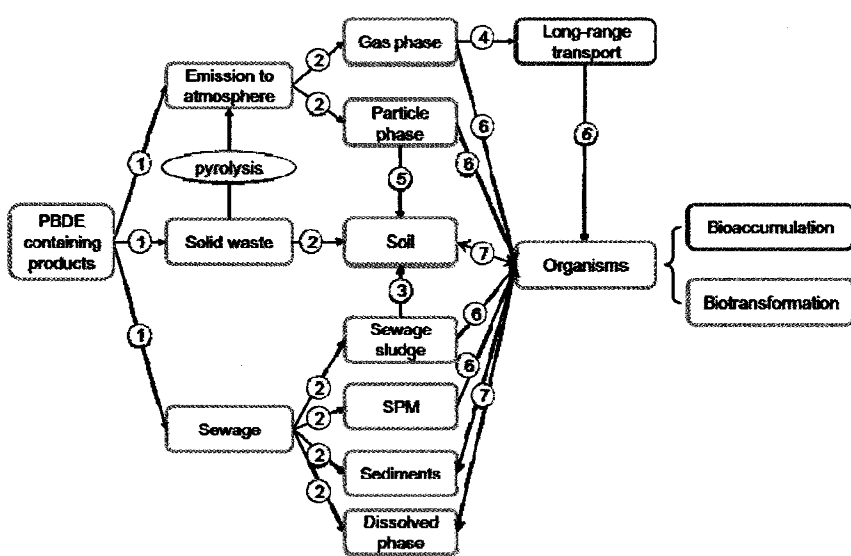


Figure 1-2: Environmental partitioning and fate of PBDEs. Concepts in orange frames will be discussed in the section of "environmental levels and trends" (Section 1.2.3.1); concepts in purple frames will be discussed under "environmental behaviour and fate" (Section 1.2.3.2); and the concept in the green frames will be discussed under "biotransformation by microorganisms" (Section 1.2.3.2.3) and "metabolism" (Section 1.2.4.4). ① PBDEs in commercial products may enter the environment through solid or aqueous wastes or by partitioning to the atmosphere from product surfaces where they are non-covalently bound [4]. ② In the atmosphere, PBDEs are either present in the gas phase or are associated with the particle phase; PBDEs can leach into soil from the solid waste; in water, PBDEs partition between the dissolved phase and solid phases (sewage sludge, SPM, sediments). ③ Sewage sludge is commonly applied to agricultural lands for fertilization, and thus may enter the soil. ④ Gas phase PBDEs undergo long-range transport. ⑤ Particle phase PBDEs undergo deposition to the soil. ⑥ Uptake of PBDEs by organisms can occur through inhalation and ingestion of solids. ⑦ Interaction of PBDEs between some environmental compartments and organisms, where biotransformation and bioaccumulation change the concentrations and forms of PBDEs.

1.2.3.1 Environment concentrations and trends

1.2.3.1.1 PBDEs in the abiotic environment

1.2.3.1.1.1 PBDEs in the atmosphere

The non-covalent nature of PBDEs in polymers allows them to easily enter the atmosphere from the product surfaces through simple partitioning via volatilization [4, 12, 22, 23]. Sources of atmospheric PBDEs, especially in the indoor environment, could also be dust formed from fragments of PBDE-treated polymers [24]. The significant associations shown between suddenly high concentrations of PBDEs in the air and large combustion events, such as a bonfire festival [25], or incineration of waste [11, 26, 27] indicate that combustion of PBDE-treated products is also a source of PBDEs entering the atmosphere.

Atmospheric concentrations of PBDEs are usually monitored using high volume air sampling or passive diffusion air sampling. The former requires a high-volume pump to actively pull air through a glass microfiber filter to collect the particle-bound PBDEs, followed by adsorbents to collect the gas-phase PBDEs. The latter generally involves collection of only the gas-phase compounds through simple steady-state partitioning onto an adsorbent, similar to the adsorbents used in the high-volume sampler; however, the passive sampling is designed to exclude the particle-associated compounds [28]. Two types of adsorbents have been used for PBDE sampling: XAD-2® resin [26, 29] and polyurethane foam (PUF) plugs [27, 28, 30-38]. Gas-phase air concentrations can be estimated from passive samples by back-calculation from concentrations of other media

using the media-air partition coefficient such as K_{FA} (film-air coefficient) [39]. Detailed atmospheric concentrations of PBDEs are summarized in Appendix I, where concentrations of ΣPBDEs, excluding BDE 209 (shown separately), and concentrations of ΣPCB in the same samples are also listed for comparison.

In general, ΣPBDEs are slightly lower but comparable to ΣPCBs in most air samples reported [36, 40]. BDE 209 generally dominates the air samples followed by BDE 47 or BDE 99, with an exception that in earlier (1997–1999) samples from Chicago, BDE 209 was present at much lower concentrations than the rest of the congeners. However, after Hoh and Hites collected more recent (2002–2003) samples at the same site showing the predominance of BDE 209, they declared that the increased usage of deca-BDE in the US was responsible for the change [33]. Indoor concentrations are always significantly higher than outdoor concentrations [41] irrespective of where samples were collected such as in industrialized urban [30], non-industrialized urban [38], suburban, or rural areas [39]. Significant positive correlations have been shown between the concentration of PBDEs in air and the number of electrical appliances and PUF chairs at the indoor location [30]. Compared to other endocrine-disrupting chemicals in the indoor atmosphere (air + dust), PBDEs were slightly lower in concentration than phthalates and alkylphenols, but comparable or slightly higher than pesticides and PCBs [42]. The high concentrations of PBDEs in indoor dust compared to importance of the gas-phase PBDEs may result from fragments of PBDE-treated polymers [43]. In addition, the trend of the atmospheric concentration of PBDEs in urban > suburban > rural areas indicated a linkage between high emission and heavy usage of PBDEs [28]. Taken together, atmospheric concentrations of PBDEs are clearly emitted from microenvironments or the

regions where they are applied or used most heavily [24]; but the occurrence of detectable PBDEs in remote areas, far from known sources, indicates the role of long-range transport in the fate of PBDEs [11, 28].

PBDEs partition between the gas- and particle-phase according to their vapour pressures, whereby lower brominated congeners[1] partition to a greater extent into the gas phase than higher brominated congeners. Therefore, it is not surprising that only the congeners having fewer than six bromine atoms can be detected using passive diffusion samples [28, 40]. Furthermore, the fact that up to 70% of the hexa-BDEs partitioning to the particle phase at 20°C [29] indicates the potential underestimation of the detectable PBDE homologues using passive diffusion sampling. The partitioning of higher brominated congeners to the particle phase may limit their long-range transport, possibly via scavenging by precipitation events which are a significant atmospheric removal pathway for PBDEs [36]. Because increasing temperature increases the vapour pressure in general, total PBDE concentrations are somewhat higher in the summer and a higher fraction is present in the gas phase, whereas in the winter PBDEs tend to partition to other environmental compartments such as soil [34]. Soil and sediment is discussed further in the next section, but there is a latitudinal fractionation of PBDEs, due to differential atmospheric transport, in sediments and soils whereby lower brominated congeners such as BDE 47 become more important in northern latitudes [44-46], whereas higher brominated congeners, such as BDE 209, diminish with increasing latitude [11].

[1] Lower brominated BDE congeners have seven or fewer bromine atoms, whereas higher brominated congeners contain more than seven bromine atoms.

1.2.3.1.1.2 Soil, sewage sludge, and sediment

Besides their wide distribution in the atmosphere, PBDEs are also detectable in the solid compartments of our abiotic environment. The concentration and trend of PBDEs in the solid compartments will be discussed in three parts: soil, sewage sludge, and sediments. Generally, the profiles of PBDEs in these samples (soil, sewage sludge, and sediments) are either similar to the penta-BDE mixture, or dominated by BDE 209, depending on local use patterns.

The concentration of PBDEs in soil is generally low, whereas aquatic sediments contain higher concentrations, and sewage sludge contains the highest (Appendix II). Despite that sewage sludge is usually applied to agricultural land for fertilization [47, 48], resulting in 2 to 3-fold increase in PBDE concentrations [49, 50], the amended agricultural soil still contains lower concentrations than sediment and sewage sludge from various regions (Appendix II) unless there is a local source to the limited area [51]. Such contributions can be localized by matching the congener profile between the suspected sites to the sources [44, 45, 52, 53]. For example, in the Arctic region, the low concentration and the congener profile of PBDEs in the soil could be attributed to the atmospheric deposition [11]. The lower concentrations in background soil are presumably due to relatively few sources of contribution (e.g. atmospheric deposition only) compared to sewage sludge that accumulates discharged PBDEs from industrial and residential sewage [24] or compared to sediments that connect to the water system which often contains wastewater from surrounding municipalities or cities.

The congener profiles of sewage sludge reflect local discharge [44, 51] and can vary among regions, with especially large differences that are observed in the percentage

contribution of BDE 209 [48]. Generally, sewage sludge samples obtained from the US had about 10-fold higher PBDE concentrations than European samples [24], probably due to larger usage in the US.

The major sources of sediment-borne PBDEs in urban and rural regions are also from industrial and residential discharges. The closer to the source of discharge, the higher the concentrations are [11, 52, 54-58]. Whereas in remote regions such as the Arctic, similar to the soil, the only source of PBDEs is likely atmospheric deposition, and thus the concentrations among various locations (Norway, Russia, and Canada) were similar [11]. Marine sediments generally had lower concentrations of PBDEs than freshwater sediments, except for where there was local contamination [11].

The few disturbances in sediments make them the best model to predict the historical time trend of PBDE usage. A study in Europe which separately analyzed the layers of sediment corresponding to different years indicated the usage of penta-BDE began in the early 1970s and declined between 1995 and 1997, whereas the usage of deca-BDE began in the late 1970s and had itself subsequently declined in the most recent years (e.g. 1995–1997) [59]. This temporal trend was also suggested by Nylund et al. who found a rapid increasing concentration of PBDEs from the 1950s in Sweden [60], and by another study of layered sediments from Greenland, which also showed increasing concentrations of PBDEs in the layers dated 1990 to 2000 [46]. These results predicted from sediment studies match well with the investigation of PBDE usage by Prevedouros et al. during these periods [61].

1.2.3.1.1.3 Water

Data on PBDE water concentrations are very limited, and most are for effluents from sewage treatment plants (STPs). However there are data for suspended particulate matter (SPM) in some surface water. This is not surprising because the low water solubility of these compounds makes PBDEs difficult to detect in the dissolved phases, whereas they tend to be adsorbed on suspended particulates particularly as the degree of bromination increases (Table 1-3).

STP effluents contain a large amount of solids and lipids, and are thus a good reservoir for PBDEs. North reported PBDE concentrations in STP effluent in California, US, which contained 273 ng/L for ΣPBDEs (mono – nona) and 1.73 ng/L for BDE 209 [62]. The sewage sludge from the same STP was also reported as 2198 ng/g for ΣPBDEs (mono – nona) and 1183 ng/g for BDE 209. The concentrations of PBDEs in STP effluents and sewage sludge from a Canadian city were reported by Rayne and Ikonomou, and ranged from 1 to 392 ng/L in the effluents and up to 2429 ng/g in the sludge [63]. STP effluent concentrations from the Netherlands were reported based on the concentration of the particulate matter (ng/g) collected by centrifugation of the effluents [64]. The median of ΣPBDE concentration (BDE 47, 99, and 153) was 22 ng/g and of BDE 209 was 350 ng/g. As a result, PBDE burdens in STP effluents were similar between two North American STPs and were 10-fold higher than the European STP; moreover, this trend is well correlated with the trend observed in sewage sludge.

Surface water concentrations from Lake Ontario and Lake Michigan have been reported at 4 to 13 pg/L and 21 pg/L, respectively, and consisted mainly of BDE 47 and 99 (>70% in Lake Ontario and >89% in Lake Michigan) [65, 66]. The dissolved BDE 47

in Lake Ontario was 0.06 to 3.6 pg/L and the particulate matter adsorbed BDE 47 was 0.05 to 0.23 pg/L. A similar ratio between dissolved to particulate matter adsorbed BDE 47 was observed in Lake Michigan where BDE 47 was 10 pg/L and 1.3 pg/L in the dissolved and particulate phases respectively [66]. Dissolved and particulate phase BDE 99 and 100 in Lake Michigan were also reported and were, respectively, 6.1 and 1.4 pg/L for BDE 99 and 1.3 and 0.18 pg/L for BDE 100. Congeners having more than five bromine atoms were not detected in either of these samples. In addition, PBDE concentrations were 1 to 2 orders of magnitude lower than the PCBs in the same sample.

Surface water samples obtained from rivers in the Netherlands were analyzed by de Boer et al. and the results revealed that suspended particulate matter (SPM) associated ΣPBDEs (BDE 47, 99, and 153) (4.6 ng/g dry weight (dw)) was much lower than BDE 209 (71 ng/g dw) [64]. This may be attributed either to the larger discharge of BDE 209 or to the lower water solubility of heavier congeners (Table 1-3). Consequently, dissolved lower brominated congeners may be more bioavailable to aquatic organisms [24].

The presence of certain PBDEs in both the dissolved and particle phases in surface water allows the estimation of water-organic carbon partition coefficients (K_{oc}) when the organic carbon content of SPM is recorded. The correlation between K_{ow} and K_{oc} can be used to examine the equilibrium partitioning of PBDEs between the dissolved and particle phases. A positive correlation with a slope of 1 would indicate the equilibrium partitioning. However, few studies have found such a correlation for PBDEs and PCBs and the authors suggested either a lack of equilibrium or partitioning to a colloidal "third" phase [66]. It is also possible that octanol is not a perfect model for SPM organic matter.

1.2.3.1.2 PBDEs in wildlife

1.2.3.1.2.1 Concentrations

Due to their high K_{ow}, PBDEs tend to accumulate in lipid-rich tissues of exposed wildlife. The concentrations of ΣPBDEs (sum of tri-hepta BDEs), BDE 209, and partial ΣPCB concentrations (the number of summed congeners varied between studies) in wildlife including invertebrates, fish, frogs, birds, terrestrial mammals, and marine mammals from North America, Europe, Asia, and the Arctic region are summarized in Appendix III. A 5-order-of-magnitude range was observed for PBDE concentrations across all the samples obtained during 1979 to 2005; the lowest occurring in suet samples from reindeer obtained in Sweden in 1986 at 0.51 ng/g lipid weight (lw) [67] and the highest in eggs from herring gull in Lake Michigan in 2000 at 1.3×10^{4} ng/g lw [68]. The corresponding ΣPCB concentrations (depending on the number of summed PCB congeners), if applicable, were 1 to 2 orders of magnitude higher than ΣPBDE. Trends in PBDE concentrations in wildlife are also noted with respect to time, region, and trophic level (e.g. biomagnification). Generally, higher concentrations are associated with more recent samples, with samples from contaminated sites, or for higher trophic level organisms.

BDE 209 was only detected in a limited number of wildlife samples compared to ΣPBDEs. It was seldom detectable in aquatic organisms [64] and piscivorous species [44] except for samples from heavily contaminated sites [69] or for oligochaetes which were suspected to be biased by ingested but not absorbed sediments [64, 70]. It was once hypothesized that BDE 209 could not be absorbed by organisms due to its large

molecular size [71] but more recent detection of BDE 209 in terrestrial animals such as birds of prey and foxes [44, 72-74] disproved this hypothesis, thus providing environmental evidence that BDE 209 is bioavailable. However, compared to the abiotic environment (sediments, sewage sludge, SPM) the concentration of BDE 209 in biological samples was relatively low [70], and compared to lower brominated congeners the accumulation of BDE 209 is minimal and does not contribute significantly to the congener profile. The difference between terrestrial animals and aquatic and piscivorous species with respect to BDE 209 accumulation may be explained by the water barrier which limits the transfer of BDE 209 from SPM and sediments to pelagic aquatic organisms at the base of the food web [44]. Hence, BDE 209 will not be included in further discussions of congener profiles in wildlife.

1.2.3.1.2.2 Congener profiles

The major components of the commercial penta-BDE mixture (BDE 47, 99, 100, 153, 154) dominate wildlife samples. BDE 183 is an indicator of the commercial octa-BDE mixture, but it either was not found or was detected at lower concentrations than penta congeners. To a large extent, this is likely a function of the smaller production of this commercial mixture which only accounted for 5% of total historical PBDE production.

In general, two distinct congener profiles were observed in wildlife: one was dominated by lower brominated congeners such as BDE 47 and 99, and the other was shifted towards heavier congeners such as BDE 153 (hexa) and BDE 183 (octa). Organisms from freshwater and marine ecosystems, including marine mammals, piscivorous species (water birds), fishes, and invertebrate species, followed the former

congener profile while terrestrial animals, including terrestrial birds[2] and terrestrial mammals, followed the latter (Figure 1-3).

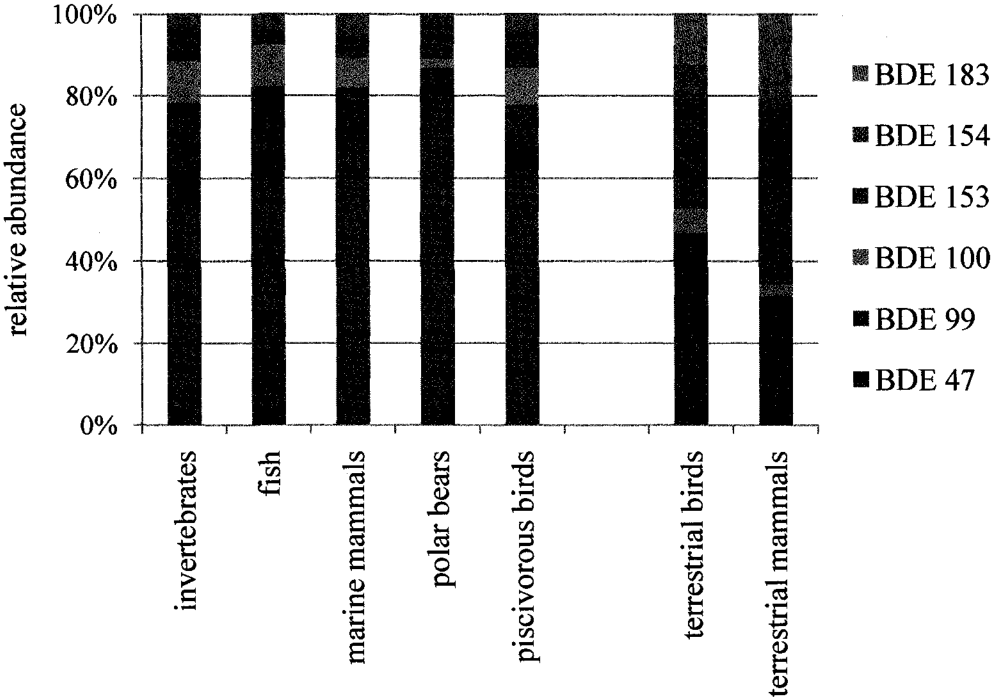


Figure 1-3: Congener profiles in wildlife. The first five columns from left exhibit average congener profiles in organisms from freshwater and marine ecosystems: invertebrates [57, 58, 64, 69, 70, 75-78], fish [51, 54, 70, 75, 76, 78-90], marine mammals [70, 76, 77, 79, 81, 83, 91-99], polar bears [77, 100, 101], and piscivorous birds [68, 79, 83, 90, 93, 101-106]; the two columns from right exhibit average congener profiles in terrestrial birds [73, 74, 102, 107] and terrestrial mammals [72].

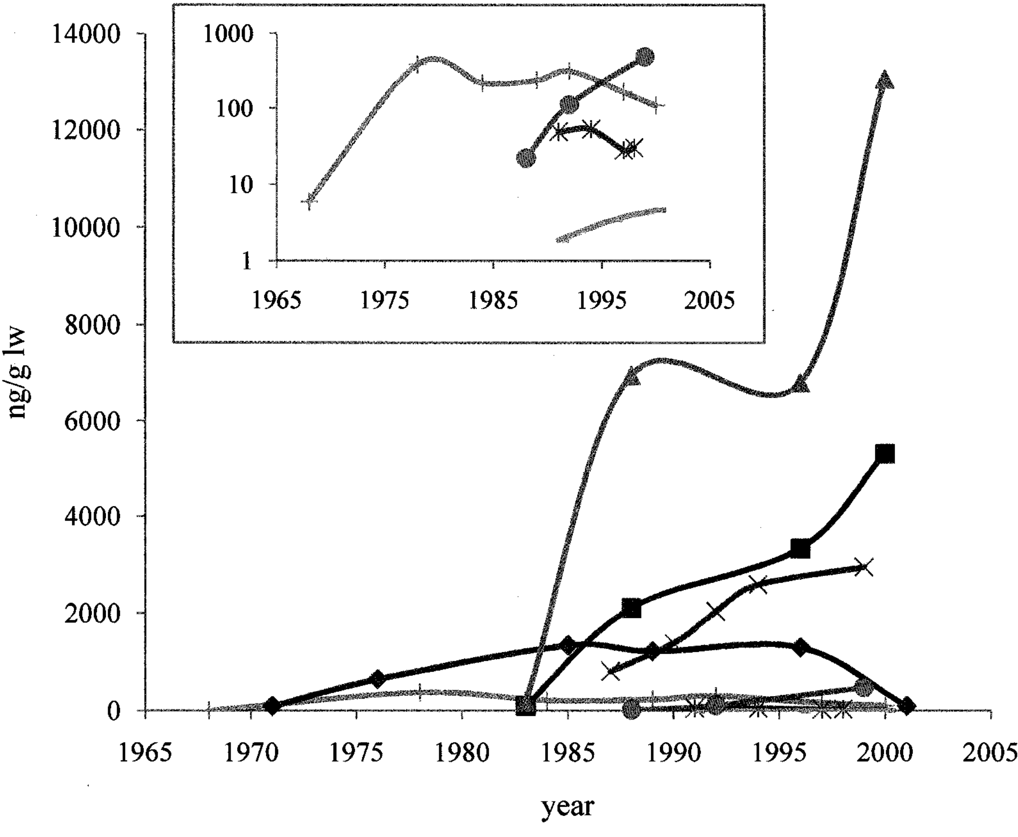
For example, piscivorous and terrestrial birds showed a distinct congener profile whereby heavier brominated congeners (i.e. BDE 153) became the major congener. The fact that terrestrial birds exhibited a higher percentage of heavier BDE 153 may be due to higher exposure [8] compared to piscivorous birds whose food sources also contain low concentrations of higher PBDEs [44]. The similar congener profile in both terrestrial

[2] In the present thesis, terrestrial birds refer to the birds that feed on terrestrial animals only.

birds and terrestrial mammals — that was shifted to heavier brominated congeners — can, therefore, perhaps be attributed to dietary exposure. Alternatively, the lower water solubility and higher atmospheric concentration of higher brominated congeners may result in higher concentration of these compounds in the terrestrial food web than in either the aquatic or marine food web. An additional consideration is that divergent biotransformation capacities between different species may also play a role in affecting PBDE congener profile of organisms. However, this could be less important in birds because common cormorants (*Phalacrocorax carbo*) in the UK and Japan exhibited different congener profile while each had profiles consistent with the type of PBDE mixtures used locally [93, 98]. Nevertheless, some species may have a distinctive metabolic capacity, such as the melon-headed whale from Japan in which tetra- and penta- BDEs were less contributive than hexa-BDEs [98], compared to most other waterborne marine mammals (Figure 1-3). Hence, even though these whales were from the same region, and thus were likely exposed to the same source of PBDEs, the congener profile in melon-headed whales was different from other whale species.

