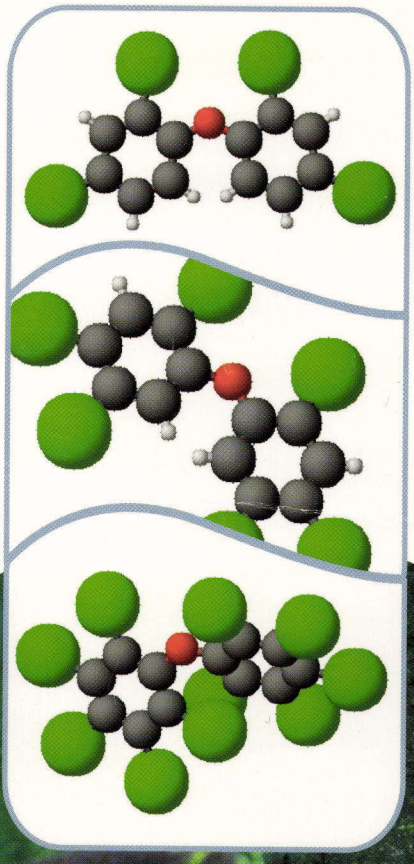
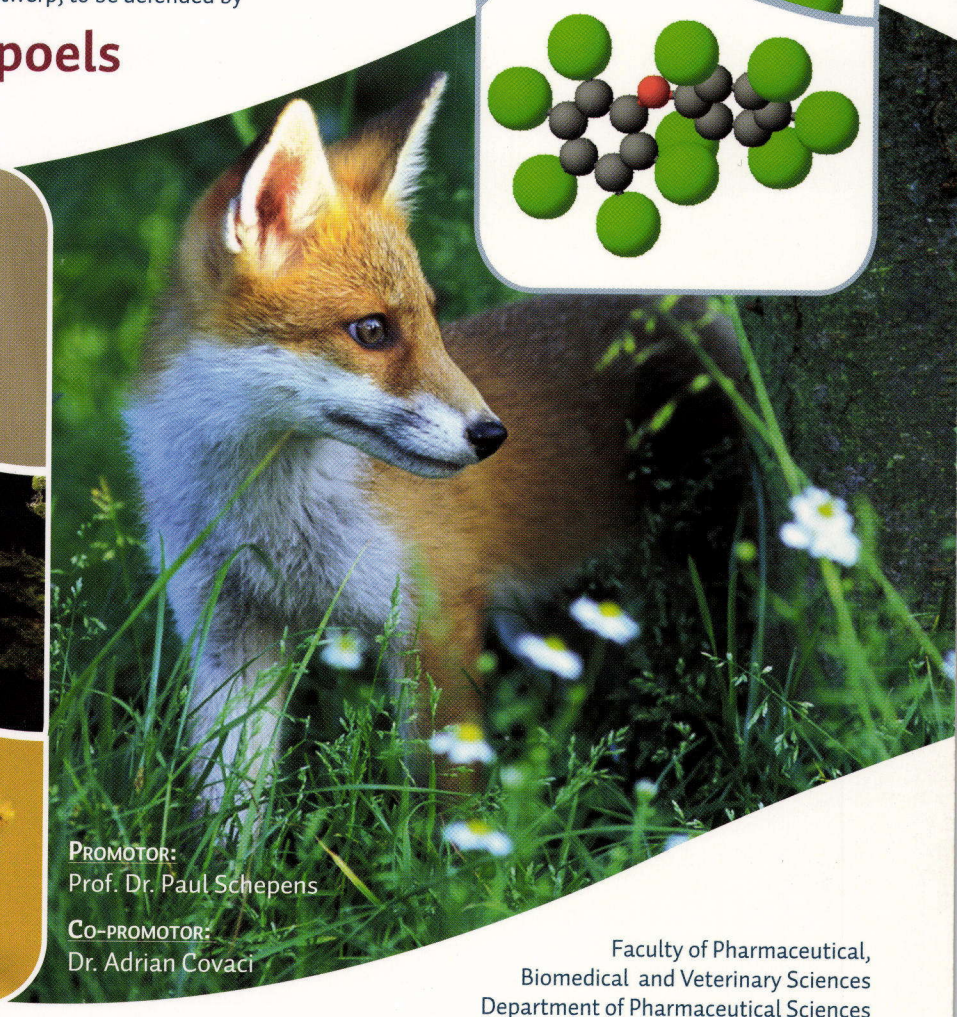
Environmental distribution of brominated flame retardants in Belgium



Dissertation for the academic degree of doctor in Pharmaceutical Sciences at the University of Antwerp, to be defended by

Stefan Voorspoels





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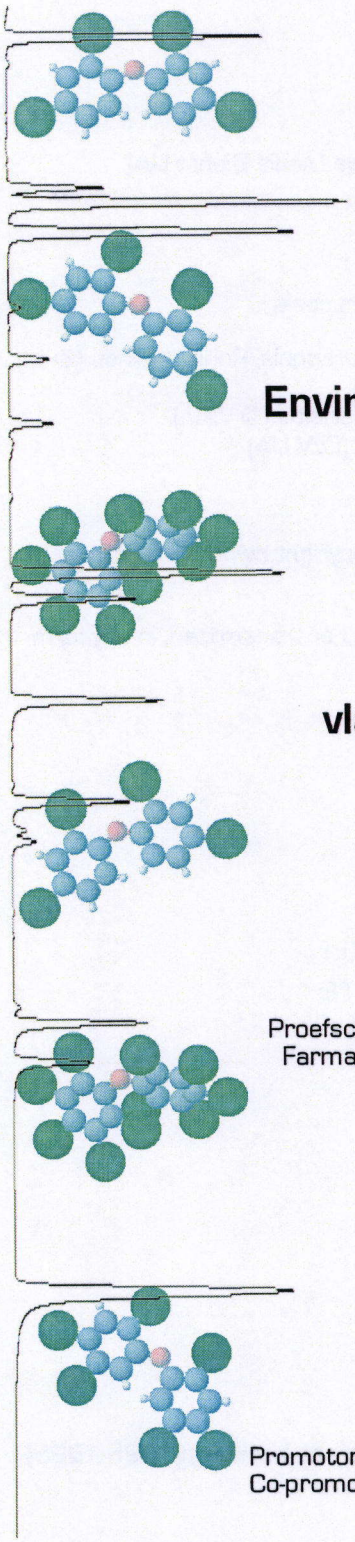

Universiteit Antwerpen

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Department of Pharmaceutical Sciences
Laboratory of Toxicology

Environmental distribution of brominated flame retardants in Belgium

Verspreiding van gebromeerde vlamvertragers in het Belgische milieu

Proefschrift voorgelegd tot het behalen van de graad van doctor in de Farmaceutische Wetenschappen aan de Universiteit Antwerpen te verdedigen door

Stefan Voorspoels

Promotor: Prof. Dr. Paul Schepens
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4. PBDEs in the Belgian terrestrial environment: Rollin Verlinde (© Vilda)
5. PBDEs and Man: Stefan Voorspoels, Koen Van Herck (CEV UA)
6. HBCDs: Karel Janák

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"Science is nothing but trained and organised common sense"

Thomas H. Huxley (1825-1895)

Abstract

Brominated flame retardants (BFRs) are a diverse group of chemicals that are used to increase fire safety. They are incorporated into a wide range of products, such as TVs, computers, household appliances, textiles and upholstery. Some of them, such as polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCD), and tetrabromobisphenol-A (TBBP-A), have led to both scientific and public concern since they have been found to accumulate in Man and wildlife. BFRs are linked to adverse physiological effects both *in vitro* and *in vivo* (e.g. interference in neurobehavioural development, foetal health and thyroid function). Adequate data on the effects are currently still insufficient to fully understand their toxicology.

This thesis presents analytical method development and measurements of BFRs in a wide range of environmental samples. Since concentrations of BFRs are low and since environmental pollution is not only restricted to these compounds, analysing these chemicals in environmental samples is a challenging task, necessitating severe quality control. Therefore, all methods that were developed and optimised in this work were finally validated through successful participation in inter-laboratory exercises.

At the start of this PhD-work, environmental occurrence of BFRs was not yet studied in Belgium. This thesis provides insight into the environmental distribution of BFRs and into the analytical aspects involved. In the first part of this thesis, the presence of PBDEs was investigated in aquatic environments. Geographical distribution and levels of PBDEs in sediments and biological samples, such as invertebrates and fish, were studied. All investigated species showed measurable levels of all contaminants. In the second part of this thesis, the presence of PBDEs was investigated in terrestrial environments. Biological samples of representative wildlife avian and mammalian top predators were investigated for PBDEs. BDE 209, a compound that is supposedly not bioavailable, was detected in the majority of the samples, which contradicts previously published statements and which suggests that legislative action is recommended for this chemical. Based on PBDE data in the prey of the investigated terrestrial predators, it was shown that PBDEs are biomagnified. In the third part of this thesis, PBDEs were investigated in relation to Man. A method for the determination of PBDEs, including BDE 209, in (human) serum was developed. Additionally, human dietary exposure was assessed by means of a market-basket study. It could be concluded that levels in food are low and comparable to other European countries. Dietary intake of PBDEs should however not be neglected. The fourth and final part of this thesis focuses on HBCD, which was investigated in selected aquatic samples.

BFR exposure to animals and humans will continue as long as these chemicals are a part of our daily life and as long as they are present as contaminants in the surrounding environment. Exposure to these compounds will not stop at the scheduled date of phase-out.

Samenvatting

Gebromeerde vlamvertragers (Brominated Flame Retardants; BFRs) zijn een diverse groep chemicaliën die worden gebruikt om de brandveiligheid te verhogen. Ze worden geïncorporeerd in vele gebruiksproducten, zoals o.a. TV's, computers, huishoudtoestellen, textiel en meubelbekleding. Sommige BFRs, zoals de polygebromeerde diphenyl ethers (PBDEs), hexabromocyclododecaan (HBCD) en tetrabromobisphenol-A (TBBP-A) hebben tot zowel wetenschappelijke als publieke bezorgdheid geleid sinds ze werden aangetroffen in mens en dier. BFRs werden in verband gebracht met schadelijke fysiologische effecten, zowel *in vitro* als *in vivo* (bv. verstoring van de normale ontwikkeling van het zenuwstelsel, van de gezondheid van de ontwikkelende foetus en van de schildklierwerking). Gegevens omtrent de effecten zijn tot op heden ontoereikend om de toxiciteit ten volle te begrijpen.

In deze thesis worden analytische methoden voor de bepaling van BFRs voorgesteld. Deze methoden werden toegepast op verschillende compartimenten van het milieu om de verspreiding van BFRs in België in kaart te brengen. Omdat de concentraties van BFRs erg laag zijn en omdat de vervuiling zich niet enkel beperkt tot deze chemicaliën alleen, is de analyse van milieumonsters een grote uitdaging. Doorgedreven kwaliteitscontrole is daarbij noodzakelijk en daarom werden alle gebruikte methoden gevalideerd door succesvolle deelname aan inter-laboratorium studies.

Bij de aanvang van dit onderzoek was de verspreiding van BFRs nog niet onderzocht in België. Deze thesis verschaft inzicht in de milieudistributie van deze polluenten en in de analytische aspecten die eraan verbonden zijn. In het eerste deel van dit werk werd gekeken naar het aquatische milieu. Geografische distributie en concentraties van PBDEs in sedimenten en biologische monsters, zowel invertebraten als vis, werden onderzocht. In alle onderzochte soorten konden PBDEs worden aangetroffen. In het tweede deel van deze thesis werd de aanwezigheid van vlamvertragers nagegaan in het terrestrische milieu. Biologische monsters van representatieve vogel- en zoogdiersoorten werden onderzocht. BDE 209, een product dat wordt verondersteld niet te bioaccumuleren, werd gedetecteerd in de meerderheid van de stalen. Dit is in tegenspraak met vroegere veronderstellingen en wijst erop dat dit product meer aandacht verdient voor wat wetgeving betreft. Verder kon ook worden aangetoond dat PBDEs onderhevig zijn aan biomagnificatie. In deel drie van dit werk werden PBDEs in relatie tot de mens nader bekeken. Hiervoor werd een methode op punt gesteld voor de analyse van PBDEs in (humaan) serum. Verder werd ook een inschatting gemaakt van de menselijke blootstelling aan PBDEs via de voeding. De concentraties in ons voedsel zijn erg laag en vergelijkbaar met andere Europese landen. De totale opname via de voeding is echter niet verwaarloosbaar. Deel vier van deze thesis is toegespitst op HBCD, waarbij enkele aquatische monsters werden onderzocht.

Mens en dier zal blootgesteld zijn aan BFRs zolang dat deze chemicaliën deel uitmaken van ons dagelijks leven en zolang ze aanwezig zijn in ons milieu. De verspreiding van dergelijke producten zal ook niet stoppen bij de aanvangsdatum van een eventueel verbod op gebruik.

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List of Publications

This thesis was based on the following scientific publications in peer-reviewed international journals. They are referred to in the text by their respective Roman numerals.

I Determination of brominated flame retardants, with emphasis on polybrominated diphenyl ethers (PBDEs) in environmental and human samples – a review
Adrian Covaci, Stefan Voorspoels, Jacob de Boer
Environ. Int. 29 (2003), 735-756

II Polybrominated diphenyl ethers, polychlorinated biphenyls and organochlorine pesticides in sediment cores from the Western Scheldt river (Belgium): Analytical aspects and depth profiles
Adrian Covaci, Adriana Gheorge, Stefan Voorspoels, Johan Maervoet, Eric Steen Redeker, Ronny Blust, Paul Schepens
Environ. Int. 31 (2005), 367-375

III PBDEs in marine and freshwater sediments from Belgium: Levels, profiles and relations with biota
Stefan Voorspoels, Adrian Covaci, Johan Maervoet, Paul Schepens
J. Environ. Monit. 6 (2004), 914-918

IV Polybrominated diphenyl ethers in marine species from the Belgian North Sea and the Western Scheldt Estuary: Levels, profiles, and distribution
Stefan Voorspoels, Adrian Covaci, Paul Schepens
Environ. Sci. Technol. 37 (2003), 4348-4357

V Levels and distribution of polybrominated diphenyl ethers in various tissues of birds of prey
Stefan Voorspoels, Adrian Covaci, Peter Lepom, Veerle L.B. Jaspers, Paul Schepens
Environ. Pollut., in press

VI Can predatory bird feathers be used as a non-destructive biomonitoring tool of organic pollutants?
Veerle L.B. Jaspers, Stefan Voorspoels, Adrian Covaci, Marcel Eens
Biol. Lett. 2 (2006), 283-285

VII Remarkable findings concerning PBDEs in the terrestrial top-predator red fox (*Vulpes vulpes*)
Stefan Voorspoels, Adrian Covaci, Peter Lepom, Sophie Escutenaire, Paul Schepens
Environ. Sci. Technol. 40 (2006), 2937-2943

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Sci. Total Environ., 366 (2006) 361-366

XI Dietary PBDE intake: A market-basket study in Belgium
Stefan Voorspoels, Adrian Covaci, Hugo Neels, Paul Schepens
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XIII Letter: Response to "HBCD: Facts and insinuations"
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Karel Janák, Adrian Covaci, Stefan Voorspoels, Georg Becher
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Determination of organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) in harbour porpoises (*Phocoena phocoena*) from the southern North Sea
Adrian Covaci, Stefan Voorspoels, Kristin Van de Vijver, Krishna Das, Jean-Marie Bouquegneau, Wim De Coen, Ronny Blust, Paul Schepens
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Stefan Voorspoels, Tinne De Mey, Adrian Covaci, Paul Schepens
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Otto Hutzinger Presentation Award

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Adrian Covaci, Lieven Bervoets, Philippe Hoff, Stefan Voorspoels, Judith Voets, Karen Van Campenhout, Ronny Blust, Paul Schepens
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Stefan Voorspoels, Adrian Covaci, Veerle L.B. Jaspers, Tom Dauwe, Marcel Eens, Paul Schepens
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Accumulation, tissue-specific distribution and biotransformation of BDE 209 in European starlings (*Sturnus vulgaris*) after exposure with silastic implants
Evi Van den Steen, Adrian Covaci, Veerle L.B. Jaspers, Tom Dauwe, Stefan Voorspoels, Marcel Eens, Rianne Pinxten
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Usefulness of bird feathers as a non-destructive biomonitor for organic pollutants
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Abbreviations

ABS	Acrylonitrile-Butadiene-Styrene
AMAP	Arctic Monitoring and Assessment Programme
AOAC	Association of Official Analytical Chemists
APCI	Atmospheric Pressure Chemical Ionisation
ASE	Accelerated Solvent Extraction
BB	Brominated Biphenyl (congener specific)
BDE	Brominated Diphenyl Ether (congener specific)
BFRs	Brominated Flame Retardants
BMF	BioMagnification Factor
BNS	Belgian North Sea
BROC	Biological Reference materials for Organic Contaminants
BSEF	Bromine Science and Environmental Forum
BTBPE	Bis(2,4,6-TriBromoPhenoxhy)Ethane
CFT	CycloDodeca-1,5,9-Triene
CHOL	Cholesterol
CMRs	Carcinogens, Mutagens or toxic regarding Reproduction
CRM	Certified Reference Material
DeBDethane	Decabromodiphenyl ethane
DCM	DiChloroMethane
DecaBDE	DecaBDE technical mixture
DDTs	1,1,1-trichloro-2,2-bis-(p-chlorophenyl) ethane and metabolites
EU	European Union
EBFRIP	European Brominated Flame Retardant Industry Panel
ECD	Electron Capture Detector
ECNI	Electron Capture Negative Ionisation
EI	Electron Ionisation
EPA	Environmental Protection Agency
ESI	ElectroSpray Ionisation
FRs	Flame Retardants
GC	Gas Chromatopgraphy
HBCD(s)	Hexabromocyclododecane(s)
HIPS	High Impact PolyStyrene
(HP)LC	(High Performance) Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
IS	Internal Standard
IUPAC	International Union of Pure and Applied Chemistry
KOH	Potassium Hydroxide
LLE	Liquid-Liquid Extraction
LOD	Limit Of Detection
LOQ	Limit Of Quantification
LRAT	Long-Range Atmospheric Transport
LRMS	Low Resolution Mass Spectrometry
LW	Lipid Weight
MASE	Microwave-Assisted Solvent Extraction
MeOH	Methanol
MeO-PBDES	Methoxy-PBDEs
ME-TBBP-A	Methyl-TBBP-A
MS	Mass Spectrometry
MSD	Mass Spectrometric Detector
NIST	National Institute of Standardisation and Technology
OctaBDE	OctaBDE technical mixture
OH-PBDEs	Hydroxy-PBDEs
OPEs	OrganoPhosphate Esters
OSPAR	OSlo-PARis commission
PBBs	PolyBrominated Biphenyls (general)
PBDEs	PolyBrominated Diphenyl Ethers (general)
PBTs	Persistent, Bioaccumulative and Toxic substances

PCBs	PolyChlorinated Biphenyls
PCDDs/PCDFs	PolyChlorinatedDibenzoDioxins/PolyChlorinatedDibenzoFuranes
PECs	Priority Existing Chemicals
PentaBDE	PentaBDE technical mixture
PHWE	Pressurised Hot Water Extraction
PL	PhosphoLipids
POP	Persistent Organic Pollutant (Stockholm Convention)
PRTR	Pollutant Release and Transfer Register
PTV	Programmable Temperature Vaporiser
QA/QC	Quality Assurance/Quality Control
QUASIMEME	Quality Assurance of Information for Marine Environmental Monitoring in Europe
REACH	Registration, Evaluation, Authorisation of Chemicals
RIVO	Nederlands Instituut voor Visserij Onderzoek (Nederland)
RoHS	Restriction on Hazardous Substances
RSD	Relative Standard Deviation
SD	Standard Deviation
SE	Scheldt Estuary
SFE	Supercritical Fluid Extraction
SIM	Selected Ion Monitoring
SPE	Solid Phase Extraction
SPI	Septum equipped Programmable Injector
SRM	Standard Reference Material
TBBP-A	TetraBromoBisPhenol-A
TG	TriGlycerides
TL	Total Lipids
TOF-MS	Time-Of-Flight Mass Spectrometry
TSCA	Toxic Substance Control Act
TTR	TransThyRethin
UK	United Kingdom
US	United States
VMM	Vlaamse Milieu Maatschappij
vPvBs	very Persistent and very Bioaccumulative substances
WHO	World Health Organisation
WW	Wet Weight

113005

1

Introduction & background information



1.1 Background

Few discoveries have had such a significant influence on the development of mankind as the skill of generating and using fire. This capability has been a basic requirement for all civilisations; Man has used fire to provide heat, to cook (otherwise uneatable) food and to extract metals from their ores. However, fire has been both a friend and an enemy of mankind throughout history. Once fire is beyond human control, its deadly and devastating natural force becomes rapidly obvious.

In search of preventive measure to increase fire safety, Man has discovered the benefits of "flame retardants". Flame retardants (FRs) are "*substances added or treatments applied to a material in order to suppress, significantly reduce or delay the combustion of the material*" (EHC-192, 1997). Such products have been used to increase fire safety throughout centuries. As early as 360 BC, vinegar was used to protect wood from catching fire (Bourbigot et al., 2004). The civilisation of the ancient Greeks, Romans and Egyptians knew the use of alum to protect wood from fire (Bourbigot et al., 2004). In more recent times, the first patent on a flame retardant was published in England in 1735 and was aimed to flame retard canvas for use in theatres and public buildings (Bourbigot et al., 2004). In 1789, the Montgolfier brothers also knew the use of FRs when they used an alum coating on their hot air balloon (Bourbigot et al., 2004). Later, in the 18th and 19th century, inorganic salts, such as ferrous sulphate and ammonium phosphate were used as FR (Hindersinn, 1990). As an example, it is known that the French King Louis XIV had the textiles in his castles treated with ammonium salts for fire protection.

As the knowledge of fire and the burning process evolved, Man developed more sophisticated FRs. Nowadays, chemicals are incorporated in the materials and they are able to prevent ignition or to slow down the initial phase of a fire. It is less likely that FRs have an influence on fires after the initial phase of their development. The use of FRs increases the escape time in case of a fire, decreases the amount of heat and toxic gasses that are released and limits the amount of material that is actually burnt.

The modern use of FRs started to increase in the 20th century, which was correlated with the increasing use of synthetic polymers (e.g. plastics). While these new materials provided many benefits, they had one problem - they were far more flammable than e.g. wood or metal, because they have a much higher carbon-hydrogen content. Therefore, polymers would catch fire more easily, produce more heat, and once alight, combust more rapidly, giving people little time to escape. FRs brought a solution to this problem and they facilitated to substantially reduce the risk of fires, providing safety at home and in public places. Furthermore, the increased use of electrical, mostly polymer-based appliances, such as TV's, computers and other electronics, has led to an enormous increase in the demand for FRs in our modern technological society.

1.2 Fire and flame retardants

Fire and combustion are gas phase reactions that involve both a fuel source and oxygen. The combustion process is characterized by four stages: (1) heating, (2) decomposition/volatilisation, (3) combustion and (4) propagation (Figure 1.1; Troitzch, 1990). Once a material is set afire, the combustion process is self-propagating and continues until one of the necessary ingredients run out; in a free-burning fire, this mostly is the burnt material (fuel).

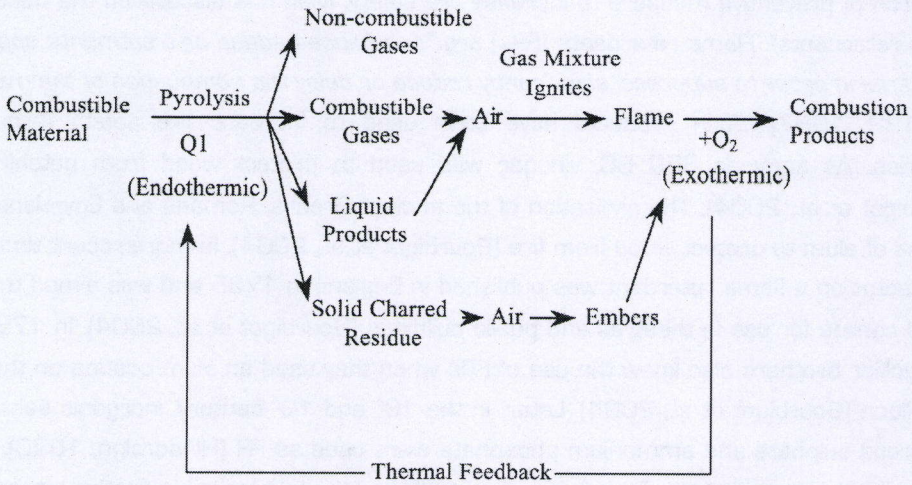


Figure 1.1. The four stages of the combustion process (Troitzch, 1990).

FRs have the ability to interfere with any of the combustion stages. Because they differ in physical and chemical properties, various FRs have different flame retarding mechanisms, capacity, and applications (EHC-192, 1997). A flame retarding compound may for example function by decomposing through endothermic processes, which cool down the burning material to temperatures below those required for pyrolysis. A different mechanism uses the ability of the chemical to form a protective coating layer of either liquid or solid state over the surface of the combusting material, which prevents oxygen and volatile combustion products to reach the flame. One very effective way of FR activity is the capture of free radicals, which are produced during the combustion process and are necessary for the propagation process. Halogens are very effective in capturing free radicals, hence removing the capability of the flame to propagate. All four halogens are effective in eliminating free radicals, but not all of the halogens are suitable for use in FRs. Fluorinated and iodinated compounds do not have the optimal decomposition properties; decomposition occurs either at too high or at too low temperatures, respectively. Consequently, only organochlorine and organobromine compounds are used as FRs. With higher trapping efficiency and lower decomposing temperature, brominated flame retardants (BFRs) have become more popular than their organochlorine counterparts.

More than 175 different commercial FRs (organic and inorganic) are available on the market (Alaee et al., 2003). There are three main families of FR chemicals, namely (1) inorganic FRs, (2) halogenated products and (3) organophosphorous and nitrogen based FRs (EHC-192, 1997). Inorganic FRs are salts, such as aluminium trihydrate, ammonium phosphate, magnesium hydroxide, borates or boric acid, and so on (EHC-192, 1997). Inorganic FRs make up 50 % of the total amount of FRs produced worldwide (EHC-192, 1997). Inorganic FRs are not only used as such, but also in combination with organic FRs, in order to enhance their flame retarding capacity. Antimony trioxide (Sb_2O_3), for example, is often used together with BFRs (EHC-192, 1997). Organic FRs are either halogenated substances, corresponding to approximately 25 % of the total amount of FRs produced, or phosphorous and/or nitrogen containing organic substances, which are estimated to 20 % of the demand of FRs (EHC-192, 1997). Examples of phosphorous containing FRs are the organophosphate esters (OPEs) that are used as well for their flame retarding as for their plasticizing properties. Next to several non-halogenated OPEs that are in use, various OPEs are substituted with halogens (EHC-192, 1997; EHC-209, 1998; EHC-218, 2000). For the reasons mentioned above, the halogenated organic FRs are either chlorinated or brominated. This latter group is discussed in detail in the chapter on BFRs (See *1.3. Brominated flame retardants*). The class of the BFRs is currently receiving most of the attention because they are characterised by their Persistent Organic Pollutant (POP)-like properties, such as persistence, lipophilicity, long-range atmospheric transport (LRAT) and toxicity. This group is also the research subject of this thesis.

1.3 Brominated flame retardants

BFRs are highly effective as FRs and economically interesting both regarding production and consumption (BSEF, 2006). BFRs are a heterogeneous group of chemicals, some of which are aliphatic, other cycloaliphatic or aromatic. Since bromine is the major component of a BFR and the component that is delivering the FR-activity, there is no particular restriction on the structure of the backbone. The main criteria for the usage of a compound as flame retardant are stability during the lifetime of the flame retarded product and compatibility with the polymer. These chemicals can be incorporated into the material to be flame retarded either by covalent bonding (reactive FR) or by simple dispersion into the matrix (additive FR) (EHC-192, 1997).

At the start of this PhD research in 2001, five types of BFRs dominated the market: three different types of technical/commercial polybrominated diphenyl ether (PBDE) products (PentaBDE, OctaBDE and DecaBDE), Hexabromocyclododecane (HBCD) and Tetrabromobisphenol-A (TBBP-A) (Figure 1.2). Following risk-assessment studies, both Penta- and OctaBDE were banned in the European Union (EU) as from August 2004 (Directive 2003/11/EC). Therefore, DecaBDE is currently the only PBDE product that is still allowed for use in the EU.

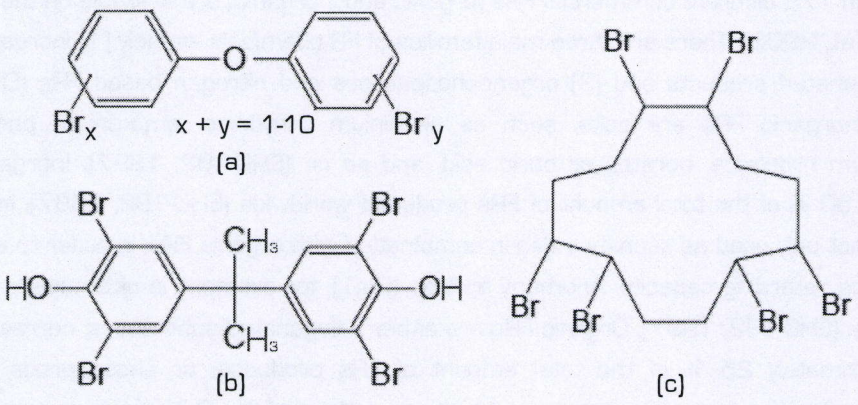


Figure 1.2. Schematic structures of (a) polybrominated diphenyl ethers (PBDEs), (b) tetrabromobisphenol-A (TBBP-A) and (c) hexabromocyclododecane (HBCD).

Apart from the ones mentioned above, other BFRs are in use, although in smaller amounts, such as Bis(2,4,6-tribromophenoxy)ethane (BTBPE), TBBP-A 2,3-dibromopropyl ether (TBBP-A-DPBE), decabromo diphenyl ethane, TBBP-A diallyl ether and pentabromo-1,4-diphenoxybenzene (Örn and Bergman, 2004). It has previously been stated that there are more than 75 different chemicals that are proposed as BFRs (Alaee et al., 2003). However, an assessment of the three major industrial BFR producers suggests that the number of different substances or classes of polybrominated chemicals is likely to be around 20 (Örn and Bergman, 2004).

During the last decade, global BFR demand has grown with a volume increase of more than 100 % (Alaee et al., 2003). The total annual market demand of BFRs in 2003 was roughly 200,000 tons (Table 1.1; BSEF, 2006). TBBP-A is the most used BFR, followed by DecaBDE and HBCD. The production and use of BFRs differs by region (Table 1.1); TBBP-A is mostly used in Asia, while the EU uses most of the HBCD. DecaBDE is used to a large extent in both the United States (US) and Asia. Interestingly, PentaBDE, which is now banned from the EU, was almost exclusively oriented at the US market (BSEF, 2006).

There is no domestic production of BFRs in Belgium. Accordingly, all BFRs in use are imported, either as the chemical itself or incorporated in flame retarded consumer products.

Table 1.1. Most recent available data on the region-specific market demand of BFRs in 2001, as released by the flame-retardant industry (tons) (BSEF, 2006).

	Europe	Americas	Asia	Rest of the world	Total
TBBP-A	11,600	18,000	89,400	600	**119,600**
HBCD	9500	2800	3900	500	**16,700**
DecaBDE	7600	24,500	23,000	1050	**56,150**
OctaBDE	610	1500	1500	180	**3790**
PentaBDE	150	7100	150	100	**7500**
TOTAL	*29,460*	*53,900*	*117,950*	*2430*	***203,740***

1.3.1 Polybrominated diphenyl ethers

PBDEs are a class of FRs with the general structure as shown in Figure 1.2. They are theoretically composed of 209 congeners, divided into 10 homologue groups (mono- to decabrominated diphenyl ethers). Only a limited number of congeners can be found in technical PBDE mixtures (EHC-162, 1994) and in the environment (de Wit, 2002; Law et al., 2003). Congeners with less than 4 bromines are rarely found in commercial mixtures (Darnerud et al., 2001). PBDEs are numbered according to the International Union of Pure and Applied Chemistry (IUPAC) system already in use for polychlorinated biphenyls (Ballschmitter and Zell, 1980; Pijnenburg et al., 1995) and thus they are referred to as numbers. The congeners that are the major constituents of the technical mixtures and most encountered in the environment, are the following: BDE 28, BDE 47, BDE 99, BDE 100, BDE 153, BDE 154, BDE 183, and BDE 209 (Table 1.2).

Table 1.2. Most environmentally relevant and investigated BDE congeners.

IUPAC number	Substitution pattern	IUPAC number	Substitution pattern
BDE 28	2,4,4'	BDE 153	2,2',4,4',5,5'
BDE 47	2,2',4,4'	BDE 154	2,2',4,4',5,6'
BDE 99	2,2',4,4',5	BDE 183	2,2',3,4,4',5',6
BDE 100	2,2',4,4',6	BDE 209	2,2',3,3',4,4',5,5',6,6'

PBDEs are lipophilic substances with high log octanol-water partition coefficients (K_{ow}) of approximately 4 to 9 (EHC-162, 1994; Braeckeveldt et al., 2003). The substances become more lipophilic with increasing degree of bromination; the tetrabrominated congener BDE 47 has an estimated log K_{ow} of 6.8, while hexabrominated BDE 153 and heptabrominated BDE 183 have a log K_{ow} value of 7.9 and 8.3, respectively (Braekevelt et al., 2003). Since (most) log K_{ow}-values are estimations, the values reported in the literature can vary to a small extent and should therefore be considered more as indicative than as being exact. The decabrominated congener, BDE 209, is the PBDE with the highest possible bromine load and

has a log K_{ow} of 8.8 (Braekevelt et al., 2003). The estimated water solubility of BDE 209 has been suggested to be 20-30 µg/l (EHC-162, 1994). At room temperature, PBDEs also have low vapour pressures, which are inversely correlated with the number of bromine substituents, i.e. higher brominated structures are less volatile. Vapour pressure of BDE 47 has been measured to be 1.9×10^{-4} Pa, while BDE 183 has a vapour pressure of 4.7×10^{-7} Pa (Tittlemier et al., 2002). These data indicate that lower brominated congeners tend to be more mobile in air and water than higher brominated congeners. The chemical characteristics of PBDEs influence their behaviour in the abiotic environment and in biological species. These chemicals' properties also have consequences regarding their sampling and analysis (See *2. Analytical methodology*).

PBDEs are industrially produced by direct bromination of diphenyl ether in the presence of aluminium tribromide or iron as catalysts (EHC-162, 1994). The production process is stopped at three different levels for production of the commercial mixtures PentaBDE, OctaBDE and DecaBDE, respectively. In contrast to the commercial PCBs produced, the technical PBDEs consist of fewer congeners (Safe et al., 1985; EHC-140, 1993). A total of 69 BDE congeners have recently been identified in technical PBDE products (Nogueiras et al., 2004).

The three technical products of PBDEs are named after the most abundant congeners in each type of mixture (Table 1.3). The different constitution of each technical mixture also results in different chemical and technical properties.

Table 1.3. The general composition of PBDE-based flame retardant commercial products given in percent of PBDE congeners present (EHC-162, 1994).

Technical product	Congener %						
	Tetra-BDEs	Penta-BDEs	Hexa-BDEs	Hepta-BDEs	Octa-BDEs	Nona-BDEs	Deca-BDE
PentaBDE	24-38	50-60	4 - 8				
OctaBDE			10-12	43-44	31-35	9-11	< 1
DecaBDE						0.3-3	97-98

PBDEs are used as additive flame retardants, which means that they are blended into the polymer or resin and that they are not chemically bound to the material. Therefore, PBDEs may migrate out of a product, either during production, storage, use or at the end of the product's life, e.g. at recycling. The fact that PBDEs are used as additives contributes to the potential for environmental and occupational exposure.

1.3.1.1 PentaBDE

The PentaBDE products consist of up to 60 % of congeners with five bromine substituents and up to 38 % of tetra-substituted congeners. Small amounts of PBDE congeners with three or six bromines are also present (Table 1.3) (EHC-162, 1994). The most abundant congeners in the PentaBDE-mixture are BDE 47 and BDE 99, which account for

approximately 75 % of the total mass, with roughly twice as much BDE 99 as BDE 47 (Sjödin et al., 1998). There are approximately equal amounts of BDEs 153 and 154 present, similar to that one of BDE 100 (EHC-162, 1994). A well-known and thoroughly investigated example of a PentaBDE technical mixture is Bromkal 70-5DE (Sjödin et al., 1998). PentaBDE is used in applications such as polyurethanes, flexible polyurethanes and rubbers (Alaee et al., 2003). Currently, pentaBDE formulations are mostly used in the US, although they are under severe legislative pressure. The EU has banned the use of these products as from 2004 (See *1.6. Legislation and policy*).

1.3.1.2 OctaBDE

The OctaBDE products consist predominantly of congeners with seven and eight bromine atoms, up to 44 % and 35 %, respectively. The OctaBDE product also contains approximately 20 % of hexaBDEs and nonaBDEs, with traces of BDE 209 (Table 1.3) (EHC-162, 1994). The major PBDE-congener present in OctaBDE is BDE 183 (Sjödin et al., 1999). OctaBDE is to a large extent used in acrylonitrile butadiene styrene plastics (ABS) (Alaee et al., 2003). The use of OctaBDE is banned by the EU from 2004. Several other countries and states are also working towards a ban on OctaBDE products (See *1.6. Legislation and policy*).

1.3.1.3 DecaBDE

The DecaBDE products consist of 98 % of one congener, namely the fully brominated BDE 209. Small amounts (< 1 %) of nonabrominated diphenyl ethers are also present in the DecaBDE technical products (Table 1.3) (EHC-162, 1994).
The largest proportion of the PBDEs in use is DecaBDE (Table 1.2). This technical mixture is used as an additive FR and it has the most variable applications, such as in high impact polystyrene (HIPS), back-coating of textiles, polyamides, polyolefins, polyethylene, polypropylene, polybutylene terephtalate, unsaturated polyesters, epoxy resins and nylon (BSEF, 2006; Alaee et al., 2003). In these different types of resins, polymers and substrates, the PBDE content can be as high as 10 % by weight (EHC-162, 1994). These different types of flame retarded products are used for the manufacturing of a wide range of consumer products, such as TV's, computers, household appliances and electronics. At this time there are no legislative measures taken to restrict or ban the production and/or use of DecaBDE (Hansen et al., 2002). This product however is subjected to a high degree of controversy due to its potential adverse effects in the environment, which is fed by research of environmental scientists worldwide. Therefore, DecaBDE has received a great deal of attention during the last years.

1.3.2 Other brominated flame retardants

1.3.2.1 Polybrominated biphenyls

Polybrominated biphenyls (PBBs) are FRs that have great structural resemblance to the notorious polychlorinated biphenyls (PCBs) (Figure 1.3). They were produced throughout the 1970s for flame retarding acrylonitrile-butadiene-styrene (ABS) plastics, coatings and lacquers, and polyurethane foam (de Boer et al., 2000). PBBs achieved worldwide notoriety in 1973 when they were accidentally used for the production of cattle feed in Michigan (Fries, 1985). This incident has a great resemblance to the Belgian "*PCB-crisis*" of 1999 (Van Larebeke et al., 2001). The Michigan incident resulted in 500 farms being quarantined and thousands of animals being destroyed. Subsequently, production of PBBs was discontinued in 1976. Commercial PBBs existed as technical mixtures, similar to PBDEs. The most common mixture used was Firemaster® BP-6, produced by the Michigan Chemical Company. Firemaster® BP-6 was a mixture of 13 congeners of PBBs, where the number of bromine atoms on the biphenyl backbone ranged from 4 to 7. The vast majority (85 %) of the Firemaster mixture was composed of two congeners, 2,2',4,4',5,5'-hexabromobiphenyl (BB-153) and 2,2',3,4,4',5,5'-heptabromobiphenyl (BB-180). PBBs continue to be detected in the environment in abiotic and animal tissue samples, even in Europe, where they were hardly ever used.

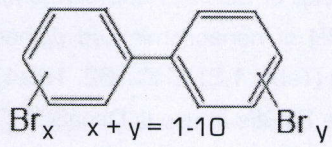


Figure 1.3. Schematic structure of polybrominated biphenyls (PBBs).

1.3.2.2 Hexabromocyclododecane

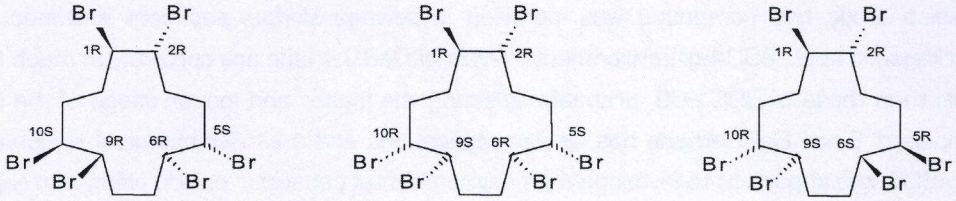
Technical HBCD is used as an additive flame retardant in the back-coating of upholstery textiles and in polystyrene foams that is used as thermal insulation in buildings and packaging material (Alaee et al., 2003; BSEF, 2006). Technical grade HBCD differs from the other major BFRs, as it is a mixture of several brominated cycloaliphatic diastereoisomers (Figure 1.4). The product is obtained via bromination of cyclododeca-1,5,9-triene (CDT) isomers. Depending on the purity of the starting material and the conditions of the industrial processes, a range of different technical products with differing isomer compositions and melting points are formed due to the presence of six stereocenters at positions 1,2,5,6,9 and 10. From all four possible CDT isomers, 16 HBCD stereoisomers may be formed, as well as six diastereomeric pairs of enantiomers and four meso forms (See *6. HBCD*).

Although technical HBCD mixtures consist mainly of three HBCD isomers, named α-, β- and γ-HBCD (Figure 1.4), more stereoisomers are present in those mixtures (Heeb et al. 2005). Heeb and co-workers (2005) reported the isolation of 8 stereoisomers from a low-melting

technical HBCD mixture. Five individual HBCD diastereoisomers, named α-, β-, γ-, δ- and ε-HBCD were isolated. The α-, β- and γ-HBCD diastereomers could each be further resolved into two peaks on a chiral, stationary phase, namely (+) and (-) α-, β- and γ-HBCD, whereas δ- and ε-HBCD both eluted as single peaks from the chiral column (Heeb et al., 2005). For these reasons, technical grade HBCD should better be referred to as "HBCDs" (***Paper XXVI***, not included in this thesis).

In the technical HBCD mixture, (+/-) γ-HBCD is the most abundant diastereoisomer (82 %) while the relative amounts of (+/-) α- and (+/-) β-HBCD were reported to be 11.8 and 5.8 %, respectively.

The δ- and ε-isomers accounted for less than 0.5 % of the total HBCD (Tomy et al., 2004; Heeb et al., 2005). These HBCD isomers are lipophilic chemicals with a log K_{ow} of 5.6 – 7.7 and a low water solubility (3.4 µg/l); they have a vapour pressure of 6.3×10^{-4} Pa (American Chemistry Council, 2003).



α-HBCD β-HBCD γ-HBCD

Figure 1.4. Schematic structure of the three major HBCD-isomers.

1.3.2.3 Tetrabromobisphenol-A

TBBP-A consists of two brominated and hydroxylated phenyl rings, each bound to a propyl group as shown in Figure 1.2. TBBP-A differs from the other major BFRs by the presence of two hydroxyl groups, which makes it a weak acid. This influences the log K_{ow} of the chemical, which was estimated between 4.5 and 5.3, rendering this product more water soluble (4.2 mg/l) than both PBDEs and HBCD (EHC-172, 1995). Due to its phenolic structure, TBBP-A can be used as a chemically bound BFR in epoxy and polycarbonate resins (Alaee et al., 2003). It is added during the polymerisation and thereby gets chemically bonded during the process. Approximately 90 % of all TBBP-A produced is used as a reactive BFR, primarily in epoxy resins used for circuit board laminates. The TBBP-A content in such products can be as high as 20 % by weight (EHC-172, 1995). TBBP-A is to a lesser extent used as an additive BFR, e.g. in ABS resins. These resins are found in a wide range of consumer products, such as telephones, refrigerators and packaging material (EHC-172, 1995).

TBBP-A is also used as an intermediate for the production of different TBBP-A derivatives (Örn and Bergman, 2004). The phenolic groups are easily used for derivatisation and subsequent modifications to form ethers, such as TBBP-A diallyl ether, diglycidyl ether and

2,3-dibromopropyl ether. This makes TBBP-A adjustable for specific applications (EHC-172, 1995). TBBP-A derivatives are also incorporated in brominated epoxy oligomers and carbonate oligomers (EHC-172, 1995). Apart from this small introduction, TBBP-A is not further discussed or investigated in this work.

1.3.2.4 Novel brominated flame retardants

Apart from the above-mentioned BFRs, novel compounds are regularly introduced onto the market. Occasionally, some studies report on previously un-investigated brominated compounds.

Due to the controversy around DecaBDE and a possible ban that might become active in the (near) future, the BFR industry has marketed alternative compounds, such as decabromodiphenyl ethane (DeBDethane; Figure 1.5). Although this chemical was introduced on the market in the early 1990's, with similar applications as BDE 209, there are currently hardly any data available about the presence of DeBDethane in the environment. Only in a Swedish study, this compound was identified in sewage sludge, sediment and indoor air (Kierkegaard et al., 2004a). Environmental levels of DeBDethane are currently at much lower levels than those of BDE 209, probably reflecting the higher and longer usage of the latter compound. Since DeBDethane has similar applications and a similar chemical structure as DecaBDE, it is important to investigate its environmental behaviour before using it to replace BDE 209. Such "*shifts in market demand*" have been seen before following the ban on Penta- and OctaBDE in the EU, when the demand of HBCD increased significantly.

Another, only recently discovered, BFR is bis(tribromophenoxy)ethane (Figure 1.5). This compound was detected in ambient air and sediment samples from the US (Hoh et al., 2005). Due to lack of reference standards, this compound was identified based on chromatographic retention time and mass spectrum. These two novel BFRs mentioned above are not the only ones that remain un-investigated. New products are introduced on a regular basis. These newer FRs were not included in this thesis and are therefore only briefly discussed.

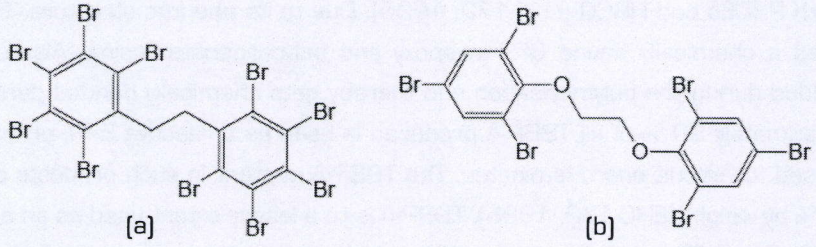


Figure 1.5. Novel BFRs: (a) Decabromodiphenyl ethane and (b) bis(tribromophenoxy)ethane.

1.4 Environmental fate and behaviour of BFRs

The massive use of BFRs (Table 1.1) has lead to their ubiquitous environmental distribution, which has raised great concern (de Wit, 2002; Law et al., 2003). Data on production and use are rather scarce, not up to date and only provided by the manufacturers themselves. Nevertheless, the market demand for BFRs has increased more than 10 % from 1999 to 2003 (BSEF, 2006). Also, considerable changes in the kinds of BFRs used and their consumption amounts occurred throughout this period. The market demand for the commercial PentaBDE and OctaBDE declined because of forced (EU) and voluntarily (US) phase-out following the global concern regarding the potential adverse environmental and health effects of these PBDEs (See *1.6. Legislation and policy*). Throughout this period, the production and demand of DecaBDE and HBCD have increased significantly.

The most common emission sources of BFRs into the environment occur at factories producing BFRs, flame-retarded polymers, and plastic products, such as electrical appliances. A Swedish survey on BFRs in river sediments surrounding a plastics factory that used TBBP-A showed significantly higher levels downstream of the factory than upstream (Sellström and Jansson, 1995). Similarly, higher levels of PBDEs and HBCD were found downstream of a textile producing plant (Sellström et al., 1998). The BFR industry has recognised this problem and voluntarily started up their so-called *product stewardship programme* in which they try to reduce this type of release during production and application processes (BSEF, 2006). A major flaw in these emission reduction measures is that they are only oriented towards producers and industrial down-stream users, end-user (the consumer) related exposure is not taken into account. Since PBDEs and HBCD are used as additive flame retardants that are not chemically bound to the products in which they are used (e.g. TV, computer), the BFRs are released into the product's surroundings while being operated. Especially electronic equipment, such as TV's and computers, contain high amounts of BFRs and are potential sources of emissions of BFRs into the environment, and humans, especially indoor. Tamade and co-workers (2002) reported that the dust collected from inside television sets, whose cabinets contained BFRs, showed levels of PBDEs and TBBP-A of the order of milligrams per gram dust. This shows that BFRs are released from products containing BFRs to surrounding indoor air. Not only consumer electronics, but also flame retarded polyurethane foam that is used for cushioning of seats and sofas was shown to be an important source of emissions of PBDEs into the environment (Hale et al., 2002). This type of usage related exposure cannot be controlled as long as these substances are used.

Once released into the environment, PBDEs largely partition to organic carbon, such as in soil, sediment and vegetation. Consequently, the overall environmental fate of these compounds depends on their behaviour in these compartments. Model results indicate that

only a small proportion of PBDEs exist in air and water, suggesting that these compounds *in theory* have limited LRAT potential (Gouin and Harner, 2003). Other models suggest that lower brominated BDE congeners have a potential for LRAT that is comparable to PCBs, of which is known that they are transported over large distances (Wania and Dugani, 2003; Watanabe and Sakai, 2003); higher brominated BDE congeners have a much lower potential to reach remote areas (Wania and Dugani, 2003). This is in accordance with the available data on the log K_{ow} and vapour pressure of these compounds (See *1.3.1. Polybrominated diphenyl ethers*) and with the field measurements in remote regions that indicate that the lighter components of commercial mixtures predominate (Wania and Dugani, 2003). Further, PBDEs may experience active surface–air exchange as a result of seasonally and fluctuating temperatures because of their physico–chemical properties. Subsequently, this may result in the potential for LRAT of the PBDEs through a series of deposition/volatilisation hops, otherwise known as the "*grasshopper effect*" (Gouin and Harner, 2003). BFRs can therefore contaminate distant regions, far away from known sources. Sufficient data is currently lacking to fully explain the occurring transport processes.

BFRs have now been detected in all environmental compartments, e.g. sediments, soils, fish, marine mammals, birds and humans (Law et al., 2003). The presence of BFRs in biological species is due to their physico-chemical properties, such as lipophilicity and resistance to degradation, which lead to bioaccumulation (Gustaffson et al., 1999). Furthermore, these compounds are passed on through the food chain and are biomagnified. Inside organisms, these lipophilic chemicals are partitioned into body lipids in organs and tissues. Apart from the abiotic environmental compartment, a great proportion of the BFRs released into the environment persist inside living organisms, where they might exert adverse health effects.

Since BFRs are susceptible to biomagnification, one important route of transfer and uptake is through the diet. Seeing that BFRs are omnipresent, they can also be found in the food we consume daily. Due to their lipophilic nature, these man-made chemicals are primarily found in lipid-rich foods of animal origin, such as meat, fish and dairy products. It has been shown that food, and more in particular food of animal origin, can be responsible for more than 90 % of the average human intake of PCBs (Liem et al., 2000). Additionally to food, other routes of exposure are currently being investigated. Because dust has been shown to be a large reservoir of BFRs (Tamade et al., 2002), more in particular in indoor environments (Harrad et al., 2004; Jones-Otazo et al., 2005), the contribution of inhalation and/or ingestion of dust to the total PBDE intake should not be overlooked. The importance of the different routes of human exposure to PBDEs is not yet completely understood and opinions about the contribution of the dietary uptake vary among publications. In general, the dietary uptake is estimated between 73 (Wijesekera et al., 2002) and 93 % (Harrad et al., 2004). The rest of the uptakes would then originate from inhalation and/or ingestion. However, the contribution

of inhalation to the total PBDE exposure is susceptible to great variance depending on several factors, such as lifestyle (indoor – outdoor), age (toddler – adult), home environment, and work place exposure (Jones-Otazo et al., 2005).
Certain animals species, such as predatory fish, carnivorous mammals and Man are at the top of their respective food chain, which makes it very likely that they ingest large quantities of these compounds through their diet. This makes top-predators very interesting study objects in regard to environmental BFR distribution.

1.5 Toxicology and metabolism

The occurrence of several classes of BFRs in the environment has become increasingly evident and presents a potential health risk to organisms exposed to these environmental contaminants. Limited toxicokinetic data on PBDEs are available in the scientific literature. There is evidence that PBDEs are bioavailable to animals and humans, although the lower brominated congeners (tetra- through hexa-substituted BDEs) appear to be more readily absorbed than the higher brominated congeners (hepta- through deca-substituted BDEs). This has been linked to the high K_{ow} and low water solubility (See *1.3.1. Polybrominated diphenyl ethers*), but also to the large molecular size of the higher brominated congeners (Kierkegaard et al., 1999; Hardy et al., 2000).

1.5.1 PBDE toxicology

In vivo experiments have demonstrated that PBDEs may cause various adverse effects, which depend on the type of product and dosing level. Generally, the technical PentaBDE products seem to cause effects at the lowest dose, whereas much higher doses were needed for effects of the DecaBDE (Darnerud et al., 2003). Indeed, DecaBDE is generally considered to have the lowest toxicity of the three major technical PBDE-mixtures. The critical effects of PentaBDEs are those on neurobehavioural development and, although somewhat less sensitive, thyroid hormones in offspring (Zhou et al., 2002; Timme-Laragy et al., 2006). Whereas OctaBDEs primarily give rise to fetal toxicity/teratogenicity in rats and rabbits, DecaBDE causes certain morphological effects in the thyroid, liver and kidney of adult animals (Darnerud et al., 2003). Carcinogenicity studies on DecaBDE have resulted in an IARC (International Agency for Research on Cancer) classification stating limited evidence for carcinogenicity of DecaBDE in experimental animals (IARC, 1990). The knowledge of effects of PBDEs on Man is still very limited (Birnbaum and Cohen Hubal, 2006); risk assessment is chiefly based on animal models. What we do know is that humans in general, at least in Western countries, are exposed to PBDEs and that human tissues contain measurable PBDE levels (Sjödin et al., 1999, Covaci et al., 2002, Schecter et al., 2003). It is currently unresolved whether these levels are high enough to cause any adverse effects in Man, but it was shown that the lowest body weight-related dose levels that cause effects in animals are much higher than available estimations of human dietary intake (Darnerud et al., 2000).

Recently, Birnbaum and Cohen Hubal (2006) concluded that further studies are needed on developmental effects, endocrine disruption, and long-term exposure. Significant insight could be obtained from additional information on environmental fate and behaviour of PBDEs, as well as population-based biomonitoring data for adults and for potentially vulnerable populations, such as children (Birnbaum and Cohen Hubal, 2006).

1.5.2 PBDE metabolism

In exposed organisms, metabolism is an important factor in determining the bioaccumulation, fate, toxicokinetics, and toxicity of contaminants. Bioaccumulation only occurs if the rate of elimination is lower than the intake. We know very little of PBDE toxicokinetics in Man, while a limited number of studies were performed on mice and rats (Orn and Klasson-Wehler, 1998; Von Meyerinck et al, 1990; Hakk et al., 2002; Mörck et al., 2003; Hakk and Letcher, 2003; Staskal et al., 2005; Chen et al, 2006; Sanders et al., 2006) and on fish (Kierkegaard et al., 1999; Stapleton et al, 2004a,b,c). Although these BFRs can be catalogued as environmentally persistent, contaminant exposure can, to a certain degree, result in the induction of Phase I cytochrome P450 monooxygenase (CYP) enzymes and Phase II conjugation enzymes (Nelson et al., 1996; Lewis et al., 1998). Phase I metabolites are subsequently metabolised via Phase II conjugation. PBDE metabolism has been investigated through *in vitro* liver microsomal studies (de Boer et al., 2000), through dosing studies with captive animals, and through studies on the presence of retained hydroxylated PBDE (HO-PBDE) and methoxylated (MeO-PBDE) metabolites in blood in wildlife species (Hakk and Letcher, 2003). However, recent studies indicate that PBDE metabolism via oxygen insertion (formation of HO-PBDEs) and reductive debromination pathways may be most common (Darnerud, 2003; Stapleton et al., 2004a,b,c). Mounting evidence supports the hypothesis that marine sponges can produce MeO-PBDEs (which are found in marine species) and that they are of natural origin (Teuten et al., 2005). Whether or not MeO-PBDEs can be biologically formed from PBDEs in marine or other animal species needs to be resolved.

Biological dehalogenation in organisms explains partly why the congener composition found in biota rarely mimics the composition of commercial mixtures; congeners that have not been reported in any commercial mixture are found in biological samples and the ratio of congeners changes (Hites 2004). For example, while the ratio of BDE 99 to 47 is nearly two in the commercial PentaBDE mixture, there is almost twice as much BDE 47 as 99 present in human breast milk (Schecter et al., 2003). In some marine mammals, such as ringed seals in northern Canada, the levels of BDE 99 are barely 10 % of those of BDE 47 (Ikonomou et al., 2002). In ***Paper IV*** (See *3.4.*), such profile shifts are investigated and are further linked to exposure level and metabolic capacity of the species (See *3. PBDEs in the Belgian aquatic environment*). This is likely due to metabolism of BDE 99, because it has been

shown that this congener is rapidly absorbed and converted to hydroxy- and debrominated (hydroxy-) metabolites (Hakk et al., 2002). In addition, the relative amounts of several other congeners change. Whether these changes are due to metabolism and clearance, or are the result of debromination from other congeners, remains to be determined. Furthermore, the biological dehalogenation of PBDEs raises question on the effectiveness of the European ban on Penta- and OctaBDE while DecaBDE is still in use.

Biologically formed metabolites can have substantial endocrine disrupting effects (Meerts et al., 2001; Lilienthal et al., 2006). HO-PBDEs are potent competitors for TTR, the plasma protein responsible for the transport of the thyroid hormone. Brominated structural analogues of T4 and T3 (Figure 1.6) can also interact with thyroid hormone receptors (Legler and Brouwer, 2003). These metabolites are overlooked in any ongoing risk-assessment study.

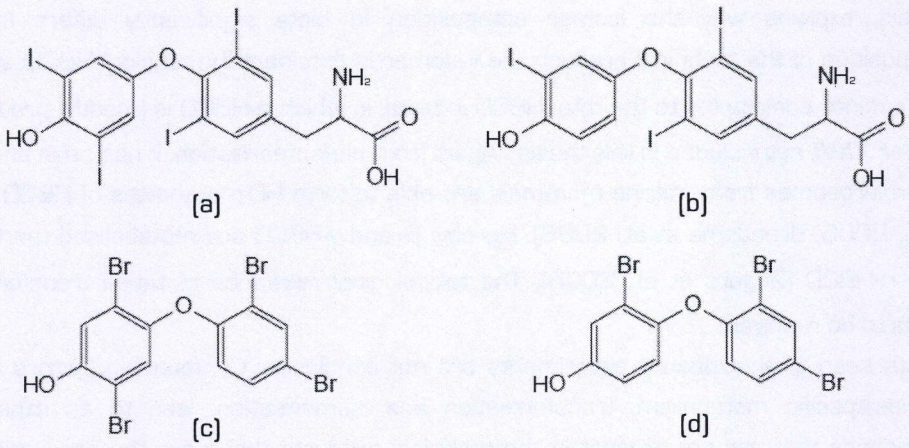


Figure 1.6. Structural resemblance of thyroid hormones and some PBDE-metabolites: (a) T4, (b) T3, (c) 4-OH-2,2',4',5-BDE 48 and (d) 4-OH-2,2',4'-BDE 17.

1.5.3 HBCD toxicology and metabolism

The toxicological database for HBCD is very limited and there is a serious lack of relevant, high-quality studies (Darnerud, 2003). The direct acute and chronic toxicity of HBCD appear to be low, but this does not rule out sub-lethal effects. HBCD has an antagonistic effect on detoxification enzymes and may also increase the toxicity of other compounds (Ronisz et al., 2001). It has also been suggested that HBCD may induce cancer by a non-mutagenic mechanism (Ronisz et al., 2001; Helleday et al., 1999) and that HBCD should be considered as an endocrine disrupting chemical because of its potential to disrupt the thyroid hormone system (Darnerud 2003; Hall et al., 2003; Sakai et al., 2003). The endocrine disrupting activity of HBCD is currently under investigation within the EU-funded FIRE project (Flame retardants Integrated Risk assessment for Endocrine effects; http://www.rivm.nl/fire). Following neonatal exposure, developmental neurotoxic effects, such as aberrations in

spontaneous behaviour, learning and memory function, can be induced (Eriksson et al., 2002; Eriksson et al., 2004) and HBCD can alter the normal uptake of the neurotransmitters in rat brain (Mariussen and Fonnum, 2003).