1.2.3.1.2.3 Temporal trends

The emission of PBDEs to the environment not only resulted in their increasing concentration over time in the abiotic environment, but also in wildlife (Figure 1-4). The concentrations and temporal trends in wildlife provide information on the local use patterns despite that differential bioavailability [71, 73, 108] and biotransformation capacity may partially change the congener profile to a certain extent.



pike, Lake Bolmen, Sweden (Kierkegaard, 2004)
guillemot eggs, Baltic Sea, Sweden (Sellstrom, 2003)
herring gull eggs, Lake Ontario (Norstrom, 2002)
herring gulls, Lake Michigan (Norstrom, 2002)
peregrin falcon, Greenland (Vorkamp, 2005)
fur seal, Pacific Coast, Japan (Kajiwara, 2004)
ringed seal, Canadian Arctic (Ikonomou, 2002)
beluga whales, St. Lawrence Estuary, Canada (Lebeuf, 2004)

Figure 1-4: Time trends of ΣPBDEs (tri- ~ hexa-) in wildlife from Europe, North America, Asia, and the Arctic region. The inset shows certain temporal trends on a log-scale. Cited authors include Kierkegaard et al. [85], Sellstrom et al. [104], Norstrom et al. [68], Vorkamp et al. [109], Kajiwara et al. [94], Ikonomou et al. [91], and Lebeuf et al. [95].

Most accumulated BDE congeners in wildlife are markers of the commercial penta-BDE mixture. In the eggs of guillemot (a permanent resident) from the Swedish coast of

the Baltic Sea, PBDE concentrations (mainly the congeners in penta-BDE formula) were low before the application of PBDEs as flame retardants, followed by a rapid increase that peaked around the mid- to late-1980s and later dropped to relatively stable concentrations in the mid- to late-1990s [104]. A similar trend was found in pike in remote Lake Bolmen, Sweden [85]. In contrast, wildlife in North America and Greenland showed a continuous increase until 2000 [68, 91, 95, 109]. The ΣPBDEs increased 8-fold in ringed seals in the Canadian Arctic from 1981 to 2000, and the doubling time for the sum of tetra-, penta-, and hexa-BDEs was estimated at 8.6, 4.7, and 4.3 years, respectively [91]. Similar increases (a 3-fold increase over a 10-year period) were observed in peregrine falcons from Greenland [109]. Lebeuf et al. reported a 22-fold increase of ΣPBDEs in beluga whales in the St. Lawrence Estuary, Canada, and estimated the doubling time of ΣPBDEs at less than 3 years [95]. The most rapid increase occurred in the Great Lakes region, whereby concentrations of ΣPBDEs in herring gull eggs increased 60-fold in Lake Ontario and 80-fold in Lake Michigan between 1981 and 2000, and the doubling time for ΣPBDEs (47, 99, 100) was estimated at 2.6 years in Lake Michigan and 3.1 years in Lake Ontario [68]. The concern that rapidly increasing PBDE concentrations may eventually surpass PCB concentrations was consequently raised [88].

The areas of North America close to PBDE manufacturing sites, such as Lake Michigan, had much higher concentrations than remote regions, and correlations between the use of penta-BDE technical formulations and PBDE concentrations (BDE 47, 99, 100, 153, and 154) in biological samples were observed in these corresponding regions. For example, the estimated consumption of penta-BDE in Europe started dropping in 1995 [61], and this was correlated with the decrease in concentrations in both herring and

guillemot eggs from Sweden (Figure 1-4) [85, 104]. Conversely, penta-BDE mixtures were still in use in North America in 2000 resulting in the highest concentration at that time (Figure 1-4) [68, 91, 95]. The influence of the voluntary phase-out of penta-BDE in North America in 2004 [18] cannot currently be assessed due to lack of more recent data.

1.2.3.1.2.4 Spatial trend

Latitudinal fractionation, as observed for PBDEs in sediments and soils [44-46], was also observed in frogs [105], whereby lower brominated congeners such as BDE 47 become more important in northern latitudes. As mentioned previously, this spatial trend likely resulted from the differential atmospheric transport based on vapour pressure of different BDE congeners.

1.2.3.1.3 PBDEs in humans

Human exposure to PBDEs may occur through food, ingestion of dust, or by inhalation; the first two may be the major routes, while dermal absorption is expected to be minimal [110, 111]. Following absorption, these high K_{ow} compounds tend to partition preferentially to lipid-rich tissues. The partitioning ratio of PBDEs between human adipose tissue, milk, and blood was close to 1:1:1 based on lipid-normalized concentrations [112, 113]. Generally, body burdens of PBDEs in the European population were 10 to 100-fold lower than in the North American population [114]. Occupational exposure of PBDEs (mainly via inhalation of particulate matter), or high consumption of PBDE-contaminated fish, may result in a significantly higher body burden than in the background population [111, 115]. No trends have been associated with age, as have been

found for PCBs, for concentrations of PBDEs in adipose tissue or breast milk samples [114, 116]. In fact, there is a lack of correlation between PCB and PBDE concentrations in humans, suggesting there are other (other than diet) important routes of exposure (e.g. inhalation) to PBDEs in the general populations [112, 116].

Compared to the general population, the developing fetus and nursing infant may be at a higher risk from PBDE exposure because animal studies have suggested several toxic effects induced by PBDE exposure specific to these life-stages, including the development of the nervous system and thyroid hormone regulation [117-120]. The detectable PBDEs in breast milk and the serum in newborns make it evident that PBDE exposure for the fetus and infant can occur via transplacental transfer and breast milk, respectively [12, 112, 115, 121]. The placenta may act as a barrier by reducing exposure to a certain extent [112], but may not be able to protect the fetus when the maternal body burden exceeds a certain limit. For example, a 70% reduction from maternal to cord blood was observed in mother and fetus pairs when the mother's plasma concentrations ranged from 6.53 to 57.9 ng/g lw [112], but comparable concentrations were observed when the mother's blood concentrations ranged from 15 to 530 ng/g lw [121]. Maternal transfer of PBDEs via breast milk has been suggested to be more pronounced than placental transfer [112]. Concentrations of ΣPBDEs (sum of BDE 47, 99, 100, 153, 154) in the breast milk from Swedish mothers ranged from 0.4 to 3.2 ng/g lw and this was similar to the milk from other European mothers [115]; whereas these concentrations are about 10 times lower than in Canadian mothers (median of BDE 47 = 13 ng/g lw), and up to 100 times lower than in US mothers (ΣPBDEs = 6.2 to 419 ng/g lw), respectively [110]. Likely as a direct result of higher maternal body burden and milk concentrations in

North Americans, fetal serum concentrations in the US were 69-fold higher than in Sweden [110]. The effort by European countries to voluntarily phase out and establish regulations against commercial penta-BDE and octa-BDE mixtures has shown encouraging results at the population and environmental exposure level as evidenced by decreasing concentrations of PBDEs in environmental samples since the mid 1990s and in breast milk in the late 1990s [110, 115].

1.2.3.2 Environmental behaviour and fate

Addressing the questions of where in the environment a chemical accumulates, and in what chemical form it resides, can assist in developing wiser uses, manufacturing and application scenarios, and effective regulations. Specifically, after their emission of from sources, PBDEs partition among various environmental compartments, including organisms, as shown in Figure 1-2. The environmental monitoring studies described in the previous sections have demonstrated some trends and behavioural characteristics of PBDEs which can be summarized, in general, may be similar to POPs with regard to environmental persistence and long-range transport [10, 11]. Another important characteristic to consider is their bioaccumulation and biotransformation capacity, and these are a focus of the present thesis. In Canada, the *Canadian Environmental Protection Act* prescribes certain conditions that must be met in order for a chemical to be labeled as persistent subject to long-range transport, or bioaccumulative.

1.2.3.2.1 Persistence and long-range transport

According to the *Canadian Environmental Protection Act*, "a substance is considered to be persistent when its half life equals to or is longer than 2 days in air, 182 days in water, 365 days in sediments and 182 days in the soil"; furthermore, "a substance may be considered as persistent in air if it is shown to be subject to atmospheric transport to remote regions such as the Arctic."

Studies on the half-life of PBDEs in environmental compartments are limited. Half-lives under solar light degradation have been studied in pure solvent or water/solvent mixtures (Appendix IV) [122, 123]. The half-lives of PBDEs varied according to the degree of bromination and the ratio of organic and aqueous composition; with the decrease in the degree of bromination and/or increase in aqueous composition, the degradation half-life of certain PBDEs increased [123]. However, this study did not demonstrate the degradation of PBDEs in pure water. The degradation in pure water has only been studied for BDE 209, and that half-life (30 to 40 h) was longer than that in any of the studies using pure solvent (0.1 to 0.5 h) [122, 123]. Although the experimental conditions such as lower light intensity could result in a longer half-life, in pure water the half-life of lower brominated congeners such as BDE 47 (half-life was 290 h in MeOH/water (80:20, v/v)) is presumably longer than the persistence criteria prescribed in the *Canadian Environmental Protection Act.*

In addition, the similar composition of PBDEs between the commercial penta-mixtures and samples of soil or sediment may be interpreted as evidence that little degradation of the congeners occurs by processes occurring during atmospheric transport or within the soil [44]. Microbial biodegradation studies have shown that the full

brominated congener, BDE 209, had a longer half-life (700 d) [124] than the *Canadian Environmental Protection Act* criteria. Moreover, the authors also suggested that the lower brominated congeners would be even more resistant to microbial degradation.

The detectable levels of PBDEs in many samples from remote environments, where few local sources of PBDEs exist, provide the most convincing evidence for long-rang transport. Atmospheric half-lives have not been measured directly, but the fact that the congener profile in soil matches the atmospheric congener profile [11] provides some evidence that PBDEs may undergo long-range atmospheric transport. In the atmosphere, the relative contribution of BDE 47 — a relatively "light" congener — to ΣPBDEs increases towards the North Pole, suggesting that the "cold condensation" effect has acted to deposit the least volatile fraction at lower latitudes, and hence to enrich the more volatile compounds in the colder areas during long-range transport [45, 46]. As such, larger PBDEs such as BDE 209 are poor candidates for long-range transport to the Arctic. This effect also resulted in the latitudinal fractionation of PBDEs which has been described in several previous sections.

1.2.3.2.2 Bioaccumulation

1.2.3.2.2.1 Bioconcentration

Bioconcentration describes the magnification of a waterborne chemical into an aqueous organism through respiration or skin absorption. It is usually measured under experimental conditions in which the aquatic organism (e.g. fish) is exposed to the chemical only through the water. The degree of bioconcentration is expressed by the

bioconcentration factor (BCF), which is defined as the concentration ratio, at steady state, of the chemical concentration in the aquatic organism to that in the water [125]. The concentration can be based on wet weight (ww).

$$BCF^{3} = C_{organism} / C_{water}$$

1.2.3.2.2.2 Biomagnification

Biomagnification describes the magnification of a chemical by an organism only through the diet, and can thus be determined in laboratory feeding experiments, but it is also applicable to field measurements when exposure through other routes (e.g. respiration or skin absorption) is thought to be negligible. The biomagnification factor (BMF) is therefore defined as the concentration ratio, at steady state, of the chemical concentration in an animal to that in its diet. BMFs are best determined under experimental conditions to minimize other exposure sources [125] and also to have high confidence in the true diet concentration, which can be difficult to assess in the field. However, many field studies use BMFs as a term to describe the magnification of a chemical from assumed predator/prey relationships. These "BMFs" are rather bioaccumulation factors (BAFs), which are described below. Only when the predator/prey relationship is simple enough (e.g. polar bear/ringed seal), and other exposure routes of the predator are negligible (e.g. respiration), can the BAFs estimated from the field studies be treated as BMFs. The concentration can be based on ww, dw, or lw. However, because increasing wet weight concentrations of some hydrophobic (and

[3] There is no unit for BCF. Units of concentrations in the organism and water are cancelled based on the assumption that the density of water is 1 g/ml.

lipophilic) chemicals in food chains may occur simply as a result of increasing lipid content of higher trophic organisms, it is often necessary to use lipid-normalized concentrations of the chemical.

$BMF = C_{organism} / C_{diet}$

1.2.3.2.2.3 Bioaccumulation

Bioaccumulation is a more general term and is "the result of simultaneous bioconcentration and biomagnification" [125]. For the waterborne organisms, BAFs are determined by the ratio, at steady state, of the chemical concentration in the organism to the chemical concentration in the water (sometimes only involving in the dissolved phase). However, BAFs can vary with a change in the relative apportionment of the chemical in the diet and exposure water. Therefore, BAFs estimated in a certain environment cannot necessarily be used to predict the concentrations in water or organisms in another environment. For terrestrial animals, BMFs best represent BAFs if the exposure routes other than diet (e.g. respiration) are negligible. The concentration can be based on ww, dw, or lw.

1.2.3.2.2.4 Food-chain bioaccumulation

Food-chain bioaccumulation describes the magnification of a chemical through the food chain and, thus, is a measure of accumulation across multiple trophic levels [125]. The food-chain bioaccumulation factor (sometimes also called the trophic magnification factor) can be estimated by the biomagnification power (*B* value) by the following equation, $c = A \cdot e^{(B \cdot \delta^{15}N)}$, where *c* is the lipid-normalized concentration (the reason why

lipid-normalized concentrations are used has been described previously in the "Biomagnification" section) in one organism for which the $\delta^{15}N$ value has been determined (as a measure of trophic level), and the A is the base concentration in the food chain [126]. A positive B value indicates that organisms located in higher trophic levels accumulate higher lipid-normalized concentration of the substances.

1.2.3.2.2.5 Practical considerations for measuring bioaccumulation

It is important to note that for all the above bioaccumulation parameters it is technically necessary to know the whole-body concentration of the target chemical. However, this is only practically feasible when an organism is small enough to be fully homogenized, and therefore would limit the study of larger wildlife such as whales or polar bears. Therefore, for lipophilic compounds, it has become common practice to lipid-normalize the concentration determined in a single tissue (or fluid) of the predator and its diet [77, 96, 100] based on the assumption that lipid-normalized concentrations between tissues are comparable, or in other words, that the whole-body lipid-normalized concentration is reflected by the lipid-normalized concentration of a single tissue sample.

However, this is not always a safe assumption, particularly in species, such as ringed seals, that undergo pronounced lipid cycles. This may result in a large variance of lipid-normalized concentrations for any contaminant, and more importantly may lead to inaccurate assessments of bioaccumulation tendencies [77, 96, 127]. In addition, the use of lipid-normalized concentrations may give inaccurate impressions of the total body burden when suspect compounds do not distribute evenly on a lw basis [77]. In this case, ww-based whole-body concentrations (predicted from lipid-normalized concentrations

and the whole-body lipid content) are estimated [77] and used to calculate or interpret the BMF.

The use of ww-based whole-body concentration for assessing bioaccumulation clearly reduces the bias caused by tissue-specific accumulation. However, the resulting BMF parameter can be misleading for lipophilic chemicals when the predator has a higher percentage of body fat than its prey. This is because the increase in concentrations of some hydrophobic chemicals in a predator organism can simply result from a higher total lipid content of the predator relative to its prey [125]. Nevertheless, the relative biomagnification ability between PBDE congeners can be compared by any of these methods.

1.2.3.2.2.6 Bioaccumulation data for PBDEs

According to the *Canadian Environmental Protection Act*, substances having log K_{ow} larger than 5 are considered to be bioaccumulative; all the major PBDEs detected in the environment belong to this category (Table 1-3). Thus PBDEs have been the subject of several bioaccumulation investigations in various species.

Though no study has estimated the BCFs and waterborne BAFs of PBDEs, they are believed to be bioavailable to aquatic organisms, through dietary intake of particulate organic matter, contaminated prey organisms or detritus [77]. However, food-chain bioaccumulation (expressed by B values) and biomagnification (expressed by lw-normalized BMFs) of PBDEs have been determined for several natural feeding relationships around the globe (Figure 1-5).

Significant bioaccumulative potential through the food chain (*B* value > 0) for lower PBDEs has been observed in the Baltic Sea food web (roach, perch, and pike) (Table 1-5), and the Atlantic Ocean food web (zooplankton, sprat, herring, and salmon) (Table 1-5). However, negative *B* values were suggested for higher brominated congeners such as BDE 203 (octa), and the *B* value was not significantly different than 0 for BDE 209 [126, 129].

Biomagnification was also observed for BDE 47 in ringed seals when interpreted by BMFs based on estimated whole-body concentrations (ww-based) and lipid-normalized tissue concentration (Table 1-5). However, BDE 100 was shown to biomagnify to a larger extent than BDE 99 in ringed seals relative to polar cod; and polar bears seemed to biomagnify only BDE 153 other than BDE 47, relative to ringed seals which were presumed to be their primary prey (Table 1-5).

Table 1-5: Bioaccumulation (expressed by BMFs and *B* values) of PBDEs.

		BDE Congeners					
	Predator/Prey	**28**	**47**	**99**	**100**	**153**	**154**
Marine BMFs	harbor seal/cod [70]	3.6	29	6.3	44	>140	5.4
	ringed seal/polar cod [77]	14	56	14	26	—	7.9
	polar bear/ringed seal [100]	—	3.9	5.8	4.7	71	—
	polar bear/ringed seal [77]	0.1	0.5	0.3	0.3	7.5	0.3
Terrestrial BMFs	Sparrowhawk /passerine [128]	4	10	20	25	21	24
	buzzard/rodent [128]	—	12	14	17	22	—
	fox/rodent [128]	—	<1	<1	<1	<1	—
B values[a]	Baltic Sea food web [126]	>0.3	>0.6	>0.6	>0.6	>0.5	>0.5
	Atlantic food web [129]	>0.1	>0.4	>0.3	>0.3	>0.2	>0.2

[a] B values are estimated from figures. — not available

Relative to the simple feeding relationships in arctic marine food webs, comparatively few accumulation measurements have been made in terrestrial food chains because the food sources for terrestrial animals are varied (in space and time) which greatly complicates the dietary assessment and, thus, the number of samples required to be collected and analyzed. Nonetheless, some data have been reported for BMFs in some terrestrial feeding relationships (Table 1-5). In two terrestrial feeding relationships, sparrowhawk/passerine and buzzard/rodent, BMFs for detectable PBDEs were all greater than one, whereas in another relationship involving a top predator (fox/rodent), foxes seemed not to accumulate PBDEs [128]. A significant linear relationship between BMFs and log K_{ow} was shown for all the detected congeners (tri- to hepta-) in sparrowhawks, whereas in buzzards the correlation was only observed for congeners having fewer than six bromine atoms [128], suggesting that buzzards may have the capacity to biotransform the higher brominated PBDEs, unlike sparrowhawks.

In general, high K_{ow} PBDEs are bioaccumulative through the food chain resulting in higher exposure to top predators in both marine and terrestrial food webs. However, the negative BMFs of polar bears and foxes indicated that these organisms might have an elevated capacity for biotransformation and elimination of PBDEs. Lower brominated congeners are more accumulative and their BMFs may be correlated with their log K_{ow}, because they are not readily biotransformed, whereas higher brominated congeners are less bioaccumulative and are more susceptible to biotransformation.

1.2.3.2.3 Biotransformation in microorganisms

Biotransformation of PBDEs has been demonstrated in various microorganisms. Microbial reductive debromination of higher brominated PBDEs was observed under anaerobic conditions (Table 1-6) [130, 131].

Table 1-6: Possible debromination pathways by microbial degradation.

Substrate (PBDEs)	**Active Microbes**	**Products**	**Ref.**
Deca-BDE	Sewage sludge from a mesophilic digester	Nona- (BDE 208 and 207) to octa-BDE congeners	[130]
Nona-BDE congeners (207 and 206)	Sewage sludge from a mesophilic digester	Octa-BDE congeners	[130]
Deca-BDE	*Sulurospirillum multivorans*	Octa- and hepta-BDE congeners	[131]
Octa-BDE mixture	*Dehalococcoides ethenogenes 195*	Hepta- to di-BDE congeners	[131]
Octa-BDE mixture	*Dehalococcoides ethenogenes 195* and *Dehalococcoides* sp. strainBAV1	Tetra- to di-BDE congeners	[131]

Anaerobic degradation of deca-BDE by sewage sludge, collected from a mesophilic digester, was observed within an 8-month incubation; the disappearance rate with the addition of primers (related compounds which may enhance the degradation of the target compound) corresponded to a pseudo-first-order rate constant of 1×10^{-3} per d [130]. Much quicker debromination of deca-BDE was observed by a dechlorinating bacterium, *Sulurospirillum multivorans*, which is responsible for dechlorinating highly chlorinated ethenes; it was able to convert deca-BDE to octa- and hepta-BDE congeners, but without further debromination [131]. Several *Dehalococcoides* species are capable of debromination of the octa-BDE mixture to much lower brominated congeners (from

hepta to di-). However, due to the toxicity of PBDEs to these bacterial species, the transformation rate of PBDEs was slow and the fastest degradation rate was limited to several hundred nanomole per year [131]. The identified debromination product congeners in sewage sludge included BDE 207 and 208 and by *Dehalococcoides* species included BDE 47, 49, 99, and 154, of which the congeners containing *ortho*-substituted bromine atoms were the most resistant to further debromination [130, 131]. No microbial debromination has been reported for penta-BDE mixture presumably due to the resistance of debromination of these lower brominated congeners.

1.2.4 Mammalian toxicological profile

The mammalian toxicological profile of lower brominated BDE congeners is discussed in the following sections. Higher brominated BDE congeners are not of the interest due to their less frequent detection, lower bioaccumulation and toxicity, and debromination to lower brominated congeners. Absorption[4], distribution, metabolism, excretion[5] (ADME), and toxic effects after oral exposure of individual congeners (BDE 47, 99, 100, and 153) or a commercial penta-BDE mixture (DE-71) in two rodent species (rats and mice) are discussed in the following sections in order.

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[4] Absorption is defined as the fraction of the dose being absorbed from the gut. The dose includes the parent compounds and their metabolites.

[5] Excretion is the removal of administered substances in the parent forms in excreta (including feces and urine in general, and other routes such as milk and bile); whereas elimination refers to all the processes that remove administered substances including excretion and metabolism.

1.2.4.1 Absorption

The individual congener exposure studies have demonstrated that all the tested congeners are bioavailable[6] and well-absorbed by the gastrointestinal tract. However, the absorption in rodents after a single exposure generally decreased with the increasing molecular weight, specifically, >75% of BDE 47 [23, 132], 50% to 80% of BDE 99 [133, 134], 73% of BDE 100 [135], and 70% of BDE 153 [136].

1.2.4.2 Distribution

Using ^{14}C-labeled BDE 99, tissue concentrations and tissue distribution were studied in rats and mice [134]. Six hours after a single oral exposure, the highest ^{14}C concentrations were observed in the liver and kidney. Between 6 and 24 h, concentrations increased in adipose tissue while decreased rapidly in other major tissues. The adipose ^{14}C concentration remained constant over the next 9 d. This result revealed the fact that adipose tissue was the major accumulation site of BDE 99 [134], in agreement with its high K_{ow}. Based on the proportion of the administered dose, the skin accumulated the second most PBDEs at 24 h, followed by the muscles and liver [132-135]. However, BDE 153 was an exception as it accumulated primarily in liver but the reason for this was not clear [136]. The accumulation potential of PBDEs in other tissues was generally low.

Repeated-dose exposure to rats and mice resulted in enhanced relative accumulation of BDE 47 and 99 in adipose tissue compared to single exposure at the same amount [132-134, 137]. Selective accumulation of PBDE congeners was observed in some tissues

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[6] The bioavailability was defined as the fraction of the administered parent compound reaching systemic circulation.

in rats. For example, BDE 47 had a greater preference for adipose tissue than BDE 99 [133]. Therefore, if only adipose tissue was measured, then BDE 99 may have been underestimated and BDE 47 overestimated in the whole body [133].

A sex difference for BDE 47 accumulation in adipose tissue, and excretion to feces, was observed whereby females accumulated more and excreted less than males [132], thus suggesting a longer whole-body half-life for BDE 47 in females [132]. However, it is important to keep in mind that females can also excrete some of the dose through the maternal transfer mechanisms during pregnancy [12]. In contrast, sex differences for either tissue distribution or excretion were not significant for BDE 99 or 153 [134, 136].

1.2.4.3 Excretion

As previously mentioned, species differences for PBDE excretion were observed. Urinary excretion of BDE 47 and 99 was different between rats and mice [132, 134, 137], whereas urinary excretion of BDE 100 and 153 and fecal excretion of BDE 47, 99 and 100 was similar between species [135-137]. The dose concentration of BDE 47 (0.1 to 1000 μmol/kg) seemed not to affect the proportion excreted [132]. In rats, excretion in the parent forms through feces was a significant route (>26% of a single dose within 24 h) for elimination of BDE congeners (BDE 47, 99, and 153), whereas their excretion through urine was limited [132, 134, 136]. In mice, the dominant fecal route of excretion was similar to rats for BDE 99 and 153; however, BDE 47 was not only greatly excreted through feces (>20% of a single dose within 24 h) but also significantly excreted through urine (>20% of a single dose within 24 h) [132, 134, 136]. Excretion of BDE 99 through feces was higher than BDE 47 in rodents (based on percentage of dose administered),

probably due to the poorer intestinal absorption of BDE 99 resulting from its larger molecular weight [133, 134, 137]. The reduced capacity of developing mice to excrete of BDE 47 may have resulted in higher concentrations in target tissues during critical windows of development [138]. A reference half-life of a tetra-BDE (not defined but likely to be BDE 47) in adipose tissue was estimated at 19 days in male rats [23, 139, 140].

1.2.4.4 Metabolism

Biotransformation of PBDEs occurring in higher organisms is more complicated than in lower organisms because of the transient nature of the metabolites of PBDEs which may be eliminated or transported into different tissues. Biotransformation processes, including oxidative debromination, hydroxylation, reductive debromination, cleavage of the ether bond, and phase II conjugation have been observed, and many products including hydroxylated PBDEs (OH-PBDEs), bromophenols, glucuronides, glutathione, and sulfate conjugates have been reported in various experimental animals [23, 132-135, 139, 141, 142]. The metabolic pathways for PBDE are summarized in Figure 1-5.

Hydroxylation of BDE 47 has been suggested to be mediated by cytochromes P450, because some of their gene expression was up-regulated in rats following repeated exposure to BDE 47 [132]. Proposed metabolism pathways of BDE 99 were more complicated than BDE 47 in rodents [133]. In addition to phase I metabolism such as hydroxylation and several phase II metabolites identified in bile, feces, and urine, debromination metabolites were also detected [134]. Fewer metabolites were identified in fecal extracts for BDE 100 compared to BDE 99 or 47, but the identified metabolites

included mono-hydroxylated, oxidative debrominated congeners [135]. Comparatively minimal metabolism of BDE 153 in rodents has been observed [136]. This could be attributed to the absence of adjacent unsubstituted carbons which is an essential feature for arene oxide formation catalyzed by cytochrome P450 [136].

Figure 1-5: Proposed metabolic pathways of PBDEs to form lower brominated congeners, mono-OH-PBDEs, di-OH-PBDEs, bromophenols, glutathione, and glucuronide conjugates.

In summary, the toxicokinetics of PBDEs are consistent with environmental observations that the lower brominated congeners are generally persistent and, to some extent, accumulative in organisms. Phase I and phase II metabolism changes the structure and properties for a fraction of the parent PBDE congeners, thus making them more water-soluble and more easily excreted. However, as will be mentioned in the following sections, these metabolic processes may also result in the bioactivation of PBDEs.

1.2.4.5 Toxic effects and the mechanisms of action

Because PBDEs are structurally similar to toxic polyhalogenated aromatic hydrocarbons, such as PCBs, dibenzo-*p*-dioxins, and dibenzofurans, concerns were raised that PBDEs may cause similar toxic effects. Nevertheless, other toxic effects, specific to PBDEs, are also important to examine. Lower brominated congeners are more toxic than higher brominated congeners in terms of acute and subacute to subchronic toxicity [9, 143], influence on hepatic enzyme activities, thyrotoxicity [117, 118], and neurotoxicity [119, 120, 144, 145]. Moreover, lower brominated congeners are more bioaccumulative than higher brominated congeners, and furthermore, higher brominated congeners can be debrominated to lower brominated congeners in mammals [71, 146] or by environmental bacteria [130, 131] as described in previous sections. Typical toxic effects of lower brominated congeners, including acute toxicity, thyrotoxicity, neurotoxicity, and hepatotoxicity, are discussed here.

1.2.4.5.1 Acute toxicity

Although lower brominated congeners are more potent toxicants than higher brominated congeners regarding in acute exposures, the effective doses required to elicit acute toxic effects are relatively high compared to the well-known toxic compounds, dioxins. For example, the oral LD_{50} of the penta-BDE mixture for female and male rats was 5.8 and 7.4 g/kg body weight (bw) respectively [14], whereas the LD_{50} range of dioxins was from microgram to milligram per kg bw level [147]. Humans and wildlife are not likely to be exposed to such high concentrations of PBDEs under any exposure scenario, except perhaps in accidental exposures. Greater focus, therefore, is placed here on the effects of these congeners in chronic low-dose exposure scenarios.

1.2.4.5.2 Affected hepatic enzyme activities

In vivo and in vitro studies have demonstrated that hepatic phase I enzymes activities, such as cytochrome P450 isozymes, can be up-regulated by PBDEs in many models (Table 1-7). As hydrophobic xenobiotics, biotransformation of PBDEs by P450 will increase their hydrophilicity, and thus induction of phase I enzyme activity may initially be thought to be a detoxification step. However, these processes may also lead to bioactivation. For instance, hydroxylated BDEs are likely to lead to thyrotoxicity as discussed in the following sections. PBDEs also induced phase II enzyme activities, such as uridinediphosphate-glucuronosyltransferase (UDPGT), and through this pathway may have cause thyrotoxicity. This will also further discussed in the following sections.

Table 1-7: Summary of in vivo and in vitro studies examining the influence of PBDEs on the activity of P450 enzymes.

Tested PBDEs	Study Object	Targets	Results	Ref.
DE-71	Male F344 rats	*CYP 1A1* gene	Up-regulation but weak compared to positive control (PCB 126).	[148]
		CYP 2B gene	Up-regulation	[148]
		CYP 3A gene	Up-regulation	[148]
	Primary rat hepatocytes	CYP 1A1 activity[a]	No induction of CYP 1A1	[149]
	Long-Evans rats	CYP 1A1 activity[a]	Significant induction in both dam and offspring at 10 mg/kg/d	[118]
		CYP 2B activity[b]	Significant induction in both dam and offspring at 10 mg/kg/d	[118]
BDE 47	Male F344 rats	*CYP 1A1* gene	Up-regulation only appeared at 100 μg/kg/d group	[148]
		CYP 2B gene	Up-regulation	[148]
		CYP 3A gene	Up-regulation	[148]
	C57BL6 mice	*CYP 1A* gene	No induction of *CYP 1A1* or *CYP 1A2* gene expression	[150]
		CYP 2B gene	Induction of *CYP 2B10* gene expression	[150]
		CYP 3A gene	Induction of *CYP 3A11* gene expression	[150]
	Primary rat hepatocytes	CYP 1A1 activity[a]	No induction of CYP 1A1	[149]
BDE 99	Male F344 rats	*CYP 1A1* gene	Up-regulation only appeared at 100 μmol/kg/d group	[148]
		CYP 2B gene	Up-regulation	[148]
		CYP 3A gene	Up-regulation	[148]
	C57BL6 mice	*CYP 1A* gene	No induction of *CYP 1A1* or *CYP 1A2* gene expression	[150]
		CYP 2B gene	Induction of *CYP 2B10* gene expression	[150]
		CYP 3A gene	Induction of *CYP 3A11* gene expression	[150]
	Primary rat hepatocytes	CYP 1A1 activity[a]	No induction of CYP 1A1	[149]
BDE 153	Male F344 rats	*CYP 1A1* gene	Up-regulation only appeared at 100 μg/kg/d group	[148]
		CYP 2B gene	Up-regulation	[148]
		CYP 3A gene	Up-regulation	[148]
	Primary rat hepatocytes	CYP 1A1 activity[a]	Weak induction of CYP 1A1	[149]

[a] CYP 1A1 activity was tested by the 7-ethoxyresorufin-O-deethylase (EROD) assay. [b] CYP 2B activity was tested by the 7-pentoxy-resorufin-*O*-dealkylase (PROD) assay.

1.2.4.5.3 Thyrotoxicity

Because PBDEs, and particularly their OH-PBDE metabolites, structurally resemble of thyroid hormones, such as thyroxine (T4) and triiodothyronine (T3) (Figure 1-6), and also because other halogenated aromatic hydrocarbons have been found to affect thyroid hormone regulations, PBDEs and their metabolites are suspected to be thyroid hormone disruptors.

Figure 1-6: Structure of T4 (upper left), T3 (upper right), and an OH-penta-BDE (lower middle).

T4 is the major thyroid hormone in the blood, whereas T3 is the biologically active form in cells. T4 can be converted to T3 by 5'-deiodinase in various tissues and in the placenta for use by the fetus [151]. Once released from the thyroid gland, thyroid hormones bind to a great extent to serum transport proteins including thyroxine binding globulin, transthyretin (TTR), or albumin. Only the free fractions of T4 and T3 are considered physiologically relevant. Thyroid hormone homeostasis is regulated by a sensitive feedback mechanism via the hypothalamic-pituitary-thyroid axis [152, 153]. Normal thyroid hormone levels in blood are essential for growth and development of tissues and for the maintenance of tissue and organ function [151]. The most critical role

for thyroid hormones is their regulation of the development of the central nervous system (CNS) in the developing fetus. Insufficient thyroid hormones during critical windows of development will result in irreversible damage [154], which may be subtle (e.g. effect on behaviour) or overt (e.g. mental retardation or abortion).

Indeed, among various PBDE toxicities, altering thyroid hormone regulation is one of the most sensitive endpoints [120]. Dose-dependent decrease of serum T4 was observed (no sex difference) in both rats and mice (weaning to adult stages) after PBDE short- or long-term exposure at relatively low doses (lowest-observed-adverse-effect-level (LOAEL) was 30 mg/kg/d in different studies) [117, 152]. Although not all the studies examined free T4 in serum or plasma, one study showed that the total and free T4 concentrations paralleled each other after PBDE exposure [155] indicating that free T4 is affected proportionally with total T4. The free T3 concentration was but not significantly affected by PBDEs, but the male was more sensitive than the female [117, 152]. However, this is not to say that PBDE exposure is less critical for tissue development or function maintenance in the female because hypothyroxinemia[7] with normal T3 levels will still leads to permanent functional abnormalities [118]. Another thyroid endpoint, thyroid-stimulating hormone (TSH), which serves to stimulate release to T4 when circulating levels are low, was not affected in all these studies [117, 143] except that it increased at a higher dose (60 mg/kg/d) in the pubertal males [152].

Neonatal and fetal stages may be more sensitive to PBDE exposure due to their developing CNS. It has been shown that PBDEs were retained in the neonatal brain during a defined critical period of brain development following maternal exposure [119].

[7] Hypothyroxinemia is characterized as low level of serum T4 with normal level of TSH.

In addition, fetal and neonatal hypothyroxinemia was observed after in utero and/or lactational exposure to PBDEs [117, 118]. Furthermore, the no-observed-adverse-effect-level (NOAEL) and LOAEL of hypothyroxinemia was respectively 1 and 10 mg/kg/d for fetuses or suckling neonates and the NOAEL was one-order-of-magnitude lower than the NOAEL in weanling or adult animals [117, 118]. Fortunately, serum total T4 of these young rats returned to and remained at the normal level after ceasing the exposure [118].

Three general mechanisms for disruption of the thyroid hormone regulation systems by PBDEs have been conceived: i) direct interference (i.e. toxicity) on the thyroid gland; ii) interference with thyroid hormone metabolizing enzymes; or iii) interference with the plasma transport of thyroid hormones [156]. Although enlarged thyroid glands were observed after PBDE exposure following high-dose (100 mg/kg/d) and subchronic exposure (90 d) [9], it was unlikely that PBDEs directly affected the thyroid gland at the previously studied doses. Several experiments have demonstrated the effects of PBDEs on thyroid hormone metabolism enzymes, particularly induction of the phase II enzyme, UDPGT [117, 118], leading to faster elimination of free serum T4 via glucuronidation.

Interestingly, PBDE metabolites have a higher affinity to the serum transport protein TTR than T4, the natural ligand [157]. Therefore, this may also serve as one of the mechanisms affecting the thyroid hormone regulation. The specific metabolites were not characterized by these authors; therefore the effective compounds remain unidentified but could be OH-PBDEs or bromophenols [157, 158]. However, the physiological relevance of this was unclear since T3 and TSH were unaffected in vivo [117, 118]. In another study, the total T4 in plasma after BDE 99 exposure was elevated in adult rats, though it returned to the control level several days after exposure was ceased [133]. The authors

attributed this to competed binding of T4 and hydroxylated BDE 99 to TTR, because this results in low protein-bound T4 which serves as a signal to the hypothalamus to send the signal, thyrotropin-releasing hormone (TRH), to the pituitary to release the TSH which triggers the synthesis of T4 in the thyroid gland. However, neither TRH nor TSH level was measured in the study.

1.2.4.5.4 Neurotoxicity

Even though no immediate effects were observed in mothers, such as on bw gain, pregnancy duration, proportion of successful deliveries, or pup sex-ratio after maternal exposure to DE-71 and BDE 99 [119, 120], prolonged effects in offspring, including hyperactivity at young adulthood [144, 159] and hypoactivity at late adulthood, were observed in perinatally exposed animals [159]. Furthermore, the effects of PBDEs on the CNS and brain function can get worse with age [119, 120, 144]. These behavioural effects may be attributed to the neurotoxicity of PBDEs in the CNS, and perhaps as a secondary effect derived from thyroid hormone disruption [120], although the latter has not been demonstrated. However, linkage between disrupted thyroid hormone (decreases in maternal serum T4 or free T4, or other indicators of thyroid abnormalities) and negative impact on the intelligence of children has been demonstrated in humans [120]. In other studies, neonatal mice exposed BDE 99 and 153 exhibited impaired cognitive abilities in the Morris water maze test[8] [120, 144].

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[8] The Morris water maze is one of the most used behavioural procedures to explore the role of the hippocampus in the formation of spatial memories. It was developed by neuroscientist Richard G. Morris in

A potential underlying mechanism of neurotoxicity can be related to the regulation of the cholinergic system which is one of the major transmitter systems that correlated closely to cognitive abilities such as reversal learning and working memory in animals [144]. Indeed, a disrupted cholinergic neurotransmitter system (significant decrease in density of nicotinic receptors in the hippocampus) in adult rodents has been observed after neonatal exposure to PBDEs [144, 160].

1.3 Mink (*Mustela vison*)

The mink is a member of the weasel family and is widespread throughout North America. Mink are shoreline dwellers and their one basic habitat requirement is a suitable permanent water area such as rivers or lakes. Mink are strictly carnivorous and are semiaquatic animals. Their diets are composed of organisms from both land and water including small mammals, such as rats and rabbits, frogs, fish, muskrats, insects, birds, and eggs. Their predators, such as owls and wolves, are generally the top predators in the terrestrial food webs, and mink have few aquatic predators. Their life span is generally 8 to 10 years.

Compared to laboratory animals, such as rats and mice, mink are in a higher trophic level and have a longer life span. Therefore they may be exposed to higher concentrations of PBDEs over a longer period of time than typical laboratory test species. In addition, because of their dietary habits, they may have a different metabolic capacity than rats and mice, possibly including a more efficient elimination of xenobiotics via

1984. It is now commonly used to test the cognitive ability of rats and mice, by testing whether they can learn to orient and swim rapidly to an invisible escape platform using distant cues.

biotransformation. However, metabolism of xenobiotics may also be a bioactivation process. This is supported by different congener profiles in DE-71 exposed laboratory rodents and farmed mink (Chapter 2). In addition, mink may respond differently to PBDE exposure than laboratory rodents. For example, cholinergic parameters were not affected in mink following chronic exposure to DE-71 [161], whereas both rats and mice were indeed affected. Taken together, the mink species is a more environmentally relevant model to study the fate of PBDEs and other accumulative organohalogens. For example, mink have previously been used to investigate environmental toxicants such as dichloro-diphenyl-trichloroethane (i.e. DDT) and PCBs [162, 163].

1.4 Objectives

Health and environmental concerns have been raised as a consequence of observations that concentrations of PBDEs were quickly increasing in biota, particularly the lower brominated congeners, and thus investigations on the environmental concentrations, toxicological effects, and fate of PBDEs continue to be undertaken. Risk assessment, regulation, and/or replacement of PBDEs with suitable alternatives depend on sound decision-making supported by scientific evidence. An understanding of how these compounds circulate between environmental components, particularly within and among relevant organisms, including bioaccumulation, excretion, metabolism, and maternal transfer, is thus a very important consideration.

Taking mink as a sentinel species and as the study subject, the present work aims i) to characterize the dietary accumulation, disposition and metabolism of PBDEs in pregnant mink and their offspring and ii) to study potential metabolic pathways for PBDEs

including debromination and hydroxylation by in vitro methods using mink intestinal microflora and hepatic microsomes.

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Chapter 2: Dietary accumulation, disposition, and metabolism of technical pentabrominated diphenyl ether (DE-71) in pregnant mink (*Mustela vison*) and their offspring[9]

2.1 Introduction

Because of their wide use in consumer products, polybrominated diphenyl ethers (PBDEs) are now ubiquitous in the abiotic environments [1, 2], wildlife [3, 4], and in humans [5-7]. This may present environmental health risks given that animal studies have demonstrated that PBDEs can cause adverse effects on reproductive capacity, thyroid hormone regulation, and on the central nervous system (CNS) [8].

Although the highly brominated deca-BDE mixture is the major commercial formulation in use today, and its primary component (BDE-209) is now the major congener in many abiotic environmental samples [1], the most prominent and frequently detected PBDEs in wildlife and human samples are the tetra- to hexa-brominated congeners, BDE 47, 99, 100, and 153 [9]. These are the major congeners in penta-BDE commercial formulations, such as DE-71, that are now largely phased out, but because of

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[9] A version of this chapter has been published as "Dietary accumulation, disposition, and metabolism of technical penta brominated diphenyl ether (DE-71) in pregnant mink (*Mustela vison*) and their offspring" in *Environmental Toxicology and Chemistry*. Available online at http://dx.doi.org/10.1897/07-487.

their persistence in the environment, the fact that they accumulate in organisms to a greater extent than more highly brominated PBDEs [10, 11], and the presumed stockpile of current-use products still containing penta-BDE mixtures, continued study of their environmental fate is warranted.

Studies on arctic marine animals, using lipid-normalized tissue concentrations as a proxy for whole-body concentration, have demonstrated that biomagnification factors (BMFs) for major BDE congeners (BDE 47, 99, 100, 153, 154) are larger than 1 (Table 1-5). These data are highly suggestive of significant biomagnification potential in these species [12, 13], but BMFs derived from monitoring studies can be uncertain due to the wide range of food sources (spatially, temporally, and ranging from plants to small animals), thus making it hard to define the true concentration in prey. Therefore, there is great value in conducting dietary accumulation studies in a controlled laboratory setting where food concentrations are constant and multiple tissues can be analyzed to ensure that the analytes are distributed evenly among tissues on a lipid-normalized basis.

Previous studies have investigated PBDE toxicokinetics in traditional laboratory animals, but these are not necessarily good models for higher trophic level wildlife. Even among common laboratory test-species, there are significant differences with regards to metabolism and excretion of specific BDE congeners [14-16]. Consistent with this observation, different wildlife also vary widely with respect to their PBDE BMFs. For example, BMFs of the predominant congeners for sparrowhawks and common buzzards were larger than 1, whereas red foxes had lower tissue concentrations than their potential prey [17].

Mink are high trophic level carnivores and are known to be susceptible to the accumulation of other persistent organic pollutants, such as polychlorinated biphenyls (PCBs) [18], and thus were chosen here as an environmentally relevant test-species to test PBDE accumulation. As part of a larger study examining the effects of DE-71 on reproduction, endocrine disruption, and development of the CNS in these animals [19, 20], we report here on the congener-specific dietary accumulation, disposition, and metabolism of DE-71 in pregnant farmed mink and their offspring.

2.2 Materials and methods

2.2.1 Chemicals and standards

Standard solutions of PBDEs including DE-71 and standard solutions containing BDE 28, 47, 66, 85, 99, 100, 153, 154, [^{13}C]BDE 138, and [^{13}C]6-OH-BDE 47 were purchased from Wellington Laboratories (Guelph, ON, Canada). The PCB standard PCB 199 was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Ottawa sand (60-100 mesh), Florisil® (60-100 mesh), hexanes (optima grade), acetone (optima grade), and methyl *t*-butyl ether (MTBE) were obtained from Fisher Scientific (Ottawa, ON, Canada), and dichloromethane (Omnisolv) was obtained from VWR (Mississauga, ON, Canada). Bio-Beads® S-X3 (200–400 mesh), for size exclusion chromatography, were purchased from Bio-Rad (Mississauga, ON, Canada). The derivatization reagent diazomethane was generated from nitrosomethylurea (Sigma Aldrich, Oakville, ON, Canada).

2.2.2 Animal treatment

Forty first-year virgin, natural dark, female mink were housed at the Michigan State University (MSU) Experimental Fur Farm (East Lansing, MI, USA) as previously described [18, 19]. This application to use animals in research (05/03-069-00) was approved by the Michigan State University Institutional Animal Care and Use Committee. Ten female mink were randomly chosen per group and each group received the standard MSU Experimental Fur Farm ranch diet spiked with one of four treatment doses of DE-71 in mink feed: 0, 0.1, 0.5, or 2.5 µg/g wet weight (ww). Samples of each treatment diet were frozen at -20°C for subsequent analysis of PBDEs. Animals commenced their respective treatment diets 5 to 7 weeks prior to breeding, through 6 weeks (on average) of gestation, and until weaning at 6 weeks post-parturition. Spiked feed and clean water were available ad libitum. Dietary intake of DE-71 was estimated from feed consumption and body weight (bw) of females recorded weekly during the first 4 weeks of treatment.