The different HBCD diastereoisomers and enantiomers that are present in the technical product have different properties regarding their solubility and partitioning behaviour, as well as their biological uptake and metabolism. Keeping this complex stereochemistry of technical HBCD in mind, it is likely that diastereo- and enantioselective processes will occur in the environment, possibly inducing relative enrichment of different stereoisomers of HBCD. At this moment there is no information on the relative toxicity of the different HBCD diastereoisomers and enantiomers. Also, until very recently, toxicological testing has been conducted solely with technical HBCD products rich in γ-HBCD, whereas internal exposure may be dominated by other isomers. Law and co-workers (2004) reported evidence for the bio-isomerisation of HBCD diastereoisomers in fish, which, in accordance to PBDEs (See higher), explains why the isomer composition in biota significantly differs from the composition of the technical product; the γ-isomer is dominant in technical HBCD, while it is only a minor contributor to the total HBCD in biota, in which α-HBCD is (mostly) predominant (***Paper XXVI***, not included in this thesis). Apart from bio-isomerisation, it has been shown that liver microsomes from marine mammals are able to form HO-metabolites of HBCD (Zegers et al., 2005; Brandsma et al., 2006). Hereby, β- and γ-HBCD are metabolised more rapidly than α-HBCD (Zegers et al., 2005). The toxicological relevance of these transformations needs to be resolved.

Effects seen after exposure experiments are not conclusive. Confounding factors, such as species-specific metabolism, transformation and isomerisation, lead to an exposure to compounds that are not present in the technical products that leave the production plant. This is further complicated by species-specific uptake, elimination and transformation.

1.6 Legislation and policy

At the start of this PhD research, no legislation existed regarding BFRs, except for PBBs. Nevertheless the lack of legislation regarding PBDEs, the Commission for the Protection of the Marine Environment of the North-East Atlantic (OSPAR) has included PBDEs on the list of chemicals for priority action to protect the marine environment in 2001 (OSPAR, 2001). Due to the increasing number of reports about the environmental presence of BFRs and their proven persistence and toxicity, regulators worldwide decided it was time to take action. In early 2003, the EU adopted a directive, which bans the marketing and use of Penta- and OctaBDE in all consumer products beginning August 15, 2004 (Directive 2003/11/EC). In addition, a separate Europe-wide ban under the Restriction on Hazardous Substances (RoHS) (which has a specific focus on electronics), will eliminate all PBDEs in electronics beginning 1st July 2006 (Directive 2002/95/EC). This European ban allows for an exemption for DecaBDE. The EU risk assessment report for DecaBDE stated however that the chemical

has a widespread occurrence in top-predators (birds), that it is potentially neurotoxic and that the formation of lower brominated diphenyl ethers is possible (Hansen et al., 2002). Furthermore, the conclusions of the risk assessment report stated that "*more data is needed*" and that "*there is a need to monitor the presence of this compound in the environment*" (Hansen et al., 2002). Nevertheless, DecaBDE successfully passed the EU risk assessment study and at this time there are no legislative measures taken to restrict or ban the production and/or use of DecaBDE (Hansen et al., 2002).
For these reasons, throughout this thesis, special emphasis was put on BDE 209, which is one of the most controversial FRs. Furthermore, the flame retardant industry still continues to claim that this product is not bioaccumulative and thus harmless.

In the US, California was the first state to ban the manufacture and commercial distribution of products containing Penta- or OctaBDE by 2008 (Renner, 2003). Although other US states have also introduced bills to control PBDE production and use, they are currently not regulated by the US government. The US Environmental Protection Agency (EPA) has been working on a voluntary phase out plan and the EPA has included PBDEs in the High Production Volume Evaluation and Testing Programmes. Nowadays, the US EPA addresses FRs under the Toxic Substance Control Act (TSCA). This requires notification ninety days prior to US manufacture or import, for any use, of the commercial products PentaBDE and OctaBDE after January 1, 2005. This action is a follow-up to the voluntarily phase-out of these chemicals by Great Lakes Chemical Corp., the only US manufacturer of PentaBDE and OctaBDE. Manufacture of both chemicals was ceased by the end of 2004.

Until now, there is no existing legislation in the Asia-Pacific region restricting the use of BFRs. However, the industry in Japan has voluntarily replaced PBDEs with other FRs (Watanabe and Sakai, 2003). Also in Japan, DecaBDE is mentioned in the Pollutant Release and Transfer Register law (PRTR law). This means that yearly reports have to be produced on volumes imported, volumes used and quantities released in the environment.
In June 2005 the Australian Ministry for Health and Ageing declared DecaBDE "Priority Existing Chemicals (PECs)". Being classified as "PEC", this BFR will be subject to an assessment of its potential effects to human health and the environment in the near future. The Australian authorities are currently compiling information on quantities and use of these substances from importers and producers, as well as general scientific information that is already available (BSEF, 2006).

Strategies and legal frameworks with which to assess the safety of HBCD in the environment vary among countries. The EU risk assessment for HBCD began in 1997, with Sweden as the lead country, and a draft risk assessment report is currently under discussion. In its current draft version, the EU risk assessment of HBCD states that there are needs for further

information and/or testing as well a need to limit the risks to environmental and human health (National Chemicals Inspectorate, 2005). Nevertheless the current lack of any restrictive legislation, the OSPAR Commission has included HBCD on the list of chemicals for priority action to protect the marine environment (OSPAR, 2001).
The US EPA is currently developing a rule that would require notification and review of several FRs, including HBCD (See *6.2.* ***Paper XII***). This is the only formal mention of HBCD in any regulatory context in the United States. In Canada, HBCD was identified for assessment over the next few years. In Japan, manufactured and imported amounts of HBCD must be reported to the government. Further, the presence of this chemical in the Japanese environment needs to be monitored. In Australia, the National Industrial Chemicals Notification and Assessment Scheme is currently updating information on use, import and production of BFRs, including HBCD, to determine the need for a full risk assessment and further regulatory activities.
To better protect its environment and citizens against the continuously increasing presence of (synthetic) chemicals, the EU has elaborated a new policy concerning newly introduced chemicals, which is known as REACH (Registration, Evaluation, Authorisation of Chemicals). By the end of 2006 or early 2007, the REACH-regulation should have taken all regulatory hurdles. REACH will consist in 3 major parts: Firstly, the producers and importers of chemicals, in quantities above 1 ton, are obliged to provide all required data on the chemicals to a Central Agency. These data is made up of a technical and security part. Secondly, the member state authorities perform an evaluation of the submitted file (available alternatives, animal testing, etc.) and an evaluation of the chemical in question, in case of any doubt concerning environmental, animal or human safety. For the most dangerous chemicals, it can be possible to work in a safe way if the appropriate measures are taken (e.g. in closed systems). If this is not possible, their use can be restricted. Finally, authorisation by the Central Agency is required for the chemicals of major concern, i.e. CMRs (carcinogens, mutagens or toxic regarding reproduction) of 1st and 2nd category, PBTs (chemicals complying to the criteria for persistency, bioaccumulation potential and toxicity), vPvBs (very persistent and very bioaccumulative) and chemicals that are known to have severe and irreversible effects on humans and the environment, such as endocrine disruptors. The producer of the chemical in question can only be granted approval if he can provide proof that the risk can adequately be controlled or the socio-economic benefits surpass the risks to a sufficiently great extent. The current REACH-draft version only necessitates the testing of the potentially most dangerous compounds. Only the future can provide an answer whether or not this REACH-approach can prevent the massive introduction of new toxic chemicals into the environment.

Until recently, no up-to-date information regarding BFRs was available for the Belgian environment. National policy was therefore not directed towards PBDEs or other BFRs. Until

now, data obtained from this PhD research were incorporated in the 2 latest issues of the yearly MIRA-T report of the Flemish EPA (VMM – Vlaamse Milieu Maatschappij) (***Papers O-XXXII – O-XXXVI***, not included in this thesis). These yearly reports function as a policy making tool for the Flemish government.

Bans and restrictions on the use of BFRs are a step towards reduction of exposure. However, the bans, (voluntary) phase-outs and legislative restrictions, which are currently in action or under discussion, will not imply that all BFR containing products are removed from the society. A vast amount of BFRs is incorporated into products that are still in use today and in the future. Unfortunately, exposure does not stop at the date of the scheduled phase-out.

1.7 Bromine Science and Environmental Forum (BSEF)

"*What exactly is the Bromine Science and Environmental Forum (BSEF)?*" The name suggests an academic body, an environmentalist organisation or a coalition bringing together scientists. In reality, BSEF has four members: the world's major producers of bromine flame retardants, based in Israel (Dead Sea Bromine Group, recently renamed ICL Industrial Products), in the US (the Great Lakes Chemical Corporation and the Albemarle Corporation), and in Japan (the Tosoh Corporation). When the commercial interests of these four companies were threatened by plans to ban some of their products from the EU market, they turned to public relations giant Burson-Marsteller for assistance. Well-experienced in running front groups, Burson-Marsteller helped to set up the Bromine Science and Environmental Forum and the PR firm has ever since run BSEF's EU operations from its office at Av. de Cortenbergh 118 in Brussels. Burson-Marsteller's Brussels office also runs several other bromine industry outfits fighting EU bans, such as the Alliance for Consumer Fire Safety in Europe (ACFSE) and the European Brominated Flame Retardant Industry Panel (EBFRIP). BSEF has commissioned more than 100 scientific studies that provided evidence that their products were not related to any adverse effects (Corporate Europe, 2006). It is surprising that hardly any study yielded results that did not fit the commercial interests of the producers of these chemicals. In reality, BSEF's main task consists in representing the interests of their members, i.e. the BFR-industry. BSEF has consistently fought against bans and restrictive regulation on BFRs, while only accepting the need for phase-out in cases when the lobbying battle was lost. Now that the battle is lost for two of their major products (Penta- and OctaBDE), they have directed all their efforts towards the remaining marketed products, i.e. DecaBDE and HBCD. To protect the market, they have an arsenal of techniques going from influencing policy makers to cast doubt on published independent scientific papers (See *6.3.* ***Paper XIII***).

1.8 Aim of this thesis

At present, legislation regarding BFRs is scarce or even lacking completely in some part of the world (See *1.6. Legislation and policy*). During the past years, scientific publications highlighting the risks associated with the use of these chemicals have increased media coverage, parliamentary discussions and general public interest. In 2001, at the start of this PhD thesis, scientific data on environmental BFR distribution was limited due to the complex nature of the analytical processes involved and the small number of laboratories that were able to come forward with reliable results. At that time, no data on BFRs from Belgium and hardly any data from Europe were available. Data on environmental occurrence, combined with toxicological studies, are of capital importance as a starting point for policy makers.

The first aim of this thesis was to establish analytical methods and procedures to obtain reliable data on environmental BFR levels and distribution. The newly optimised methods should then be validated through participation in international inter-laboratory exercises (See *2.5. Quality assurance and quality control*).

The second aim was to apply the newly developed methodology to samples from various environmental compartments, both abiotic and biotic. The obtained data would usefully add to the growing European and global database of environmental distribution of BFRs. Finally, human exposure to BFRs would be assessed. This can be done by analysis of human samples as well of human food items, since food is regarded as a primary route of exposure to these chemicals.

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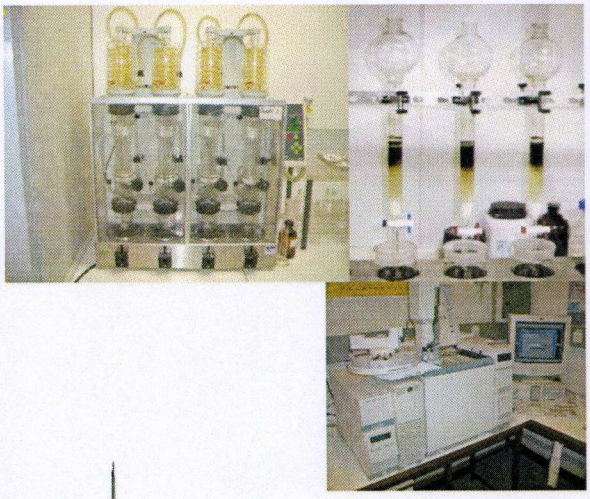
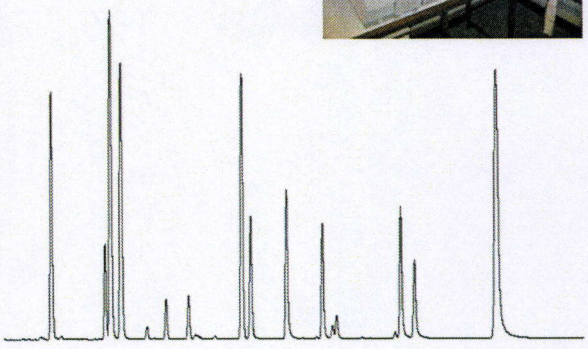
2

Analytical methodology





2.1 Introduction

Analytical procedures for the determination of organic pollutants consist generally of several equally important steps: sample collection, extraction, clean-up, chromatographic separation, identification and quantification. Each step of the procedure is directly influenced by the chemical characteristics of the chemical to be determined and by the sample matrix that is analysed. The BFRs under study in the present thesis are highly lipophilic compounds with a high potential for accumulation in the organic fraction of the environment, including fatty tissues of animals and humans. To investigate the presence, distribution and fate of BFRs in the (Belgian) environment, possible target matrices are the following: soils, sediments, and different (lipid rich) tissues of various species of animals such as fish, birds, mammals and humans. To compensate for the lipid content of certain organs/species, results of lipophilic compounds such BFRs are often expressed on a lipid weight basis. Advantages and disadvantages of this approach will be addressed later on in ***Papers IV, V, and X*** (See *3.4, 4.2, 5.3*).

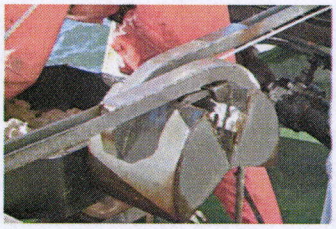
After a representative sample selection and collection, the samples are homogenised. Then the lipids and lipophilic compounds of interest need to be separated from other interfering matrix components using the appropriate extraction technique. This step also works as a first pre-concentration of the analytes of interest. Because gas chromatography (GC) is commonly used for quantification of these analytes, lipids need to be eliminated; lipids are incompatible with the GC sample introduction procedure and they significantly reduce the lifetime of the capillary GC column used for the PBDE analysis. This is done in the so-called clean-up step. During the chromatographic procedure, the different compounds are separated and can be individually identified and quantified. ***Paper I*** summarises techniques, methods and procedures that are used for determination of PBDEs. In this chapter, more emphasis will be put on the specific techniques used in our studies that make part of this thesis.

2.2 Sampling

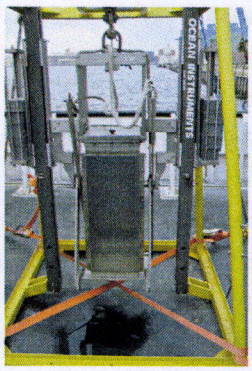
Sampling is the first step in the analytical procedure and of capital importance for the outcome of the study. Sampling location, sample type and amount are crucial parameters that can influence the outcome of the study to a great extent.

In ***Papers II and III*** (See *3.2 and 3.3*), sediments were sampled with the two most routinely used methods, i.e. box corer and a Van Veen sediment grabber (Figure 2.1). Samples taken with a box corer can be fractionated regarding depth and depth profiles can be established. Depth profiling is useful to assess time-trends of pollution. Sediments settle down at a fairly constant rate, which enables estimation of the age of the sediment depending on the sampling depth. The use of a box corer is more complicated than a Van Veen grabber. The latter instrument is a simple device that is easily manipulated and is therefore the most

widely used. Depending on its size, such grabber has a sampling depth of at least 15-20 cm; up to a depth of 20 cm, the sediment is mixed and homogenised. Therefore, depth profiling is not possible. For legacy pollutants, such as PCBs and DDTs, Van Veen sampling is an often-used procedure. For PBDEs, which were only introduced quite recently into the environment, this is another issue. The results of ***Paper II*** (See *3.2*) indicate that PBDEs are mostly found in the top layer (1-2 cm) of the sediment. If the widely used Van Veen grabber is applied (See *3.3.* ***Paper III***), this results in a vast dilution of the top layer and further an underestimation of the exposure of aquatic biota. Furthermore, since PBDE levels are rather low in non-exposed marine locations, this can result in a high number on non-detects. From these two studies (***Papers II and III***), it could be concluded that even an established and widely used sampling procedure is not always applicable and that results can be biased to a great extent based on the sampling technique.



(a)



(b)

Figure 2.1. The most commonly used sediment sampling devices: (a) Van Veen grabber and (b) box corer

The physico-chemical properties of PBDEs make that these compounds can be found in a wide variety of biological samples (de Wit 2002; Law et al., 2003). Due to their mobility through the food web, sample selection is crucial when studying samples of animal origin. Samples should be selected based on their nature (lipid-rich organs such as liver, muscle, adipose tissue) and the animal's position in the food web. Therefore, top-predators are preferred for their ability to gather large quantities of pollutants throughout their lifetime. On the other hand, levels in prey species are generally relatively low, which is often a challenge to quantify, but which can provide insight into the transfer of PBDEs through the food web (See *4.5.* ***Paper VIII***).

Sample selection can also be based on the importance in relation to humans, e.g. fish species that are suited for human consumption (See *3.4.* ***Paper IV***) or processed foods that are major constituents of the human diet (See *5.4.* ***Paper XI***). Food is considered a major route of transfer of BFRs and has already been investigated in other parts of the world (Bocio et al., 2003; Ohta et al., 2002; Huwe et al., 2004; Domingo 2004; Harrad et al., 2004; Schecter et

al., 2004). Most research on PBDE pollution in food for human consumption has been performed in the US (Schecter et al., 2004; Hites et al., 2004, Huwe et al., 2004).
Concerning the assessment of human exposure, analysis of human samples is preferred. Such samples are rather difficult to collect because of practical and ethical reasons. Tissues are only available from deceased individuals, but serum is more easily accessible. PBDE levels in serum are very low due to its low lipid content. Very sensitive methods are therefore needed to analyse PBDEs in serum (See *5.2.* ***Paper IX***).
In general, it can be concluded that sampling is a critical but a rather unpredictable step in the entire procedure. Because it is mostly done by volunteers and external collaborators, the sampling protocol needs to be comprehensive. Biological samples of wildlife are often not available as expected and the sampling campaign often spans several years (See *4.2.* ***Paper V****, 4.3.* ***Paper VI****, 4.4.* ***Paper VII*** *and 4.5.* ***Paper VIII***). Studies are therefore often adapted to the sample availability.

2.3 Sample preparation

2.3.1 Extraction

Since organs and tissues cannot be analysed "*as such*", the analytes of interest need to be separated from the matrix. This first separation step is done by means of an extraction with organic solvents. The resulting extract does not only contain the analytes of interest, but also other lipophilic compounds and extractable lipids. The latter have to be eliminated (See *2.3.3. Clean-up*) before instrumental analysis can take place (See *2.4. Instrumental analysis*). Depending on the sample matrix, an appropriate extraction technique needs to be applied. Internal standards (IS) are added prior to extraction to enable quality control and quantification (See *2.5. Quality assurance and quality control*). The different extraction techniques used are discussed below and a summarising workflow is presented in Figure 2.2.

Before extraction, solid samples were dried. In ***Papers II and III*** (See *3.2 and 3.3*), drying of the sediments was achieved by evaporation of water, while organs and tissues (See *3.4.* ***Paper IV****, 4.2.* ***Paper V****, 4.4.* ***Paper VII****, 4.5.* ***Paper VIII****, 6.4.* ***Paper XIV***), and food items (See *5.4.* ***Paper XI***) were easily dried chemically by grinding with anhydrous sodium sulphate, which binds water irreversibly. Drying is primarily done for convenience and method optimisation. Dry samples are more easily manipulated and facilitate the use of a Soxhlet extraction method. The absence of water in the samples makes the sample matrix more accessible to organic solvents. Dried sediments were also sieved (180 µm stainless steel sieve) before extraction. This was done in order to remove debris and all solid particles of organic origin and to only retain the sediment particles.

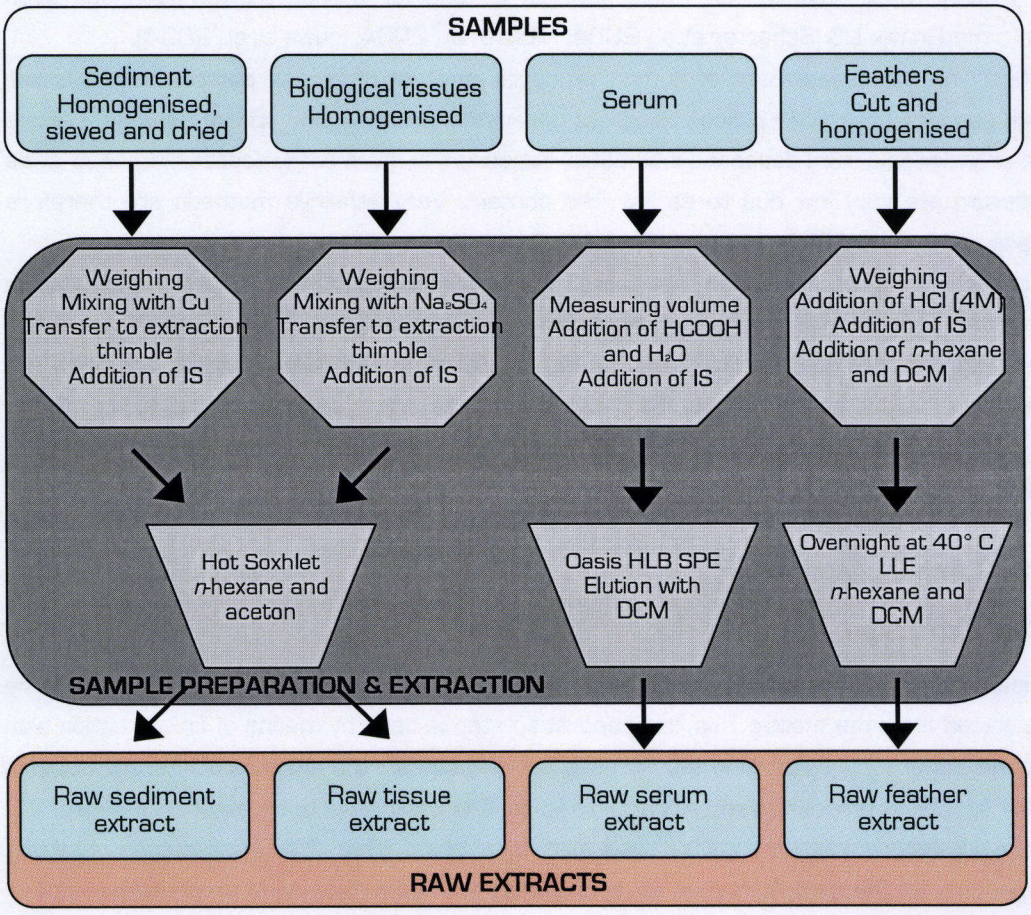


Figure 2.2. Workflow of the extraction procedures.



Figure 2.3. The automated solvent extraction system

Subsequently, the dried samples were Soxhlet-extracted in a Büchi B-811 automated extraction device (Büchi, Switzerland; Figure 2.3). This apparatus is able to heat the upper part of the solvent in which the sample is submerged (hot Soxhlet) and therefore allows for a reduction of the extraction time from 24h to 2h. The solvent used for the extraction of BFRs was a mixture of *n*-hexane:acetone (3:1; *v/v*). This solvent mixture was previously found to give the highest extraction recovery, regardless of the matrix used (de Boer et al., 2001; Manirakiza et al., 2001). An overview of other possible extraction methods, such as supercritical fluid extraction (SFE), accelerated solvent extraction (ASE), microwave-assisted solvent extraction (MASE), pressurised hot water extraction (PHWE) and traditional methods for solid-liquid extraction, is given in ***Paper I***. All these above mentioned techniques have their

advantages and disadvantages, but we preferred using the Büchi system for the extraction of solid material because of the good results we obtained with this apparatus.
Traditionally, human blood analyses of lipophilic compounds have been performed by liquid-liquid extraction (LLE) of serum or plasma with organic solvents (Sjödin et al., 1999; Sjödin et al., 2001; Hovander et al., 2002). After protein denaturation, both the lipids and lipophilic contaminants are partitioned to the organic layer. Extraction with organic solvents is a time-consuming process, which often requires relatively large amounts of solvents and analyst skills. A good alternative to LLE of serum is solid phase extraction (SPE). SPE has been previously suggested for the analysis of BFRs in human plasma or serum (Thomsen et al., 2001a). More recently, semi-automatic high-throughput methods for extraction and clean-up of human serum have been developed (Sjödin et al., 2004). In those methods, extraction and clean-up was performed on an automated modular SPE-system and a two-layer disposable silica and silica/sulphuric acid cartridge (See *2.3.3. Clean-up*).

Paper IX (See *5.2*) describes a newly optimised SPE-method for the PBDE determination in serum. This method allowed downscaling and substantially reduced solvent consumption. In that method, serum (2 – 4 ml) was mixed with 2 ml formic acid (to remove proteins) and 3 ml water (to reduce viscosity) and was then sonicated. The sample was consecutively loaded on a washed and conditioned Oasis HLB SPE cartridge. After washing of the cartridge, the analytes were eluted with 3 x 3 ml dichloromethane (DCM). This method was successfully used for the analysis of serum of birds of prey (See *4.2.* ***Paper V***).

Other matrices that are of interest regarding chemical analysis are hair and bird feathers. Levels in tissues and organs can give more information about both exposure and possible effects in the organism itself. Tissues and organs are only available through so-called *invasive sampling* and therefore the sampling is restricted primarily to deceased or killed individuals. On the other hand, hair and feathers can be easily collected without invoking any harm to the sampled animal, while these matrices are able to provide insight in the pollutant (i.e. PBDEs) exposure. In ***Paper VI*** (See *4.3*), we successfully applied a modified extraction method to feathers and we were able to determine their PBDE content. In that method, feathers were cut into small pieces and homogenised. The samples were consecutively incubated overnight at 40° C with hydrochloric acid (4M) and a mixture of *n*-hexane and dichloromethane (4:1, *v/v*). The aqueous layer was extracted twice with the solvent mixture.

2.3.2 Lipid determination

Due to the lipophilic nature of PBDEs, results are mostly lipid-normalised. This necessitates the determination of the lipid content of the analysed biological samples, such as organs and serum. For those samples that allowed Soxhlet extraction, the lipid determination was performed on an aliquot of the extract (≈1/5th). The lipid constitution of serum samples was

enzymatically determined by a third party clinical laboratory. Based on those results, the total lipid content of serum was estimated using a formula that was available in literature (Philips et al., 1989). In ***Paper X*** (See *5.3*), the commonly applied formula of Philips and co-workers (1989) was put into question and was revised based on a large serum lipids data set. Errors due to calculations of lipid content in serum can possibly lead to a underestimation by 20 % of the lipid-normalised serum levels of lipophilic pollutants.

2.3.3 Clean-up

Clean-up of crude extracts can be considered as the removal of interfering substances (e.g. lipids and sulphur), necessary to achieve good GC/MS performance. The clean-up techniques that were used were both dependent on the matrix as on the pollutants that were present and/or targeted in the samples. An overview of all techniques that are commonly used in BFR-analysis is given in ***Paper I*** at the end of this chapter.
Clean-up used in this work was based on silica gel in various forms: acidified, base and activated. Acidified silica gel was prepared by drop-by-drop addition of 25 ml of sulphuric acid to 50 g of silica, which was pre-extracted with *n*-hexane, under constant stirring. This resulted in a free-flowing acidified silica gel, containing 48 % (*w/w*) sulphuric acid. To prepare base silica gel, a solution of 16.5 g KOH in 80 ml MeOH was added to 50 g of pre-extracted silica gel. The mixture was regularly shaken until all MeOH was evaporated. This resulted in a free-flowing base silica gel, containing 25 % (*w/w*) KOH. Activated silica was prepared by heating pre-extracted silica gel at 200° C for 12h.

For most extracts, a clean-up, consisting of acidified silica, was adequate to remove all interfering lipids. The crude extract was passed over a 10-ml cartridge filled with 8 g of acidified silica gel (48%, *w/w*). On contact with the strong acid present in the silica gel, lipids were degraded, hydrolysed and adsorbed to the silica. The analytes of interest are not affected due to their strong resistance to chemical breakdown. The analytes of interest were consecutively eluted from the cartridge with *n*-hexane and DCM. This set-up was capable of removing approximately 200 mg lipids.

Some extracts required additional treatment (i.e. serum, liver, sediments). Serum often contains rather large quantities of cholesterol that is not adequately removed by acidified silica gel (See *5.2.* ***Paper IX***). Therefore, an extra layer of activated silica gel, which strongly retains cholesterol, was added to the clean-up cartridge. Furthermore, extractions done by means of the Oasis HLB SPE cartridges often lead to water retention. Adding a layer of anhydrous Na_2SO_4 to the clean-up cartridge can retain the water-residue (See *5.2.* ***Paper IX***). Sediments do not only require the removal of lipophilic co-extracted substances, but also of elemental sulphur, which is often present in high amounts in anoxic sediments (See *3.2.* ***Paper II*** *and 3.3.* ***Paper III***). Sulphur interferes with the GC/MS determination but can be

removed by adding copper powder to the extraction thimble and/or the clean-up cartridge. Not only biological or natural substances can interfere with the analyte determination, but also the presence of specific pollutants, such as HBCD (See *6. HBCD*). HBCD is thermally unstable and may degrade upon injection in a GC-system. This results in several specific break-down products that can co-elute with PBDEs of interest (See *2.4.1.1. Gas chromatography*). This necessitates the removal of HBCD prior to injection into the GC/MS system. Some sediment-samples analysed in ***Papers II and III*** (See *3.2 and 3.3*) contained high amounts of HBCD, which were removed by addition of an additional layer of base silica to the clean-up cartridge. Also a limited number of biological samples sometimes requires additional base silica clean-up. A workflow of the various clean-up procedures is presented in Figure 2.4.