Offspring were completely weaned by 6 weeks of age, at which time all adult females and six offspring (6-week-old kits) per treatment group were necropsied. Ten offspring from each treatment group, except at the highest dose (because there were no offspring in this group), were maintained on their respective treatment diets until approximately 27 weeks of age at which time these offspring (7-month-old juveniles) were all necropsied. Urine and feces from the juveniles were collected 5 or 6 d before necropsy by suspending a screen with a plastic bag attached from the bottom of the cage for 24 h. The feces were collected from the top of the screen while urine was collected in the plastic bag to minimize any cross-contamination. Whole-body and major organ weights were recorded at necropsy. Samples of plasma, whole liver, hind femur muscle, abdominal fat, and

centrifugation (2000 × g for 5 min), the organic phase was transferred to another test tube. This procedure was repeated again after the addition of 10 ml of hexane:MTBE (9:1) to the lower layer, and the two organic phases were combined and evaporated to dryness. The residue was weighed to calculate the lipid content, dissolved in 3 ml of dichloromenthane:hexane (1:1, v/v); fractionated by size-exclusion in vertical glass columns (55 cm × 27 mm inner diameter (i.d.)) packed with 60 g of Bio-Beads S-X3. Size-exclusion columns were eluted with dichloromethane:hexane (1:1, v/v), and the first 140 ml fraction, containing lipids, was discarded and the second 220 ml fraction, containing analytes, was collected, reduced, and split into two aliquots.

The first aliquot was to be analyzed for parent PBDEs and was further cleaned by passing through glass columns (30 cm x 10 mm i.d.) packed with 8 g of 1.2% deactivated Florisil, eluted with 100 ml of hexanes, reduced to dryness, and solvent-exchanged into 500 μl toluene. An instrument performance internal standard, 10 μl accepted of 100 ng/ml PCB 199, was added before analysis.

The second aliquot was analyzed for OH-PBDEs. A second internal standard, [^{13}C]6-OH-BDE 47 (2 ng) was added prior to fractionation of parent and OH-PBDEs. The fractionation method has been described by Verreault et al. [22] but with minor modifications here. Briefly, 6 ml of KOH solution (1 M in 50% ethanol) was added to the extract and the mixture was vortexed for 30 s, allowing OH-PBDEs to partition to the aqueous phase. The aqueous fraction was then acidified by 1 ml of 3 M H_2SO_4 and back-extracted with 10 ml hexane:MTBE (9:1, v/v) three times. The extract was then washed with water until the pH exceeded 5.0, and solvent-exchanged to hexane. OH-PBDEs were derivatized by diazomethane at room temperature for at least 3 h. The solvent was then

whole brain were collected and initially frozen at -80°C but were later shipped on dry ice and stored at -20°C (brain tissues were stored at -80°C) until the time of PBDE analysis. Three mink from each of the maternal and kit groups, and four mink from the juvenile group were analyzed for PBDEs or OH-PBDEs. Only adult females that had corresponding kits and surviving juveniles were chosen for analysis.

2.2.3 Analytical methods

All tissues were homogenized by mortar and pestle in liquid nitrogen and extracted by accelerated solvent extraction (ASE 200, Dionex, Sunnyvale, ON, Canada). Fecal samples were extracted identically to tissue samples except that they were homogenized at room temperature. Prior to sample extraction, each ASE cell was pre-cleaned by the following procedure: two cellulose filters were placed in each ASE cell (22 ml), the void volume filled with Ottawa sand, and the cell was washed with dichloromethane:acetone (1:1, v/v) at 100°C and 1,500 psi with a 5 min heat-up period and three static cycles. Depending on the sample size, up to 3 g of sample (0.5 g of fat) was weighed, dispersed in pre-washed Ottawa sand in a pre-washed aluminum weighing boat, loaded into the pre-washed ASE cell, and spiked with the internal standard ([^{13}C]BDE 138). Samples were extracted by the same method used for ASE cell washing. After high temperature and pressure ASE extraction, tissue samples with Ottawa sand were disposed as chemical wastes. The extracts were reduced to dryness under a gentle stream of nitrogen. The removal of coextracted water and particles has been described by Saito et al. [21] but briefly, 10 ml of hexane:MTBE (9:1, v/v) and 10 ml of 1% KCl in 0.1 M H_3PO_4 (w/v) aqueous solution were added to the residue, and the solution was mixed thoroughly. After

reduced to dryness and analytes dissolved in 100 μl of toluene. For urine (2 to 5 ml) and pooled plasma (5 g) samples, both internal standards were added prior to extraction. Then the samples were extracted three times by liquid-liquid extraction using 5 ml of hexane:MTBE (1:1, v/v) followed by fractionation and derivatization as described above.

Parent PBDEs, derivatized OH-PBDEs, and internal standards were analyzed by gas chromatography/mass spectrometry (GC/MS) using an HP6890 gas chromatograph (Agilent, Palo Alto, CA, USA) with a DB-5MS capillary column (25 m × 0.25 mm i.d. × 0.25 μm film thickness, J&W Scientific (Folsom, CA, USA)) and helium as the carrier gas (2 ml/min) coupled to an HP5973N mass spectrometer (Agilent) operated in electron impact ionization mode. The injections (1 μl) were made in splitless mode at 230°C. The GC oven temperature was initially 110°C, ramped to 250°C at 32.5°C per min, then to 275°C at 10°C per min and to 325°C at 20°C per min, followed by a 5 min hold. The transfer line and ion source temperatures were held at 300 and 150°C, respectively, and the electron energy was always 69.6 eV. Identification and quantification of PBDEs and OH-PBDEs including QA/QC criteria are described in detail in Appendices V and VI.

The concentrations of BDE 28, 47, 66, 85, 99, 100, 153, and 154 were quantified by their relative response to [^{13}C]BDE 138. Internal standards also acted as a recovery surrogate and were quantified by their relative response to the instrumental performance internal standard. Only the samples for which the absolute recovery of internal standard ranged between 70 and 100% were considered valid and included in data analysis; invalid samples were reprocessed. The sum of eight quantified BDE congeners was considered to be the total amount of PBDEs in DE-71, because they accounted for approximately 96% of DE-71 by weight according to supplied specifications (Wellington Labs). The entire

method was verified and monitored every five batches using the certified reference material (WMF-01, fish, Wellington Laboratory). The method detection limit (MDL) was determined based on the minimum concentration per g (for tissues and feces) or per ml (for urine) of sample required to reach the instrumental quantification limit (signal to noise ratio ≥ 10). For statistical analysis, congener concentrations below the MDL were estimated as half the MDL (ng) / sample weight (g). Statistical analysis was performed by GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA) and Microsoft Excel 2003 (Redmond, WA, USA). The Student's *t*-test, or one-way analysis of variance followed by Tukey's test, was applied to test for statistical differences ($\alpha = 0.05$) of concentrations between tissues or age groups.

2.3 Results and Discussion

2.3.1 Method validation

All analyte concentrations in certified standard material were quantified within the range of reference values and their relative standard deviations were less than 10%. BDE 47 (and sometimes BDE 99) was detectable in control animal samples; however, the concentrations were always less than 10% of the lowest dose group, therefore concentrations were not corrected for control concentrations.

2.3.2 Exposure scenario

The exposure scenarios, including exposure route and duration, were different for the three different age groups of mink: adult females, kits and juveniles. The adult females were exposed to DE-71 in their diets 6 weeks before gestation (on average), throughout the 6 weeks of gestation (on average), and during 6 weeks of lactation. Kits received DE-71 indirectly through their mother during in utero and lactation, and the subsequent juveniles, in addition to their exposure as kits, were exposed to DE-71 in their diets for an additional 21 weeks.

The measured concentration of DE-71 in the nominal diets of 0.1 and 0.5 μg/g were 0.078 ± 0.004 ($n = 3$, mean ± standard deviation) and 0.45 ($n = 1$) μg/g ww respectively, and the composition of major BDEs was 35, 44, 11, 3.8, and 3.5% for BDE 47, 99, 100, 153, and 154, respectively. Due to decreased food consumption at the two higher doses (0.5 and 2.5 μg/g), and reproductive toxicity at the highest dose (2.5 μg/g), only samples collected from the lowest dose (0.1 μg/g) were considered in this paper to reflect PBDE disposition in healthy animals. Since 0.5 μg/g was the lowest-observed-adverse-effect-level (LOAEL, reduced food consumption), these tissue concentrations may be useful as critical concentrations for any future biomonitoring and risk assessment in mink. For this purpose, the tissues (liver, muscle, and fat) of one adult female from the 0.5 μg/g dose group were analyzed for parent PBDEs and whole-body concentrations calculated (Figure 2-1A).

Assuming that juveniles consumed feed at the same rate as the adult female, the daily intake of total PBDEs for mink in both age groups was approximately 0.01 mg/kg/d at the 0.1 μg/g dose. The daily consumption rates for neonatal mink, during lactation, were not

determined directly (i.e. milk samples were not obtained); in a previous study, however, the average milk consumption during lactation by mink kits was 29.0 g/d and the fat content of milk was 30% [23]. It was assumed that mink milk would share the same lipid-normalized total PBDE concentrations as adipose tissue (900 ± 140 ng/g, mean ± standard deviation, $n = 3$) given that PBDE concentrations in human breast milk and adipose tissue were also similar [24, 25]. Therefore, the average daily intake of total PBDEs during lactation was approximately 0.015 mg/kg bw in the 0.1 μg/g dose group.

2.3.3 Tissue distribution and whole-body concentrations

Concentrations of BDE congeners in liver, muscle, and fat for the three age groups (adult females, kits, and juveniles) were analyzed. Wet weight tissue concentrations were significantly higher in fat than in liver or muscle. This was in agreement with previous studies in rodents [14-16] and, as expected, revealed that these hydrophilic compounds tend to accumulate in lipid-rich tissues. Lipid-normalized concentrations in tissues of adult females, kits, and juveniles are shown (Figures 2-1B–D), with the exception of BDE28 and 66, which were detected but minor. For all congeners detected, the lipid-normalized concentrations in all age groups for these tissues were not statistically different except for a few exceptions in kits. In kits the concentrations of BDE 47 in liver were significantly lower than in fat, and BDE 99 in kit liver was significantly lower than in either fat or muscle. The similarity of lipid-normalized concentrations in different tissues was also observed in a field study in wild birds and rodents [17, 26] and suggests that, in general, accumulation of PBDEs is lipid content dependent.

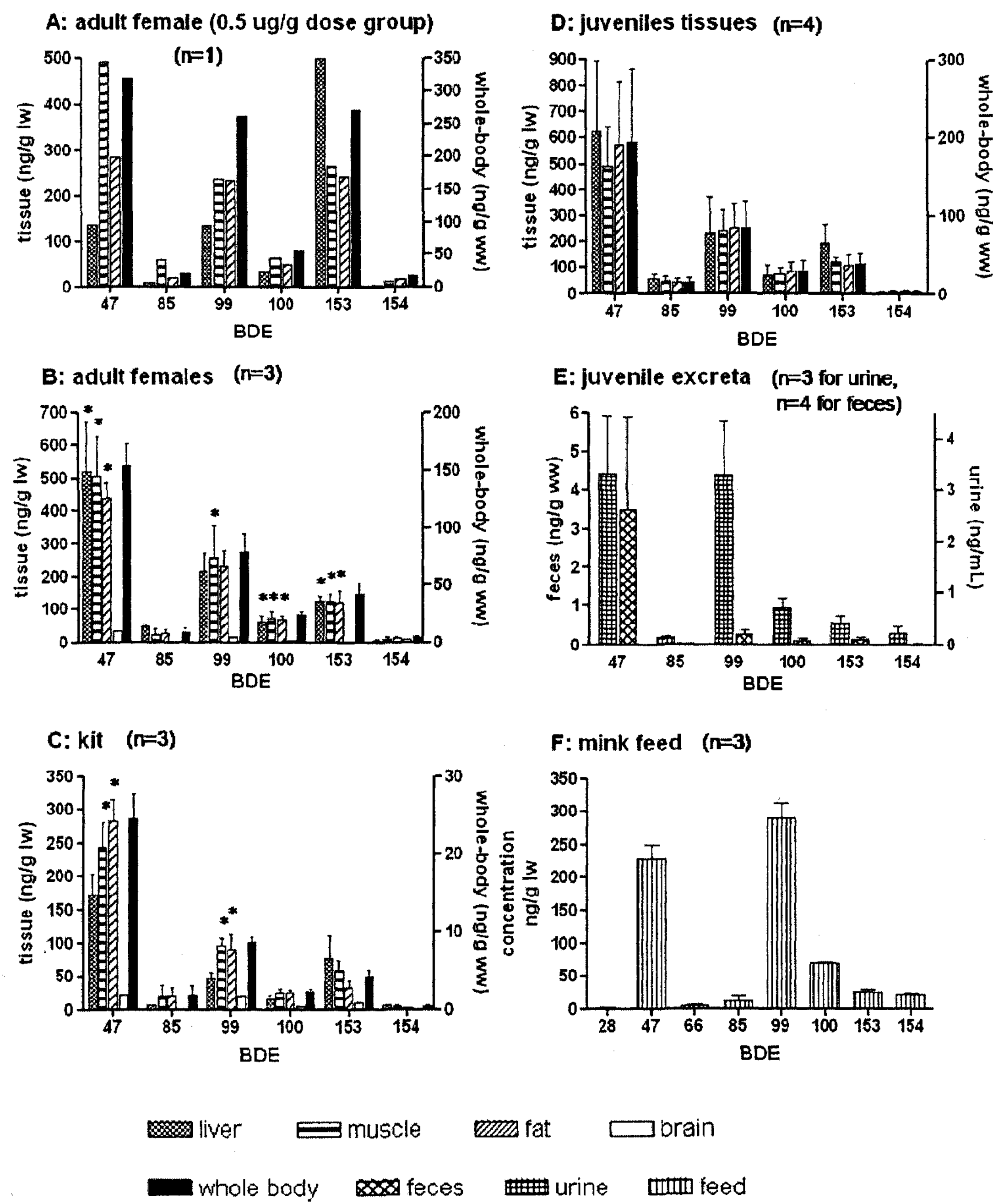

Figure 2-1: PBDE congener concentrations (ng/g lipid weight (lw)) of tissues (liver, muscle, fat, and brain) and the whole-body concentrations (ng/g ww) of adult females from (A) 0.5 μg/g dose group (*n*=1) and (B) 0.1 μg/g dose group (*n*=3); also from (C) kits, (D) juvenile tissues from 0.1 μg/g dose group (*n*=4), (E) juvenile excreta from 0.1 μg/g dose group (*n*=3 for urine, *n*=4 for feces) and (F) mink feed (*n*=3). Error bars represent one standard deviation. *Lipid-normalized concentrations in the brain were significantly lower than other tissues ($p<0.05$).

However, plasma and brain tissue were exceptions. The lipid-normalized ΣPBDE concentration in the plasma of adult females was 406 ng/g lw (three pooled samples) and 337 ng/g lw (average of concentrations from female (n = 5) and male (n = 5), n = number of pooled samples) in juveniles. The plasma concentrations were 2- to 4-fold lower than in the liver, muscle, and fat of adult females, and were 3- to 6-fold lower in juveniles. Furthermore, lipid-normalized concentrations in the brain of adult females were 2- to 5-fold lower than those in the plasma, and kit brain concentrations were 2- to 6-fold lower than those in all other kit tissues (Figures 1B and C).

Lower PBDE concentrations in the brain were also observed experimentally in rodents (ww-based) [27, 28] and in wild mice and voles (lipid-normalized) [17]. This suggests that the blood-brain barrier, which is known to protect the CNS from xenobiotic exposure [29], limits the diffusion of PBDEs into the CNS. Moreover, unlike liver, muscle, and fat, in which maternal concentrations (lw-based) were significantly higher than those of kits, brain concentrations were similar between age groups. In other words, the kits had a higher proportion of the total PBDE body burden in brain tissue compared to their mothers, presumably because development of the blood-brain barrier was not complete until after birth. In rats, for example, the development of the blood-brain barrier begins in the embryo but is not completed until weaning [29, 30].

Whole-body concentrations were reconstructed from measured tissue concentrations (muscle, liver, and abdominal fat), predicted skin subcutaneous fat concentrations, and the relative tissue weight to the bw. It has been demonstrated that skin accumulates higher PBDE concentrations (presumably equivalent on lw basis) of PBDE-derived radioactivity than liver or muscle in experimental mammals, especially after repeated

doses [14-16]. In mink, skin subcutaneous fat tends to accumulate lipophilic substances such as DDT [31]; it accounts for 17% of the bw in female mink and 21% in male mink [32] and thus could not be discounted here. For BMF calculation purposes, we assumed that skin subcutaneous fat had an equal lipid-normalized PBDE concentration to abdominal fat. This was justified given that there were no statistically significant differences among lipid-normalized BDE congener concentrations in adipose, liver, and muscle. Concentrations of all other tissues and organs were assumed to be negligible because of their minor contribution to the overall mass of the animal. The relative tissue weight was estimated based on previous studies on mink and other animals (i.e. rabbits and rats) [31-33].

Among juveniles, it is notable that a consistent trend was observed whereby the two females had higher whole-body concentrations for each congener than the two males. However, statistical significance could not be tested due to the small sample size ($n = 2$ for each sex) (Figure 2-2A), thus female and male juvenile data were combined for analysis in all of the following sections. The lower whole-body concentrations in male juveniles were due, at least partially, to more rapid growth dilution because male mink grow faster than female mink after 6 weeks of age [34], and at the end of the present study had significantly higher bw than females. The concentration differences could not otherwise be explained by differential metabolism or excretion since the general rank of the major BDE congeners (Figure 2-2A) was not different between the sexes and no overt differences were seen between female and male excretion (Figures 2-2B and C). The difference between the sexes is similar to that shown in rodents, in which the difference

diminished with increasing degree of bromination [14-16] from BDE 47 (tetra) through BDE 99 (penta) and BDE 153(hexa) (Figure 2-2A).

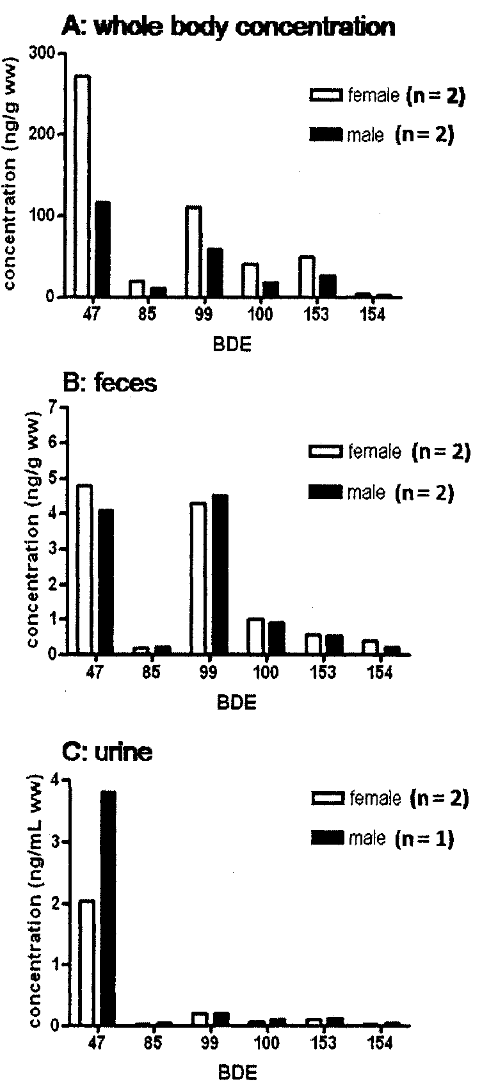

Figure 2-2: Concentrations of PBDE congeners in female (n = 2) and male juvenile mink (n = 2). Means are shown, but due to the limited number of samples no statistical analysis was performed.

2.3.4 Maternal transfer of PBDEs

In the present study, the pathways of maternal transfer to the kits included in utero and lactational transfer. The percent maternal transfer was defined in the present study as

the kit's body burden at weaning divided by the sum body burden of the kit and its respective mother at weaning. Ww-based whole-body concentrations in kits at weaning were always less than concentrations in the corresponding mother, and the average maternal transfer for total PBDEs was 6.2 ± 1.3%. In fact, PBDEs may accumulate to a higher extent in kits than their mothers in some specific tissue such as plasma. This result has been observed in mice [35]. Congener-specific maternal transfer was calculated for those congeners that were always above the MDL, and a plot of maternal transfer versus the number of bromine atoms revealed a statistically significant inverse linear relationship, suggesting that maternal transfer favoured the lower brominated BDEs (Figure 2-3). This general trend was also suggested by Meironyte-Guvenius et al. based on paired analysis of human maternal and cord blood samples [25].

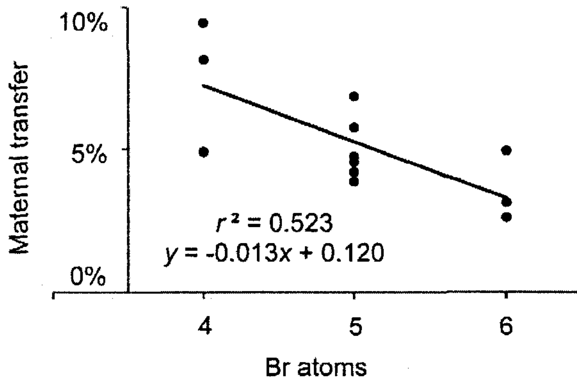

Figure 2-3: Maternal transfer favoured the lower brominated PBDEs, as demonstrated by the statistically significant negative slope ($p < 0.05$), when calculated for each PBDE congener and plotted versus the number of bromine atoms.

Human studies also indicate that the placenta acts as a barrier to PBDEs, and the fetus does not accumulate a greater concentration than the mother, as determined by comparing cord blood to maternal blood [25, 36]. For species without a placenta, such as birds, the

PBDE concentrations in birds and their eggs are similar based on lipid-normalized concentrations [17]. In this study, although newborns were not analyzed at birth, the bulk of the kits' body burden at weaning was expected to be resulted from lactational transfer, rather than transplacental transfer. This is because kits are only approximately 10 g at birth [34, 37], and assuming a worst-case scenario in which the kits shared the same concentration of PBDEs as their mother at parturition, the total mass of PBDEs received through in utero exposure could be no greater than 3 μg. Given that mink kits had accumulated an average of 21 μg total PBDEs by the time of weaning, the contribution of in utero exposure must account for less than 15% of the body burden by this stage. This is not to say, however, that in utero exposure is not toxicologically significant; if indeed the kits shared the same concentration as their mothers at or before birth (312 ng/g bw), this is far higher than measured at weaning (41.7 ng/g bw). Additionally, although the kits had a lower body burden at weaning, some tissues (such as plasma) may accumulate higher levels of PBDEs than their mothers [35].

2.3.5 Biomagnification factors (BMFs)

When reporting accumulation factors from laboratory studies it is important to consider whether the exposure was long enough to allow steady-state concentrations to be achieved. In the present study, we did not have sufficient animals to prove this by sampling at several different times, thus we can only assume that juveniles approached steady-state after 21 weeks of dietary exposure. For this reason, and because growth dilution was occurring to some extent, BMFs presented here may be somewhat underestimated. Adult females were excluded from BMF analysis because of the complex

physiological changes during pregnancy, including transplacental transfer of PBDEs, which would result in underestimation of whole-body concentrations.