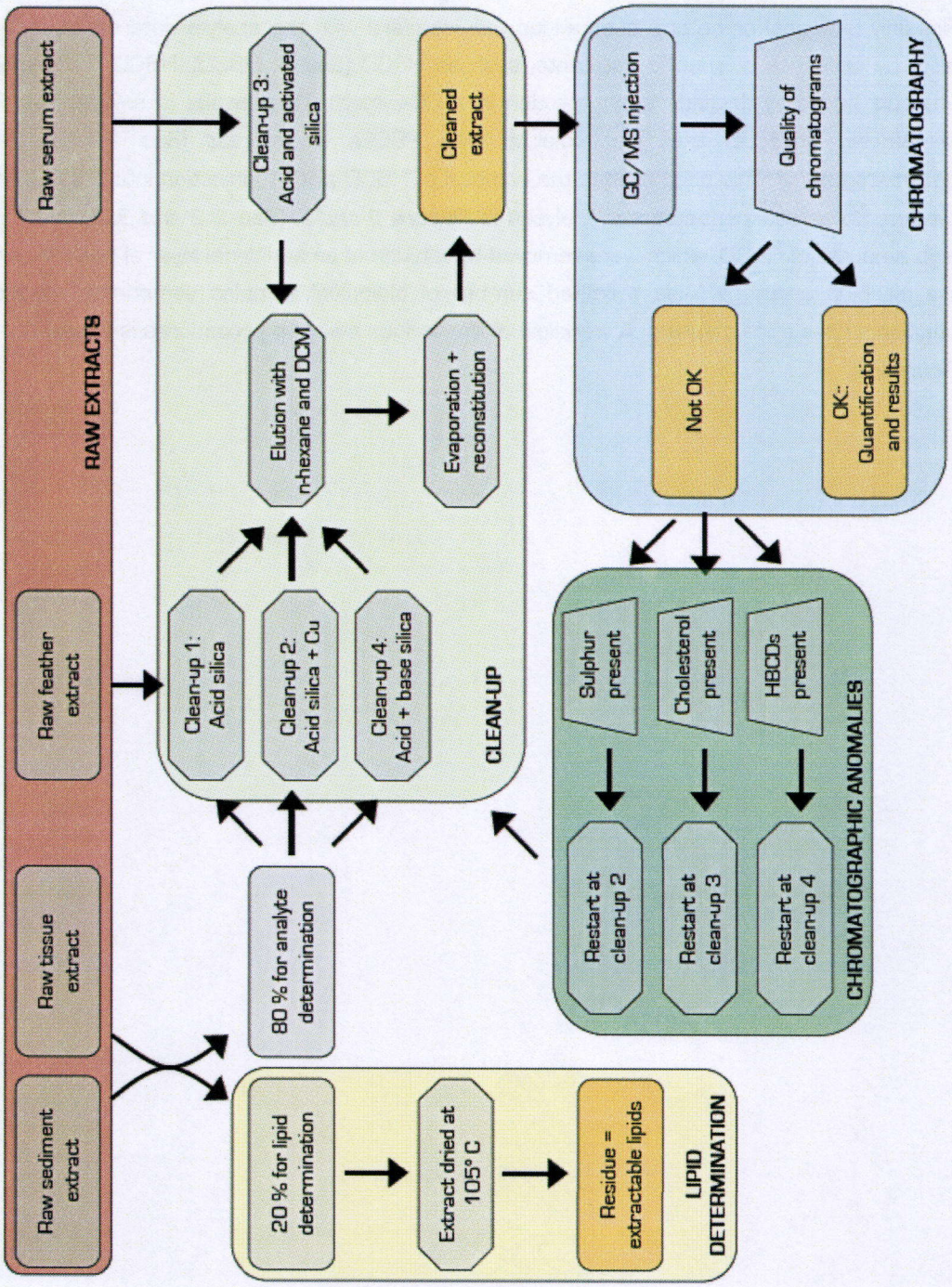


Figure 2.4. Flow chart of the various clean-up procedures.

2.4 Instrumental analysis

2.4.1 GC/MS

For analysis of most BFRs in environmental samples, GC-techniques are preferred to obtain adequate separation of the different analytes. Due to the complex composition of environmental samples, GC coupled to a mass spectrometry detector (MSD) is preferred (See *2.4.1.2. Mass spectrometry*). Both electron ionisation (EI) and electron capture negative ionisation (ECNI) can be used. These two techniques do not have the same sensitivity for PBDEs. Therefore, the MS was operated in ECNI mode in order to obtain the highest possible sensitivity.

2.4.1.1 Gas chromatography

GC and capillary columns enable the separation of different volatile and lipophilic chemical compounds in complex sample extracts. Liquid chromatography is preferred for certain compounds, such as HBCD, which is thermolabile and cannot be resolved on a GC-column, (See *2.4.2. LC/MS, 6.2.* ***Paper XII****, 6.3.* ***Paper XIII*** *and 6.4.* ***Paper XIV***). In this work, all analyses were performed by GC-techniques, except for the determination of HBCD in ***Paper XIV*** (See *6.4*).

Several injection techniques are available for optimal GC separation of BFRs, such as the split/splitless injector, programmable temperature vaporiser (PTV) injector, septum equipped programmable injector (SPI) or on-column injectors, where split/splitless and on-column type injection techniques are most commonly used (***Paper I***). Split/splitless injectors are considered robust, and still perform well if injected extracts are dirty, but temperature-induced degradation of analytes may occur if the injector temperature is too high. The technique is also known to discriminate analytes with high molecular weight. The first drawback can be compensated by lowering the injector temperature, but this will discriminate even more high molecular weight analytes. This can be overcome by application of a pressure pulse (Björklund et al., 2004). The issue of discrimination of high molecular weight analytes is of special importance when analysing BFRs with high bromine content, such as nona-BDEs and BDE 209. However, the PTV injector is a versatile tool since most of its injection parameters can be modified to optimise the transfer of BFRs from the liner to the column. All analyses in this work were done using a PTV injector, which was optimised for low to high brominated BFRs (See *2.5. Quality assurance and quality control*).

PBDEs with a low to medium degree of bromination (tri- to hepta-BDEs) usually are analysed on capillary columns, such as the DB-1 or DB-5 (J&W), CP-sil 8 (Chrompack) or AT-5 (Alltech). In order to achieve enough resolution for all congeners of interest, columns of 30 to 50 m with diameters < 0.25 mm are recommended (***Paper I***), but not necessary. Short, narrow-bore columns with thin film (such as a 10 m × 0.10 mm × 0.10 μm HT-8 (SGE) capillary column) can provide adequate resolution, combined with extensive reduction of the

analysis time (Figure 2.5; See *3.4.* ***Paper IV***). Due to the drawbacks of short, narrow-bore capillary columns (i.e. low column capacity, sensitivity to residual lipids), most of the lower brominated BDE-analyses in this work were done by means of a 25 m x 0.22 mm x 0.25 μm HT-8 capillary column (SGE).

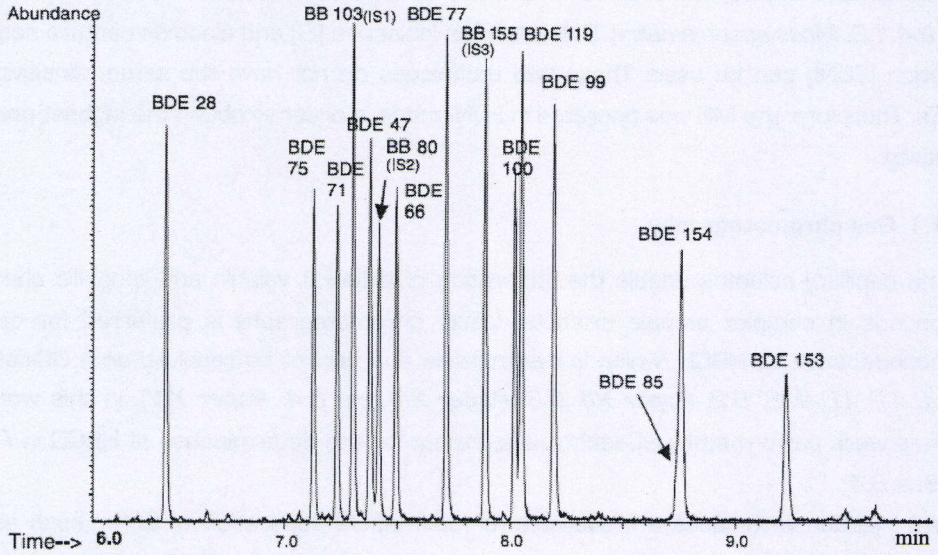


Figure 2.5. ECNI-MS chromatogram (*m/z* 79/81) of a standard mixture of PBDEs on a 10 m x 0.10 mm x 0.10 μm HT-8 (SGE) capillary column (***Paper I***).

For the analysis of PBDEs with high degree of bromination, such as BDE 209, short columns (preferable 10-15 m) with thin film thickness (0.1-0.2 μm) are recommended in order to reduce the residence time in the column (Björklund et al., 2004; de Boer et al., 2001). Especially BDE 209 should receive special attention because of its sensitivity to heat and the higher susceptibility for degradation in the GC-system. For such reasons, BDE 209 does not elute from a HT-8 column, nor do any degradation products. The analysis of BDE 209 seems to necessitate specific column stationary phases, as was also shown by Björklund and co-workers (2004), who studied the influence of column stationary phase on the response of BDE 209. Even columns with comparable stationary phases from different manufacturers can have varying results concerning BDE 209 elution (Björklund et al., 2004).

All BDE 209 analyses that were performed in our laboratory were obtained by means of a 9 to 15 m x 0.18 mm x 0.10 μm AT-5 capillary column (Alltech). The GC method used was optimised in order to reduce the residence time of this compound in the column (***Paper I***). This approach greatly reduces resolution, but separation is not very critical for BDE 209, as most of the other PBDEs will elute earlier in the chromatogram and will not interfere with the peak of BDE 209 (Allchin et al., 1999). Additionally, BDE 209 can be characterised by its distinct mass spectrum in comparison to other PBDEs. Whereas for lower brominated PBDEs only the bromine trace can be monitored (*m/z* 79 and 81), BDE 209 additionally has

other mass fragments that can be monitored (See *2.4.1.2. Mass spectrometry*). The AT-5 column provides excellent performance for the analysis of BDE 209 if appropriate quality control measures are taken. This column however does not resolve BDE 154 and BB 153, and BDE 153 and TBBP-A (***Paper I***). Samples containing BB 153 are therefore better not analysed on an AT-5 column. More co-elutions occur during GC-separation (***Paper I***), but they can be overcome by the use of MSD. Column selection is always a matter of compromises; the perfect column for BFR analysis doesn't exist yet.

As mentioned earlier, BDE 209 is a BFR that deserves special attention, both concerning regulation (See *1.6. Legislation and policy*) and chemical analysis. Apart from the crucial column selection, also the oven programming and injector condition can compromise the analysis of BDE 209 or even render it impossible. Since BDE 209 is a thermolabile product, the GC temperature programme is a determining factor for quantification of BDE 209. Degradation does, to some extent, always occur in the GC-system. The degree of degradation can vary greatly depending on the system status, oven temperature and the residence time in the column (unpublished test results). Fast temperature programming leads to less degradation (See *3.4.* ***Paper IV***), but can hamper the analysis of complex samples because resolution is sacrificed (Table 2.1).

Table 2.1. Degradation of BDE 209 (expressed as percentage remaining) on a 15 m x 0.18 mm x 0.10 µm AT-5 column at different column loadings (pg) and oven ramping programmes.

Loading (pg/oc)	8	13	25	50	85	125	2500	5000
Slow*	77	75	84	91	93	91	99	99
Fast*	90	92	92	92	92	95	99	99

* Slow ramp: 10 °C/min; Fast ramp: 75°C/min

Additionally, it should be taken into account that the degradation, to a minor extent, also depends on the injected amount of BDE 209: degradation is less pronounced for higher column loadings (Table 2.1). The issue of degradation in the GC system can be overcome by using a carbon labelled IS (^{13}C-BDE 209), which is subject to the same degradation as the native compound. The amount of IS that is added to the sample should be well adapted to the expected BDE 209 levels, since degradation is dependent on the amount of BDE 209 injected.

In order to maintain an adequate analyte transfer to and through the column, all injector parts, the liner and column (head) need to be completely free from activated sites. Therefore, the liner needs to be replaced often and the column is shortened on a regular basis. A dirty liner and/or column head can lead to debromination (degradation) of BDE 209. Upon injection of BDE 209, other BDE compounds are formed in the inlet system, such as BDE 206, BDE 207 and BDE 208. In an optimised GC system, this degradation is only of minor importance and perfectly under control (Figure 2.6a). A contaminated injection system leads to significantly higher formation of debromination products (Figure 2.6b), which can interfere with the quantification of BDE 209. Because degradation of BDE 209 can be compensated

for with the proper use of IS, this degradation theoretically only interferes with the limit of quantification (LOQ).

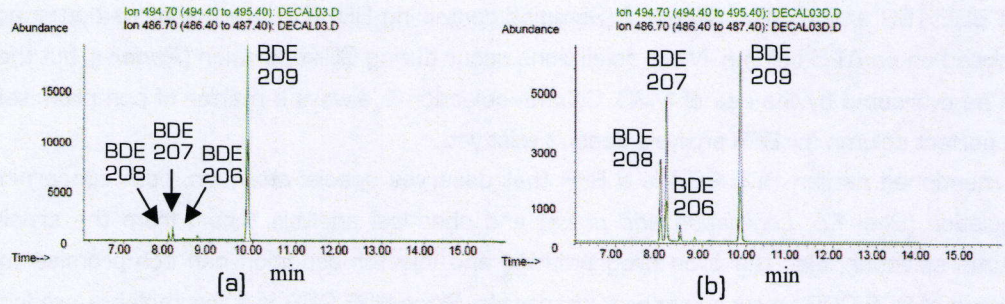


Figure 2.6. ECNI-MS ion chromatogram of a standard mixture of BDE 209 and on a 12 m × 0.18 mm × 0.10 μm AT-5 (Alltech) capillary column; (a) clean system with minor degradation, (b) degradation is substantial after injection of 100 liver extracts (= dirty liner).

Paper XII (See *6.2*) summarises the current state-of-the-art of the HBCD analysis, which is focussed on LC/MS(MS), the analysis technique of preference. Analysis of HBCD is possible on a GC-system, but the isomer specific analysis cannot be performed. Technically produced HBCD is an isomer mixture (mainly α, β, and γ) that is thermally labile at the temperature needed for elution of the GC-column. Temperatures exceeding 160 °C result in interconversion of the HBCD isomers, which results in a broad unresolved peak (***Paper I***). Therefore, separate determination of the three isomers by GC is currently not possible and only the total amount of HBCD can be determined. Furhtermore, it has been shown that pure HBCD undergoes decomposition by elimination of HBr at temperatures above 240°C (Larsen et al., 1986; Barotini et al., 2001). Furthermore, an activated liner and dirty column head may result in breakdown of HBCD, resulting in an additional unresolved peak area that may co-elute with BDE 47 (Figure 2.7), the major congener in most environmental samples. Seeing the high concentrations of HBCD that are often encountered in environmental samples, some extracts may require an additional clean-up step to remove HBCD, in order not to interfere with determination of PBDEs (See *2.3.3. Clean-up, 3.2.* ***Paper II*** *and 3.3.* ***Paper III***).

Optimisation of the GC-system can render this technique useful for the determination of total HBCD (the sum of all 3 major isomers, namely α, β, and γ), as apparently the response factors of the three diastereomers do not differ very much (QUASIMEME, 2002). Therefore, the thermal exposure of HBCD must be minimised during analysis. Cold on-column injection, short GC columns, thin film stationary phases, and high flow rates are several measures to minimize the risk of thermal degradation and to reduce the elution temperature of HBCD. Additionally, similarly as for determination of BDE 209, the GC-system needs special attention concerning the condition of the liner and column to eliminate break-down of HBCD and to limit broadening of the unresolved chromatographic HBCD-peak. Not surprisingly, partial

breakdown and even complete loss of HBCD have been reported in GC systems (Eljarrat et al., 2004).

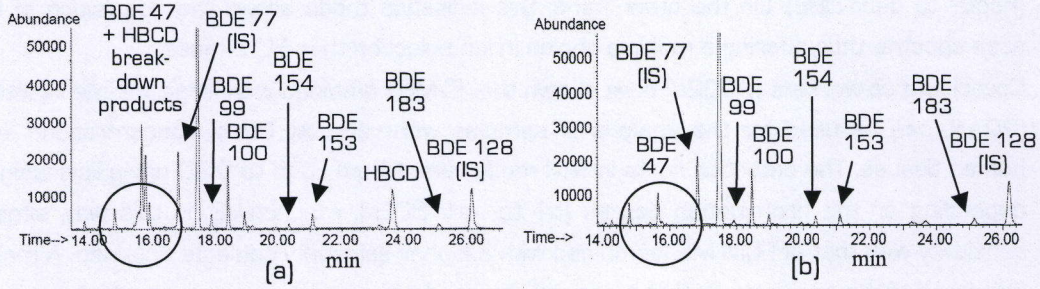


Figure 2.7. Co-elution of HBCD breakdown products with BDE 47 on a 25 m x 0.22 mm x 0.25 μm HT-8 capillary column; (a) sample with high amount of HBCD, (b) sample with no HBCD.

A GC-method was developed for HBCD determination and its usefulness was demonstrated by successful participation in an international inter-laboratory study (See *2.5.6. International inter-laboratory exercises*). A 10 m x 0.18 mm x 0.10 μm AT-5 capillary column was used for the separation of HBCD from the other BFRs. Apart from successful participation in the inter-laboratory exercise, this method has not been applied to any environmental samples yet.

2.4.1.2 Mass spectrometry

The most widely used GC-detectors for the BFR determination are mass spectrometers operated either in ECNI or in EI mode (de Boer et al., 2000). Recently, time-of-flight mass spectrometry (TOF-MS) has been applied for BFR analysis in both human serum and milk samples (Focant et al., 2004). Low-resolution MS (LRMS) is commonly used for routine analysis of PBDEs because the ease of use, and relatively low purchase and maintenance cost compared to high resolution MS (HRMS). The first two world-wide interlaboratory studies on BFRs (de Boer and Cofino, 2002) have shown that that laboratories using HRMS may be able to obtain somewhat lower detection limits, but a superior performance for HRMS could not be demonstrated. A similar conclusion was reached by Thomsen and co-workers (2002) who stated that GC/ECNI-LRMS and GC/EI-HRMS are equally well suited for the determination of PBDEs in biological samples, as well as in standard solutions, with respect to response, detection limits and repeatability at the pg-level. Therefore, all further discussion is focused on LRMS. Occasionally, electron capture detectors (ECD) have been used for specific applications, but these are not preferable. The different detection methods are discussed in detail in ***Paper I***.

In EI-MS, the major ions formed from PBDEs are the M^+ and the $[M-2Br]^+$, which can be used for their identification and quantification (Sellström, 1999). Major advantages of this technique are the selectivity and the possibility to use ^{13}C-labelled IS for a more precise quality control of the procedure. This is not possible for the ECNI-MS, since generally only the Br^- ions

(*m/z* = 79 and 81) can be monitored. EI-MS is not routinely used for PBDE analysis, because of its relatively low sensitivity, especially for the analysis of higher brominated BDE congeners (hepta- to deca-DBE). On the other hand, this ionisation mode allows the acquisition of full scan spectra, thus offering a multiple choice in ion selection than ECNI mode.

Covaci and co-workers (2002a) have shown that EI-MS combined with large volume injection (20 µl), can be used for the analysis of samples with very low PBDE concentrations, e.g. human tissues. The detection limits in SIM mode varied from 0.05 to 0.30 ng/g lipid weight, depending on the bromination degree (tri- to hexa-BDEs), respectively. In this way, similar sensitivity with that of ECNI-MS, combined with a higher selectivity could be obtained. A major drawback of this technique is that it can only be used with very clean extracts, which can only be obtained following a time-consuming sample pre-treatment.

Being a very sensitive technique, ECNI-MS is preferred for the determination of PBDEs and other bromine containing organic compounds. Bromine shows a typical ^{79}Br (50.5 %) and ^{81}Br (49.5 %) isotope distribution pattern (Sellström, 1999), which can be monitored by ECNI-MS. For this reason, the mass spectrum of PBDEs consists only of *m/z* = 79 and *m/z* = 81. This approach reduces selectivity, but has the advantage of high sensitivity, which is more than 10 times better compared to ECD (Pijnenburg et al., 1995).

Since BFRs generally have a high bromine content, ECNI-MS is very suitable for these compounds. However, the low selectivity of ECNI-MS does not allow for mass spectrometric resolution of co-eluting compounds. This can cause quantification problems when using certain GC-columns (See *2.4.1.1. Gas chromatography*).

Occasionally the selectivity of ECNI-MS can be enhanced when other mass fragments can be monitored, as for the fully brominated diphenyl ether congener BDE 209 (Lepom et al., 2002). This congener allows monitoring of *m/z* = 484.7 and 486.7, while for the mass labelled analogue (^{13}C-BDE 209), that can be used as IS, the major ions are *m/z* = 494.7 and 496.7. Björklund and co-workers (2003) have shown how to use this isotopic dilution in ECNI-MS analysis of BDE 209. In the present work, isotopic dilution was optimised and applied for all BDE 209 analyses. This approach can overcome or limit most pitfalls that are encountered in the analysis of BDE 209.

The spectra and data obtained using ECNI are strongly dependent on the instrumental conditions, i.e. type of moderating gas, gas pressure, the instrument used and the ion source temperature (Buser, 1986; Stemmler et al., 1988a; Stemmler and Hites, 1988b). Other critical parameters that influence reproducibility are the temperature and pressure in the ion source, the purity of the moderating gas, the type of instrument used and its tuning conditions (Stemmler and Hites, 1988b).

2.4.2 LC/MS

In contrast to GC, HBCD isomers can easily be separated using reversed-phase high performance liquid chromatography (HPLC; Figure 2.8a). HPLC, coupled to a MSD is a versatile tool for the isomer-specific determination of HBCD in environmental samples (See *6.4.* ***Paper XIV***). HPLC on chiral, permethylated β-cyclodextrin columns has been successfully employed to separate the enantiomers of the (+/-)α-, (+/-)β- and (+/-)γ-HBCD diastereomers (Heeb et al., 2005; See *6.4.* ***Paper XIV***). Using chiral LC/MS(MS), it is possible to fully separate the three pairs of enantiomers of HBCD in a single analysis, with limits of detection for the different isomers between 10 and 20 pg injected on column (Figure 2.8b; See *6.4.* ***Paper XIV***). Matrix components that co-elute with the analytes may lead to serious ion suppression of the primary ion $[M\text{-}H]^-$, resulting in strongly decreased sensitivity. This problem might be avoided by thorough sample clean-up to remove interfering components before HPLC analysis. Furthermore, it is essential to use isotopically labelled IS to compensate for potential variations in sensitivity during and between sample runs. Both ^{13}C-labelled and ^{2}H-labelled standards of α-, β- and γ-HBCD have recently become commercially available. In LC/MS, the use of other IS must be discouraged.

The detection of HBCD following HPLC-separation is slightly different than following GC-separation, since coupling LC with ECNI-MS is not possible. HPLC is generally coupled to electrospray ionisation (ESI) or atmospheric pressure chemical ionisation (APCI) mass spectrometry, which is a versatile tool for the isomer-specific determination of HBCD in environmental samples. However, the sensitivity of LC/MS is lower than that of GC/ECNI-MS, and it has meant that HBCD could only be detected in environmental samples with relatively high contamination levels. Recently, a sensitive and selective method using LC/ESI-MS(MS) and single reaction monitoring for the transition $[M\text{-}H]^-$ (m/z 640.6) → Br^- (m/z 79 and 81) has been described by Budakowski and Tomy (2003). Using a triple-quadrupole instrument, they obtained a limit of detection (LOD) of 4-6 pg γ-HBCD in a standard solution injected on-column. This method was further optimised and was applied to a selected sample set of various benthic species and fish from the Scheldt Estuary, which is presented in ***Paper XIV*** (See *6.4*). The optimisation of both the LC and MS conditions resulted in separation of the six diastereoisomers and a LOD of 0.5 pg for γ-HBCD in standard solutions and 5 – 10 pg for γ-HBCD in fish extracts, injected on-column.

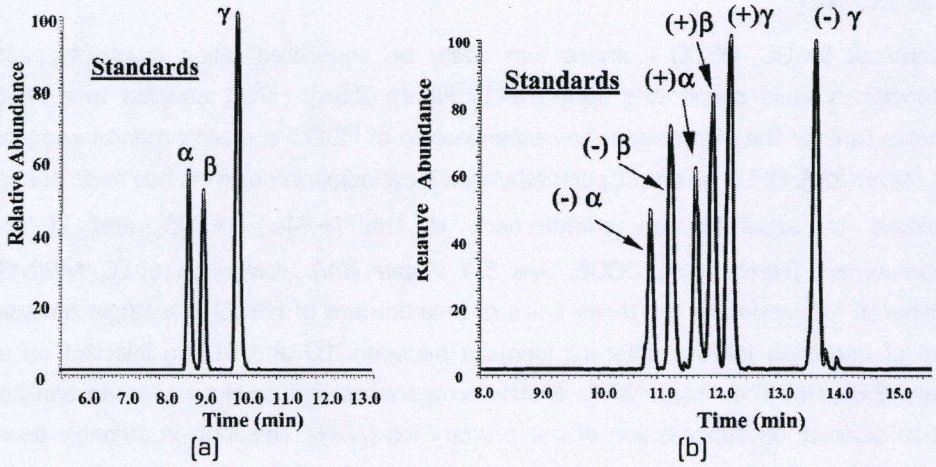


Figure 2.8. LC-separation of (a) the three major HBCD isomers on a Symmetry C_{18} (2.1 × 150 mm, 5μm) column and (b) the six major HBCD diastereomers on a chiral Nucleodex beta-PM (4.0 × 200 mm, 5μm) column.

2.5 Quality assurance and quality control

2.5.1 General

Quality assurance and quality control (QA/QC) of (chemical) analysis has become increasingly important. To be able to compare data generated independently by different laboratories throughout the world, quality assurance is essential. The need for strict QA/QC is also dictated by the severe consequences that might follow the inclusion of research results in official reports, such as e.g. the MIRA-T report, which is issued on a yearly basis by VMM (Vlaamse Milieu Maatschappij, Flemish EPA; ***Papers O-XXXII and XXXVI***, not included in this thesis). Finally, official reports might have important economic consequences for both manufacturers and down-stream users if the administration ever decides to retract products from the market, as has already happened for e.g. Penta- and OctaBDE technical mixtures. Extensive QA/QC is necessary for reliable analysis of chemicals and for BFRs in environmental samples, such as in this thesis.

Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Parameters that need attention and optimisation and/or characterisation are the following: selectivity, precision (repeatability, intermediate precision, and reproducibility), accuracy, linearity, range, limit of detection (LOD), limit of quantification (LOQ), and robustness. Selectivity is already discussed in *2.4.1.2. Mass spectrometry*. The other relevant parameters are discussed below.

2.5.2 Calibration curve and linearity

Of all compounds that were analysed in this thesis, authentic reference standards were used for identification and quantification during the GC/ECNI-MS analyses. Analysis of complex environmental samples often implies extensive sample pre-treatment. To overcome inherent drawbacks of such complex procedures, i.e. reduced recovery of analytes, the use of internal or surrogate standards, which are added at the start of the procedure, is recommended. Throughout this work, different IS have been used, depending on the commercial availability of "new" BDE standard solutions. All IS that were used, were compounds that were not present in the analysed samples. In ***Papers II, III, and IV*** (See *3.2, 3.3 and 3.4*), BB 103 and BB 155 were used for the quantification of brominated analytes. When more individual congeners of analytical purity became commercially available, the methods were further optimised and BDE 77 and BDE 128 were used as IS in ***Papers V, VI, VII, VIII, XI*** (See *4.2, 4.3, 4.4, 4.5 and 5.4*), while BDE 104 and BDE 140 were used in ***Paper IX*** (See *5.2*).

Standard curves were based on peak areas and amount ratios of standard and IS. All standard curves for quantification of PBDEs were generated with at least five points and all curves were linear. The linearity was assessed by means of an ANOVA lack-of-fit test during method validation. Correlation coefficient ($R^2 > 0.995$) was used as quality check of the calibration curves during analyses.

Environmental analyses are characterised by the large concentration range that can be encountered in one sample batch. This is demonstrated in ***Paper V*** (See *4.2*), where the PBDE concentrations in the bird population under study spanned more than four orders of magnitude. In such cases, two or more overlapping calibration curves were created.

2.5.3 Precision

Requirements regarding precision (repeatability, intermediate precision and reproducibility) are dependent on the type of analysis and the analyte concentration, i.e. pharmaceutical, biological, food and environmental. For pharmaceutical analyses, a precision of 0.1 % may be required, while for environmental analyses a precision up to 20 % can be common. For analyte concentrations between 1 and 10 ppb, the range in which most of the BFR-analyses are situated, a method relative standard deviation (RSD) between 20 and 30 % is considered acceptable (Horwitz et al., 1980). The methods used in this thesis have a method RSD < 15 % (intermediate precision) for all tested matrices and congeners, except BDEs 183 and 209 (Table 2.2). Apart from validation, this parameter was evaluated with every sample batch that was analysed. Replicate analyses of selected samples were performed throughout each batch, together with laboratory reference material, whenever it was available.

Table 2.2. Intermediate analytical precision (RSD) of the different congeners in various matrices.

	BDE 28	BDE 47	BDE 99	BDE 100	BDE 153	BDE 154	BDE 183	(^{13}C)BDE 209
Sediment	14	4	2	7	3	2	10	13
Fish	14	4	9	4	5	4	29	-
Liver	9	3	2	2	4	2	8	15
Serum	10	9	6	5	4	9	12	17

Since results of lipophilic pollutants, such as PBDEs, are commonly expressed on a lipid weight basis, lipid determination is a crucial step that can influence the final reported value to a great extent if not done properly. Intermediate precision of the gravimetrical lipid determination as used in this work was < 5 %.

2.5.4 Limit of quantification and background concentrations

The common approach for environmental analysis is to measure concentrations as low as technically possible. Therefore, the LOQ needs to be well defined. In general, much higher LOQs are encountered when environmental samples are analysed than when standard solutions are injected. Since BFRs are used in everyday products that surround us, it is not remarkable that they are present everywhere, such as in indoor air and dust in laboratories (Thomsen et al., 2001b; Wilford et al., 2005). If proper measures are taken, the laboratory background concentrations are low and reproducible. In that case, the procedural background determines the LOQ of the PBDE congeners.

For PBDE analysis by hot Soxhlet extraction, BDE 28 and BDE 183 were not present in procedural blanks, while levels for BDEs 47, 99, 100, 153 and 154 ranged from 0.01 to 0.20 ng per extraction, depending on the congener. BDE 209 background levels are significantly higher than those of the other congeners (around 0.50 ng per extraction), which hampers the analysis of this compound even more. SPE-extraction, as used for serum samples (See *4.2.* ***Papers V*** *and 5.2.* ***Paper IX***), is much less subjected to background contamination. This allows for a more than 10-fold reduction of the LOQ for serum samples compared to samples analysed by hot Soxhlet.

This implicates varying LOQs depending on the congener and sample intake. If no measurable amount of an analyte was present in the background, the LOQ was determined by the system performance, which was assessed empirically for each congener. Different amounts of the congeners were injected in 5-fold and the average injection precision (RSD) was determined (around 2 %). The precision of the injection was dependent on the amount injected and on the congener. In general, an instrumental LOQ of 5 pg on column could be attained for a cut-off RSD of 2 %. (Figure 2.9). This approach was seldom applicable, due to the presence of BFRs in the laboratory environment.

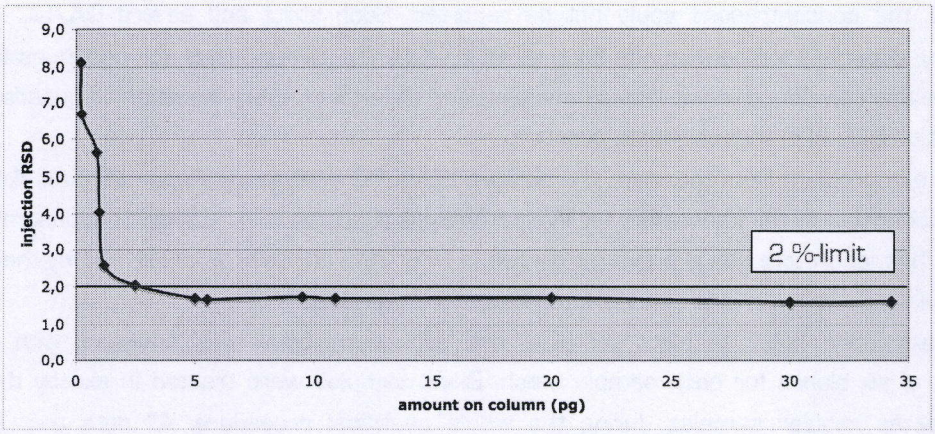


Figure 2.9. The precision of the automatic GC-injector depends on the injected amount of standard. Although the instrument's sensitivity allows for a 10-fold reduction, the LOQ of BDE 47 is set at 5 pg in order to keep the RSD on the injection below 2 %.

When the analytes were present in the procedural blanks, this was taken into account for determining the LOQ. Thomsen and co-workers (2003) suggested setting the LOQ to "*the average of the measurements in blanks added with three times the standard deviation (SD) of the blank measurements*". Different solutions for this problem have been suggested, such as setting the LOQ at twice the blank measurement (Päpke et al., 2004) or even at five-fold the blank measurement (Sellström, 1999).

In this work, a similar approach was followed. Since blanks are dependent on the laboratory conditions, they can be held relatively constant throughout the analysis of a sample batch (RSD < 30 %) if appropriate measures are taken. Therefore, the average blank was subtracted from the untreated GC/MS-data and a cut-off value of 3 times the SD of the blank was used. This approach ensured both that blank contribution to the reported results was minimised and that there was 99 % certainty that any reported concentration was not originating from a blank.

It is evident that reduction of blank interference is crucial for lowering the LOQ of the methods. Therefore, a lot of effort was invested both in maximal reduction of background levels and in keeping the blanks at a constant level. Each step of the procedure was investigated and optimised for blank reduction. Some specific measures that were taken are the following: rinsing of the Büchi apparatus between runs, pre-extracting the Soxhlet extraction thimbles, heating Na_2SO_4 at 600°C for 6 hours, pre-extracting silica, using glassware that is exclusively dedicated to BFR analysis, and specific procedures for cleaning the glassware.

For most congeners, the LOQs that were obtained were acceptable. Laboratory background levels of BDE 209, together with the low sample amount sometimes available, occasionally increased the LOQ to unacceptably high levels, such as in the small rodent samples in ***Paper VIII*** (See *4.5*). In that study, BDE 209 could be measured in several samples, but due to the

high LOQ, the concentrations could not be reported. Such strict and severe QA/QC is necessary, especially for compounds such as BDE 209. The BFR-industry still continues to claim that this compound is not bioaccumulating and thus takes every occasion to question any result on BDE 209 that scientists produce.

To tackle this problem from the start, the positive BDE 209 analyses in ***Paper VII*** (See *4.4*) were all confirmed by the Laboratory for Water Analysis (German EPA - Umweltbundesamt) in Berlin. This laboratory has a proven expertise in BDE 209 analysis, as confirmed by their successful participation in international inter-laboratory exercises.

In each extraction batch of eight samples, one procedural blank was analysed, with a minimum of six blanks for each sample batch. Blank samples were treated in exactly the same way as regular samples during the whole analytical procedure. All data that is presented was blank corrected (i.e. the average blank amount was subtracted) and was above the LOQ, unless stated otherwise.

2.5.5 Recovery and accuracy

Recovery of analytes following extraction is a good indicator of the quality of the data that is produced. This parameter was checked on a regular basis in each sample batch. Recovery of the analytes was validated through spiking of known amounts of authentic reference standards to samples, which were consecutively extracted. The recovery could then be calculated, taking into account the incurred analyte. As mentioned earlier, samples that do not contain PBDEs are rare and often not available at all. Recovery can be divided into *real recovery* (i.e. absolute recovery) and *apparent recovery* (i.e. recovery in relation to the IS) (Burns et al., 2002). The first is the absolute amount of the congener that can be retrieved after analysis. This is verified against a reference standard that is added to the extract prior to injection into the GC/MS. Ideally, the "absolute recovery" should lie between 70 and 120 % for environmental analyses in the ppm-range. AOAC recommendations state that the desired recovery may be dependent on the analyte concentration in the samples (AOAC, 2002). In some cases, recoveries as low as 40 % may still be acceptable. The methods used in the present study pass these criteria for most congeners, with typical recoveries between 80 and 100 % for all matrices (Table 2.3 and 2.4).

The apparent recovery was consistently around 100 %, except for serum. No correction for recovery was done, except for BDEs 153, 154 and 183 in serum. Estimated correction factors often have a high relative uncertainty, whereas uncorrected results usually have the smaller relative uncertainty associated with volumetric and instrumental measurements (Thompson et al, 2002).

Table 2.3. Method recoveries in % (RSD) of the different BDE congeners in various

	BDE 28	BDE 47	BDE 99	BDE 100	BDE 153	BDE 154	BDE 183	BDE 209
Sediment	85 (16)	89 (3)	88 (5)	88 (1)	89 (4)	93 (5)	83 (5)	87 (19)
Fish	94 (5)	93 (8)	93 (4)	92 (2)	105 (3)	101 (2)	80 (34)	80 (32)
Liver	90 (2)	88 (7)	84 (8)	89 (3)	84 (7)	90 (2)	88 (5)	82 (18)
Serum	84 (10)	88 (9)	88 (6)	84 (5)	82 (4)	74 (9)	66 (12)	-

Table 2.4. Method recoveries in % (RSD) of the different IS in various matrices.

	BB 103	BB 155	BDE 77	BDE 128	BDE 104	BDE 140	^{13}C BDE 209
Sediment	89 (6)	84 (7)	-	-	-	-	89 (16)
Fish	92 (4)	89 (5)	-	-	-	-	81 (31)
Liver	-	-	82 (9)	88 (7)	-	-	80 (19)
Serum	-	-	-	-	84 (8)	95 (5)	64 (17)

Absolute accuracy of the methods could not be assessed because certified reference material (CRM) for PBDEs is currently not available. Method performance was tested by participation in inter-laboratory exercises, based on a consensus value, derived from the results of the participants.

2.5.6 International inter-laboratory exercises

Internationally organised inter-laboratory exercises and CRM are suitable tools to evaluate the quality of the reported data of a laboratory (Päpke et al., 2004). The first world-wide inter-laboratory exercise on PBDEs was organised between November 1999 and April 2000 and included five biological samples, two sediments and two standard solutions (de Boer and Cofino, 2002). These materials were sent to 26 participants in nine different countries. Results were returned from 18 laboratories. Only the results for environmentally most relevant congener (BDE 47) were acceptable with RSDs ranging from 17 to 40 %. Results of all the other reported PBDEs (99, 100, 153, and 154) showed that a further improvement of the analyses was needed. The BDE 209 analysis was not under the control by the participating laboratories. The conclusion of that study stated that "*the performance of the laboratories can substantially be improved by ensuring a better calibration, good blanks, a better GC resolution, better internal standards, and protecting BDE 209 from high temperatures, incoming daylight and UV light*" (de Boer and Cofino, 2002).

CRM is an invaluable tool for validation of the accuracy of analytical methods. As the state-of-the-art in PBDE analysis progressed, between 2001 and 2003, an EU-funded project (BROC – Biological Reference Materials for Organic Contaminants; GRD2-2000-30019) assessed the feasibility of the determination of PBDEs by 15 expert laboratories, including the Toxicological Centre of the University of Antwerp. The study included an inter-laboratory test on PBDE determination in an unknown solution, fresh fish tissue and sediment. All major congeners were included in the study (BDEs 28, 47, 99, 100, 153, 154, 183, and 209 (only

for sediment]). The results of this feasibility study should have led to the preparation and certification of the corresponding reference material, but unfortunately up to this date, no CRM for PBDEs is available.

IUPAC guidelines describe the use of z-scores for the assessment of accuracy in inter-comparison exercises (Thompson and Wood, 1993). This index assesses the difference between the results of each participant and the exercise assigned value and can be used, with caution, to compare performance on different analytes and on different materials.
Performance of a laboratory in a PBDE inter-laboratory exercise is commonly expressed by means of z-scores. All z-scores discussed below are derived from the following formula:

$$\text{z-score} = [\text{bias estimate}]/[\text{performance criterion}]$$

The z-scores are commonly calculated using a fixed performance criterion of 20 % deviation of the Exercise Assigned Value or Exercise Mean. The z-scores calculated can be interpreted as shown in the following examples:

+1 → laboratory result is 20 % higher than the assigned value
-2 → laboratory result is 40 % lower than the assigned value

Of all the inter-laboratory exercises discussed below, only the exercise for serum organised by the Arctic Monitoring and Assessment Programme (AMAP) used a CV of 30 % for the calculation of the z-scores, probably because of the very low levels that were present. From a scientific point of view, IUPAC allows a classification of $z \leq 1$ as "*excellent*", $z \leq 2$ as "*satisfactory*", z between 2 and 3 as "*questionable*", and $z \geq 3$ as "*unsatisfactory*".
All methods applied in the present work were validated through participation in international inter-laboratory exercises. Table 2.5 gives a brief overview of all exercises in which was participated. Results on each sample matrix are briefly discussed below.

Table 2.5. Overview of inter-laboratory exercises that were used for the validation of the methods.

Organiser	Year	Matrices	Compounds[1]	Outcome[2]	Reference
BROC	2002	Sediment, fish	All PBDEs	++	Lohman et al., 2003
Quasimeme /RIVO/BSEF, 2nd Round	2001	Sediment, sediment extract, solution, fish	All PBDEs	++	De Boer and Wells, 2002
Quasimeme /RIVO, 3rd Round	2003	Sediment, solution, fish	All PBDEs	++	de Boer and Wells, 2006
NIST	2003	Marine mammal blubber	All PBDEs, BB 153	++	Kucklick et al., 2005
NIST	2004	Sediments	All PBDEs	++	Stapleton et al., 2005
NIST	2005	Marine mammal blubber	All PBDEs, BB 153	++	Report is still in draft
AMAP, 1st Round	2005	Serum	BDEs 47, 99	-	AMAP, 2005a
AMAP, 2nd Round	2005	Serum	BDEs 47, 99	++	AMAP, 2005b
AMAP, 3rd Round	2005	Serum	BDEs 47, 99	++	AMAP, 2005c
AMAP, 1st Round	2006	Serum	BDEs 47, 99	++	AMAP, 2006
Norwegian Institute of Public Health	2005	Solution, fish oil	HBCD	++	Haug et al., 2006

[1] Compounds reported by the Toxicological Centre; "all PBDEs" = BDEs 28, 47, 99, 100, 153, 154, 183, and209.

[2] Based on the IUPAC classification; ++ = excellent, + = satisfactory, - = unsatisfactory

2.5.6.1 Sediments

In support of environmental monitoring measurement programmes, the National Institute of Standards and Technology (NIST, Gaithersburg, MD, US) conducts inter-laboratory comparison exercises to provide a mechanism for participating laboratories/monitoring programmes to evaluate the quality and comparability of their performance in measuring selected organic contaminants in environmental samples. In 2004 they organised an inter-laboratory exercise for the determination of PBDEs in sediment samples. At the start of this exercise, only very few inter-comparison exercises on the measurements of PBDEs in environmental samples had been conducted and the major exercise (de Boer and Cofino, 2002) that was conducted had shown large variations in measurement values. This exercise organised by NIST was set up exclusively for the determination of PBDE congeners in marine sediments (Table 2.5; Stapleton et al., 2005). Only twelve laboratories from six different countries participated, including the Toxicological Centre of the University of Antwerp.

Participants were asked to quantify thirty-four individual PBDE congeners in two sediment Standard Reference Materials (SRM 1941b and SRM 1944). These SRMs were certified for organochlorine compounds. A majority of the laboratories supplied results for the eight most common PBDE congeners measured in environmental samples (BDE 28, BDE 47, BDE 99, BDE 100, BDE 153, BDE 154, BDE 183 and BDE 209). In general, data obtained for individual PBDE congeners were normally distributed, except for BDE 209. Several laboratories reported BDE 209 values that were significantly outside the range of values reported by a majority of the participating laboratories. The analysis of this congener still seemed not to be fully under control by some laboratories.

BDE 209 was the dominant congener measured in both sediment SRMs and thus is similar to patterns observed in environmental sediment samples (Hites, 2003). The majority of the z-scores, based on 20%, for each laboratory are within ± 2. Z-scores calculated for BDE 209 among the laboratories displayed the widest variation (0.00 to 52.5), pointing out at the problems associated with the analysis of this compound.

In SRM 1941b, sulphur was very abundant and this compromised the analysis of some congeners. Our z-scores for the analysis of BDEs 28, 47, 99, and 100 were below 1 for this SRM. Due to clean-up problems, no results were reported for BDEs 153, 154 and 183. For SRM 1944, we reported all major congeners. In this sediment, the z-scores for all congeners, except BDE 28, was below 1. For BDE 28, the z-score was below 2, which is still considered satisfactory (See higher). The overall performance of the Toxicological Centre in this inter-laboratory exercise can be considered "*excellent*" and the results of the sediment analyses are of high quality. Figure 2.10 shows the performance (z-scores of major congeners) of all participating laboratories for SRM 1944.

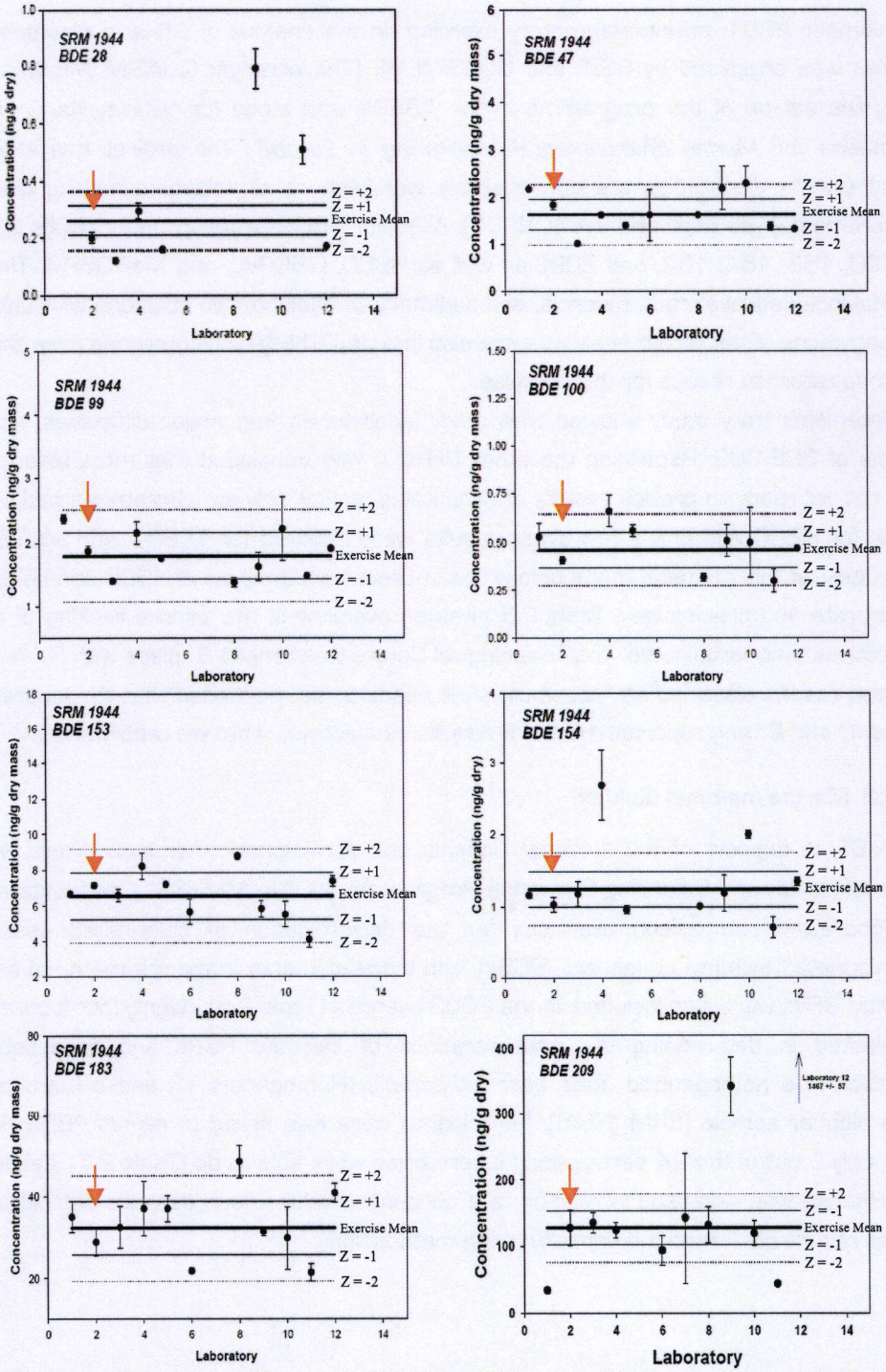


Figure 2.10. Z-score plots of the major BDE-congeners of all participating laboratories for SRM 1944. The Toxicological Centre was assigned code "*2*" (indicated by the *red arrow*). All z-scores were below 1, except for BDE28, for which it is below 2.

2.5.6.2 Fish

In November 2001, an inter-laboratory exercise on the analysis of BFRs in environmental samples was organised by BSEF and QUASIMEME (The acronym QUASIMEME was used during the set up of the programme in the 1990's and stood for "*Quality Assurance of Information for Marine Environmental Monitoring in Europe*". The project has kept this acronym, while the studies are now available worldwide to all scientists making chemical measurements) (de Boer and Wells, 2002). All major PBDEs were targeted (BDEs 28, 47, 99, 100, 153, 154, 183, and 209), as well as HBCD, (TBBP-A), and Me-TBBP-A. The test material included lake trout, mussels, and sediment. In addition, two solutions with unknown concentrations of the target analytes were also included. Thirty-six laboratories from thirteen countries returned results for this exercise.

This inter-laboratory study showed that most laboratories had major difficulties with the analysis of BDE 209. Regarding the other BFRs, it was concluded that most laboratories were not yet ready to provide results of acceptable quality; only six laboratories had sent in results for HBCD and only a few single results were received for TBBP-A and Me-TBBP-A. Fortunately, at this moment, methodology has improved and analysis of HBCD can be done in an accurate and precise way. Table 2.6 gives an overview of the z-score ranking of all the laboratories that participated. The Toxicological Centre was ranked 3rd place with 87 % of the reported results classified as "*satisfactory*". It needs to be mentioned that the laboratories ranked 1st and 2nd only reported 16 and 8 results, respectively, while we reported 31.

2.5.6.3 Marine mammal blubber

The NIST, in support of the National Oceanic and Atmospheric Administration's Marine Mammal Health and Stranding Response Programme (NOAA/MMHSRP), conducts annual inter-laboratory comparison exercises for the determination of chlorinated pesticides, polychlorinated biphenyl congeners (PCBs), and trace elements in marine mammal tissues. Recently, BFRs were also included. In the 2003 exercise (Table 2.5), twenty-four laboratories participated in determining the concentrations of selected PCBs and organochlorine pesticides in a homogenized polar bear fat sample (Homogenate VI) and a homogenized whale blubber sample (SRM 1945). Participants were also asked to report PBDE results, which only 5 out of the 24 participating laboratories were able to do (Table 2.7). Laboratory performance was assessed in relation to a consensus value. Z-scores were not calculated, but our results confirmed the capacity of the methodology.

Table 2.6. Overview of the z-score ranking of all participants in the 2001 inter-laboratory exercise that was organised by QUASIMEME (BFRs in fish; de Boer and Wells, 2002). The Toxicological Centre was assigned code "*Q512A*" (highlighted in *grey*).

Labcode	NObs |Z| < 2[1]	Possible % |Z| < 2[2]	Labcode	NObs submitted[3]	Actual % |Z| < 2[4]
Q332A	29	76	Q311A	16	100
Q512A	27	71	Q319A	8	88
Q509A	26	68	Q512A	31	87
Q251A	25	66	Q509A	32	81
Q534A	23	61	Q332A	36	81
Q104C	22	58	Q127A	28	79
Q127A	22	58	Q251A	33	76
Q532A	21	55	Q522A	12	75
Q539A	21	55	Q104C	31	71
Q509B	19	50	Q520A	19	68
Q523A	19	50	Q117A	24	67
Q527A	19	50	Q539A	32	66
Q533A	19	50	Q509B	29	66
Q524A	17	45	Q533A	29	66
Q117A	16	42	Q524A	26	65
Q311A	16	42	Q525A	14	64
Q314A	15	39	Q534A	36	64
Q153A	14	37	Q523A	30	63
Q520A	13	34	Q527A	31	61
Q535A	13	34	Q314A	25	60
Q531A	12	32	Q538A	17	59
Q530A	11	29	Q535A	23	57
Q528A	10	26	Q532A	38	55
Q538A	10	26	Q371B	14	50
Q519A	9	24	Q519A	18	50
Q252A	9	24	Q530A	22	50
Q525A	9	24	Q531A	26	46
Q529A	9	24	Q153A	33	42
Q275A	8	21	Q528A	26	38
Q041C	8	21	Q130B	6	33
Q319A	7	18	Q529A	29	31
Q371B	7	18	Q526A	25	28
Q526A	7	18	Q401C	30	27
Q130B	2	5	Q275A	33	24
Q410A	2	5	Q516A	10	20
Q516A	2	5	Q410A	13	15

[1] NObs |Z| < 2: Total number of satisfactory observations (|Z| < 2) from each laboratory; [2] Possible % |Z| < 2: Total number of satisfactory observations as a % of the total number of determinands with assigned values; [3] NObs submitted: Number of datasets submitted by each laboratory for each determinand with an assigned value; [4] Actual % |Z| < 2: % of observations submitted that were satisfactory (|Z| < 2); de Boer and Wells, 2002.

Table 2.7. Results (ng/g wet weight) of the 5 laboratories that participated in the 2003 NIST inter-laboratory exercise. The Toxicological Centre was assigned code "*18*" (highlighted in *grey*). n.a. = not available

SRM 1945

	Laboratory					Consensus		
Compound	**2**	**7**	**16**	**18**	**22**	**Mean**	**1SD**	**n**
BDE 28	20.2	n.a.	n.a.	1.24	n.a.			
BDE 47	44.4	39.2	33.0	35.6	33.5	36.9	4.7	5
BDE 99	17.9	17.9	16.4	16.9	16.0	17.0	0.9	5
BDE 100	13.5	7.17	8.57	8.73	8.92	9.17	2.4	5
BDE 153	8.47	6.39	7.54	7.42	8.04	7.54	0.78	5
BDE 154	23.1	11.1	10.8	10.7	12.1	12.9	5.4	5
BDE 183	6.16	n.a.	n.a.	1.55	1.66			
BDE 209	n.a.	n.a.	n.a.	n.a.	n.a.			
BB 153	n.a.	n.a.	n.a.	3.18	n.a.			

Homogenate IV

	Laboratory					Consensus		
Compound	**2**	**7**	**16**	**18**	**22**	**Mean**	**1SD**	**n**
BDE 28	n.a.	n.a.	n.a.	n.a.	n.a.			
BDE 47	n.a.	6.10	4.10	4.48	4.38	4.76	0.90	4
BDE 99	n.a.	0.97	0.36	0.41	0.37	0.53	0.3	4
BDE 100	n.a.	0.29	0.33	0.38	0.26	0.32	0.05	
BDE 153	n.a.	0.66	0.70	1.1	0.77	0.80	0.18	
BDE 154	n.a.	0.01	<0.15	0.18	0.04			
BDE 183	n.a.	n.a.	n.a.	0.27	n.a.			
BDE 209	n.a.	n.a.	n.a.	n.a.	n.a.			
BB 153	n.a.	n.a.	n.a.	3.0	n.a.			

2.5.6.4 Serum

Several times a year, AMAP organises inter-laboratory exercises for pollutants in serum. Recently, also PBDEs were included. The Toxicological Centre participated to three rounds in 2005 and to the first round of the 2006 AMAP ring test for persistent organic pollutants in human serum. At the time of the first round in 2005, the method for PBDEs in serum was not fully optimised. As a result, the outcome of the first round was "*unsatisfactory*" (Table 2.5). At the time the second round was organised, the method was adapted and results were "*excellent*". In the latest exercise, the first round of 2006, ten laboratories reported results for PBDEs. This exercise included PBDEs, PCBs and organochlorine pesticides. Being the two congeners of major importance in human serum, only BDE 47 and 99 were included in the exercise. Since the PBDE-levels in the serum were not certified, all PBDE-concentrations are given an assigned value.

Also in this exercise, the performance of the laboratories is expressed by means of z-scores (See higher). For both congeners, the z-score of the Toxicological Centre was below 1, which classifies our result as "*excellent*". Additionally, the organisers used a scoring system based on the z-scores and the number of reported congeners (Taylor et al., 1986). Taking into account the 33 other results (several PCB congeners and organochlorine pesticides) that

were reported additionally to PBDEs, the Toxicological Centre scored "100%" according to this performance indicator. Results of the AMAP 2006 first round inter-laboratory exercise are given in Table 2.7; z-scores are not included in the table.

Table 2.7. Individual results of all reporting labs for BDE 47 and 99 in the first round of the AMAP serum ring test of 2006. The Toxicological Centre was assigned code "*2036*" (highlighted in *grey*).

	BDE 47 (ng/ml)		
ID	W-06-01-b47	W-06-02-b47	W-06-03bB47
2003	0.68	0.51	0.34
2015	0.75	0.54	0.37
2023	0.64	0.54	0.38
2036	0.73	0.47	0.32
2038	0.74	1.58	0.83
2043	0.86	0.58	0.48
2046	0.84	0.58	0.39
2047	0.93	0.64	0.45
2049	0.96	0.63	0.44
Mean	0.79	0.65	0.44
CV	14%	50%	34%
N	9	10	10
Median	0.75	0.56	0.39
Assigned value	**0.75**	**0.56**	**0.39**

	BDE 99 (ng/ml)		
ID	W-06-01-b99	W-06-02-b99	W-06-03-b99
2003	0.54	0.81	0.37
2015	0.48	0.71	0.34
2023	0.43	0.76	0.36
2036	0.48	0.72	0.32
2038	0.51	2.13	0.69
2043	0.55	0.77	0.35
2046	0.55	0.77	0.36
2047	0.56	0.86	0.36
2049	0.65	0.95	0.43
Mean	0.53	0.93	0.39
CV	12%	46%	27%
N	9	10	10
Median	0.54	0.79	0.36
Assigned value	**0.54**	**0.79**	**0.36**

2.6 Paper I

Determination of brominated flame retardants, with emphasis on polybrominated diphenyl ethers (PBDEs) in environmental and human samples – a review

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Determination of brominated flame retardants, with emphasis on polybrominated diphenyl ethers (PBDEs) in environmental and human samples—a review

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Abstract

Analytical methods for the determination of brominated flame retardants (BFRs), with a special emphasis on polybrominated diphenyl ethers (PBDEs) are reviewed. A number of procedures, which can be applied to the analysis of PBDEs and polybrominated biphenyls (PBBs), and in some cases for hexabromocyclododecane (HBCD), in environmental and human samples are described. Because several BFRs, such as tetrabromobisphenol-A (TBBP-A), BDE 209 and, to some extent, HBCD, may require a different approach, specific advice on their analysis is given separately when needed. Sample pretreatment, extraction, cleanup and fractionation, injection techniques, chromatographic separation, detection methods, quality control and method validation are discussed. For each topic, an overview is given of the current status of the field and recommendations for an appropriate analytical approach are presented.
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Keywords: Brominated flame retardants; Polybrominated diphenyl ethers; Methods; Analysis; Review

1. Introduction

Flame retardants are chemicals that are added to polymers which are used in plastics, textiles, electronic circuitry and other materials to prevent fires (WHO/ICPS, 1994, 1997). Included among the different flame retardants are inorganic chemicals (such as antimony oxides), organic phosphate esters with or without halogens, and chlorinated and brominated organic compounds (WHO/ICPS, 1997). The most used brominated flame retardants (BFRs) are polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCD), tetrabromobisphenol-A (TBBP-A) and polybrominated biphenyls (PBBs). Similarly to other persistent organic pollutants (POPs), these BFRs (except TBBP-A) appear to be lipophilic and bioaccumulate in biota and humans (de Wit, 2002).

Due to the observed increasing temporal trends in humans (Meironyté et al., 1999) or biota (Sellström, 1996; Ikonomou et al., 2002a; Luross et al., 2002), BFRs are being determined in a growing number of laboratories (de Boer and Cofino, 2002). Analytical methods for the determination of BFRs have shown a rapid development over the last 5–6 years and were in most cases based on established methods for chlorinated pollutants, such as polychlorinated biphenyls (PCBs). However, results of the first two international intercalibration exercises (de Boer and Cofino, 2002; de Boer et al., 2002) demonstrated that analytical methods for the determination of BFRs in environmental and human samples need further improvement.