Wet-weight biomagnification factors (*ww*BMFs) were calculated by dividing reconstructed whole-body concentrations of juveniles by the measured food concentration (Table 2-1). Mean *ww*BMFs ranged from 1.3 for BDE 154 to 13 for BDE 153; only BDE 47 and 153 had *ww*BMF significantly greater than 1. The rank order, from most to least accumulative, was BDE 153 > 47 > 28 > 100 > 99 > BDE 154, and was evidently not a simple function of the number of bromine atoms. This general trend for accumulation potential of BDE congeners has also been demonstrated in several food webs [10, 11].

Table 2-1: BMFs of PBDEs reported in the literature and from the present study based on either lipid-normalized or whole-body concentrations.

	Predator/Prey	**BDE 28**	**BDE 47**	**BDE 99**	**BDE 100**	**BDE 153**	**BDE 154**
		BMFs based on lipid-normalized concentrations (*lw*BMFs)					
Marine food web	Harbor seal/cod [9]	3.6	29	6.3	44	>140	5.4
	Polar bear/ringed seal [13]	0.1	0.5	0.3	0.3	7.5	0.3
	Polar bear/ringed seal [12]	—	3.9	5.8	4.7	71	—
Juvenile mink	Mink/mink feed (*n* = 4, mean ± 95% confidence interval)	1.2 ± 0.8	2.6 ± 1.7	0.9 ± 0.5	1.2 ± 0.9	5.2 ± 2.7	0.5 ± 0.3
		BMFs based on ww-based concentrations (*ww*BMFs)					
Marine food web	Polar bear/ringed seal [13]	0.16	0.4	0.29	0.23	5.2	0.25
Juvenile mink	Mink/mink feed (n=4, mean ± 95% confidence interval)	5.6 ± 4.2	7.1 ± 5.4	2.4 ± 1.6	3.5 ± 2.6	13 ± 7	1.3 ± 0.8

— Not available.

For lipophilic contaminants, increases in the wet-weight concentration up a food chain (e.g. *ww*BMF > 1) can occur as a simple result of a relatively higher lipid content in

the consumer, and thus may be misconstrued as biomagnification. In this study, the whole-body mink lipid content was approximately 3-fold higher than the lipid content of spiked food, and thus a better measure of true biomagnification (e.g. fugacity driven) is the lw-normalized BMF (*lw*BMF) [38]. The *lw*BMFs were calculated by dividing the mean lipid-normalized abdominal fat concentrations by the measured lipid-normalized food concentrations (Table 2-1). The rank order of congener-specific *lw*BMFs is identical to the rank order of *ww*BMFs, but as expected *lw*BMFs are approximately 3-fold lower. The *lw*BMFs also allow a comparison from this study to field observations of various predator/prey relationships in which lipid-normalized BMFs were reported (Table 2-1). The experimental *lw*BMFs for mink agree with observations in the marine food web, whereby all studies indicate that BDE 153 is the most accumulative congener through dietary exposure when measured (Table 2-1).

2.3.6 Interpretation of PBDE profiles

Congener profiles of PBDEs were examined in each tissue and at each life-stage. As described previously, although juveniles had undergone indirect exposure (i.e. lactation) as kits and direct exposure through food, the accumulation of PBDEs from the latter exposure was much more significant as indicated by the low body burden at 6 weeks of age (~21 μg) compared to the accumulation during the 21 weeks of dietary exposure (~760 μg). Consequently, the exposure scenario for juveniles was not dramatically different from that for adult females and this is reflected in the similar BDE congener profile (Figures 2-1B and D). Despite the congener profile in kit liver being different from either muscle or fat, the whole-body profile for kits was also similar to adult

females and juveniles (Figure 2-1C). There was, thus, no apparent difference caused by the life-stages. In mink, the general rank-order of major BDE congener concentrations was: BDE 47 > 99 > 153 > 100, 154. However, this rank-order was clearly different from food (BDE 99 > 47 > 100 > 153, 154) and urine and feces (BDE 47, 99 > 100, 153, 154).

The relative abundance of BDE 47, a tetra-BDE, was highest in urine followed by whole-body, feces, and food. In contrast, the two penta-BDEs, BDE 99 and 100 showed the opposite trend. The relative proportion of BDE 99 and 100 significantly decreased from diet to whole body. Given that the tetra-BDE was less prominent than penta-BDEs in food and that it seemed to be selectively excreted relative to penta-BDEs, it is initially counterintuitive that it became more prominent than penta-BDE in the whole-body after long-term exposure. However, these results are most likely reflective of the debromination of penta-BDEs as has been reported by Stapleton et al. for fish [39], but penta-BDEs may also undergo metabolic oxidative debromination [16]. Furthermore, the ratio of BDE 99 to BDE 100 was similar in the feed and feces (4.4 ± 0.7, mean ± standard deviation, $n = 4$), but significantly lower in the whole-body of mink (3.0 ± 0.3, mean ± standard deviation, $n = 4$). These results suggest that BDE 99 is more vulnerable to metabolism than BDE 100, and that the biotransformation product of penta-BDEs may be BDE 47.

Despite BDE 153 and 154 having very similar concentrations in food, these hexa-BDE congener concentrations diverged in all samples of mink. For example, BDE 153 was an order of magnitude higher than BDE 154 in whole-body samples and the relative concentration of BDE 153 increased from 3.8 ± 0.3% in diet to 11 ± 2% in the whole body. The apparent preferential accumulation of BDE 153 cannot be attributed to any

debromination of higher congeners because DE-71 has only two hepta-BDEs in the mixture that account for less than 0.15% by mass. Furthermore, this could not be explained by more efficient excretion of BDE 154 because it was below the MDL in 66% of urine samples and 50% of feces samples, whereas BDE 153 was always at least two times higher than the method quantification limit in the same samples. Therefore it can only be concluded that BDE 154 was being metabolized to a much greater extent than BDE 153, and this fact explains the large difference in their BMFs. This is reasonable given the tri-*ortho* substituted bromine atoms present in BDE 154 but not in BDE 153, thus making BDE 154 more vulnerable to P450 oxidation or debromination [12]. Presumably, the *meta-para* substitution of bromine atoms in BDE 153 also makes it more recalcitrant to metabolism than BDE 154.

Some other DE-71 feeding studies are available with which to compare the BDE profile changes observed here. The most recent study involves rats orally exposed to DE-71 for 21 d at 0.12 μg/kg/d [40]. The congener profile in food was similar to ours (BDE 99 > 47 > 100, 153, 154), but the consequent congener profile in rat carcasses (BDE 99 > 47, 100 > 153 > 154) was quite different than that in the whole body of mink (BDE47 > 99 > 153 > 100 > 154). The difference in the congener profile between these two species could either be due to the difference in daily dose or to different metabolic capacities. In the present study, mink were exposed to a dose two orders of magnitude higher than the rat study; however, no toxicity was evident in the present work and the metabolic capacity was unlikely to have been overwhelmed here given that even 1000× higher doses in mink, in a different study, produced a similar profile [20]. Therefore, the

differences are most likely due to species differences in biotransformation, indicating that rats do not provide a good model for this environmentally relevant species.

2.3.7 Metabolites of PBDEs

Congener profile changes suggested significant metabolism of several congeners and this was supported by metabolite detection. Methoxy-PBDEs were not detectable in any sample, but OH-PBDEs were detected in the plasma, liver, and feces of juveniles in the 0.1 μg/g dose group (Figure 2-4). Concentrations of OH-PBDEs were below MDLs in muscle, abdominal fat, and urine. MDLs, based on [^{13}C]6-OH-BDE 47 response, were 0.03 ng/g for tissue and fecal samples and 0.01 ng/ml for urine and plasma samples. In plasma, liver, and feces, five mono-hydroxylated tetra-BDEs (OH-tetra-BDEs, peaks A-F, Figure 2-4), five mono-hydroxylated penta-BDEs (OH-penta-BDEs, peaks G-K, Figure 2-4), and two mono-hydroxylated hexa-BDEs (OH-hexa-BDEs, peaks L and M, Figure 2-4) were observed. Other unlabeled peaks (Figure 2-4) were present in control animals, and are not PBDE metabolites. One of these OH-tetra-BDE congeners was identified as 6-OH-BDE 47 (peak A, Figure 2-4) based on the same retention time as the internal standard, [^{13}C]6-OH-BDE 47. Although not detectable in the fat of the low-dose group (0.1 μg/g), OH-PBDEs were observed in the fat extract of an adult female from the higher-dose group (0.5 μg/g) (Figure 2-4). Unlike parent PBDEs, the OH-PBDEs profiles varied among plasma, liver, fat and fecal samples (Figure 2-4) as previously noticed by Orn and Klasson-Wehler [41]. Therefore, biomonitoring of OH-PBDEs should consider tissue-dependent accumulation potential.

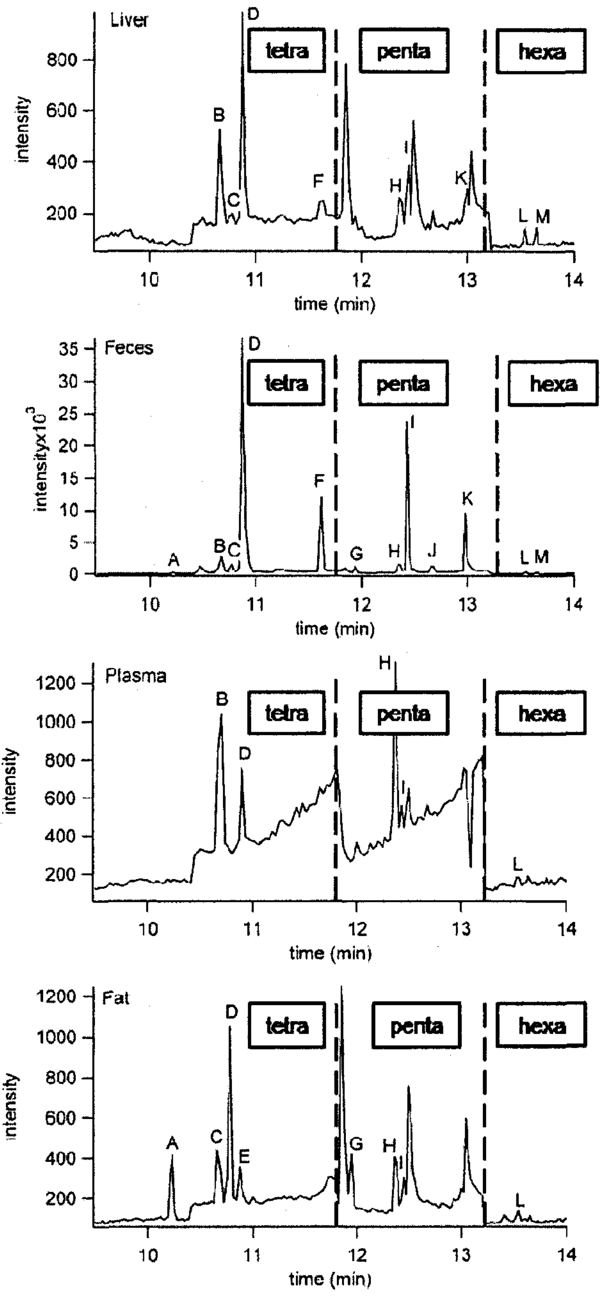

Figure 2-4: GC/MS chromatograms (SIM) for OH-tetra-BDEs (*m/z* of [M^+] is 513.7), OH-penta-BDEs (*m/z* of [M^+] is 595.6), and OH-hexa-BDEs (*m/z* of [M^+] is 673.5) in the derivatized extract of liver and feces (female, 0.1 μg/g dose group shown), pooled plasma (5 individual males, 0.1 μg/g dose group), and abdominal fat (female, 0.5 μg/g dose group). There was no visible difference in hydroxylated brominated diphenyl ether profiles between the males and females for any sample. The juvenile female plasma congener profile (not shown) was identical to the male. Chromatograms of control samples were shown in Appendix VII.

In order to estimate the importance of the hydroxylation pathway, a semi-quantitative approach was used to calculate the total contribution of detected OH-PBDEs to the mass balance by assuming that the instrumental response of the unknown OH-tetra-BDEs was equivalent to 6-OH-BDE 47. The total concentration of all quantifiable OH-tetra-BDEs was 9.75 ng/g in feces ($n = 1$), 0.307 ng/g in liver ($n = 1$), 0.254 ng/g in pooled male plasma (from five male juveniles), and 0.418 ng/g in pooled female plasma (from five female juveniles). No standard was available for semi-quantification of OH-penta-BDEs, but because their total peak areas were the same order of magnitude as those of OH-tetra-BDEs, their overall contribution to the mass balance was presumed to be similar to the OH-tetra-BDEs. The low concentration of OH-PBDEs that accumulated in the mink body was likely due to their higher solubility, brought upon by the hydroxyl group moiety. However, since OH-PBDEs were only analyzed in tissues of plasma, liver, muscle, and fat, we cannot exclude the possibility that other tissues contained significant quantities of these metabolites.

2.3.8 Metabolism versus excretion

Compared to parent PBDEs, OH-PBDEs are more structurally similar to thyroid hormones (Figure 1-6). Accordingly, they are more likely to cause thyrotoxicity via interaction with the thyroid hormone transport proteins in plasma (Section 1.2.4.5.3). Previous studies have demonstrated that PBDE metabolites, but not parent PBDEs, bind with greater affinity to transthyretin than thyroxine (T4) [42]. This may displace T4 from its proteins, thus lowering the circulating levels of T4 since, in its free form, it may be rapidly eliminated through glucuronidation pathways [43, 44]. Therefore, the relative

extent of metabolism to excretion is an important consideration to understand the mechanism(s) of PBDE elimination.

In the present study, parent and OH-PBDEs were analyzed in excreta to compare the importance of excretion and biotransformation. In the female juvenile mink, the total concentration of parent PBDEs in feces was 14.8 ng/g, comprised of 6.31 ng/g from tetra-BDEs. The estimated concentration of all OH-tetra-PBDEs in feces of the same mink was 9.75 ng/g, which was higher than the parent tetra-BDE concentrations and within a factor of two of the total PBDEs. In contrast to feces, parent PBDEs but not OH-PBDEs were detected in urine. Using average volumes or mass of excretion (44 to 72 ml/kg/d urine, and 14 to 56 g/d feces), the total daily mass of parent PBDEs and OH-BDEs excreted in urine and feces were compared (Figure 2-5). Overall, the amount of OH-tetra-BDEs in excreta was substantial but was less than parent PBDEs and accounted for 28 to 32% of the excreted fraction on a mass basis. Although OH-penta-BDEs could not be quantified, they likely contribute to a similar proportion of the mass balance as OH-tetra-BDEs. Furthermore, bromophenols [45] and phase II metabolites such as glutathione or glucuronide conjugates [16] were not monitored here but have been detected in urine samples from PBDE-exposed rats and mice. Therefore the total metabolism may be greater than estimated here by hydroxylation alone. Moreover, some of the parent PBDEs measured in feces may represent the non-absorbed fraction from diet, or may be debromination products of higher parent PBDEs. Taken together, metabolism is likely just as important as excretion for PBDE elimination in mink.

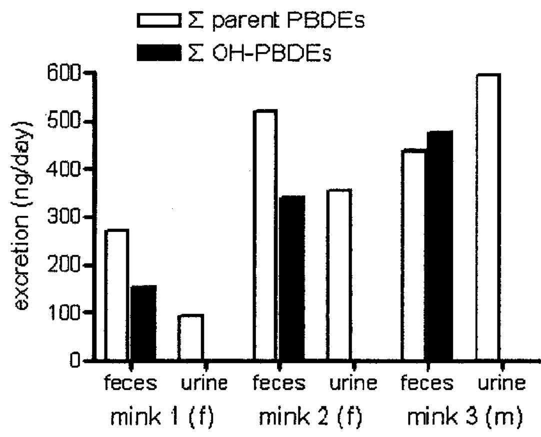

Figure 2-5: Daily excretion of total parent PBDEs and total OH-PBDEs in excreta of juvenile female (f) and male (m) mink.

2.4 Conclusions

The present study found similar lipid-normalized concentrations of PBDEs in most tissues of adult mink, with the exception of brain tissue which was lower in adult females and kits, presumably because of the blood-brain barrier; albeit due to its incomplete development in kits the blood-brain barrier provided less protection than in adults. Dietary exposure to DE-71 resulted in significant biomagnification for some PBDEs in mink, and lipid-normalized whole-body BMFs were highest for BDE 47 and BDE 153, thus validating many environmental biomonitoring data. Maternal transfer to the fetus favoured lower brominated BDE congeners, lactational transfer was much greater than transplacental transfer, and kit whole-body PBDE concentrations were lower than in corresponding adult females. Metabolism clearly limited biomagnification of some PBDEs and was an important elimination pathway that requires further study to understand the mechanisms of PBDE degradation.

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Chapter 3: Metabolic pathways of polybrominated diphenyl ethers (PBDEs)

3.1 Introduction

In Chapter 2, the different congener profiles in food, whole-body, and excreta of mink indicated that the elimination pathways (i.e. excretion, and/or biotransformation) of DE-71 PBDE congeners differed. Furthermore, we suggested that selective metabolism among congeners may be one of the reasons for this. According to the congener profile change and the detected metabolites in Chapter 2, we considered two general metabolic pathways for the elimination of PBDEs, oxidative metabolism (formation of hydroxylated PBDEs (OH-PBDEs)) in the liver, and reductive debromination in the intestines.

The organ responsible for hydroxylation of PBDEs was presumably the liver, such that it expresses the largest amount of cytochrome P450 enzymes that are well-known to catalyze oxidative biotransformation of xenobiotics [1]. P450 enzymes are located in the membrane of the endoplasmic reticulum, and thus can be studied by isolating the microsome fraction of cells, including hepatocytes.

Reductive debromination of PBDEs (i.e. with no simultaneous oxidation), on the other hand, is generally considered to be a reductive process, and thus is most likely to occur within reducing environments of the body. The oxidation-reduction potential becomes more negative along the length of the gastrointestinal tract and there is a

dramatic decrease starting in the cecum which results in a large increase in the number of anaerobic bacteria here [2]. The tetra- to penta-brominated congeners are generally more accumulative (Chapters 1 and 2) and toxic (Chapter 1) than higher brominated congeners. Therefore it is important to characterize the extent of debromination because of potential bioactivation, and also because debromination of the higher brominated congeners, rather than excretion, may have caused a systematic bias leading to overprediction of the accumulation potential of lower brominated BDEs.

Hydroxylation of PBDEs may facilitate their elimination, but this is not necessarily a detoxification pathway. The structures of hydroxylated PBDE metabolites are similar to those of thyroid hormones (Figure 1-9) and they have been shown to interfere with thyroid hormone homeostasis. This effect — of low circulating thyroxine (T4) concentrations — has been shown for individual PBDE congeners and for commercial PBDE mixture exposure in rodents [3-5]. The reduced T4 concentrations may arise by several possible mechanisms. For example, PBDEs may induce hepatic uridinediphosphate-glucuronosyltransferase (UDPGT) [3, 4], which may cause increased phase II conjugation and elimination of T4. Alternatively, reduced T4 may result from competitive binding of OH-PBDEs to transthyretin (TTR) [6] thus displacing T4 from its carrier protein and facilitating elimination of the free T4. A combination of these two mechanisms may also occur.

Taken together, there is value in conducting in vitro metabolism experiments with PBDE congeners in order to better understand the altered BDE profiles and hydroxylated metabolites in mink (Chapter 1). Here we focus on the hepatic microsomal fraction of mink liver and intestinal microflora (from rat) to the study hydroxylation of specific BDE

congeners. In the microflora we tested for debromination using the same DE-71 mixture as in Chapter 2. For the microsomes, because of possible ambiguity from a combination of hydroxylation and debromination reactions occurring simultaneously [7], we tested three pure BDE congeners: BDE 47, 99, and 154. BDE 47 was chosen because of its significant accumulation in the mink body, whereas BDE 99 was hypothesized to be subject to debromination, and we also hypothesized that BDE 154 was significantly metabolized compared to the other major hexa-congener, BDE 153.

3.2 Materials and Methods

3.2.1 Chemicals

The commercial penta-BDE mixture (DE-71) and individual PBDE congeners including BDE 47, 99, and 154 were purchased from Wellington Laboratories (Guelph, ON, Canada). All solvents were Optima grade and were purchased from Fisher Scientific (Ottawa, ON, Canada). Sucrose and HEPES were also purchased from Fisher Scientific. Mannitol, ethylene glycol tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), ethoxy-resorufin, and pentoxy-resorufin were purchased from Sigma-Aldrich (Oakville, ON, Canada). The NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) regenerating system was purchased from BD Biosciences (Oakville, ON, Canada). The derivatization reagent diazomethane was generated from nitrosomethylurea (Sigma Aldrich, Oakville, ON, Canada).

3.2.2 Intestinal microflora metabolism test

3.2.2.1 Experimental design

Intestinal microflora mediated anaerobic degradation of PBDEs was studied for DE-71 in rat cecal microflora. VPI buffer[10] was used for preparation of cecal homogenates and also as the incubation buffer. After the buffer was prepared, dissolved O_2 was purged by 30% CO_2 and 70% N_2 for 20 min, and 10 ml of resazurin (100 mg/L) was added as a redox indicator (i.e. turns pink in presence of oxygen). The prepared buffer (pH 7.14) was then distributed into 200 ml serum bottles, purged again by the same gases as above, and autoclaved for 20 min. The substrate, 100 μl of 6.3 μg/ml of DE-71, was spiked into another five serum bottles: three replicate samples, one positive control, and one negative control. A blank was prepared by spiking 100 μl of hexane into another serum bottle. These bottles were then covered by sponge stoppers, thus allowing the evaporation of solvent overnight in the fume hood. After solvent evaporation, serum bottles were transported into the anaerobic chamber and sealed with a rubber stopper, crimp-sealed by aluminum caps, and filled with 50 ml of medium buffer using syringes.

Cecal homogenate was prepared from cecal content obtained from a healthy male Sprague-Dawley rat. After it was sacrificed by CO_2, both sides of its cecum were tied and removed to maintain the internal anaerobic condition. The cecum was then transported on ice into the anaerobic chamber. Cecal content (7.04 g) was transferred into a tube containing 10 ml of VPI buffer. The tube was then vortexed for homogenization of

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[10] The VPI buffer is a typical type of anaerobic incubation medium. It consists of 0.1 g $CaCl_2$/L, 0.2 g $MgSO_4$/L, 0.5 g KH_2PO_4/L, 5.0 g $NaHCO_3$/L, and 1.0 g NaCl /L.

intestinal microflora, and 1 ml of this homogenate was inoculated by syringe into the triplicate samples, the positive control, and the blank. In the negative control, boiled cecal homogenate was added. Finally, 3 μl of 1,2,4-trichlorobenzene (TCB) was spiked into the positive control bottle because it has been shown to be metabolized to di- and mono-chlorobenzenes in rat cecal microflora [8, 9]. All the bottles were wrapped with aluminum foil and incubated on a vibrating shaker at 37 °C. Samples were taken at 16 h, 24 h, 7 d, and 30 d. After each sampling time, the serum bottles were shaken, and 5 ml of medium was collected by a syringe and placed into 5 ml of hexane:methyl-*t*-butyl ether (MTBE,1:1, v/v) to quench the reactions and to enable extraction.