This paper aims to give an overview of available methods for the determination of PBDEs, PBBs, HBCD and TBBP-A (in the text referred to as BFRs) in environmental (water, air, sediment, sewage sludge, biota) and human (blood, milk, tissue) samples and to discuss associated specific aspects. A number of procedural steps with their limitations and advantages are described below. Different combinations of steps can result in a number of procedures, which can be applied to the analysis of PBDEs and PBBs, and in some cases for HBCD, in environmental matrices. Some BFRs, such as TBBP-A, BDE 209 and, to some extent HBCD, may require a different approach. Specific advice on the analysis of these BFRs will be given separately when needed. Whichever procedure is applied, each laboratory must demonstrate the validity of the entire procedure. In addition, the participation in interlaboratory tests is encouraged.

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2. Sample pretreatment

In general, the methods used for the determination of BFRs in different matrices are very sensitive and thus able to detect extremely low amounts of compounds. However, BFRs are present in the environment (de Wit, 2002) and therefore contamination during sample pretreatment may easily occur.

Drying of solid samples (soil, sediment, sewage sludge) by evaporation of water is primarily done for convenience. Dry samples are more effectively homogenised, allowing accurate sub-sampling for parallel analyses for other determinants (e.g. organic carbon). In addition, storage and transport may be easier. The absence of water in the samples avoids laborious extraction with separation funnels and makes the sample matrix more accessible to organic solvents. As an alternative to drying through evaporation, several methods can be applied for water binding. Chemical drying of samples can be performed by grinding with anhydrous Na_2SO_4. Intensive grinding and the addition of sufficient quantity of drying salt to obtain a free-flowing powder are of vital importance for a complete extraction. Drying with water-adsorbing materials (alumina, silica, etc.) may be an alternative, but in this case water is not bound irreversibly and can easily be released only when polar solvents are used for extraction. The use of a mixture of less polar solvents (e.g. hexane, dichloromethane) may help to avoid these problems. Freeze-drying (water evaporation below 0 °C under vacuum conditions) can also serve for sample drying (Smedes and de Boer, 1997).

During evaporation of water from sediment or soil samples at room temperature or in a heated oven (<40 °C), both losses and uptake of compounds from air may occur (de Boer et al., 2001). In addition, cross-contamination cannot be excluded. Validation should ensure that evaporation of analytes and cross-contamination are minimised.

Sampling of indoor air (Sjödin et al., 2001a) involves either passive/diffusive samplers or active pumping of air through glass filters (for BFRs associated with airborne particles) or polyurethane foam adsorbents (for BFRs present in the gas phase). Exposure of polymeric SPE cartridges to the drying process in parallel with the samples provides a sensitive test for the evaluation of laboratory air contamination with PBDEs (Thomsen et al., 2001a).

3. Extraction

The extraction procedure is dependent on the sample matrix; different methods are used for solid or liquid samples. Sample intake usually varies between 0.5 and 50 g.

3.1. Solid samples

The following considerations apply for a large range of solid matrices, including soil, sediment, sewage sludge, adsorbent materials used for air sampling, but also biotic samples (tissues, eggs) which were previously dried by Na_2SO_4 homogenisation or freeze-drying.

During the extraction step, the contaminants are isolated from the matrix and transferred to an organic solvent. The yield of the extraction procedure is determined by several factors such as the solubility of analytes in the extraction mixture, the accessibility of the extraction solvent to the matrix and the extraction time. The solubility of analytes determines the equilibrium that can be reached in a certain stage of the extraction, while accessibility and extraction time are more related to the kinetics of the extraction. A nonpolar organic solvent, like hexane, may have a high solubility for most BFRs, but does not have access to the inner part of biotic tissues or sediment/sewage sludge organic matter (containing many polar groups like amines, phenols and carboxilic acids). For wet samples (sediment, sewage sludge, soil), lipophilic contaminants have to desorb from the matrix to the water phase before they can be transferred to hexane. This process is extremely slow (10–30 days, especially in aged samples) and therefore, the extraction efficiency using hexane alone can be very low (Gevao et al., 2001).

Liquid–solid extraction, using Soxhlet apparatus, is a widely used standard technique and despite recent advances in techniques (such as supercritical fluid extraction, accelerated solvent extraction or microwave-assisted extraction—see below), it is still attractive due to its general robustness and low cost (Tables 1–3). Typical solvents used for the extraction of BFRs from soil, sediment, sewage sludge and dried biota are hexane, toluene, hexane/acetone mixtures, or dichloromethane. Binary solvent mixtures (combination of a nonpolar and a polar solvent) are most commonly used for their known extraction efficiency, especially for biota samples (de Boer et al., 2001). Hexane/acetone mixtures in different proportions (1:1 or 3:1, v/v) were found to give the best recoveries for extraction of BFRs (de Boer et al., 2001). The extraction times may vary between ca. 6 and 24 h depending on the extraction procedure and the sample intake. Two Soxhlet types are available: the regular one and the so-called hot Soxhlet, in which the solvent is distilled into the extraction chamber, which is heated below the boiling point of the solvent. In this way, the sample is permanently in contact with hot, but not boiling solvent, thus accelerating the desorption and finally, the extraction process. The use of hot Soxhlet results in a reduction of the extraction time, together with a comparable extraction efficiency (Covaci et al., 2002a).

Wet sediment can also be extracted using a two-step Soxhlet extraction (Nylund et al., 1992). To penetrate the organic matter and to extract the water, the addition of a water-miscible solvent (methanol or acetone) is needed. Next, the sample is extracted with a mixture of hexane/acetone, after which the extracts are combined (Nylund et al., 1992).

For BDE 209 (sensitive to UV light), special precautions should be taken. Incoming sunlight into the laboratory as

well as possible UV light from fluorescent tubes should be blocked. This can be done by placing UV filters on the windows of the laboratory and under the fluorescent lights. BDE 209 may easily adsorb to small dust particles in the laboratory. An ioniser should be installed, which attracts small dust particles by static electricity and keeps the air as clean as possible (QUASIMEME, 2002). In addition, all open glassware should be covered, e.g. by aluminium foil, to prevent dust particles to enter the solutions or samples. When using Soxhlet extraction, the coolers should be thoroughly rinsed with toluene before and after extraction, as traces of BDE 209 originating from a previous extraction could be present. Otherwise, false positive results can easily be obtained, as blanks do not always correct for this problem because the contamination degree of the different coolers may be very different (QUASIMEME, 2002).

Extraction of BFRs with organic solvents from a chromatographic column filled with homogenised sample (see Section 2) has been used for fish and marine mammal tissues (Haglund et al., 1997; Alaee et al., 2001a; Hale et al., 2001; Manchester-Neesvig et al., 2001; Ikonomou et al., 2002a,b; Luross et al., 2002), and human adipose tissue (Meneses et al., 1999). Despite its simplicity, the method uses large volumes of organic solvents that have to be further evaporated and disposed off.

Ultra-sonication was used in a small number of laboratories for the extraction of PBDEs from polyurethane foams used for air sampling (Sjödin et al., 2001a) or from high-impact polystyrene (Hamm et al., 2001). However, this technique has not found a wide application area for BFRs. Lower extraction recoveries than with the Soxhlet extraction were obtained. The method is laborious because several subsequent extractions are needed and because the contact time (up to 24 h) should be high enough.

New extraction techniques such as accelerated solvent extraction (ASE) or microwave-assisted extraction (MAE) are currently applied in a number of laboratories. Although higher costs are involved compared with Soxhlet extraction, these techniques have the advantage of lower solvent consumption, which makes the long-term cost lower and the procedures more environmentally friendly. They also result in reduced extraction time and they can be highly automated (Björklund et al., 2000a). ASE has the possibility of in-line lipid destruction (Björklund et al., 2001a). ASE and MAE have been used for the extraction of anthropogenic and natural brominated compounds from marine mammals (Herzke et al., 2001; Vetter, 2001, 2002; Vetter et al., 2001) and from lyophilised human milk (Guillamon et al., 2002). However, while recoveries of di- to hepta-BDEs and di- to hepta-BBs were >65%, very low recoveries (0–40%) were obtained by ASE for BB 209, BDE 209 and HBCD, probably due to excessive adsorption in the extraction system or the tubing (Kemmlein, 2000).

Supercritical fluid extraction (SFE) with solid-phase trapping has been used for the extraction of PBDEs and PBBs together with PCBs and chlorinated benzenes from sediment samples with CO_2 as the supercritical fluid (Hartonen et al., 1997). Before extraction, the sediment was mixed with copper powder and Na_2SO_4. Better results were obtained using CO_2 with modifiers (methanol, acetone and diethylamine) than with only CO_2 or Soxhlet extraction. Moreover, the extracts were much cleaner, eliminating the use of an additional cleanup step. In other SFE applications, PBDEs were extracted from human adipose tissue (Lindström et al., 1996; Hardell et al., 1998) and whale blubber (van Bavel et al., 1999a,b) using CO_2 as extracting medium. However, at least for sediments, de Boer et al. (2001) have shown that, despite higher recoveries for BDE 47, the coefficient of variance was unacceptably high, possibly indicating that SFE is less reproducible than Soxhlet extraction.

Extraction with pressurised hot water (PHWE) has also been used for the analysis of several brominated analytes from sediment (Hyötyläinen et al., 2001). The extracted analytes were trapped into a solid-phase trap (Tenax™ TA), from which they were eluted with a pentane/ethyl acetate mixture after drying the trap with nitrogen. Also in this case, no additional cleanup was needed before GC-MS analysis.

3.2. Liquid samples

Liquid–liquid extraction (LLE) has been applied for river and seawater samples, by using hexane/acetone mixtures (Luckey et al., 2001). LLE using hexane/acetone (Darnerud et al., 1998) has been used for the determination of PBDEs in milk samples, while hexane (Ohta et al., 2002) was used after saponification of human milk with ethanolic KOH. A mixture of chloroform/methanol/water has been preferred for the extraction of PBDEs from mussels (Booij et al., 2002). Similar procedures involving protein denaturation with HCl/*iso*-propanol (Sjödin et al., 1999, 2001b; Hovander et al., 2002a,b) and extraction with hexane/methyl *tert*-butyl ether have been used for the determination of neutral and phenolic-brominated compounds from human serum.

PBDEs have been extracted from human milk with a mixture of lipophilic gel and formic acid (Meironyté et al., 1999). The milk samples were incorporated into the gel by shaking the mixture for 2.5 h. The mixture was transferred to a glass column and after removal of interferences with water/methanol and methanol/dichloromethane/hexane mixtures, the analytes of interest were eluted from the gel with acetonitrile.

Solid-phase extraction (SPE) has been used for the determination of acidic and neutral BFRs from human plasma (Thomsen et al., 2001b,c). The plasma was first mixed with a formic acid/*so*-propanol mixture in an ultrasonic bath, then diluted and transferred to a conditioned SPE column (polystyrene–divinylbenzene, Isolute ENV+®). After washing the column with a water/*iso*-propanol mixture, the lipids were directly decomposed onto the column by addition of concentrated sulphuric acid. After further

washing with water, acetate buffer and a water/methanol mixture, the analytes were eluted from the dried cartridges with dichloromethane/ethanol. For human milk (Thomsen et al., 2002a), a similar procedure was applied, but in this case, a SPE column with a copolimeric sorbent of divinylbenzene-*N*-vinylpyrrolidone (Oasis™ HLB) was used in order to increase the recoveries of phenolic BFRs. Furthermore, an additional cleanup step was required due to higher amounts of co-extracted lipids than in the case of human plasma.

4. Cleanup and fractionation

The crude extract requires a cleanup as many other compounds (e.g. humic acids, lipids) can be co-extracted with the analytes. Extracts from sediments, sewage sludge or soil samples may contain sulphur that has to be removed. Several methods such as the reaction with tetrabutyl ammonium sulphite (Jensen et al., 1997) or Cu treatment (Covaci et al., 2002b) can be used. Biota extracts usually contain high concentrations of lipids that should be removed by destructive or nondestructive methods, prior to the gas chromatographic separation of BFRs. As the BFR concentrations are usually related to the amount of lipids, the lipid content is often measured gravimetrically prior to the cleanup, or determined separately by a total lipid determination (Bligh and Dyer, 1959; de Boer, 1988; Smedes, 1999). For serum or plasma samples, the lipid determination can be conveniently done on separate aliquots by enzymatic tests (Thomsen et al., 2001b,c).

4.1. Nondestructive lipid removal

Several techniques such as gel permeation chromatography (GPC) and column adsorption chromatography are usually used for the elimination of lipids and other high molecular weight compounds that can cause serious interferences during the GC separation of BFRs.

GPC is widely used for the primary cleanup of biological samples (Haglund et al., 1997; Asplund et al., 1999; Alaee et al., 2001a; de Boer et al., 2001; Dodder et al., 2002) (see Tables 1–3). The separation of interferences is usually done using polystyrene–divinylbenzene copolymeric columns. While self-packed columns are still used (Asplund et al., 1999; Meironyté et al., 1999; Alaee et al., 2001a), nowadays, pre-packed HPLC columns allow a better separation reproducibility, combined with a considerable reduction in solvent consumption (Haglund et al., 1997; de Boer et al., 2001). Dichloromethane or mixtures of dichloromethane/hexane or ethylacetate/cyclohexane are applied as eluents. However, this technique does not separate BFRs from other organohalogenated compounds, and often, an additional cleanup step is used for further purification of the extract (Asplund et al., 1999; de Boer et al., 2001) or for fractionation into different classes.

Neutral adsorbents, such as silica gel, alumina or Florisil® are widely used for the cleanup (de Boer et al., 2001; Law et al., 2002). When GPC is applied, further extract purification by Florisil® (Haglund et al., 1997; Norstrom et al., 2001) or silica gel (de Boer et al., 2001; Hale et al., 2001; Vetter, 2001; Ikonomou et al., 2002a,b) is necessary for further extract purification (Tables 1–3). When GPC is not used, a combination of two adsorbents is recommended for an efficient cleanup. Silica and Florisil® have a relatively low fat retaining capacity compared with alumina (Smedes and de Boer, 1997; de Boer et al., 2001).

Silica gel allows the fractionation of the extract according to the polarity of different classes of compounds. PBDEs normally elute in the second, more polar (pesticide) fraction after elution with hexane/diethyl ether or hexane/dichloromethane over silica gel columns (de Boer, 1989, Darnerud et al., 1998). PBBs and BDE 209 are normally eluting in the first fraction, together with PCBs. For β-HBCD (one of the three diastereoisomers of HBCD), larger elution volumes of more polar solvent mixtures (hexane/diethyl ether) are necessary (QUASIMEME, 2002).

4.2. Destructive lipid removal

Sulphuric acid treatment, either directly to the extract (Watanabe et al., 1987; de Boer et al., 2001; Johnson and Olson, 2001) or via impregnated silica columns (Covaci et al., 2002a; Huwe et al., 2002; Ikonomou et al., 2002a), is the most commonly applied lipid removal destructive method (Tables 1–3). It has been shown that PBBs, PBDEs and HBCD are stable under strong acidic conditions (de Boer et al., 2001, QUASIMEME, 2002). Furthermore, the lipid destruction with concentrated acid may allow the removal of a higher lipid amount than adsorption chromatography. The direct use of concentrated acid needs several extraction and centrifugation steps, thus making the procedure labour intensive and time-consuming. In contrast, acidified silica gel should be handled with extreme care and inhalation of fine acid containing particles should be avoided.

A similar approach makes use of silica gel impregnated with alcoholic KOH (Christensen and Platz, 2001; Christensen et al., 2002; Ikonomou et al., 2002a) or of a multilayer column with neutral silica, acidified silica and basic silica (Hagenmaier et al., 1992). While PBBs, PBDEs and TBBP-A are stable to basic conditions, HBCD is degraded (de Boer et al., 2001) and cannot be measured. Basic conditions can be used also during the saponification of biota samples (heating with ethanolic KOH) (Ohta et al., 2002). However, the conditions for saponification are critical as too high temperatures and too long saponification times may cause degradation of highly brominated PBDEs and PBBs (de Boer et al., 2001; Ohta et al., 2002), leading to an underestimation of their concentration and an overestimation of lower brominated PBDEs and PBBs.

Three lipid removal methods (GPC, acidified silica column and sulphuric acid treatment) were investigated

Table 1
Analysis (extraction/cleanup/GC/detection) of abiotic samples (air, water, sewage sludge, sediment)

Sample	Compounds	Sampling/pretreatment	Extraction	Cleanup	Detection	Rec. (%)	References
Indoor air	PBDEs, TBBP-A, PBBs	Ioniser, aluminium cup	(1) Particles wiped with glass wool in DCM (2) Dissolution in Hex	(1) Partitioning with KOH/EtOH (2) Derivatisation with CH_2N_2 (3) H_2SO_4 conc.	ECNI-MS	n.r.	Bergman et al., 1997
Indoor air (electronic plant)	PBDEs, TBBP-A	Glass fiber and polyurethane foam plug	Ultra sonication with D	(1) Partitioning with KOH/EtOH (2) Derivatisation with CH_2N_2 (3) Silica/H_2SO_4 column	ECNI-MS	60–97	Sjödin et al., 2001a
Laboratory air	PBDEs, TBBP-A	SPE column adsorbent	–	(1) Elution with D/M = 7:3 (2) Derivatisation with CH_2N_2	ECNI-MS	n.r.	Thomsen et al., 2001a
Outdoor air	PBDEs, PCBs	Quartz fibre filters and XAD-2 adsorbers	Soxhlet (24 h) with Hex/Acet = 1:1	Silica gel, elution with Hex (PCBs, DDE), and Hex/DCM = 1:1 (PBDEs, OCPs)	EI-MS	~ 100, ~ 70 for BDE 209	Strandberg et al., 2001
Arctic air	PBDEs	Glass fiber and polyurethane foam plug	(1) Hex and DCM (2) Tol (for BDE 209)	–	EI-HRMS	n.r.	Alaee et al., 2001c
Water	PBDEs	Quartz fibre filters	XAD-2 adsorbers	Elution with Hex	EI-HRMS	n.r.	Luckey et al., 2001
Sewage sludge	PBDEs, PBDDs, PBDFs, PCBs	Drying, powdering	Soxhlet (18 h) with T	(1) Silica/acid silica/silica, elution with Hex/DCM = 4:1 (2) Alumina + $AgNO_3$, elution with Hex/Acet = 96:4 (3) GPC, Biobeads SX3, elution with CY/EtAc = 1:1 (4) Basic alumina, elution with Hex/DCM = 1:1	EI-MS	n.r.	Hagenmaier et al., 1992
Sewage sludge	PBDEs, HBCD, TBBP-A, PCBs	Centrifugation	(1) 60 min with 40 ml Acet (2) 50 ml 0.2 M NaCl in 0.1 M Na_2HPO_4 (3) 30 min with 40 ml Hex/Acet = 3:1 and 10 ml Hex/DE/U = 90:10:2	(1) i-PrOH/TBA-sulphite (2) Washing with water (3) Treatment with conc. H_2SO_4	ECNI-MS	106–140	Nylund et al., 1992; Sellström et al., 1998
Sediment	PBDEs	Air drying, homogenisation, mixing with Na_2SO_4	Soxhlet 4 h, Hex/Acet = 1:1	(1) Alumina, elution with Hex (2) Silica, elution with Hex	ECD, confirm EI-MS	n.r.	Allchin et al., 1999
Sediment	PBDEs	Drying	Soxhlet 6 h, Hex/Acet = 3:1	(1) GPC for S removal (2) Silica, elution with Hex (3) Treatment with conc. H_2SO_4	ECNI-MS	n.r.	de Boer et al., 2001
Sediment	PBBs, PBDEs	Drying, mixing with Cu	SFE/CO_2 (20 min static, 40 min dynamic), 120 °C, C_{18} trapping, elution with Hp/EtAc = 98:2	–	EI-MS	n.r.	Hartonen et al., 1997
Sediment	PBDEs	Mixing with Cu	Soxhlet 2 h, Hex/Acet = 3:1	(1) Cu powder for S removal (2) Acidified silica, elution with Hex/DCM = 1:1	ECNI-MS	~ 85%	Covaci et al., 2002b
Sediment	BFRs	Drying	PHWE (40 min), water 325 °C, 120 bar, trapping Tenax™, elution with Pent/EtAc	–	EI-MS	n.r.	Hyötyläinen et al., 2001

Solvents: Hex—hexane, Acet—acetone, CY—cyclohexane, DCM—dichloromethane, DE—diethyl ether, U—undecane, Hp—heptane, EtAc—ethyl acetate, Pent—pentane, i-PrOH—2-propanol, AcN—acetonitrile.
n.r. —not reported.

Table 2
Analysis (extraction/cleanup/GC/detection) for biota samples (mussels, fish, marine mammals, birds, eggs, meat)

Sample	Compounds	Pretreatment	Extraction	Cleanup	Detection	Rec. (%)	References
Benthic org. (crab, sea star, shrimps)	PBDEs	Homogenisation mixing with Na_2SO_4	Soxhlet (2 h), Hex/Acet = 3:1	Acidified silica, elution with Hex	ECNI-MS	>80	Voorspoels et al., 2002
Mussels	PBDEs	–	SLE with DCM/MeOH/H_2O	Silica, elution with P	ECNI-MS	n.r.	Booij et al., 2002
Mussels, marine fish	PBDEs	Mixing with Na_2SO_4	Soxhlet (6 h), Hex/Acet = 4:1	Multilayer silica column and alumina	ECNI-MS	>75	Christensen et al., 2002
Fish	PBDEs	Homogenisation	LSE with Hex/Acet and Hex	(1) Treatment with conc. H_2SO_4 (2) Florisil®, elution with Hex	ECD or EI-MS	>70	Watanabe et al., 1987
Fish	PBDEs	Mixing with Na_2SO_4	Soxhlet (16 h), DCM/Hex = 1:1	(1) Florisil®, elution with Hex/DE = 94:6 (2) Treatment with conc. H_2SO_4	AED	>80	Johnson and Olson, 2001
Fish, marine mammals	PBDEs	Mixing with Na_2SO_4	Soxhlet (6 h), Hex/Acet = 3:1	(1) GPC for lipid removal (2) Silica, elution with Hex (3) Treatment with conc. H_2SO_4	ECNI-MS	n.r.	de Boer et al., 2001
Fish	PBDEs	Mixing with Na_2SO_4	Soxhlet (24 h), Hex/Acet = 1:1	(1) GPC for lipid removal (2) Silica, elution with DCM	EI or ECNI-MS	70–120	Dodder et al., 2002
Fish	PBDEs	Mixing with Na_2SO_4	Column extraction with 300 ml DCM	(1) GPC on Biobeads SX3, eluent DCM/Hex (2) Silica, elution with DCM	EI-HRMS	65–120	Alaee et al., 2001a
Fish	PBDEs	Mixing with Na_2SO_4	Column extraction with DCM	(1) GPC for lipid removal (2) Silica, elution with Hex	ECD	71–106	Manchester-Neesvig et al., 2001
Salmon (muscle, eggs)	PBDEs, MeO-PBDEs		SLE with Hex/Acet and Hex/DE	(1) GPC for lipid removal (2) KOH/EtOH partitioning neutral compounds (3) Silica, elution with Hex (4) HPLC, NO_2-column phenolic compounds (5) Derivatization with CH_2N_2 (6) Treatment with conc. H_2SO_4	ECNI-MS	80–90	Asplund et al., 1999
Fish, fish feed	PBDEs	Mixing with Na_2SO_4	Soxhlet (2 h), Hex/Acet = 3:1	Acidified silica, elution with Hex	EI-MS, ECNI-MS	>80	Jacobs et al., 2002a
Fish oil	PBDEs	Dilution in Hex	–	Acidified silica, elution with Hex	ECNI-MS	>80	Jacobs et al., 2002a,b
Seal, herring	PBDEs, MeO-PBDEs	Mixing with Na_2SO_4	Column extraction with 300 ml Hex/Acet = 7:3 and 300 ml Hex/DE = 9:1	(1) GPC on PL-gel, eluent Hex/DCM = 1:1 (2) Florisil, elution with Hex/DCM = 1:1	EI-MS	50–115	Haglund et al., 1997
Porpoise blubber, fish, cormorant	PBDEs	Mixing with Na_2SO_4	Soxhlet (4 h), Hex	(1) Alumina, elution with Hex (2) Silica, elution with Hex	ECNI-MS	n.r.	Law et al., 2002; Allchin et al., 1999

Whale blubber	PBDEs	Homogenization with Na_2SO_4, mixing with alumina	SFE with CO_2, 40 °C, 281 bar, trap C_{18}, elution with 2 ml Hex and 2 ml DCM	–	EI-MS	n.r.	van Bavel et al., 1999a
Porpoises, crab, sole	PBDEs	Homogenization with Na_2SO_4	Column extraction with 250–300 ml, DCM/Hex = 1:1	(1) Multicolumn with acid silica–silica–basic silica, elution with DCM/Hex = 1:1 (2) Alumina, elution with DCM/Hex = 1:1	EI-HRMS	40–120	Ikonomou et al., 2002a
Marine mammals	PBDEs, natural Br comp.	–	MAE with EtAc/Hex = 1:1	(1) GPC (2) Silica, elution with Hex	ECNI-MS	75–89	Vetter, 2001; Vetter et al., 2001
Fish, bird eggs	PBDEs	Homogenization with Ultra-Turrax	SLE, Hex/Acet (1:2.5) and Hex/DE (9:1)	(1) Treatment with conc. H_2SO_4 (2) GPC, eluent DCM/Hex = 1:1 (3) Silica, elution with Hex and Hex/DE = 4:1	ECNI-MS	39–65	Jansson et al., 1991
Bird eggs	PBDEs	Mixing with Na_2SO_4	ASE (3 × 15 min, with Hex/Acet = 1:1)	(1) GPC (2) Silica, elution with Hex/Tol = 6:4	EI-LRMS	n.r.	Herzke et al., 2001
Bird eggs	PBDEs	Mixing with Na_2SO_4	Soxhlet with D/H = 1:1	(1) GPC (2) Florisil®, elution with Hex	EI-HRMS	n.r.	Norstrom et al., 2001
Chicken fat	PBDEs	Dissolution in DCM	–	(1) Acid silica, elution with Hex (2) Multicolumn with acid silica–silica–basic silica, elution with DCM/Hex = 1:1 (3) Alumina, elution with DCM/Hex = 1:1	EI-HRMS	>75	Huwe et al., 2002
Fish, meat, vegetables	PBDEs	Freeze-drying	(1) Saponification with KOH/EtOH (2 h) (2) LLE with Hex Soxhlet (5 h) with Tol	(1) Multicolumn with $AgNO_3$/silica–acid silica–silica–basic silica, elution with DCM/Hex = 5:95 (2) Active carbon, elution with DCM/Hex = 1:3	EI-HRMS	>80	Ohta et al., 2002

Solvents: Hex—hexane, Acet—acetone, DCM—dichloromethane, DE—diethyl ether, EtAc—ethyl acetate, Pent—pentane, i-PrOH—2-propanol, AcN—acetonitrile, Tol—toluene, MeOH—methanol, EtOH—ethanol.
n.r.—not reported.

Table 3
Analysis (extraction/cleanup/GC/detection) of human tissues and fluids (adipose tissue, serum, milk)

Sample	Compounds	Sample pretreatment	Extraction	Cleanup	Detection	Rec. (%)	References
Adipose tissue	PBDEs	Drying with Na_2SO_4	Soxhlet (2 h), Hex/DCM/Acet = 3:1:1	(1) Acid silica (2) Acid silica/alumina, elution Hex	EI-MS	81–102	Covaci et al., 2002a,c
Adipose tissue	PBDEs	Drying with Na_2SO_4 mixing with alumina	SFE with CO_2, 40 °C, 300 atm, trapping on PX21/C_{18}, elution with Hex/DCM	–	EI-MS, TOF-MS	n.r.	van Bavel et al., 1999b
Adipose tissue/ liver	PBDEs	Homogenisation	Ultra Turrax extraction with i-PrOH/Hex = 2:3 and Hex	(1) Lipide × 5000 partitioning (after mixing with i-PrOH, formic acid) (2) Column elution with MeOH in H_2O and AcN (3) Alumina and silica columns, elution with Hex (4) GPC on bio-beads SX-3	EI-HRMS	57–84	Meironyté et al., 2001
Adipose tissue	PBDEs	Homogenisation	Soxhlet (24 h), Tol	(1) Silica gel (2) Activated carbon (Carbopack C) (3) Activated alumina	EI-HRMS	42–104	Strandman et al., 1999
Human breast adipose tissue	PBDEs	Homogenisation	Hex/DCM	(1) GPC (2) Florisil®, elution with Hex	ECNI-MS	n.r.	She et al., 2000; Petreas et al., 2002
Serum	PBDEs	Acetic acid addition	Hex/DCM	(1) Florisil, eluted with Hex/DCM	Dual GC-ECD	n.r.	Petreas et al., 2002
Serum	PBDEs	HCl and i-PrOH addition	Hex/MBTE = 1:1	(1) Washing with KCl solution (2) KOH/EtOH partitioning (3) Treatment with conc. H_2SO_4 (4) Acid silica, elution with H	ECNI-MS	69–95	Sjödin et al., 1999
Serum	PBDEs, TBBP-A	(1) Formic acid/ i-PrOH (4:1) (2) Ultra sonication (3) Dilution with H_2O	SPE on Isolute ENV + ® (200 mg, 6 ml)	(1) Lipid decomposition with conc. H_2SO_4 (2) Wash with H_2O, acetate buffer and H_2O/MeOH (3) Elution with DCM/MeOH = 1:1	ECNI-MS	56–111	Thomsen et al., 2001b
Milk	PBDEs	Homogenisation, mixing with formic acid and Lipide × 5000	(1) Wash with MeOH/H_2O (2) Elute with AcN	Alumina and silica columns, elution with Hex GPC on Bio-beads SX-3, elution with Hex/DCM	EI-HRMS	57–84	Meironyté et al., 1999
Milk	PBDEs, TBBP-A	(1) Formic acid/i-PrOH (4:1) (2) Ultrasonication (3) Dilution with H_2O	SPE on Oasis™ HLB (500 mg, 6 ml)	(1) Lipid decomposition with conc. H_2SO_4 (2) Wash with H_2O, acetate buffer and H_2O/MeOH (3) Elution with DCM/MeOH = 1:1	ECNI-MS	49–83	Thomsen et al., 2002a
Milk	PBDEs	Saponification with ethanolic KOH	LLE with Hex	(1) Multilayer column acid silica–silica–base silica, elution with Hex	EI-HRMS	>80	Ohta et al., 2002
Milk	PBDEs	–	LLE with Hex/Acet	(1) Conc. H_2SO_4 (2) GPC (3) Florisil®, elution with H	EI-HRMS	85–110	Ryan and Patry, 2000

Solvents: Hex—hexane, Acet—acetone, DCM—dichloromethane, MTBE—methyl *tert*-butyl ether, i-PrOH—2-propanol, AcN—acetonitrile, Tol—toluene, MeOH—methanol.
n.r.—not reported.

for the analysis of neutral and phenolic BFRs from plasma (Hovander et al., 2002a). The acidified silica gel column provided higher recoveries for acid-stable (both phenolic and neutral) compounds and can be most easily automated for high-throughput applications.