3.2.2.2 Analytical methods

Before extraction, 200 μl of 25 ng/ml of [^{13}C]BDE 138 (internal standard) was added into the solvent layer of the mixture. An exhaustive extraction was then carried out with hexane:MTBE three times. The extracts were combined and evaporated to about 3 ml for Florisil chromatography, using conditions as described in Chapter 2 (Section 2.2.3). For the final analysis of PBDE samples, the eluents were then evaporated to dryness and dissolved in 100 μl of toluene. PBDE samples were analyzed by gas chromatography/mass spectrometry (GC/MS), also as described in Chapter 2 (Section 2.2.3). The same steps were used for TCB analysis, except that after the Florisil chromatography, the eluent was evaporated to 10 ml. The concentrations of TCB were semi-quantified by peak areas detected by gas chromatography/electron capture detection (GC/ECD) with a DB-5MS capillary column (25 m × 0.25 mm i.d. × 0.25 μm film thickness, Agilent J&W Scientific, Palo Alto, CA, USA) and helium as the carrier gas (2

ml/min). The injections (1 μl) were made in splitless mode at 230°C. The GC oven temperature was initially 80°C and was ramped to 180°C at 5°C per min.

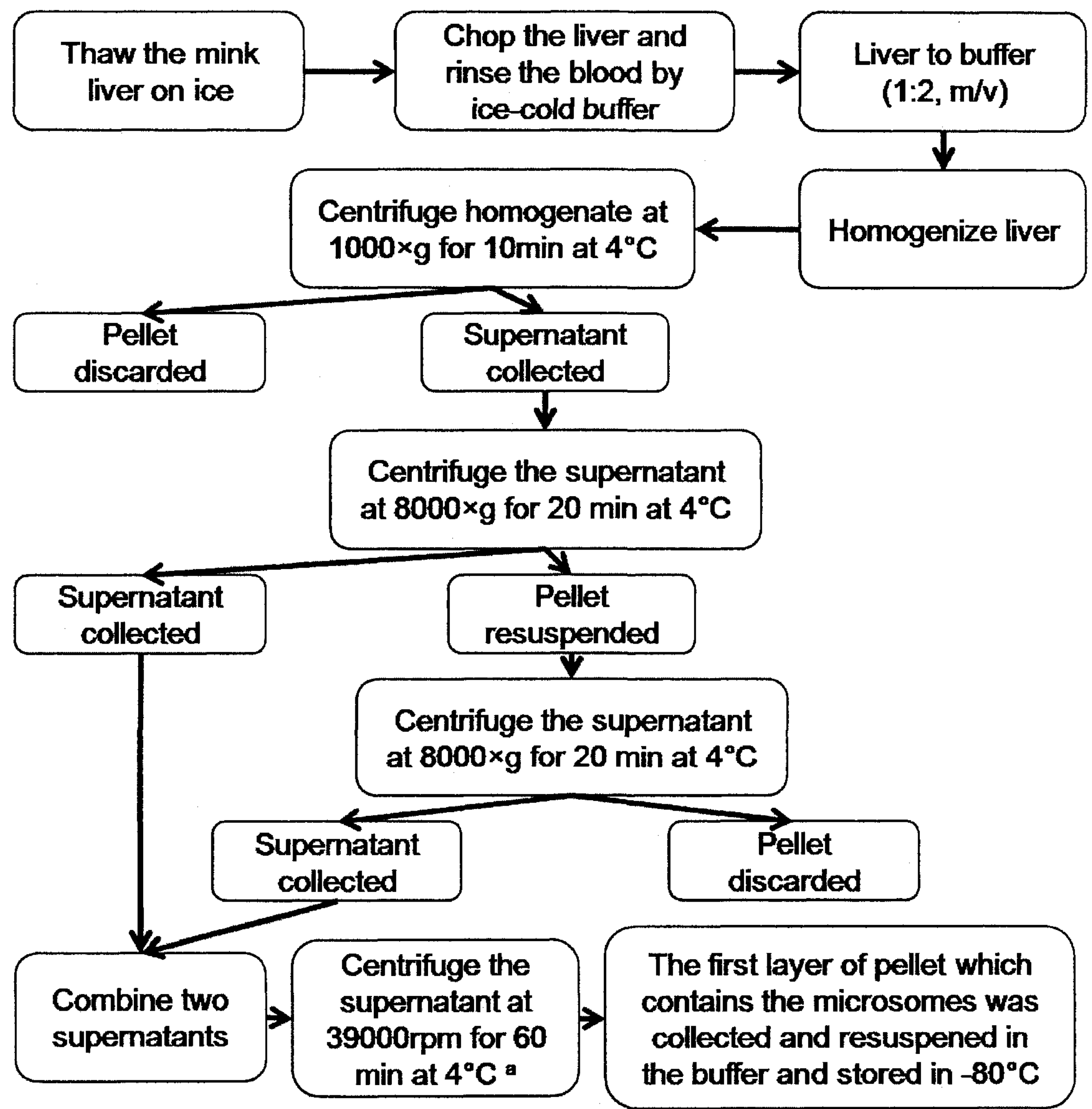
3.2.3 PBDE depletion by mink hepatic microsomes

3.2.3.1 Hepatic microsomes preparation

Mink liver was taken from healthy control mink (the same colony of mink examined in Chapter 2) housed at the Michigan State University Experimental Fur Farm (East Lansing, MI, USA). Immediately after the mink had been euthanized, the liver was collected, flash-frozen in liquid nitrogen, and a portion shipped on dry ice to the University of Alberta. Upon arrival, the liver was then stored at -80°C until the preparation of hepatic microsomes.

Rat livers were obtained from healthy rats that had been euthanized by CO_2 gas, cardiac punctured, and exsanguinated for training purposes at the University of Alberta Health Sciences Laboratory Animal Services. Livers were collected and placed into a -80°C freezer until the preparation of hepatic microsomes.

Hepatic microsome preparation buffer consists of sucrose (125 mM), mannitol (125 mM), EGTA (1 mM), and HEPES (5 mM). The procedures for extraction of hepatic microsomes from the whole liver are shown schematically in Figure 3-1. The total protein concentration of hepatic microsomes was determined by Bradford Reagent (Sigma, Saint Louis, MI, USA) using BSA protein (Sigma) as the protein concentration standard.



[a] this procedure utilized a Beckman Model L7 preparative ultracentrifuge, classified R. The rotor model was Type 55.2 Ti; 39000rpm is approximately 100,000 ×g

Figure 3-1: Procedures of hepatic microsome extraction.

3.2.3.2 Validation of microsomal activity

The quality of extracted mink (or rat) hepatic microsomes was examined by ethoxy-resofurin-*O*-deethylase (EROD) (biomarker for CYP 1A, the most abundant subfamily) and 7-pentoxy-resorufin-*O*-deethylase (PROD) (biomarker for CYP 2B, the subfamily hypothesized to be important in PBDE metabolism) assays at a single time-point (10 min) by comparison to a blank and to a negative control. Because microsomal activity severely decreased after several freeze-thaw cycles (determined with rat hepatic microsomes), only the mink hepatic microsomes after one freeze-thaw cycle are reported in this thesis for PBDE incubation.

The standard incubation mixture contained ethoxy-resorufin (1 μM) or pentoxy-resorufin (10 μM), each spiked separately as substrate, hepatic microsomes (1 mg/ml for mink, 0.15 and 1 mg/ml for rat, quickly thawed in 37°C water bath) and 30 μl of NADPH regenerating system in a final volume of 500 μl of 50 mM phosphate buffer (pH 7.8). Ethoxy-resorufin (0.1 mM) was dissolved in acetone and the pentoxy-resorufin (1 mM) was dissolved in dimethyl sulphoxide (DMSO). The final concentration of delivery solvent in the reaction medium was 1%, v/v. The NADPH regenerating system consisted of 5 μl of Solution B (40 U/ml glucose-6-phosphate dehydrogenase in 5 mM sodium citrate) and 25 μl of Solution A (26.1 mM $NADP^+$, 66 mM glucose-6-phosphate, and 66 mM $MgCl_2$ in H_2O). After pre-incubation at 37°C for 1 min, the reaction was initiated by addition of the NADPH regenerating system.

The mixtures were incubated at 37°C for 0 and 10 min and the reactions were terminated with 500 μl of ice-cold methanol by vortexing. After cooling on ice for 5 min, the samples were centrifuged at 4°C at 6000×g for 5 min. The supernatant was filtered,

and EROD and PROD activities were determined by semi-quantification of resorufin product. Samples were injected without chromatography delivered in methanol, and the peak areas of resorufin were generated by fluorescence detector. The excitation and emission wavelengths were set at 560 nm and 585 nm, respectively [10]. For the hepatic microsomal activity, a negative control was also used to control for any background fluorescence of the substrate and microsomes. This contained all components, except NADPH which was added after termination of the reaction. For mink hepatic microsomal activity, a blank and a negative control were applied to control the background fluorescence from substrates or microsomes, respectively, whereby microsomes were absent from the incubation medium of the blank, and substrates were absent from the negative control.

3.2.3.3 Depletion of individual PBDE congeners by mink hepatic microsomes

The experimental incubation mixtures were identical to EROD and PROD assays except that individual PBDE standards were spiked instead of ethoxy-resorufin or pentoxy-resorufin. Individual PBDE standards were dissolved in acetone at different concentrations (Table 3-1) selected based on a previous study examining the depletion of individual PBDEs by beluga whale microsomes [11]. The final concentration of acetone in the reaction medium was 1%. To control the introduction of any airborne contaminants during incubation, a blank sample consisting of hepatic microsomes, NADPH regenerating system, and incubation medium was also conducted. To control for the degradation of PBDEs under mechanisms other than biotransformation, triplicate negative controls for each congener were also incubated with the samples. In the negative

controls, boiled hepatic microsomes were added instead of live microsomes. After pre-incubation at 37°C for 1 min, the reaction was initiated by addition of the NADPH regeneration system. The mixtures were incubated at 37°C for 24 h and were subsequently terminated with 500 μl of ice-cold methanol with vortexing.

Table 3-1: Concentration of individual PBDEs in the standard solution and their final concentrations in the reaction medium.

	BDE 47	BDE 99	BDE 154
Commercial standard in nonane (μM)	103	88.5	77.7
Stock solution in acetone (μM)	5.97	5.04	2.72
Final concentration in the reaction medium (μM)	0.0597	0.0504	0.0272
Final concentration of acetone in the reaction medium (v/v)	1%	1%	1%
Final concentration of nonane in the reaction medium (v/v)	0.058%	0.057%	0.035%

Prior to PBDE extraction, 2 ng of [^{13}C]BDE 138 (internal standard) was added to the reaction mixture. The samples were then extracted three times by 500 μl of hexane, combined, and reduced to 100 μl for GC/MS analysis (as described in Chapter 2, Section 2.2.3). Hydroxylated metabolites were analyzed to examine the possible biotransformation pathways. The replicated samples and corresponding negative controls (n = 3) were combined respectively, derivatized by diazomethane, and analyzed as previously described for hydroxylated PBDE analysis (Chapter 2, Section 2.2.3).

3.2.3.4 Data analysis

Results are reported as the fraction of PBDE congener depleted during the 24 h time of the microsomal assay in the following manner. First, the relative ratio between the individual BDE congener and the internal standard was determined by:

$$\text{ratio} = \frac{\text{Peak area}_{\text{x}}}{\text{Peak area}_{\text{MBDE 138}}}$$

where x is a BDE congener, either from a negative control or a sample. Second, the fraction of congener remaining was determined by:

$$\text{fraction of congener remaining}_{\text{sample}} = \frac{\text{ratio}_{\text{sample}}}{\sum_1^n \text{ratio}_{\text{negative control}} / \text{n}}$$

In order to perform the statistical analysis, the fractions of congener remaining were also calculated for negative controls to generate the variance of negative controls.

$$\text{fraction of congener remaining}_{\text{negative control}} = \frac{\text{ratio}_{\text{negative control}}}{\sum_1^n \text{ratio}_{\text{negative control}} / \text{n}}$$

F-tests were used to determine whether the variances between samples and negative controls were statistically different. Student's *t*-tests were used to determine whether the samples were significantly lower than the corresponding negative control, assuming a one-tailed distribution and a maximum probability of a type-I error set to $\alpha = 0.05$.

3.3 Results and discussion

3.3.1 Gut microflora results

During the 30-d incubation period, anaerobic conditions were maintained as indicated by the unchanged colour of resazurin in all bottles. The total concentrations of DE-71 fluctuated between samples taken at different time intervals, but no trend between the concentration and time interval was found. This variation was most likely attributable to the lack of consistency in sample collection because it was difficult to obtain a representative sample from the heterogeneous medium with a syringe following shaking. Therefore, the relative concentration of congeners in DE-71 was examined, rather than the absolute concentrations, to evaluate the extent of metabolism.

The relative concentration of BDE 47 (Figure 3-2) in samples collected at 16 h was similar to the DE-71 standard, although it was slightly increased after 24 h and 7 d incubation, possibly as a result of debromination from higher brominated congeners (e.g. BDE 99). However, the relative concentration of BDE 47 decreased to similar levels as in DE-71 again at 30 d. A decreased relative concentration of BDE 99 appeared at 7 d, consistent with the increase in BDE 47 at this time, but then increased again by 30 d. Statistical analysis showed there was no significant trend for the relative concentrations of all these DE-71 congeners over time. Therefore, intestinal microflora mediated metabolism was deemed to be insignificant in the present study.

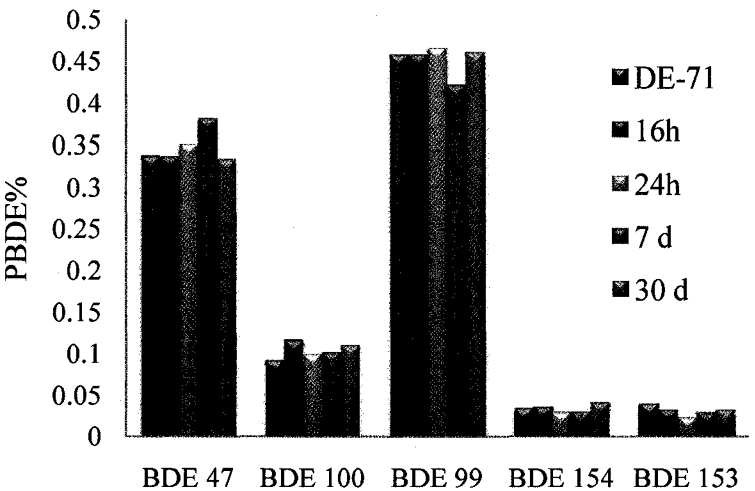


Figure 3-2: Relative concentrations of PBDEs in pure DE-71 standard and in anaerobic incubations with mink intestinal microflora at various times ($n = 1$).

Explanations for the negative findings in this study include possible non-viability or non-activity of the intestinal microflora, or the short time frame of this experiment. For example, pure cultures of anaerobic bacteria (*Sulfurospirillum multivorans* and *Dehalococcoides*), which are specifically responsible for dehalogenation, took more than 1 month to debrominated PBDEs in a past study [12] (Table 1-6). Moreover, no microbial debromination has been reported for penta-BDE mixture presumably due to the resistance to debromination of these lower brominated congeners (Chapter 1). However, the passage of ingesta through the gastrointestinal tract of mink takes less than 5 h [13], thus a longer incubation time would be irrelevant. These results make it difficult to explain the substantial congener profile changes in mink feces compared to their feed (Chapter 2).

The role of the positive control incubation, with TCB, was to validate whether rat cecum microflora were viable. Unfortunately, we did not have a strong positive control to prove the viability or activity of intestinal microflora. Although a decreasing trend was

observed for TCB over time, this decrease may have resulted from the unexpected adsorption of TCB to the rubber stopper (Figure 3-3). Therefore we cannot rule out the possibility that the anaerobic bacteria were not viable or active in this experiment.

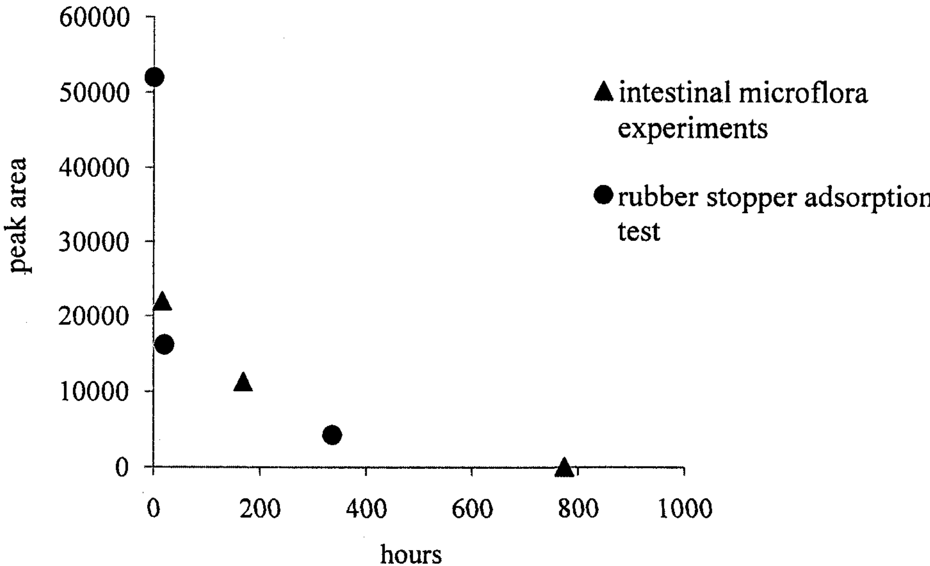


Figure 3-3: Rubber stopper adsorption test was carried out using the same conditions as intestinal microflora experiments except that the incubation buffer was changed to deionized water, and the incubation was carried out under aerobic conditions.

Besides intestinal microflora, cells of the intestines may also be able to metabolize PBDEs. In a recent study using carp intestinal microflora and intestinal microsomes to study the debromination of BDE 99 (penta) to BDE 47 (tetra), it was shown that microsomes but not microflora mediated the debromination process [14]. However, this study also did not have a strong positive control and did not prepare cecum in an anaerobic chamber to validate their negative result in their microfloral study. Therefore, in future studies, I suggest that mink intestinal microsomal studies be conducted, in addition to considering repeating these microflora tests with more reliable tests of microflora viability and activity. Moreover, carp intestinal or hepatic microsomes

mediated debromination was only observed in the presence of dithiothreitol (DTT), an electron donor, in the incubation buffer [14]. Thus DTT may serve as an important cofactor for debromination and should be included in the incubation buffer in the future work.

Not all dehalogenation reactions occur under anaerobic conditions. For example, thyroid hormones undergo deiodination (i.e. from thyroxine to triiodothyronine) by deiodinases enzymes that are present in several organs, including the liver [15]. Because of the similarity between certain PBDEs and thyroid hormones, this metabolic route has been proposed as a possible mode for debromination of PBDEs [14]. Furthermore, deiodinases may be yet another target for thyroid hormone disruption since PBDEs have been shown to decrease the activity of these enzymes due to competitive or non-competitive interaction [14].

3.3.2 Hepatic microsome study

3.3.2.1 Validation of P450 activity

3.3.2.1.1 Optimization of assay condition

Optimization of buffer conditions for P450 enzyme activity was conducted by the EROD assay using rat hepatic microsomes. Background fluorescence signal appeared in the blank when NADPH was added after inactivation of rat hepatic microsomes, but it was less than 1% of the peak area of the sample incubated for 1 min.

To validate our methods, the effects of delivery solvent and EDTA on P450 activity were examined in the present study. Due to the ability of acetone to enhance hydrocarbon hydroxylase activity [16], the final acetone concentration in our medium was optimized using EROD assay response. The highest conversion of exthoxy-resorufin to resorufin (i.e. largest peak area) was observed when the medium contained 1% of acetone compared to 0.4, 2, and 5%. Therefore, 1% of acetone was applied in all subsequent assays.

EDTA (e.g. 1 mM) containing buffer has been used in previous PBDE metabolism studies [17]. However, serving as a chelating agent, excessive EDTA may chelate Mg^{2+}, which is the cofactor for NADPH, thereby affecting the function of NADPH and further inhibiting the activity of P450 enzymes. In the present study, we observed 10-fold inhibition of P450 activity when the incubation buffer contained 1 mM EDTA. Therefore, EDTA was excluded from our incubation assays. Sharing the same chelating properties as EDTA, EGTA, which was introduced into THE incubation buffer by the addition of hepatic microsome (used in the hepatic microsome preparation buffer), may also affect the function of NADPH regeneration system. However, the positive control result showed that the P450 activity was sufficient to conduct the metabolism study in the presence of EGTA at the above concentration, thus EGTA was not avoided.

3.3.2.1.2 EROD, PROD in mink liver microsomes

No fluorescent signals were detected in the blank and negative control samples, whereas significant peak areas of resorufin were present in both EROD and PROD assays, indicating there were no interferences from either substrates or microsomes and,

furthermore, that the mink hepatic microsomes were still active after one freeze-thaw cycle. In order to ensure the mink hepatic microsomes were valid for each following metabolism experiment, THE PROD assay was used as the positive control to represent all other P450 isozymes.

3.3.2.2 Mink hepatic microsomes

3.3.2.2.1 Metabolism of PBDEs by mink hepatic microsomes

Some dissipation of BDE congeners was observed in negative controls. Because hepatic microsomes in negative controls were heat-inactivated, the dissipation of PBDEs must have been due to physical loss processes, such as binding to the plastic vials. In order to quantify the effect of enzyme-mediated biotransformation, the average fraction of congener remaining in negative controls was set arbitrarily to 1. The percent congener remaining for BDE 47, 99, and 154 was 0.89 ± 0.10, 0.95 ± 0.13, and 0.66 ± 0.14 (mean $\pm$ SD), respectively (Figure 3-4). However, statistical analysis demonstrated that BDE 47 and BDE 99 were not significantly depleted by mink hepatic microsomes. Under the same conditions, 0.34 of BDE 154 was significantly depleted ($p < 0.05$). No lower brominated congeners or methoxylated PBDEs were observed in any of these samples.

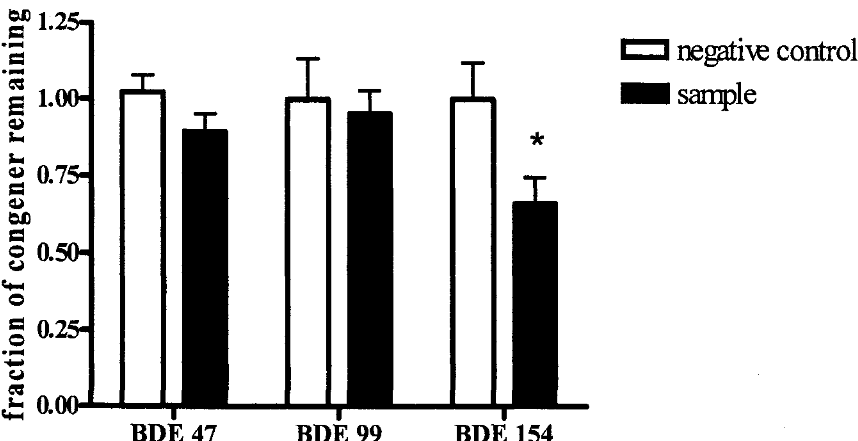


Figure 3-4: Fraction of BDE congener concentrations remaining after a 24-h incubation with mink hepatic microsomes. Error bars represent ± standard deviation for replicate assays ($n = 3$). * Statistically significant depletion ($p < 0.05$ with a one-tailed Student's t-test).

After analysis for parent, debrominated, and methoxylated PBDEs, the triplicates from the incubation of each congener were combined, derivatized by diazomethane, and concentrated for analysis of hydroxylated metabolites including OH-PBDEs and bromophenols. No OH-PBDEs were found for BDE 47 and 99, whereas two peaks were detected in the BDE 154 samples that were not present in the negative controls (Figure 3-5). Based on the mass-to-charge (*m/z*) ratio (*m/z* 673.5 and *m/z* 671.5 for confirmation), these two peaks very likely corresponded to OH-hexa-BDEs, although no standard was available for confirmation. The larger peak was also detected in mink tissue extracts (peak M in the liver extract, Figure 2-4). Bromophenols were monitored in the present study by their expected *m/z* ratios: *m/z* 186 for mono-bromophenols, *m/z* 266 for di-bromophenols, and *m/z* 343 for tri-bromophenols. Although peaks corresponding to bromophenols were detected in PBDE samples, they were not above the response detected in negative controls. Therefore, we cannot conclude the formation of

bromophenols in the present study methods. Clean-up procedures may help to eliminate these interferences having the same *m/z* ratio as bromophenols.

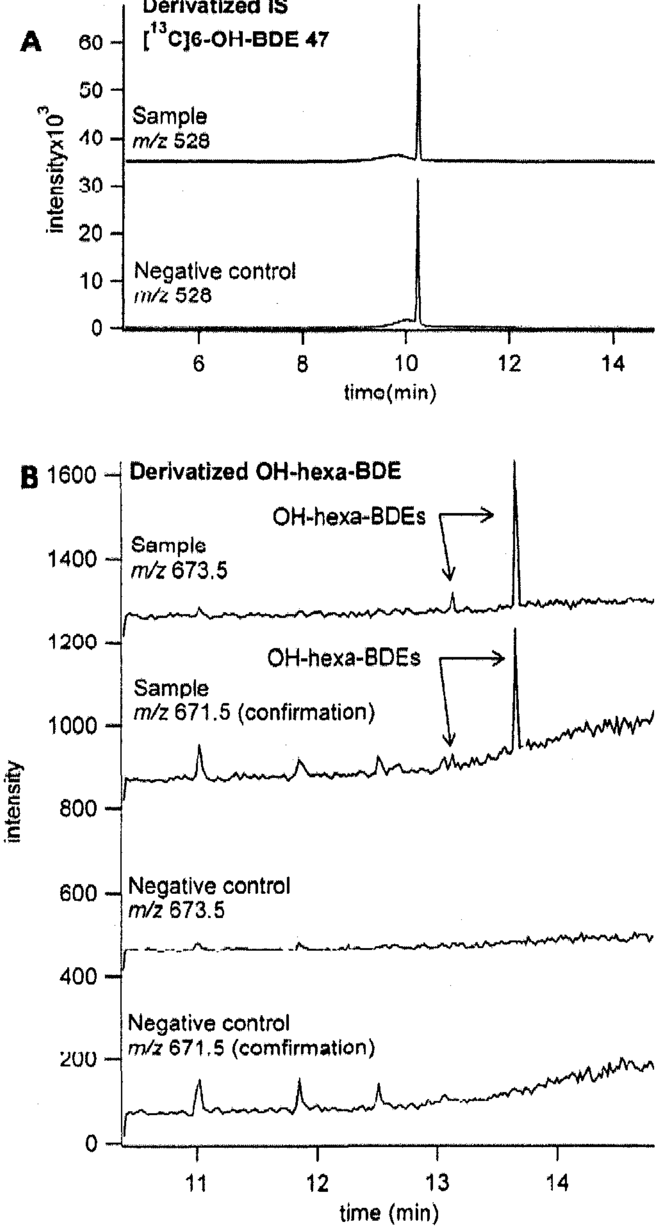


Figure 3-5: GC/MS chromatograms (SIM) of diazomethane derivatized extracts from an in vitro depletion assay of BDE 154 with mink hepatic microsomes. (A) Derivatization efficiency of internal standards was comparable between the sample and negative control in terms of the peak intensities. (B) Two peaks representing OH-hexa-BDEs, pointed out by arrows, were eluted in the derivatized sample but not in the negative control.

No significant depletion of BDE 47 and 99, and lack of any detectable metabolites, suggested that these two congeners were not metabolized by mink hepatic microsomes within 24 h under the present experimental conditions. Optimized buffer conditions for P450 activity was used for the present study. However, this condition may not be appropriate for other enzymes such as deiodinases which have been proposed to be able to debrominate higher brominated PBDEs [17] and which are present in several organs, including the liver [15]. Indeed, under an optimized buffer condition for deiodinases (inclusion of 10 mM of DTT), BDE 99 was significantly debrominated to BDE 47 in carp hepatic microsomes [14].

The previous mink in vivo study (Chapter 2) suggested that the significant increase of BDE 47 in mink tissues, relative to their feed, was likely due to the debromination of BDE 99. Consequently, I suggested that optimized buffer conditions for deiodinases should be developed and applied with mink hepatic microsomes to complement the current data reported here. In addition, intestinal cells were also suggested to be responsible for the debromination of PBDEs, thus intestinal microsomal studies should also be conducted for debromination under the same optimized buffer conditions for deiodinases. Similarly for BDE 154, hydroxylation may be only one of the possible metabolic elimination pathways, and debromination may occur under suitable experimental conditions.

A semi-quantitative mass balance of BDE 154 was performed in the following manner. In each replicate, 1.4×10^{-11} mol of BDE 154 was added, and the average depletion rate was 34%; therefore, 4.6×10^{-12} mol of BDE 154 was metabolized in the average assay. Assuming the two OH-hexa-BDEs present the only fate for BDE 154,

their mass could be equated to 4.6×10^{-12} mol of OH-hexa-BDEs. Because we combined triplicate incubations for analysis, the concentration of OH-hexa-BDEs increased by a factor of 3. Therefore, the final concentration of derivatized OH-hexa-BDE was 93 ng/ml in the final extract that was analyzed by GC/MS, similar to the concentration of the internal standard. Accounting for the fact that the response in GC/MS decreases with increasing degree of bromination (I assumed one order of magnitude decrease from tetra (i.e. the internal standard) to hexa BDEs), the combined peak area of these two OH-hexa-BDEs is still an order of magnitude smaller than the internal standard. Therefore, it is reasonable to suggest that there is a lack of mass balance in our study, and thus that other metabolic routes are operative for BDE 154.

The lack of mass balance in studies of PBDE depletion by hepatic microsomes has also been observed in beluga hepatic microsomes whereby the peak height of the OH-di-BDE metabolite of BDE 15 was only 1.6-fold higher than the detection limit (1 ng/ml) when 63% of BDE 15 was metabolized [11] (theoretical concentration assuming 100% conversion would be 91.5 ng/ml of the OH-di-BDE). These authors used a chemical ionization source for MS detection, hence they attributed the lack of mass balance to undetectable metabolites which were fully debrominated via hydroxylation to non-detectable (i.e. non-halogenated) fraction. However, this was not the case in the present study, because we used an electron ionization source with the same detection limit for OH-PBDEs (1 ng/ml), and neither debrominated, and nor any hydroxylated or oxidative debrominated BDEs were detected. Therefore full debromination was unlikely the cause of the lack of mass balance during the metabolism of BDE 154 in the present study. Neither could it be attributed to the phase II metabolism by UDPGTs because no

glucuronide substrate was added. However, we cannot rule out the possibility that endogenous glutathione [14] may have conjugated some of the epoxide intermediate (Figure 1-5). Non-extractable metabolites that are covalently bound to macromolecules such as lipids or proteins [18] present in hepatic microsomes may also result in the lack of mass balance here.

3.3.2.2.2 Selective metabolism of PBDEs in different species

Differences in PBDE profiles have been observed between different species exposed to PBDEs in laboratory studies. For example, following DE-71 exposure in the diet, body congener profiles of PBDEs were different between mink and rats (Chapter 2). This may result from the species dependent selective uptake or elimination of certain PBDE congeners. Elimination involves both excretion and metabolism. Selective excretion of certain PBDEs has previously been demonstrated [7, 19, 20], and the present study further provided evidence that selective metabolism of certain PBDEs can also play a role in altered tissue congener profiles relative to the ingested food.

Such in vitro results are useful for explaining observations in the real world. For example, from beluga whales to their primary prey (cod) [21] all congeners (including BDE 99, 100, 153, and 154) except BDE 47 increased their relative concentrations (Figure 3-6) [22, 23]. Disregarding differential uptake and elimination pharmacokinetics, this result was consistent with the observation in vitro, with beluga whale hepatic microsomes whereby only BDE 47 was significantly metabolized.

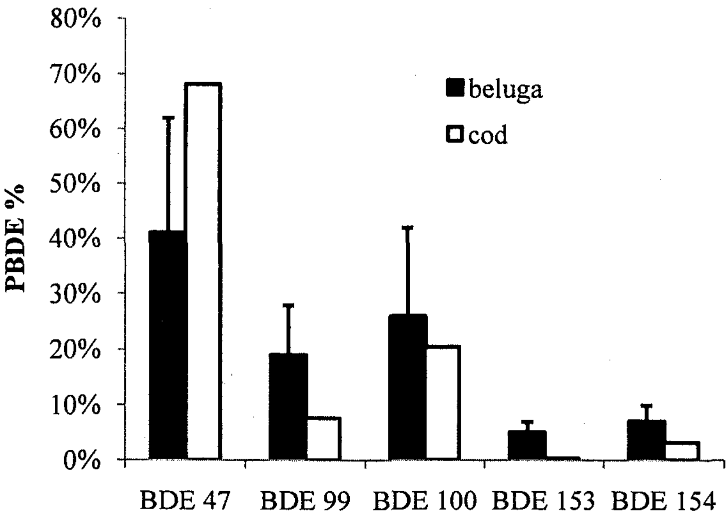


Figure 3-6: Relative concentration of PBDEs in beluga whale [22] (black columns represent means, and bars represent standard error of the mean) and cod [23] (white columns represent means).

Similarly, as we mentioned in the Chapter 2, the most significant changes with regard to the congener profile change in mink was the divergent behaviour between two hexa-BDEs, BDE 153 and 154 (Figure 2-1 D and F). The significant metabolism of BDE 154 observed in the present work provided evidence that, indeed, selective metabolism of BDE 154 could lead to the low accumulation of BDE 154 relative to BDE 153 in vivo. We would, however, need to repeat the current experiments with BDE 153 to be absolutely certain of this.

3.4 Conclusions

No significant biotransformation processes were observed in the microfloral study. However, no solid conclusion can be elucidated based on this result because of the weak positive control and lack of viability and activity data. The most significant finding of

this study was that BDE 154 can be significantly depleted by mink hepatic microsomes, and that two OH-hexa-BDEs were found as its metabolites, one of which matched the one detected in vivo. Under the same conditions, BDE 47 and 99 were not significantly depleted and no metabolites were observed. The lack of mass balance in BDE 154 metabolism may have been due to epoxidation and the phase II metabolism by glutathione-S-transferases using endogenous glutathione as the cofactor. Two more aspects need to be studied further: i) the study of intestinal microflora mediated biotransformation needs an effective positive control study or elseintestinal anaerobic bacteria may be purified and concentrated for the study, and ii) the buffer conditions need to be optimized for debromination, catalyzed by deiodinase enzymes, in hepatic and intestinal microsomes.

3.5 References

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Chapter 4: Conclusions and future work

Taking the sentinel species, mink, as the study subject, this thesis has demonstrated the whole-body bioaccumulation, disposition, and metabolism of a commercial penta-BDE mixture (DE-71). Further, the thesis research also addressed possible metabolic pathways of polybrominated diphenyl ethers (PBDEs) including debromination and hydroxylation by in vitro methods using mink intestinal microflora and hepatic microsomes. I review these contributions in section 4.1. In section 4.2, I describe several areas for future work.

4.1 New findings and their implications

4.1.1 Biomagnification factors of PBDE congeners

The bioaccumulation of PBDEs has been studied for years, and many field studies have suggested PBDEs are bioaccumulative in animal tissues. Studies in the present thesis (Chapter 2) have, for the first time, quantitatively estimated BMFs of individual PBDEs in a controlled experiment with an environmentally relevant species, and demonstrated that PBDEs are accumulative on a whole-body basis. This validated the observations from many field studies that PBDEs are accumulative in animal tissues. The accumulation potential varied between individual PBDEs; lower brominated congeners

were generally more bioaccumulative than higher brominated congeners, with the exception of BDE 153.

4.1.2 Hydroxylation of PBDEs by mink hepatic microsomes

Hydroxylation of BDE 154 by hepatic microsomes is, for the first time, demonstrated in the present thesis (Chapter 3). BDE 154 was significantly depleted by mink hepatic microsomes, and two hydroxylated BDE 154 metabolites were generated. Because the incubation buffer was optimized for cytochomones P450, these enzymes were most likely responsible for the biotransformation (i.e. oxidation) of BDE 154.

4.2 Future work

Exploring metabolic pathways was one of the objectives of this thesis, and I have demonstrated hydroxylation of BDE 154 by hepatic microsomes. However, debromination and hydroxylation of other BDE congeners was not observed. The following two sections detail several improvements in experimental design for future debromination and hydroxylation studies.

4.2.1 Reductive debromination study

A reductive debromination study by intestinal microflora was described in Chapter 3. However, appropriate incubation conditions for intestinal anaerobic bacteria may still need to be optimized. The major concern was the viability or activity of intestinal microflora. Therefore, a test of the viability or activity of intestinal microflora is

considered to be necessary before conducting metabolism studies to aid the final interpretation. Because the complex components accompanying intestinal microflora which may interfere with common bacteria viability tests, the positive control (TCB) used in the studies described in Chapter 3 may still be good for demonstrating the viability of intestinal microflora, especially anaerobic bacteria responsible for dehalogenation. TCB may not be a good quantitative positive control because it partitioned out of the aqueous homogenate. However, the detection of di- or mono-chlorobenzenes, the presumed dehalogenated metabolites of TCB, would nonetheless be indicative of viable bacteria capable of dehalogenation.

Several improvements in the optimization of anaerobic incubation need to be considered. First, mink large intestinal content (can be obtained from intestines after cecum) should be used instead of rat cecal content to minimize the difference in metabolic profiles between species. Second, the incubation medium may include more nutrients (other than inorganic salts contained in VPI buffer) for intestinal anaerobic bacteria growth (i.e. yeast extracts, bactopeptone, and glucose) [1]. The concentration of PBDEs should be kept low so as not to cause toxic effects or to impede bacterial growth. Third, in order to minimize the dilution of bacteria, the dilution of intestinal content may be waived, and larger amounts of intestinal content may be added to the incubation medium. Fourth, dithiothreitol (DTT), serving as an electron donor, may be included in the incubation medium to mediate the reductive process of debromination. If debromination is achieved in future intestinal microfloral studies, isolation of the specific intestinal bacteria that metabolize PBDEs may be conducted by adding PBDEs as one of the ingredients in a selective plate culture [1].

Besides intestinal microflora, intestinal microsomes may also be studied for debromination of PBDEs. Optimized buffer conditions for deiodinases should be developed and applied, and reverse triiodothyronine may serve as the positive control for activity of the deiodinases [2]. Again, DTT as the electron donor should be included in the incubation buffer.

4.2.2 Hydroxylation study

Although I have successfully demonstrated hepatic microsome mediated hydroxylation of BDE 154 (a hexa-BDE) under the present study conditions, several improvements and complementary studies are still necessary. First of all, as depletion of PBDEs in negative controls was observed in the present study (Chapter 3), materials other than plastic, such as Teflon® or glass, may be considered for the reaction vessels, to minimize the interaction between plastic and PBDEs. Second, complementary studies to explore the lack of mass balance may examine the following aspects. Metabolic pathways other than hydroxylation, such as phase II metabolism (formation of glutathione conjugates), cleavage of the ether bond (formation of bromophenols), and hydroxylation associated with debromination (hydroxylated lower brominated BDEs) may also be examined. The present study (Chapter 3) suffered interferences while trying to identify bromophenols in samples and negative controls. Therefore the analytical method (i.e. performing a clean-up procedure) may be specifically optimized for determination of bromophenol. Determination of phase II metabolites, especially glutathione conjugates, may explain the lack of mass balance. Because of the good solubility of glutathione

conjugates, development of an analytical method based on high performance liquid chromatography coupled with tandem mass spectrometry is necessary.

Furthermore, the metabolic manner may be studied by paired BDE congeners having similar bromine substitution. For example, BDE congeners having uncompleted *meta-para* bromine atom substitution (e.g. BDE 47) may be compared to BDE congeners having full *meta-para* bromine atom substitution (e.g. BDE 77) to test the hypothesis that the *meta-para* substitution of bromine atoms in BDE 153 makes it more recalcitrant to metabolism than BDE 154 (Section 2.3.6).

4.3 References

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Appendices

Appendix I: Concentrations of PBDEs in the atmosphere.

Time	Region	Status	ΣPBDEs (pg/m³)	BDE 209 (pg/m³)	ΣPCBs (pg/m³)	Ref.
High volume						
94–95	Dunai, Canada	remote	14	<0.1	34	[1, 2]
	Alert, Canada	remote	240	<0.1		
	Tagish, Canada	remote	428	<0.1		
97–99	the Great lakes region	remote	5.5	<1.0	140	[3]
	the Great lakes region	rural	11	<1.0	595	
	Chicago, USA	urban	52	0.3	3199	
01	the Baltic Sea		3.7	6.1	7.4	[4]
00	Peterborough, Canada		295		315	[5]
00	England, UK	rural	12			[6]
	England, UK	remote	2.6			
00–01	Tokyo, Japan	urban	15			[7]
01–02	incineration plant, UK		6.3	10.4		[8]
01	Torna, UK		3.5	6.5		
01–02	Birmingham, UK	outdoor	18			[9]
02–03	Birmingham, UK	indoor	726			
02–03	Chicago, USA		31	71		[10]
	Lake Michigan, USA		14	1.8		
	Cocodrie, USA		12	3.8		
04	Guangdong, China	urban	88.8	263.8		[11]
	Guangdong, China	industrial	229.6	749.8		
05	Ispra, Italy		91	5		[12]
Passive diffusion						
00	Poland		4.97		18.69	[13]
	Iceland		1.65		10.53	
	Russia		2.45		197	
	Finland		3.4		19.68	
	Netherlands		6.12		32.48	
	Sweden		3.27		62.11	
	EIRE	remote	1.6		4.76	
	Germany		1.7		22.36	
	Italy	urban	10.13		135.71	
	Italy	rural	2.3		52.43	
	Spain		4.47		23.49	
	UK	rural	8.03		12.3	
	UK	urban	25.61		140.22	
00–01	Alert, Canada	remote	4		14	[14]
	Toronto, Canada	urban	27		670	

Time	Region	Status	ΣPBDEs (pg/m^3)	BDE 209 (pg/m^3)	ΣPCBs (pg/m^3)	Ref.
00–01	Toronto		15		n.a.	[15]
02–03	Ottawa	outdoor	2.2			[16]
		indoor	120			
04	China	rural	7.29		9.29	[17]
	China	urban	2.55		18.82	
	Singapore		3.74		4.21	
	Korea		1.75		7.01	
	Japan		3.81		12.53	
Estimated						
01	Toronto, Canada	outdoor	4.8			[18]
		indoor	42.1			
Time	Region	Status	ΣPBDEs (ng/g)	Octa-BDE (ng/g)	BDE 209 (ng/g)	Ref.
Vacuum dust						
02–03	Ottawa, Canada	indoor	900		630	[19]
04	Washington, DC, USA	indoor	2816	84	1350	[20]
n.a.	Dallas, USA	indoor	1842		665	[21]

Appendix II: Concentrations of PBDEs in sediments, sewage sludge, and soils.

Time	Location	Status	ΣPBDEs ng/g dw	BDE 209 ng/g dw	ΣPCBs	Ref.
Sediments						
87	Sweden	marine	2.9 [a]			[22]
98	High Arctic, Canada	marine	0.122	0.046		[23]
99	Indiana, USA	lake	7	30		[24]
99	the Netherlands	n.a.	2.05	22		[25]
00	Demark	freshwater	0.92	2.5		[26]
		marine	0.25	2.2		
00	Atlanta, USA	pond	0.25			[27]
		stream	17.2			
00	Greenland	lake	0.012		0.177	[28]
n.a.	Portugal	river basin	1.68			[29]
		coastal	0.37			
01	the Netherlands	estuary	22	272		[30]
02	Spain	river		5.47		[31]
02	Spain	river	5.2	3.9		[32]
03	Northern Norway	marine	0.25			[2]
				0.42		
n.a.	Ireland	harbor	1.75	<0.1		[33]
n.a.	Pearl River Delta, China	marine	3.62			[34]
04	Hong Kong, China	marine	10	0.18		[35]
04	Korea	marine	0.1	4.26		[36]
95	Visda River, Sweden	river	26 [a]	75 [a]		[37]
04–05	Lake Maggiore, Italy	lake	5.2	0.5		[12]
n.a.	India	wetland	0.62			[38]
Sewage sludge						
87	Sweden		38 [a]			[22]
n.a.	Germany		8.37			[39]
97–98	Sweden		200	220		[40]
99	the Netherlands		22	350		[25]
n.a.	USA, several states		1564	368		[41]
n.a.	California, USA		2198	1183		[42]
02	Kelowna, Canada		2429	In ΣPBDE		[43]
n.a.	Denmark		238	248		[44]
Soil						
00	Sweden, agricultural land	ref. site	0.103		0.865	[45]
		fertilized	0.68		2.55	
00	Atlantic coast, USA	polluted	13.6			[27]
00	Sweden, agricultural land	ref. site	0.076	0.076		[46]

Time	Location	Status	ΣPBDEs ng/g dw	BDE 209 ng/g dw	ΣPCBs	Ref.
		fertilized	0.2	0.62		
n.a.	UK, rural/remote	woodland	2.5			[47]
		grassland	0.61			
	Norway	woodland	0.97			

n.a. not available. [a] unit: ng/g ignition loss

Appendix III: The concentrations of ΣPBDEs (sum of tri-hepta BDEs), BDE 209, and partial ΣPCB concentrations (the number of summed congeners varied between studies) in wildlife including invertebrates, fish, frogs, birds, terrestrial mammals and marine mammals from North America, Europe, Asia, and the Arctic region.