For BDE 209, toluene, dichloromethane, or mixtures of acetone and hexane should be used as a solvent as much as possible, as it may not be well soluble in other solvents such as *iso*-octane. It should be avoided that the extracts would be evaporated until dryness, because BDE 209 may not completely re-dissolve after that step even when using toluene. In general, PBBs and PBDEs adsorb to glass more tenaciously than PCBs (de Boer, 1999). For TBBP-A, strong adsorption to glass was observed when hexane was used as solvent, while no adsorption was seen with methanol (Sjödin, 2000).

4.3. Fractionation of phenolic BFRs

TBBP-A may be separated from the extract that contains neutral and phenolic BFRs by treatment with an alcoholic KOH solution (Hovander et al., 2002a). The aqueous solution is then acidified (pH = 1) and TBBP-A is extracted with hexane and methyl *tert*-butyl ether (Sjödin et al., 1999; Hovander et al., 2002a,b). Before analysis, TBBP-A is derivatised to its diacetylated derivative. Although TBBP-A is more suited for HPLC analysis, it may be analysed by GC without derivatisation, but the column quality is essential and active sites should be absent (QUASIMEME, 2002). Dimethylated TBBP-A, also found in sediments, follows the cleanup scheme for PBDEs (Sellström and Jansson, 1995). Hydroxylated PBDEs and 2,4,6-tribromophenol (another BFR and also the major breakdown product of TBBP-A) are separated in a similar way as TBBP-A and then derivatised with diazomethane. Methoxy-BDEs, which were first identified in Baltic salmon, are found in the neutral fraction (Haglund et al., 1997; Asplund et al., 1999).

5. Injection techniques

The two most frequently used injection systems are splitless and on-column injection (Table 4).

In splitless injection, the transfer of the analytes into the analytical column is controlled by the volume of the liner and by the injected volume. When a liner is too small, memory effects can occur, while very large liner volumes can cause a poor transfer of early eluting compounds. Normal injector temperatures are between 250 and 300 °C. For octa- to deca-BDEs, some degradation might occur if the residence time in the liner is too long, while discrimination is possible when the residence time is too short. A pressure pulse (pulsed splitless) may help to improve the injection performance. Small injection volumes (typically 1 μl) are preferred, but larger volumes can help to lower the detection limit (see below). The use of an autosampler is a prerequisite for obtaining an acceptable reproducibility.

For BDE 209, it is possible to differentiate if possible degradation has occurred during the sample preparation (UV decomposition) or during injection. The presence of nona-, octa- and eventually other lower brominated PBDE congeners may indicate that degradation occurred during sample preparation. The presence of a hump or a rising baseline in the chromatogram before BDE 209 may indicate degradation during injection. A dirty GC system (liner or column) may lead to partial or complete losses of HBCD isomers.

On-column injection has been successfully applied for the accurate introduction of BFRs into the GC column (Sjödin et al., 1999; Alaee et al., 2001a; Björklund et al., 2001b). The extract is injected directly into the column (Alaee et al., 2001a) or in a glass insert fitted into a septum-equipped programmable injector kept at low temperature (Sjödin et al., 1999; Björklund et al., 2001b). No significant degradation of higher brominated PBDEs, including BDE 209, was observed. However, the extracts should be very clean, as otherwise the GC column can quickly deteriorate. Thus, it is mandatory to use a retention gap, but care should be taken as some coatings can cause excessive degradation of highly brominated PBDEs. Björklund et al. (2000b) have observed that the highest degree of degradation for BDE 209 was observed for untreated, intermediate polar and polar retention gaps. The best results were obtained with a Restek™ Siltek deactivated retention gap.

Other techniques, such as temperature-programmed or pressure-programmed injection may have additional advantages, but should also be thoroughly optimised before use. Injections of large volumes of extracts, up to 20 μl (Covaci et al., 2002a) or 50–100 μl (Björklund et al., 2001b) may be good options for the determination of PBDEs from samples with low concentrations such as human tissue or air. In these cases, the extracts should be very clean to reduce interferences during the GC separation.

For BDE 209, a pressure-pulse injection should be used to reduce the exposure to high injector temperatures. Also, the maximum injection temperature should not be higher than 300 °C. Alternatively, on-column injection may be used. This technique requires very clean extracts, because otherwise the first part of the column may become dirty soon, which will result in deteriorated chromatograms.

6. GC separation

Although 209 BDE congeners, numbered in an identical way as PCBs (Ballschmiter and Zell, 1980; Pijnenburg et al., 1995), are theoretically possible, only a small number can be found in technical PBDE mixtures (WHO/ICPS, 1994). While profiles of PBDEs in sediments tend to match the profiles of technical mixtures (penta-, octa-, and deca-), their distribution in biota is altered, presumably by selective uptake, metabolism or degradation (de Wit, 2002). There-

Table 4
Injection and GC parameters for BFR analysis

Sample	Compounds	Injection (Temperature)	Column	Dimensions	Detector	References
Air	Tetra- to deca-BDE, TBBPA	On-column (60 °C)	DB5-HT	15 m × 0.25 mm × 0.10 μm	ECNI-MS	Sjödin et al., 2001a
Sediment, fish	(1–2) Tetra- to octa-BDEs	(1–2) Splitless, 1 μl (270 °C)	(1) HP-5	(1) 50 m × 0.2 mm × 0.25 μm	Dual ECD	Allchin and de Boer, 2001
	(3) BDE 209	(3) Splitless (110 °C)	(2) HP-1701	(2) 60 m × 0.25 mm × 0.25 μm		
			(3) HP-5	(3) 25 m × 0.2 mm × 0.33 μm		
Sediment, mussels	(1) Tetra- to hexa-BDEs	Splitless	(1) DB-5	(1) 60 m × 0.25 mm × 0.25 μm	ECNI-MS	Christensen and Platz, 2001
	(2) BDE 209	Cold splitless (110 °C)	(2) DB-1	(2) 15 m × 0.25 mm × 0.10 μm		
Mussels	Tri- to deca-BDEs	Splitless (270 °C)	CP Sil-8	25 m × 0.25 mm × 0.25 μm	ECNI-MS	Booij et al., 2002
Fish	Di- to deca-BDEs	Splitless, 2 μl (275 °C)	DB-1	15 m × 0.25 mm × 0.25 μm	ECNI-MS	Akutsu et al., 2001
Crab, fish	Tri- to hepta-BDEs	Cold splitless, 1 μl (100 °C)	HT-8	10 m × 0.10 mm × 0.10 μm	ECNI-MS	Voorspoels et al., 2002
Fish	Mono- to hepta-BDEs	On-column, 1 μl (100 °C)	RTX-5	60 m × 0.25 mm × 0.25 μm	EI-HRMS	Alaee et al., 2001a
			DB-1701	60 m × 0.25 mm × 0.25 μm	EI-HRMS	Lurose et al., 2002
Fish	(1) Tetra- to hepta-BDEs	Splitless	(1) DB-5MS	(1) 30 m × 0.25 mm × 0.25 μm	(1) EI-LRMS	Strandberg et al., 2001;
	(2) BDE 209		(2) DB-5MS	(2) 15 m × 0.25 mm × 0.25 μm	(2) ECNI-MS	Dodder et al., 2002
Salmon	Tri- to hexa-BDEs	n.s.	DB-5	30 m	GC-ECD	Manchester-Neesvig et al., 2001
Fish	Tetra- to hexa-BDEs	Splitless	DB-5	60 m × 0.25 mm × 0.32 μm	GC-ELCD, EI-LRMS	Hale et al., 2001
Fish, human milk, vegetables	Tri- to hexa-BDEs	Splitless, 2 μl (260 °C)	SPB-5	30 m × 0.32 mm × 0.25 μm	EI-LRMS	Ohta et al., 2002
Seal, fish, crab, porpoise	(1) Mono- to nona-BDEs	Splitless, 1 μl (300 °C)	(1) DB-5	(1) 30 m × 0.25 mm × 0.25 μm	EI-HRMS	Ikonomou et al., 2002a
	(2) BDE 209		(2) DB-5-HT	(2) 15 m × 0.25 mm × 0.10 μm		
Porpoise, cormorant	(1) Tetra- to octa-BDEs	(1) PTV (50 °C)	(1) DB-5	(1) 50 m × 0.2 mm × 0.25 μm	(1) ECNI-MS	Law et al., 2002;
	(2) BDE 209	(2) PTV (70 °C)	(2) HP-1	(2) 15 m × 0.25 mm × 0.10 μm	(2) ECD	de Boer et al., 2001
Whale	Tetra- to hexa-BDEs	Splitless, 2 μl	DB-5	n.s.	EI-LRMS	Lindström et al., 1999
Chicken fat	Mono- to deca-BDEs	On-column	DB-5MS	30 m	EI-HRMS	Huwe et al., 2002
Human adipose	Tri- to hepta-BDEs	Large volume, 20 μl (70 °C)	AT-5	10 m × 0.10 mm × 0.10 μm	EI-MS	Covaci et al., 2002a
Milk	Tri- to hexa-BDEs	Splitless	Quadrex 007-5	25 m × 0.32 mm × 0.25 μm	EI-HRMS	Meironyté et al., 1999
Milk, serum	Tri- to hepta-BDEs	Pulsed splitless, 1.5 μl (250 °C)	CP Sil-5CB	30 m × 0.25 mm × 0.25 μm	ECNI-MS	Thomsen et al., 2001b,c

n.s.—not specified.

fore, the contamination with PBDEs is preferably monitored by the determination of selected individual congeners. Several congeners, such as BDEs 28, 47, 99, 100, 153, 154 and 183 are frequently detected, while BDE 209 was found in sediment (Allchin et al., 1999; de Boer et al., 2003), but also in human serum (Sjödin et al., 1999) and Peregrine falcon eggs (Sellström et al., 2001). This means that single-capillary column GC may offer sufficient resolution for a congener-specific PBDE determination. In order to achieve enough separation between BDE congeners and possible interferences, there is a need for using sufficiently long columns (30–50 m) and small diameters (≤ 0.25 mm). Good resolution may also be obtained by using narrow bore columns (internal diameter = 0.1 mm) (Covaci et al., 2002a).

PBDEs can relatively easily be determined on nonpolar or semi-polar columns such as 100% methyl-polysiloxane type (DB-1) and 5% phenyl-dimethyl polysiloxane type (DB-5, CP-Sil 8 or AT-5). The most frequently used lengths are 25 to 60 m (de Boer et al., 2001; Alaee et al., 2001a; Hale et al., 2001; Booij et al., 2002) (Table 4). Several other columns, such as 10 × 0.10-mm internal diameter 8% phenyl-polycarborane-siloxane HT-8 (Voorspoels et al., 2002) or AT-5 (Covaci et al., 2002a), 60 m 14% cyanopropyl-phenyl 86% dimethyl-polysiloxane (CP-Sil 19, HP-1701 or DB-1701) (Allchin et al., 1999; Alaee et al., 2001a) were successfully used. All these columns display a good resolution for most compounds, but each type of column should first be tested for possible co-elutions of target compounds, internal standards and other compounds present in the sample. The impact of the co-elutions depends on the sample cleanup, pollutant load in the sample, chromatographic system and detection method.

Hale et al. (2001) have reported the co-elution of CB 194 and BDE 100 on a 60 m DB-5 column. Alaee et al. (2001b) have indicated that there are potentially 10 BDE congeners that can co-elute with organochlorine pesticides or PCBs, with a particular concern addressed to CB 180 and BDE 47. While presenting problems when ECD is used, co-elutions between PCBs and PBDEs can be solved by MS detection either in selective ion monitoring (SIM) or full scan. More important is the co-elution on DB-5 or CP-Sil 8 (Fig. 1) of BB 153 and BDE 154, as well as the co-elution of BDE 153 and TBBP-A. BB 153 can be found in the American environment and population (Sjödin et al., 2001b) and in some marine mammals (de Boer et al., 1998). Using a HT-8 column, BB 153 and BDE 154 do not co-elute, but now BDE 85 co-elutes partially with BDE 154 (Figs. 2 and 3). Ikonomou et al. (2002a,b) reported the co-elution of lower brominated congeners, BDEs 28 and 33, on a 30-m DB-5 column. Recently, Vetter (2002) has shown that BDE 99 may co-elute with a natural bromine-containing compound, while Asplund (personal communication) has suggested that BDE 47 and BDE 99 may interfere with breakdown products of HBCD.

BDE 209 should receive special attention because of its sensitivity for higher temperatures and the higher susceptibility for degradation in the GC system. The GC column should be relatively short, preferably 10–15 m, to reduce as

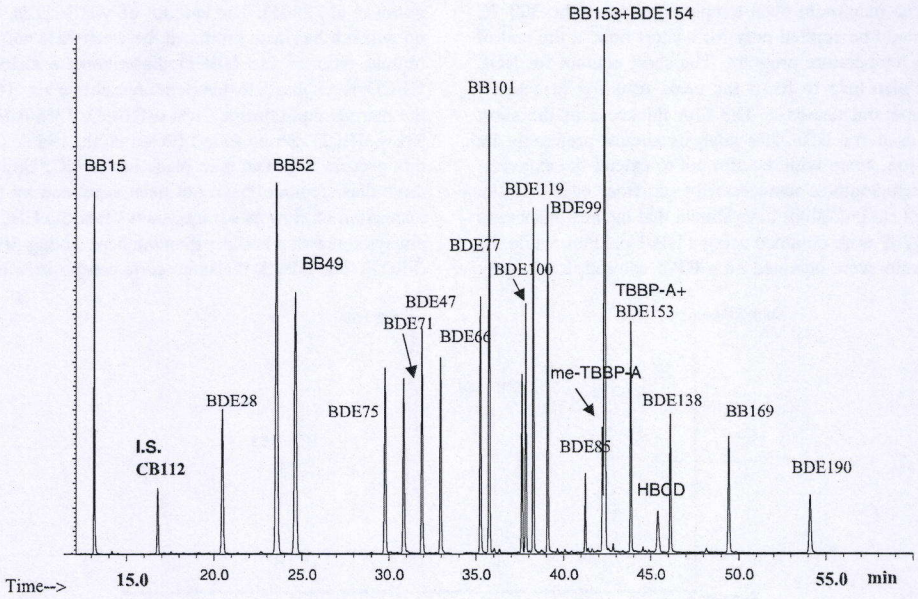

Fig. 1. ECNI-MS chromatogram of a standard mixture of BFRs on a 50 m × 0.2 mm × 0.25 μm CP-Sil 8 column (1 μl injected in pulsed splitless, injector temperature: 275 °C, oven program: 90 °C, stay 3 min, with 30 °C min^{-1} to 210 °C, stay 20 min, with 5 °C min^{-1} to 315 °C).

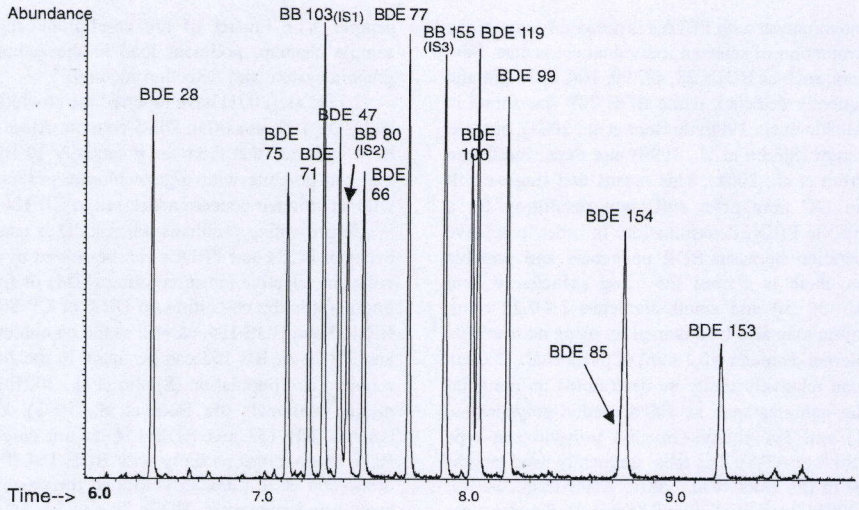

Fig. 2. ECNI-MS chromatogram of a standard mixture of BFRs on a 10 m × 0.10 mm × 0.10 μm HT-8 column (1 μl injected in cold splitless, injector temperature: 100 °C, stay 0.05 min, with 720 °C min^{-1} to 280 °C, oven: 90 °C, stay 1.5 min, with 40 °C min^{-1} to 220 °C, with 25 °C min^{-1} to 270 °C, with 40 °C min^{-1} to 290 °C, stay 10 min).

much as possible the residence time of BDE 209 (de Boer et al., 2001). This means that the analysis of BDE 209 should occur separately from the analysis of the other PBDEs. However, the separation is not very critical, as most of the other PBDEs will elute earlier in the chromatogram and will not interfere with the peak of BDE 209 (Allchin et al., 1999). The maximum oven temperature should be 300 °C and it should be applied only for a short time at the end of the oven temperature program. The short column for BDE 209 will also help to focus the peak, resulting in a better peak shape and response. The film thickness of the short column used for BDE 209 analysis should preferably be 0.1–0.2 μm, again with the aim not to extend the exposure to high temperatures unnecessarily (de Boer et al., 2001). Björklund et al. (2000b) have shown that the best responses for BDE 209 were obtained using a DB-1 column, while the worst results were obtained on a HP-1 column. It is highly possible that BDE 209 is sensitive to the type of column used, although batch to batch variations in the selectivity cannot be neglected.

HBCD can be determined by GC/MS, but it is more problematic than that for most PBDEs. Technical HBCD consists of three diastereomers: α-, β-, and γ-HBCD (Barontini et al., 2001). The amount of γ-HBCD is, depending on where it has been produced, between 72% and 89%. The mutual ratio of the HBCD diastereomers changes when HBCD is exposed to temperatures above ca. 160 °C. At the thermal equilibrium, 78% α-HBCD, 13% β-HBCD and 9% γ-HBCD can be found (Peled et al., 1995). Obviously, this process will also take place in the GC. Until now, the three diastereomers have not been separated by GC. Close inspection of chromatograms shows that the HBCD peak is always somewhat broader than the near eluting PBDE peaks (Fig. 1). The HBCD diastereomeric composition in environ-

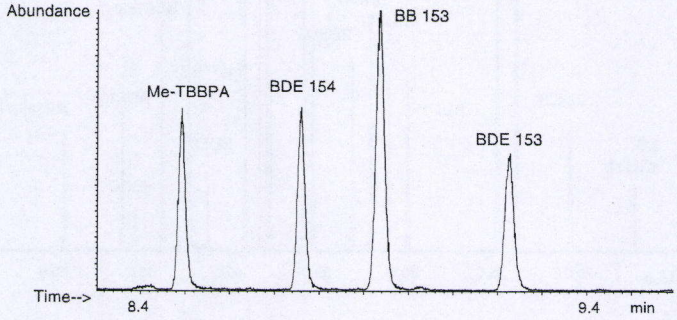

Fig. 3. Complete separation of dimethyl-TBBPA (Me-TBBPA), BDE 154 and BB 153 on a 10 m × 0.10 mm HT-8 column (same conditions as for Fig. 2).

mental samples may also differ from that of the technical HBCD mixture. However, as apparently the response factors of the three diastereomers do not differ very much (QUASIMEME, 2002), HBCD can be quantified as total HBCD by GC/MS. However, uncertainties are larger compared to those obtained for a number of PBDEs, which is proved by larger standard deviations in quality charts (ca. 25–30%). When analysing HBCD by GC/MS, the liner should always be very clean (changed after each sample batch), and the injected extracts should also be as clean as possible. A better alternative for the HBCD determination may therefore be electrospray LC/MS in which the diastereomers can be separated relatively easily using a C_{18} column and MeOH/H_2O gradients (QUASIMEME, 2002). However, no suitable internal standard could be found and the sensitivity is up to 1000 times lower compared with GC/MS in electron capture ionisation mode. This method is only applicable for the analysis of highly polluted samples such as marine mammals, fatty fish, sediment or sewage sludge.

With the ongoing production, it may be expected that at least for the coming decade and presumably for a longer period, BFRs may continue to draw the attention of policy makers and, consequently, of environmental laboratories. This will result in a more intensive use of GC/MS, and for some BFRs (e.g. HBCD and TBBP-A), in the use of LC/MS techniques. Given the simultaneous presence of BFRs and other halogenated contaminants in environmental samples, multi-dimensional GC techniques may become more important. Good progress has been made during the last 5 years with comprehensive two-dimensional GC (GC × GC) (Phillips et al., 1999; de Geus et al., 2000; Kinghorn et al., 2000; Ledford and Billesbach, 2000). Other complex mixtures of halogenated contaminants, such as toxaphene, PCBs and dioxins and furans have been analysed already with GC × GC (de Geus et al., 1998; Korytar et al., 2002). GC × GC-ECD or GC × GC/ToF (Time of Flight)-MS determinations of BFRs are expected to appear soon in literature.

7. Detection

The most widely used detectors for the BFR determination are mass spectrometers operated either in electron capture negative ionisation (ECNI) or in electron ionisation (EI) mode (de Boer et al., 2000). Occasionally, electron capture detectors (ECD) have been used for specific applications. The different detection methods are discussed below. The advantages and drawbacks of mass spectrometric detection modes for BFRs are summarised in Table 5.

7.1. Mass spectrometric detection (MS)

A distinction has to be made between low resolution MS (LRMS) and high resolution MS (HRMS). The former can be more easily routinely applied, while the latter requires more experienced users and is much more costly and labour intensive. HRMS has a number of advantages over LRMS (such as increased sensitivity and selectivity), but is almost exclusively operated in EI mode. For LRMS, ECNI, in addition to EI, can be applied to obtain an increased sensitivity for higher brominated compounds.

Table 5
Advantages and drawbacks of different detection techniques for BFRs

Detection	Advantages	Drawbacks
ECD	purchase cost maintenance cost ease of use	fair sensitivity for BFRs instability of linear range very low selectivity
EI-LRMS	facilitates the use of labelled standards good selectivity	low sensitivity
ECNI-LRMS	good sensitivity good selectivity for brominated compounds	frequent source maintenance required
EI-HRMS	good sensitivity very good selectivity	purchase cost maintenance cost difficult to use higher "down-time"

7.1.1. Electron impact ionisation (EI-MS)

In EI-MS, the major ions formed from PBDEs are the M^+ and the $[M-2Br]^+$, which can be used for their identification, and quantitation (Sellström, 1999). This ionisation technique facilitates the analysis of BDE congeners in the presence of possible co-eluted compounds (such as PCBs). EI-LRMS is not routinely used for the PBDE analysis, because of its relatively low sensitivity, especially for the analysis of higher brominated BDE congeners (hepta- to deca-DBE). However, this ionisation mode allows the acquisition of full scan spectra, thus offering a multiple choice in ion selection than ECNI mode. However, ECNI is more selective towards aromatic brominated compounds.

EI-LRMS has been used by Dodder et al. (2002) for the analysis of PBDEs (except BDE 209) in fish samples from the North-Eastern United States, while Hamm et al. (2001) have measured tri- to hepta-BDEs in polystyrene plastics. Covaci et al. (2002a) have shown that EI-LRMS combined with large volume injection (20 μl), can be used for the analysis of samples with very low PBDE concentrations, e.g. human tissues. The detection limits in SIM mode varied from 0.05 to 0.30 ng/g lipid weight, depending on the bromination degree (tri- to hexa-BDEs), respectively. In this way, similar sensitivity with that of ECNI-LRMS, combined with a higher selectivity, could be obtained by EI-LRMS. The ions typically monitored in EI-LRMS analysis are summarised in Table 6. Moreover, due to the possibility of measuring the molecular ion and other important fragments, EI-LRMS is the technique of choice for the identification of mixed organohalogenated compounds (Vetter, 2002). Another advantage of EI-MS is that it allows the use of ^{13}C-labelled internal standards for a more precise determination of the recovery of the entire analysis. This is

Table 6
Ions monitored in different BDE detection techniques

Detection mode	Homologue group	Ion	Analyte	Ions monitored (*m/z*)	Reference
EI-LRMS	tri-BDEs		BDE 28	406; 408	Covaci et al., 2002a
	tetra-BDEs		BDE 47, 66, 71, 75, 77	484; 486	Covaci et al., 2002a
			BDE 47	483.7; 485.7	Hardell et al., 1998; Meneses et al., 1999
		$[M-2Br+2]^+$		325.9	Dodder et al., 2002
		$[M+4]^+$		485.7	Dodder et al., 2002
	^{13}C-tetra BDEs		^{13}C-BDE 47	496; 498	Covaci et al., 2002a
	penta-BDEs		BDE 85, 99, 100, 119	564; 566	Covaci et al., 2002a; Meneses et al., 1999
		$[M-2Br+2]^+$, $[M-2Br+4]^+$		403.8; 405.8	Dodder et al., 2002
	^{13}C-penta BDEs		^{13}C-BDE 99	576; 578	Covaci et al., 2002a
	hexa-BDEs		BDE 138,153,154	484; 486	Covaci et al., 2002a
		$[M-Br+2]^+$, $[M-2Br+4]^+$		481.7; 483.7	Dodder et al., 2002
		M^+		641.5; 643.5	Meneses et al., 1999
	^{13}C-hexa BDEs		^{13}C-BDE 153	496; 498	Covaci et al., 2002a
	hepta-BDEs	$[M-2Br+4]^+$, $[M-2Br+6]^+$	BDE 183, 190	561.6; 563.6	Dodder et al., 2002
ECNI-LRMS	Tri-nona-BDEs	$79Br^-$		79	de Boer et al., 2001; Vetter, 2001; Booij et al., 2002
		$81Br^-$		81	Vetter, 2001; Booij et al., 2002
		$[79Br79Br]^-$		158	Vetter, 2001
		$[79Br81Br]^-$		160	Vetter, 2001
		$[1H79Br79Br]^-$		159	Vetter, 2001
		$[1H79Br81Br]^-$		161	Vetter, 2001
	BDE 209	$[M-C_6Br_5]^+$	BDE 209	487	Booij et al., 2002
				486.4; 488.4	Christensen and Platz, 2001
		Br^-		79; 81	Dodder et al., 2002
	^{13}C-BDE 209	$[M-C_6Br_5]^+$		498.7; 500.7	Lepom et al., 2002
	TBBP-A	$79Br^-$, $81Br^-$	TBBP-A	79,81	Sellström, 1999
	HBCD	$79Br^-$, $81Br^-$	HBCD	79; 81	Sellström, 1999
EI-HRMS	mono-BDEs	M^+, $[M+2]^+$		247.9837; 249.9817	Alaee et al., 2001a; Luross et al., 2002; Huwe et al., 2002
	di-BDEs	$[M+2]^+$, $[M+4]^+$		327.8922; 329.8903	Alaee et al., 2001a; Luross et al., 2002
		M^+, $[M+2]^+$		325.8942; 327.8922	Huwe et al., 2002
	tri-BDEs	$[M-2Br]^+$, $[M-2Br+2]^+$		245.9680; 247.9661	Alaee et al., 2001a; Luross et al., 2002
		$[M+2]^+$, $[M+4]^+$		405.8027; 407.8007	Huwe et al., 2002; Haglund et al., 1997
	tetra-BDEs	$[M-2Br]^+$, $[M-2Br+2]^+$		323.8785; 325.8765	Alaee et al., 2001a; Luross et al., 2002
		$[M+2]^+$	BDE 47	483.713	Thomsen et al., 2002b
		$[M+2]^+$, $[M+4]^+$		483.7132; 485.7112	Alaee et al., 2001a; Luross et al., 2002; Huwe et al., 2002
	^{13}C-tetra BDEs	$[M+2]^+$, $[M+4]^+$		495.7534; 497.7514	Huwe et al., 2002; Thomsen et al., 2002b
	penta-BDEs	$[M-2Br+2]^+$, $[M-2Br+4]^+$		403.7870; 405.7850	Alaee et al., 2001a; Luross et al., 2002; Huwe et al., 2002
		$[M-2Br]^+$	BDE 85,99,100	401.789	Thomsen et al., 2002b
		$[M+4]^+$, $[M+6]^+$		563.6217; 565.6197	Huwe et al., 2002; Haglund et al., 1997
	^{13}C-penta BDEs	$[M-2Br+2]^+$, $[M-2Br+4]^+$		415.8273; 417.8252	Huwe et al., 2002
		$[M+4]^+$, $[M+6]^+$		575.6619; 577.6599	Huwe et al., 2002

Table 6 (*continued*)

Detection mode	Homologue group	Ion	Analyte	Ions monitored (*m/z*)	Reference
EI-HRMS	hexa-BDEs	$[M-2Br+2]^+$, $[M-2Br+4]^+$		481.6975; 483.6955	Alaee et al., 2001a; Luross et al., 2002; Huwe et al., 2002
		$[M-2Br+4]^+$	BDE 138,153,154	483.713	Thomsen et al., 2002b
		$[M+4]^+$, $[M+6]^+$		641.5322; 643.5302	Huwe et al., 2002; Haglund et al., 1997
	^{13}C-hexa BDEs	$[M-2Br+2]^+$, $[M-2Br+4]^+$		493.6975; 495.6955	Ryan and Patry, 2000
	hepta-BDEs	$[M-2Br+4]^+$, $[M-2Br+6]^+$		561.6060; 563.6040	Alaee et al., 2001a; Luross et al., 2002; Huwe et al., 2002
	octa-BDEs	$[M-2Br+4]^+$, $[M-2Br+6]^+$		639.5165. 641.5145	Huwe et al., 2002
	nona-BDEs	$[M-2Br+6]^+$, $[M-2Br+8]^+$		719.4250; 721.4230	Huwe et al., 2002
	BDE 209	$[M-2Br+6]^+$, $[M-2Br+8]^+$		797.3355; 799.3335	Huwe et al., 2002

not possible for the ECNI-MS, since generally only the Br^- ions ($m/z=79$ and 81) are monitored.

Due to the complexity and the analysis cost of EI-HRMS, the use of this technique is not so widely spread. However, HRMS is preferred in principle over LRMS for its higher sensitivity (compared with EI-LRMS) and selectivity (compared with ECNI-LRMS). The first two worldwide interlaboratory studies on BFRs (de Boer and Cofino, 2002) have shown that that laboratories using EI-HRMS may be able to obtain somewhat lower detection limits, but a superior performance for HRMS could not be demonstrated. A similar conclusion was reached by Thomsen et al. (2002b) who stated that GC/ECNI-LRMS and GC/EI-HRMS are equally well suited for the determination of PBDEs in biological samples, as well as in standard solutions, with respect to response, detection limits and repeatability at the pg-level. Both techniques can be used complementarily when confirmation of the results obtained by GC/ECNI-LRMS is necessary. The ions monitored in EI-HRMS are similar to those in EI-LRMS, but the target mass fragments can be determined much more accurately, which adds considerably to the selectivity of the technique (Table 6).