Species	Tissue	Time	Region	Status	ΣPBDEs ng/g lw	BDE 209 ng/g lw	ΣPCBs µg/g lw	Ref.
Porpoise, dolphins, and whales								
porpoise	B	91–93	costal BC, Canada	M	530			[48]
porpoise	B	96–99	UK	M	642			[49]
harbor porpoise	B	99	North Sea	M	1534			[50]
finless porpoise	B	00–01	Hong Kong, China	M	380	< DL	10	[51]
spinner dolphin	B	90–92	India	M	6.8	< DL 0.5	1.6	[52]
pacific white-sided dolphin	B	99	Japan	M	690	< DL 0.5	8.7	[52]
humpback dolphin	B	00–01	Hong Kong, China	M	1600	< DL	72	[51]
long-finned pilot whale	B	94	Faroe Island, Atlantic	M	1610			[53]
melon-headed whale	B	01	Japan	M	320	< DL 0.5	24	[52]
Stejneger's beaked whale	B	00–01	Japan	M	530	< DL 0.5	19	[52]
beluga whale	B	98	Svalbard	M	161			[54]
beluga whale	B	88	St. Lawrence Estuary, Canada	M	22			[55]
		92–93			111			
		97–99			485			
killer whale	B	05	Hokkaido, Japan	M	320	<0.5	44	[56]
Seals								
grey seal	B	79–85	Baltic Sea	M	690			[57]
grey seal	B	81–88	Baltic Sea	M	468			[58]
fur seal	B	91	Sanriku, Japan	M	49	< DL 0.5	3.4	[59]
		94			53	< DL 0.5	2.7	
		97			28	< DL 0.5	2.6	
		98			30	< DL 0.5	2.2	
ringed seal	B	01	East Greenland	M	36	n.m	0.84	[60]
ringed seal	B	81	Svalbard	M	48.7			[57]
ringed seal	B	99	Svalbard	M	18.3			[54]
ringed seal	B	03	Svalbard	M	59.08			[61]

Species	Tissue	Time	Region	Status	ΣPBDEs ng/g lw	BDE 209 ng/g lw	ΣPCBs µg/g lw	Ref.
ringed seal	B	81–88	Baltic Sea	M	379			[58]
harbor seal	B	99	North Sea	M	323			[50]
grey seal	L	81–88	Baltic Sea	M	18			[58]
ringed seal	L	81–88	Baltic Sea	M	43			[58]
Polar bears								
polar bear	P	02	Svalbard	M	533			[62]
polar bear	A	02–03	Svalbard	M	30	0.09		[61]
polar bear	A	02	Svalbard	M	49.8	< 0.05		[63]
polar bear	A	99–01	East Greenland	M	69.6	< 0.05		[63]
Fish								
shorthorn sculpin	L	01	East Greenland	M	7		0.115	[60]
shorthorn sculpin	L	00	Southern Greenland	M	18.6		0.352	[26]
whiting fish	L	99	North Sea	M	108			[50]
whiting fish	L	01	North Sea	M	145			[64]
common sole	L	01	North Sea	M	21			[64]
common sole	L	92	costal BC, Canada	M	22			[48]
		00		M	70			
Arctic char	L	01	Greenland	M	23			[65]
brown trout	L	01	mountain lake, Europe	FW	55			[65]
Arctic char	L	01	mountain lake, Europe	FW	16			[65]
herring	MU	81–88	Baltic Sea	M	15			[58]
herring	MU	00	North Sea	M	61			[50]
salmon	MU	91	Baltic Sea	M	298			[58]
whiting fish	MU	99	North Sea	M	51			[50]
whiting fish	MU	01	North Sea	M	90			[64]
common sole	MU	01	North Sea	M	36			
anadormous arctic char	MU	01	East Greenland	M	10	n.m	0.18	[60]
whitefish	MU	86	Sweden	FW	22.2			[66]
Arctic char	MU	87	Sweden	FW	464			[66]
trout (*Salmo trutta*)	MU	88	Sweden	FW	270			[66]
pike	MU	95	Visda River, Sweden	FW	275	trace		[37]
rainbow trout	MU	99	Washington State, USA	FW	4150			[67]
carp	MU	99	Indiana, USA	FW	1630	<175		[24]

Species	Tissue	Time	Region	Status	ΣPBDEs ng/g lw	BDE 209 ng/g lw	ΣPCBs μg/g lw	Ref.
brown trout	MU	01	mountain lake, Europe	FW	34			[65]
Arctic char	MU	01	mountain lake, Europe	FW	14			[65]
Arctic char	MU	01	Greenland	M	41			[65]
whiting fish	MU	92	Columbia River, Canada	FW	66			[68]
		95			615			
		00			628			
		92			56			
		00			154			
salmon	W	99	Baltic Sea	M	49			[69]
salmon	W	99	Atlantic, Norway	M	13			[69]
polar cod	W	01	Svalbard	M	3.55			[54]
polar cod	W	03	Svalbard	M	1.25	0.2		[61]
smelt	W	94	Lake Superior	FW	150	< 24		[24]
smelt	W	95	Lake Ontario	FW	240	< 21		[24]
salmon	W	99	Lake Michigan	FW	2440		43.1	[70]
lake trout	W	97	Lake Superior	FW	1973			[71]
			Lake Huron		204			
			Lake Erie		94			
			Lake Ontario		362			
large mouth bass	W	99	Detroit river, MI, USA	FW	163			[72]
carp	W	99	Detroit river, MI, USA	FW	40.7			[72]
bluegill sunfish	W	00	mid-Altlantic region, USA	FW	1333			[27]
Crab, Mussels, shrimp, and sea stars								
crab		94–95	costal BC, Canada	M	320			[48]
crab		01	North Sea	M	48			[64]
crab	SA	99	North Sea	M	56			[50]
mussel		00	Denmark	M	11.9			[73]
blue mussel		02	Southern Greenland	M	5.5			[26]
shrimp		01	North Sea	M	6.7			[64]
shrimp	W	99	North Sea	M	53			[50]
sea star	PC	99	North Sea	M	35			[50]

Species	Tissue	Time	Region	Status	ΣPBDEs ng/g lw	BDE 209 ng/g lw	ΣPCBs μg/g lw	Ref.
Frogs								
frog	L	98–00	Sweden	T	4074			[74]
Birds								
glaucous gull	P	04	Svalbard	M	1338			[62]
osprey	MU	82–86	Sweden	M	1940			[66]
starling	MU	87	Sweden	T	44			[66]
glaucous gull	L	99	Bear Island	M	54			[75]
cormorant	L	99–00	UK	FW	580			[49]
cormorant	L	00	Japan	FW	1400		24	[76]
common buzzard	L	01–03	Belgium	T	70	24		[77]
sparrowhawk	L	01–03	Belgium	T	12	< DL		[77]
owl	L	01–03	Belgium	T	250	< DL		[77]
sparrowhawk	L	04–06	Beijing, China	T	3313	249		[78]
little owl	L	04–06	Beijing, China	T	1933	96		[78]
common buzzard	L	04–06	Beijing, China	T	20	71		[78]
guillemot	W	99	Baltc Sea	M	231			[69]
guillemot	W	99	Atlantic, Norway	M	76			[69]
cormorant	egg	00	Japan	FW	930		20	[76]
black guillemot	egg	01	East Greenland	M	80		1.64	[60]
guillemot	egg	71	Baltic Sea	M	95.1			[57]
		76			656			
		85			1348			
		89			1225			
		96			1307			
		01	Baltic Sea	M	101			
peregrine falcon	egg	91–99	Sweden	M	4070	110		[79]
peregrine falcon	egg	87–88	Greenland	M	813	6.85		[80]
		92			2037	8		
		99–00			2690	11		
little owl	egg	98–00	Belgium	T	110		2.6	[81]
herring gull	egg	83	Lake Ontario	FW	101			[82]
		88			2110			
		96			3337			
		00			5307			
herring gull		83	Lake	FW	189			[82]

Species	Tissue	Time	Region	Status	ΣPBDEs ng/g lw	BDE 209 ng/g lw	ΣPCBs μg/g lw	Ref.
			Michigan					
		88			6950			
		96			6804			
		00			13058			
herring gull		83	Lake Huron	FW	324			[82]
		88			940			
		96			3990			
		00			6684			
Terrestrial mammals								
moose	MU	85–86	Sweden	T	1.7		0.396	[83]
raindeer	suet	86	Sweden	T	0.51		0.05	[83]
red fox	A	03–04	Belgium	T	2.2	<3.7		[84]
rodents	MU	01	Belgium	T	7.1			[84]

Note: B=Blubber, L=liver, P=plasma, A=adipose; P=plasma, MU=muscle, W=whole body, SA= soft abdomen, PC= pyloric caeca, M=marine; FW=freshwater, T=terrestrial

Appendix IV: Solar light degradation of PBDEs.

Congeners	**Solvent**	**Light source**	**Decomposition rate constant $k \times 10^{-5}$ (s^{-1})**	**Half-life (h)**	**Ref.**
BDE 209 (deca-)	Hexane	Sunlight (summer)	186	0.1	[85]
		Sunlight (winter)	111	0.2	[85]
	Tetrahydrofuran (THF)	UV light within sunlight region	83	0.2	[86]
	MeOH	UV light within sunlight region	65	0.3	[86]
	MeOH/water (80:20, v/v)	UV light within sunlight region	40	0.5	[86]
	Water	Artificial light in sunlight region		30-40	[87]
BDE 208 (nona-)	MeOH/water (80:20, v/v)	UV light within sunlight region	17	1.1	[86]
BDE 203 (octa-)	MeOH/water (80:20, v/v)	UV light within sunlight region	3.7	5.0	[86]
BDE 183 (hepta-)	MeOH/water (80:20, v/v)	UV light within sunlight region	0.67	29	[86]
BDE 154 (hexa-)	MeOH/water (80:20, v/v)	UV light within sunlight region	0.33	58	[86]
BDE 99 (penta-)	MeOH/water (80:20, v/v)	UV light within sunlight region	0.3	64	[86]
BDE 47 (tetra-)	MeOH/water (80:20, v/v)	UV light within sunlight region	0.07	290	[86]

Appendix V: Extracted ions for quantification and confirmation.

PBDE type	*m/z*	*m/z* identity	Theoretical Abundance[c]
tri-BDEs	405.8[a]	M+2	0.333
	407.8	M+4	0.326
tetra-BDEs	483.7	M+2	0.225
	485.7[a]	M+4	0.329
penta-BDEs	563.6	M+4	0.278
	565.6	M+6	0.272
	403.8[a]	$[M-2Br]^+$	n.a.[b]
hexa-BDEs	641.5	M+4	0.211
	643.5	M+6	0.275
	405.8[a]	$[M-2Br]^+$	n.a.[b]
[^{13}C]hexa-BDEs	655.6	M+6	0.211
	495.6[a]	$[M-2Br]^+$	n.a.[b]

[a] Peak areas of these ions are integrated for quantification, while other ions are used for confirmation.
[b] [M-2Br]+ is a fragment of the molecules; its theoretical abundance depends on the ionization energy.
[c] The theoretical abundance is expressed as a fraction to 1.

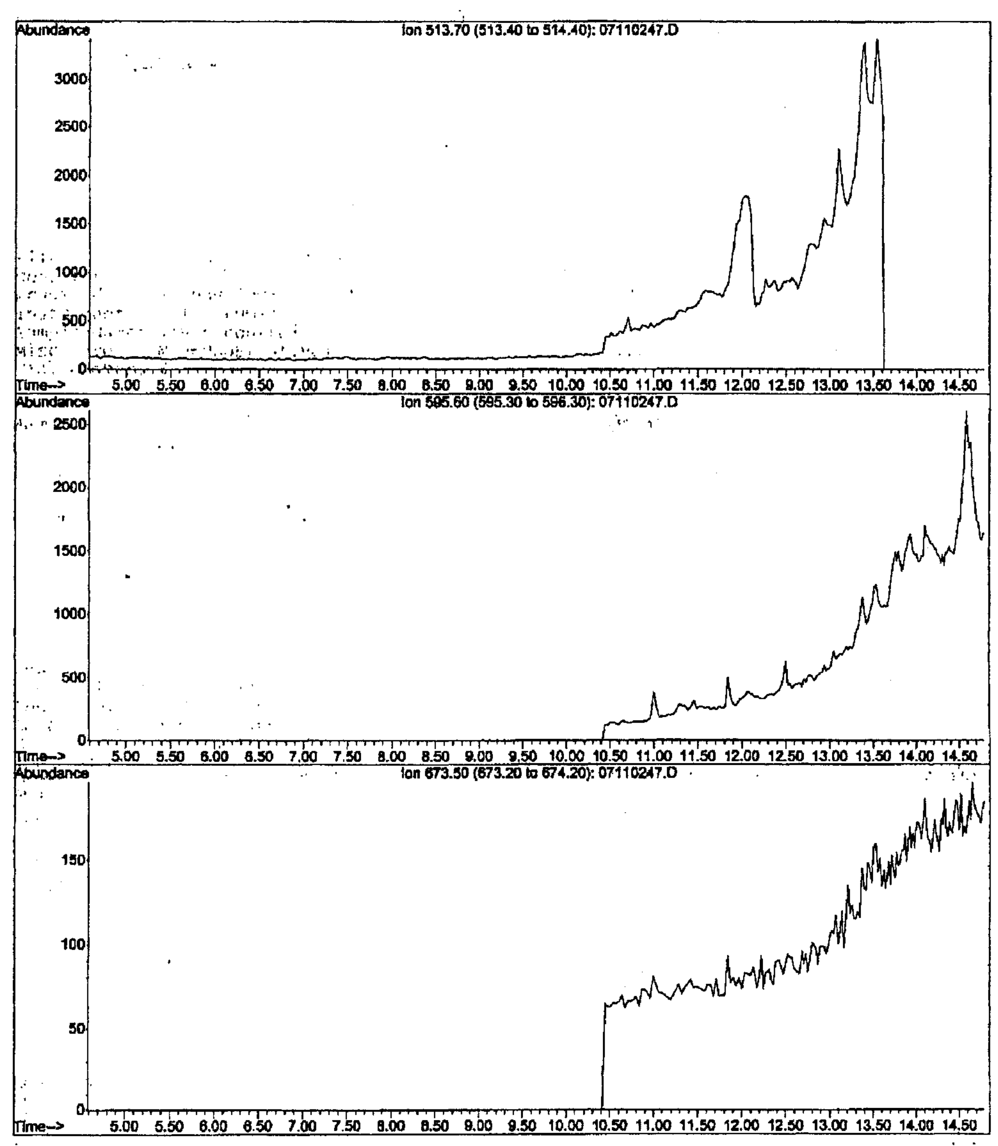
Appendix VI: Theoretical ion abundance ratios and QC limits.

PBDE type	*m/z* forming ratio	Ratio	lower QC limit	upper QC limit
tri-BDEs	(M+2)/(M+4)	1.03	0.88[a]	1.18[a]
tetra-BDEs	(M+2)/(M+4)	0.70	0.60[a]	0.81[a]
penta-BDEs	(M+4)/(M+6)	1.03	0.88[a]	1.18[a]
	$([M]^+)/([M-2Br]^+)$[b]	The peak areas are acceptable when the relative standard deviation of these ratios (n $\geq$ 3) is less than 10%.		
hexa-BDEs	(M+4)/(M+6)	0.77[a]	0.65[a]	0.89[a]
	$([M]^+)/([M-2Br]^+)$[b]	The peak areas are acceptable when the relative standard deviation of these ratios (n $\geq$ 3) less than 10%.		

[a] This criteria is defined in EPA Method 1614 as ± 15%

Appendix VII: GC/MS chromatograms (SIM mode) for OH-tetra-BDEs (*m/z* of $[M^+]$ is 513.7), OH-penta-BDEs (*m/z* of $[M^+]$ is 595.6), and OH-hexa-BDEs (*m/z* of $[M^+]$ is 673.5) in the derivatized extract of liver, feces, and pooled plasma from the control groups.

```
File        : D:\MSDCHEM\1\DATA\BDE71102.S\07110247.D
Operator    :
Acquired    : 22 Nov 2007   10:52      using AcqMethod PBDE20S
Instrument  :    Instrumen
Sample Name: liver control
Misc Info   : Mth=PBDEtest;Vol=1;Tray=Tray2;Inj=GC Inj1;...
Vial Number: 3
```



```
File        : D:\MSDCHEM\1\DATA\BDE71102.S\07110248.D
Operator    :
Acquired    : 22 Nov 2007  11:10     using AcqMethod PBDE20S
Instrument  :   Instrumen
Sample Name: feces control
Misc Info   : Mth=PBDEtest;Vol=1;Tray=Tray2;Inj=GC Inj1;...
Vial Number: 4
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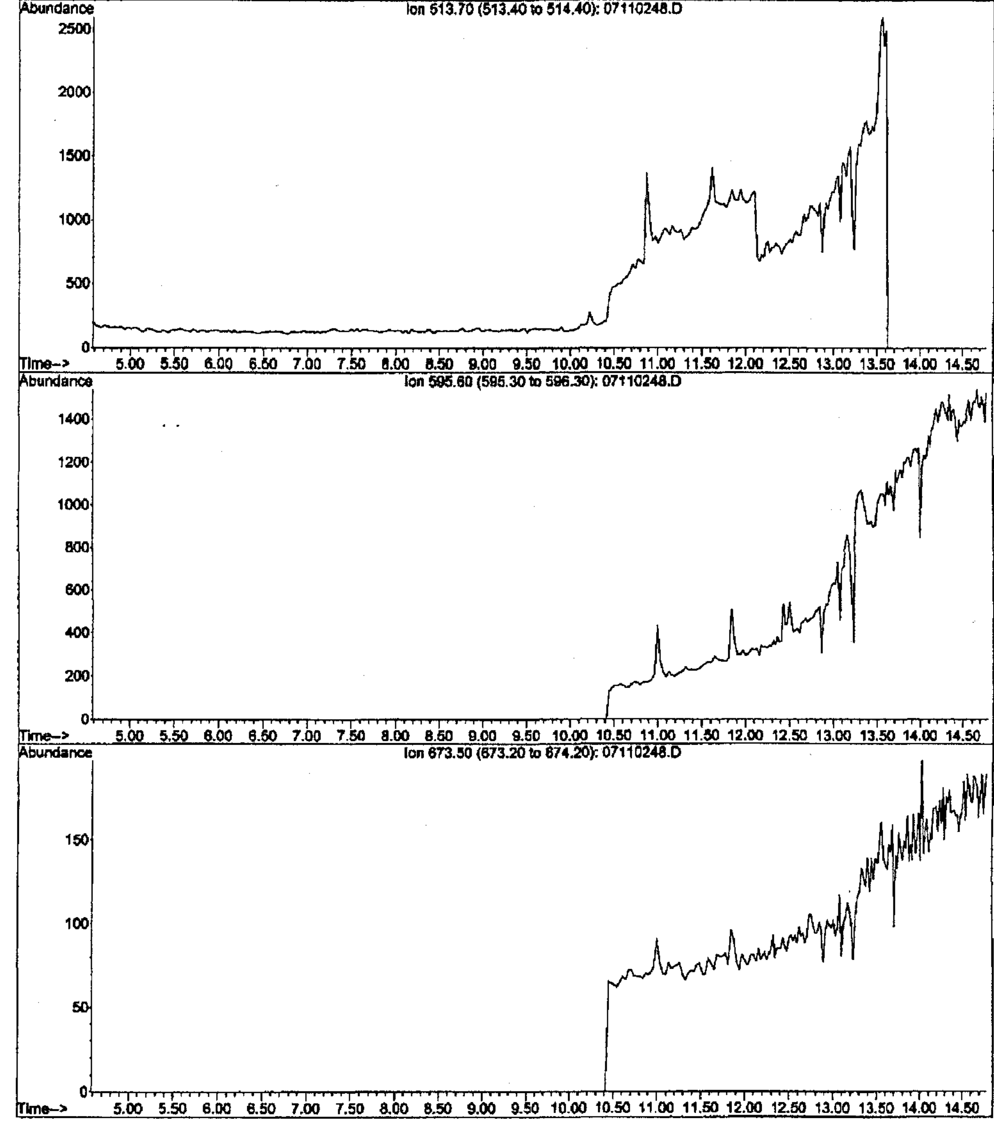
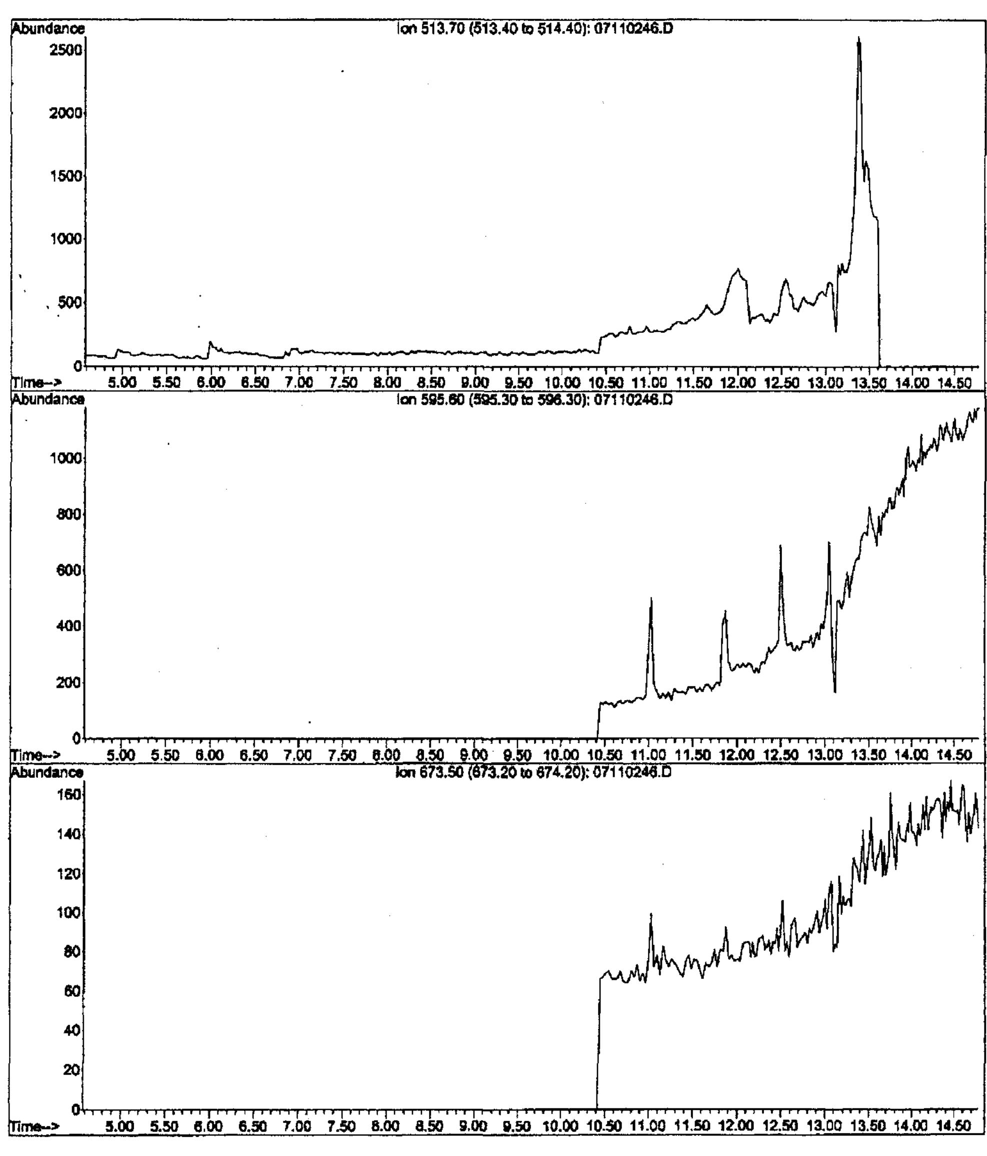


File : D:\MSDCHEM\1\DATA\BDE71102.S\07110246.D
Operator :
Acquired : 22 Nov 2007 10:34 using AcqMethod PBDE20S
Instrument : Instrumen
Sample Name: plasma control
Misc Info : Mth=PBDEtest;Vol=1;Tray=Tray2;Inj=GC Inj1;...
Vial Number: 2



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