7.1.2. Electron capture negative ionisation LRMS (ECNI-LRMS)

Electron capture ionisation is a "soft" ionisation technique that takes advantage of the interactions between thermal energy electrons and electrophilic molecules, such as PBDEs (Ong and Hites, 1994). In ECNI, the low-energy electrons (thermal electrons) generated by interactions between a high-energy electron beam and a moderating gas (see Table 7), react with the analytes to form negative ions (Dougherty et al., 1972). The electron energy should be very low to facilitate electron capture, and the specific energy required for electron capture depends on the molecular structure of the analyte (Ong and Hites, 1994).

Being a very sensitive technique, ECNI-MS is widely use for the determination of low amounts of BFRs in various samples (Sellström et al., 1993; Asplund et al., 1999; Sjödin et al., 1999; Alaee et al., 2001a; Thomsen et al., 2001b,c; Vetter, 2001; Booij et al., 2002). Brominated compounds show a typical ^{79}Br (50.5%) and ^{81}Br (49.5%) isotope distribution pattern (Sellström, 1999) (Table 6). Occasionally other mass fragments can be monitored, such as *m/z* 484.7 and 486.7 for BDE 209 and *m/z* 498.7 and 500.7 for ^{13}C-BDE 209 (Lepom et al., 2002). The benefits of ECNI are efficient ionisation, higher sensitivity and less fragmentation than EI or positive-ion CI. There is also a greater sensitivity for PBDEs, owing to their high bromine load. The sensitivity for these compounds is more than 10 times better compared to ECD (Pijnenburg et al., 1995).

The spectra and data obtained using ECNI are strongly dependent on the experimental conditions, e.g. type of reagent gas, reagent gas pressure, the instrument used and the ion source temperature (Buser, 1986; Stemmler et al., 1988; Stemmler and Hites, 1988). The most common experimental conditions are given in Table 7. PBDE residues in guillemot eggs showed an increase in levels of BDE 47, an unidentified Pe-BDE (probably BDE 100) and BDE 99 of, respectively, 10–35%, 25–80% and 0–20% after reanalysis using ammonia as reagent gas instead of methane (Sellström, 1996). Critical parameters which influence reproducibility are the temperature and pressure in the ion source, the purity of the moderating gas, the type of instrument used and its tuning conditions, and the concentration of the analytes (Stemmler and Hites, 1988).

7.2. Electron capture detector (ECD)

Although ECD is known to be very sensitive for other organohalogenated compounds (such as PCBs and organochlorine pesticides), for BFRs, it has been proven useful only in applications where concentrations were relatively

Table 7
Operation parameters of different detection modes used in BDE analysis

Detector	Reagent gas	Electron energy (eV)	Quadrupole temperature (°C)	Ion source temperature (°C)	Resolution	Reference
EI-LRMS	n.a.	70	n.s.	250	n.a.	Alaee et al., 2001a
EI-LRMS	n.a.	70	n.s.	220	n.a.	Allchin et al., 1999
EI-LRMS	n.a.	70	150	230	n.a.	Covaci et al., 2002a
EI-LRMS	n.a.	70	n.s.	250	n.a.	Ohta et al., 2002
EI-HRMS	n.a.	30–40	n.a.	270	10000	Alaee et al., 2001a
EI-HRMS	n.a.	70	n.a.	250	8000	Haglund et al., 1997
EI-HRMS	n.a.	n.s.	n.a.	n.s.	2500	Huwe et al., 2002
EI-HRMS	n.a.	39	n.a.	310	10000	Ikonomou et al., 2002a
EI-HRMS	n.a.	38	n.a.	320	5000	Ohta et al., 2002
EI-HRMS	n.a.	30–70	n.a.	265	5000	Thomsen et al., 2002b
ECNI	methane	n.s.	n.s.	150	n.a.	Alaee et al., 2001a
ECNI	methane	70	n.s.	150	n.a.	Asplund et al., 1999
ECNI	methane	n.s.	160	210	n.a.	Booij et al., 2002
ECNI	methane	n.s.	n.s.	270	n.a.	Christensen and Platz, 2001
ECNI	methane	70	n.s.	150	n.a.	Sellström et al., 1993
ECNI	methane	70	n.s.	200	n.a.	Sjödin et al., 1999, 2001
ECNI	methane	235	106	250	n.a.	Thomsen et al., 2001a,b
ECNI	methane	n.s.	100	150	n.a.	Vetter, 2001
ECNI	isobutane	70	n.s.	180	n.a.	Akutsu et al., 2001
ECNI	n.s.	133	n.s.	150	n.a.	Öberg et al., 2002

n.a.—not applicable; n.s.—not specified.

high (Allchin et al., 1999; Manchester-Neesvig et al., 2001). Despite its relative low purchase and maintenance cost, combined with a relatively good sensitivity for compounds with four or more bromine atoms, several drawbacks have reduced its application area. One major problem is that the sensitivity is not influenced only by the bromine/halogen load, but also by the substitution pattern of the rings (Sellström, 1999). This results in unequal responses for the different congeners. Furthermore, ECD is known for being linear only over a limited concentration range (Sellström, 1999). Another drawback is the lack of selectivity. Any halogen-containing molecules will produce a signal and therefore PBDE analysis can be influenced, especially when PCBs are present at high concentrations (Alaee et al., 2001a). This limits the use of the ECD to samples where there has been verified, (e.g. by random screening by MS), that co-eluting compounds are not present. Many of these co-elutions can be avoided by good selection of the column and oven temperature. Some applications described the measurement of BDE 209 in sediments on a short column (Allchin et al., 1999), while dual ECD systems have been used for the determination of PBDEs in human milk (Darnerud et al., 1998).

7.3. Other detectors

Other detectors such as electrolytic conductivity detector (Hale et al., 2001) and atomic emission detector (Johnson and Olson, 2001) have found only a limited area of application due to their low sensitivity and/or selectivity. These detectors, together with ECD, are used especially for screening purposes.

8. Quality assurance/quality control

Quality Assurance (QA) is a set of procedures, which include the quality control (QC) activities that are undertaken to affirm the quality of obtained data. As a general rule, 20–25% of the analysis time should be spent on the quality of analyses. To assure sufficient quality, a number of measures should be taken during the pre-analysis quality control (or validation) and in-process quality control. These measures can be divided into three major areas: calibrants, analytical procedure control, system performance/long-term stability.

8.1. Calibrants

8.1.1. Standard solutions

Until some years ago, individual BDE congener standards were not available and technical mixtures (such as Bromkal 70-5 DE) were used instead. Due to an exponential increase in BFR research in the last 10 years, approximately 40 individual or mixtures of native and 13 $^{13}C_{12}$-labelled BDE congeners are now available as standard solutions. The main providers are Cambridge Isotope Laboratories (Andover, MA, USA), Wellington Laboratories (Guelph, Ontario, Canada) and AccuStandard (New Haven, CT, USA). TBBP-A and all three isomers of HBCD are also available in solution.

Simultaneous preparation of two independently prepared dilutions, storage of calibration solutions in ampoules or firmly closed vials in cool dark place and regular checking of weight loss, which should be maximum 2% for 6–9-month period, are minimum requirements for adequate

storage of standard solutions (Wells et al., 1992). For BDE 209, it is recommended to store the solutions at +4 °C. For dilutions, it is recommended to work with volumes sufficiently large to minimise the accuracy errors. When working with organic solvents, all volumes should be controlled by weight to avoid errors due to change in the ambient temperature (de Boer et al., 1996).

8.1.2. Internal and syringe standards

A known amount of internal standard (IS) added at the beginning of the procedure, and/or a syringe standard (SS), added to the final extract before the injection, should always be used to compensate for the losses throughout the analytical procedure and for inter-injection fluctuations. Extensive sample preparation, which is often needed, also demands for this approach. Depending on the target compounds, several IS or SS can be applied. A suitable IS or SS should not be present in the sample, should combine chromatographic and physical properties similar to those of the analytes and should not co-elute with the analytes or with compounds suspected to be present in the samples (such as PCBs). Table 8 lists the most common IS and SS used for the determination of BFRs. Some BDE congeners (such as BDE 77, 116 and 126) are most likely to combine all the above mentioned characteristics (de Boer and Cofino, 2002). Preferably, the selected IS should be a ^{13}C-labelled analogue, but this limits the choice of detection to EI-MS (except for BDE 209, see below). Although more native BDE congeners are

Table 8
Internal and syringe standards in BFR analysis (indicative non-limiting list)

Unlabelled		$^{13}C_{12}$-labelled	
Compound	Reference	Compound	Reference
Internal standards (IS)			
BDE 49	Schröter-Kermani et al., 2000	BDE 28	Ryan and Patry (2000); Norstrom et al., 2001
BDE 71	Christensen et al., 2001	BDE 47	Ryan and Patry (2000); Covaci et al., 2002a
BDE 85	Darnerud et al., 1998	BDE 77	Meironyté et al., 1999; Thomsen et al., 2001b
BDE 119	Schröter-Kermani et al., 2000	BDE 99	Ryan and Patry (2000); Covaci et al., 2002a
BDE 128	Sjödin et al., 2001	BDE 100	Norstrom et al., 2001
BDE 138	Hagmar et al., 2000; Jakobsson et al., 2002	BDE 126	Norstrom et al., 2001; Easton et al., 2002
BDE 140	Schröter-Kermani et al., 2000	BDE 153	Ryan and Patry (2000); Covaci et al., 2002a
BB 77	Thomsen et al., 2001b	BDE 154	Ryan and Patry (2000); Ohta et al., 2002
BB 103	Voorspoels et al., 2002	BDE 183	Ryan and Patry (2000); Norstrom et al., 2001
BB 209	de Boer et al., 2001; Booij et al., 2002	BB 157	Öberg et al., 2002
CB 53	Sellström et al., 1993; Allchin et al., 2001	CDE 77	Alaee et al., 2001a
CB 112	de Boer et al., 2001	CDE 105	Alaee et al., 2001a
CB 155	Allchin and de Boer, 2001	CDE 128	Alaee et al., 2001a
CB 198	Allchin and de Boer, 2001; Law et al., 2002	CDE 156	Alaee et al., 2001a; Dodder et al., 2002
CB 204	Hale et al., 2001	CDE 170	Alaee et al., 2001a
CB 207	Christensen et al., 2002	CDE 194	Alaee et al., 2001a; Dodder et al., 2002
Dechlorane 603	Sellström et al., 1993	CB 153	Haglund et al., 1997
QCB[a]	Hale et al., 2001	CB 180	Burreau et al., 2000
		CB 209	Hardell et al., 1998; Meneses et al., 1999
Syringe standards (SS)			
BDE 15	de Boer and Cofino, 2002	BDE 28	de Boer and Cofino, 2002
BDE 138	de Boer and Cofino, 2002	BDE 47	de Boer and Cofino, 2002
BDE 190	de Boer and Cofino, 2002	BDE 77	Alaee et al., 2001a; Christensen and Platz, 2001
TBB[b]	Thomsen et al., 2001b	BDE 99	de Boer and Cofino, 2002
BB 209	de Boer and Cofino, 2002	BDE 126	de Boer and Cofino, 2002
CB 30	de Boer and Cofino, 2002	BDE 153	de Boer and Cofino, 2002
CB 112	de Boer and Cofino, 2002	BDE 154	de Boer and Cofino, 2002
CB 198	de Boer and Cofino, 2002	BDE 183	de Boer and Cofino, 2002
CB 200	de Boer and Cofino, 2002	BDE 209	Christensen and Platz, 2001
CB 204	de Boer and Cofino, 2002; Hale et al., 2001	CDE 77	de Boer and Cofino, 2002
CB 209	de Boer and Cofino, 2002	CDE 156	de Boer and Cofino, 2002
3-MSF-CB174	de Boer and Cofino, 2002	CDE 194	de Boer and Cofino, 2002; Huwe et al., 2002
Aldrin	de Boer and Cofino, 2002	CB 111	de Boer and Cofino, 2002
Dechlorane 603	Sellström et al., 1998	CB 153	Easton et al., 2002
TCT[c]	de Boer and Cofino, 2002	CB 180	de Boer and Cofino, 2002
TBP[d]	Thomsen et al., 2001b	CB 194	de Boer and Cofino, 2002
		OCDD	de Boer and Cofino, 2002

[a] Pentachlorobenzene.
[b] 1,3,5-Tribromobenzene.
[c] Tetrachloro-*p*-terphenyl.
[d] 2,4,6-Tribromophenol.

available, the choice of appropriate IS may still be very problematic and is highly dependent on the detection method used (de Boer and Cofino, 2002). The use of BDE 138 should be discouraged, since it has been shown that the technical mixture Bromkal 70-5 DE contains this congener (Sjödin et al., 1998). Alternatively, PBBs that are not present in the Firemaster® mixtures can be used. Covaci et al. (2002b) have used BB 80, 103 and 155 as IS for each level of bromination of PBDE homologues. BB 209 has been proposed as IS for the determination of BDE 209 in sediments. Although, BB 209 was produced commercially until 2000 in small amounts in France (de Boer et al., 2000), it could not be identified in a large number of sediment and biota samples from the Netherlands (de Boer et al., 2003). Alternatively, the use of ^{13}C-BDE 209 as IS for BDE 209 analysis by ECNI is possible, when ions 484.7/486.7 and 498.7/500.7 are monitored for BDE 209 and ^{13}C-BDE 209, respectively.

8.2. Analytical procedure

Because method validation (including all essential steps) is matrix-specific, it should be performed for each compound and matrix to be investigated. For the analysis of BFRs, this validation should also include the cleanup and lipid determination, since excessive cleanup is often needed.

8.2.1. Extraction/cleanup/detection

The efficiency of the cleanup method can be considered sufficient if recovery of the analytes is sufficiently high (>70%, preferably >90%), if the obtained chromatograms have a good baseline, peak shape, resolution and if compounds which might deteriorate the column are not present in the extract. Suitable column dimensions (length: 25–50 m; internal diameter $\leq$ 0.25 mm) should ensure a sufficient resolution of BFRs. Relatively high selectivity is obtained using mass spectrometric detection systems, both in EI or ECNI mode. Monitoring the ion intensity ratios in EI-MS provides a supplementary assurance on identification. If the isotopic ratio of the quantitation and confirmation ion differ more than 15% (Hamm et al., 2001; Dodder et al., 2002; Luross et al., 2002) or 20% (Covaci et al., 2002a), the obtained data is questionable and should not be used. For ECNI-LRMS, relatively good selectivity is obtained by measuring the bromine trace (m/z=79 and 81) and possible co-elutions are limited to compounds yielding bromine fragments upon ionisation.

8.2.2. Recovery

The performance of the analytical method should be assessed by measuring the recovery of all analytes and internal standards after extraction, cleanup and any other sample pretreatment steps in relation to a volumetric injection standard added just before final GC analysis. The most frequently used recovery standards are given in Table 8. Absolute recoveries between 70% and 120% are acceptable for PBDEs (Dodder et al., 2002). The recovery can be influenced by a variety of parameters, like adsorption to glass (higher brominated BDEs), UV-degradation (BDE 209) or losses caused by evaporation (BDE 28). Some of these losses can be minimised by using amber glassware and limiting the daylight exposure time of extracts to a minimum. Recovery measurements of spiked samples (especially solid samples, such as sediments and sewage sludge) may be higher than the "real recovery" of the analytes in real samples due to the possible easier extraction from these spiked samples, since they are not incorporated in the matrix.

8.2.3. Procedural blanks

The use of plastics should be reduced to a minimum in the determination of BFRs, since they can contain a wide range of these compounds. Moreover, significant concentrations of BDE 47 and BDE 99 have been identified in laboratory air (Thomsen et al., 2001a). This problem can be solved by analysing procedural blanks and by correcting the concentrations found in the samples, if necessary. However, this method can only be applied if the blank values are relatively constant. Dodder et al. (2002) applied a correction for procedural blanks if the blank value was between 10% and 20% of the measured value in the sample. If the procedural blank value was less than 10%, no corrective action was taken. Data was excluded from further consideration if the blank value exceeded 30% of the sample measurement. Other corrections for procedural blanks found in literature consist of a subtraction of the average plus three times the standard deviation (Stapleton and Baker, 2001).

8.2.4. Limits of detection and quantification

The limit of detection (LOD) and the limit of quantification (LOQ) as defined by IUPAC (1976), may be estimated in different ways. The first manner is by extrapolating the concentration that would result in a signal-to-noise ratio (S/N) of 3 and 10 for LOD and LOQ, respectively. Estimating LOD and LOQ using standard solutions should be discouraged, while extrapolating results for low-level contaminated or spiked samples is recommended. In other cases, LOQs are estimated from blank samples. Quantification is only performed if the analyte response was at least twice the blank sample value (Sjödin et al., 1999). Therefore, LOQ is directly related to the blank sample levels of each compound. Sjödin et al. (2001a,b) and Jakobsson et al. (2002) have set the LOQ at five times the blank level, if blank interference was present. This correction method resulted in many unusable data, since blank interference raised the LOQ to higher levels than those present in most samples. It must be clearly stated that LOD and LOQ are highly dependent on the sample amount used.

8.2.5. Matrix effects and washing procedures

Thomsen et al. (2002a) have observed that the analyte response can be affected by the matrix residues that are

present in the extract. This is probably caused by an increased protection of the analytes from adsorption in the injector when matrix residues are present in the extract and results in more analytes being introduced into the chromatographic column. Sellström et al. (1998) have reported a similar effect for fish tissue for which the recovery of BDE 209 was higher when a matrix was present. If relative recoveries from spiked samples are acceptable and not statistically different from recoveries calculated from standard solutions, calibration curves can be made from standard solvent-based solutions (Thomsen et al., 2002a). If not, the matrix effect should be investigated and if pronouncedly present, calibration curves should be established by means of spiked samples.

Thomsen et al. (2001a,b, 2002a) reported that all glassware was washed in 2.5% RBS 25 foaming cleaner, rinsed with distilled water, and subsequently heated at 450 °C for 4 h. Pre-cleaning with an organic solvent (e.g. hexane or toluene) may further improve the washing procedure. Glassware that cannot be washed after use, e.g. Soxhlet coolers, should be thoroughly rinsed with one or more organic solvents to prevent cross-contamination (QUASIMEME, 2002).

8.3. System performance

The precision of an analytical method (ISO, 1989) may be considered at three levels: repeatability (same operating conditions over a short time), intermediate precision (within-laboratory variation) and reproducibility (precision between laboratories). For the determination of BFRs, the addition of IS, evaporation steps, extract handling and standard solution stability are critical steps. The imprecision level is highly dependent on the analyte and ranges between 10% and 20% for tri- to hepta-BDEs and is around 25% for BDE 209 and HBCD. The first world-wide interlaboratory studies on BFRs showed that laboratories should improve their method precision (de Boer and Cofino, 2002; de Boer et al., 2002).

The method stability and reliability (maintenance of its performance over time) should be assessed through regular analysis of standard solutions, procedural blanks, duplicate samples and laboratory reference materials (LRMs), used for construction of control charts.

The accuracy (ISO, 1989) is related to a value that is accepted either as a conventional true value (using spiked samples) or an accepted reference value (using certified reference materials—CRMs). Unfortunately, CRMs are not yet available for PBDEs, but this is foreseen in the near future. Two reference matrices (fish tissue and sediment) will become available through financing of the European Communion (BROC Workshop, 2002). Until then, lake trout (available from Cambridge Isotope Laboratories) used in the second inter-laboratory study on BFRs can be used as reference material by the participating laboratories. Results can be compared with the mean values obtained by the different participating laboratories (informational or consensus value).

8.4. First and second world-wide interlaboratory study on PBDEs

The participation in interlaboratory tests facilitates the evaluation and assessment of the overall method performance. More details of analytical methods were collected during the first and second international interlaboratory study (ILS) on BFRs (de Boer and Cofino, 2002; de Boer et al., 2002). In both studies, biota and sediment samples, together with standard solutions containing unknown concentrations of BFRs were provided to the participants. A wide variety of extraction and cleanup methods, injection techniques, oven programmes and IS were applied by the participants. In general, results for BDE 47 were satisfactory with a range of relative standard deviations (RSD) of 17–40%. Results for BDE 99 were worse than for BDE 47 in the first ILS, but a substantial improvement has been observed in the second ILS. The results of the BDEs 100, 153 and 154 (RSD 25–48%) were acceptable given their generally low concentrations. The analysis of BDE 209 is not under control by the majority of the participating laboratories. The RSD values for the three sediment samples analysed in these two studies varied between 48% and 78%. The RSD values obtained for the biota samples were even larger. However, it will be very important to establish if BDE 209 is present at all in the biota samples. Further improvements in cleanup and GC analysis, as discussed in this paper, will be necessary. TBBP-A and HBCD were included in the last interlaboratory study, but only a few data sets were received, as most participating laboratories were still in the process of setting up methods for these BFRs in their laboratories.

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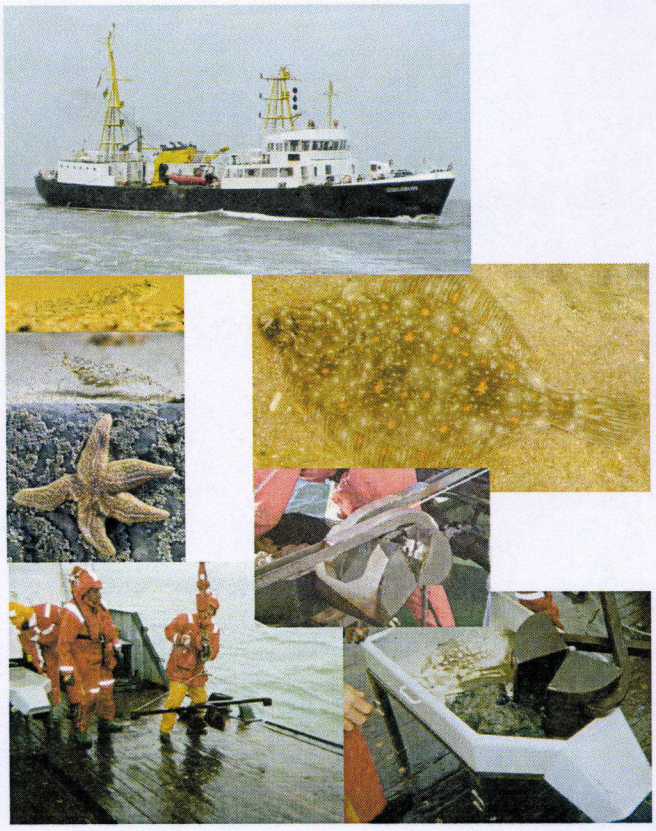
3

PBDEs in the Belgian aquatic environment



3.1 Introduction

The seas, oceans and sediments, particularly estuarine sediments, are the major sinks of persistent organic pollutants, including PBDEs, in the aquatic environment (Allchin et al., 1999; de Boer et al., 2001). The water solubility and vapour pressure of PBDEs are very low causing them to be adsorbed rapidly onto solid particles of sediment and soil when released into the environment (Watanabe and Sakai, 2003; Kuosmanen et al., 2002). The study of sediments is an important step in mapping pollution distribution and possible exposure pathways to various aquatic organisms, since contaminants in the sediments can still be bioavailable to sediment-dwelling organisms (Pruell et al., 1993; Reid et al., 2000).
This chapter focuses on PBDE levels and distribution in the Belgian aquatic environment. Although Belgium is a rather small country, it is highly trafficked and densely populated, which makes it an area of a high potential pollutant input. This raises interest regarding pollution research. Therefore, both sediment and biological samples from this area were investigated.

In ***Paper II***, a rapid and simple method for the determination of PBDEs in sediments was optimised and applied to two sediment cores from the river Scheldt near Antwerp. Such sediment cores are interesting study objects that contain temporal information on PBDE levels; based on the sedimentation speed and the sampling depth, slices can be dated and historical deposition of PBDEs in the riverbed can be studied. In our study, PBDEs were mainly present in the top-layer of the core, which is in accordance with the increase in the production and use of PBDEs in the last decades and with the high PBDE levels observed in surface sediments from other PBDE-polluted rivers, such as the river Tees, UK (Allchin et al., 1999). The intensive use of DecaBDE technical mixture was evidenced by the high concentrations of BDE 209.

Paper III had a closer look at the PBDEs in sediments of the Belgian North Sea (BNS), the Western Scheldt Estuary (SE) and a selection of freshwater inland watercourses located in the Scheldt basin. These locations are of high interest because the drainage basin of the river Scheldt covers a very densely populated and highly industrialised area of Northern France, Western Belgium and the South-Western Netherlands (Baeyens et al., 1998). It is expected that the input of pollutants in this river is substantial. Additional interest is generated by the fact that the Scheldt discharges into the North Sea, which is one of the most densely trafficked seas and furthermore a very extensively exploited fishery region. Pollutants, such as PBDEs, coming from the Scheldt will be dispersed in the narrow southern North Sea basin, where they can accumulate.
Our analyses revealed that PBDE concentrations in the BNS were low and that many measurements were below the LOQ for most congeners, except BDE 209, which was present in more than 80 % of the samples. Furthermore, BDE 209 was the most abundant

congener in most sediments that were analysed. This is in accordance with the results of ***Paper II***. Concentrations around harbours were found to be tenfold higher than at the other locations in the BNS. Sediments upstream the SE were approximately 50 times higher than at downstream locations, pointing at PBDE input more upstream. When looking at the freshwater inland locations, these sediments were more polluted with PBDEs than the samples from the BNS and SE. Such high concentrations in inland watercourses were also observed by others and is likely related to the heavy industrialisation and dense population of this region and thus caused by the high impact of human activity (Christensen and Platz, 2001). Additionally, several pollution hotspots with elevated PBDE-levels at several inland locations were encountered.

The combined results of ***Paper II*** and ***Paper III*** indicate that the most common used sampling technique for sediment, a Van Veen surface sediment sampler, is not the technique of choice when targeting PBDEs. Due to the sampling depth of the grabber (approximately 20 cm), the top-layer (1-2 cm) of the sediment is diluted to a too great extent, which has lead to a high number of non-detects in the samples from the BNS and to relatively low levels for the other locations. To ensure that only the top layer of the sediment is sampled, preferentially a box-corer sampler should be used. Unfortunately, results of ***Paper II*** were not yet available when the sediments of ***Paper III*** were sampled. The use of the Van Veen sampler in ***Paper III*** explains why the levels in the sediments were significantly lower than those in ***Paper II***, although the samples of both studies were sampled at neighbouring locations in the SE. However, sampling by means of a Van Veen surface sediment sampler does only influence total levels. PBDE patterns are not altered and can still provide very useful data.

Paper IV covers the biological part of the PBDE pollution in both BNS and SE. As mentioned before, the physico-chemical properties of PBDEs lead to bio-concentration and biomagnification, as they are lipophilic and extremely resistant to degradation (Gustafsson et al., 1999). The toxicological aspect together with steadily increasing environmental levels make it likely that also (marine) species might experience adverse effects from these compounds (Darnerud, 2003; Lebeuf et al., 2006; Timme-Laragy et al., 2006). Because aquatic organisms are also subjected to bio-concentration in addition to bioaccumulation through the diet, they are very efficient in accumulating these compounds (Hale et al., 2001). Several benthic species (invertebrates and fish) were sampled at the same locations in the BNS and the SE as in ***Paper III***. Since benthic organisms are at the base of the marine food chain (e.g. goby, starfish, shrimp, etc.) and are also consumed by humans (e.g. crabs, shrimp, dab, sole, etc.), studies on these organisms can be used to obtain valuable information about the availability of these pollutants to higher trophic levels. Benthic organisms are known to be very sedentary, which makes them very good sentinel species. This data can therefore also be used to establish geographical trends in pollution. In analogy with the results of ***Paper III***,

levels of PBDEs were significantly higher in the SE than in the BNS. Furthermore, concentrations were inversely correlated with the distance to Antwerp, pointing at a substantial PBDE input further upstream the river.

Contrasting the results of the sediments, BDE 209 was generally not present in the biological samples, except in eight pooled fish liver samples. It needs to be mentioned that the levels of BDE 209 in the biological samples were very low. Nevertheless, the presence of this congener is remarkable because it is believed not to bio-accumulate (See *1. Introduction and background information*). At the time this study was performed, only very few studies were published demonstrating that aquatic biota are able to accumulate BDE 209 (Lepom et al., 2002).

3.2 Paper II

Polybrominated diphenyl ethers, polychlorinated biphenyls and organochlorine pesticides in sediment cores from the Western Scheldt river (Belgium): Analytical aspects and depth profiles

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Polybrominated diphenyl ethers, polychlorinated biphenyls and organochlorine pesticides in sediment cores from the Western Scheldt river (Belgium): analytical aspects and depth profiles

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Abstract

A rapid and simple analytical method for the determination of organochlorines, such as polychlorinated biphenyls (PCBs) and selected organochlorinated pesticides (OCPs) and organobromines, such as polybrominated diphenyl ethers (PBDEs), in sediment samples was optimised using CRM 536 (PCBs in freshwater sediment). The method involved a hot Soxhlet extraction that reduced the extraction time to 2 h. Elemental sulphur, which is present in sediments and may interfere during the analysis, was removed by means of copper powder added to the sediment during extraction and into the clean-up cartridge. The analysis of PCBs and OCPs was accomplished by gas chromatography with electron capture or mass spectrometric detection. Similar quantitative results for PCB congeners in CRM 536 were obtained using a 50-m capillary column and a 10-m narrow bore column suited for fast analysis. The analysis of PBDEs was done by mass spectrometry in negative chemical ionisation mode.

Concentrations of organic pollutants in two sediment cores (approximately 50 cm depth) from the Scheldt river (south of Antwerp, Belgium) showed a relative steady state for PCBs and DDTs, with a slight decrease in the top layers, suggesting a slight decline in their concentrations due to restrictions in their usage. On the contrary, PBDEs were showing an increase in their concentrations in the top layers (up to 270 and 8400 ng/g dry weight for sum of tri- to hexa-BDE congeners and for BDE 209, respectively). This suggests an increasing trend in the concentrations of PBDEs in the Belgian environment.
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Keywords: Sediment core; Scheldt river; PBDEs; PCBs; OCPs; Method optimisation

1. Introduction

Despite their ban or restricted use (UNEP, 2003), polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) are among the most prevalent environmental pollutants and can be found in various environmental compartments, as well abiotic (air, water, sediments, soil) (de Boer et al., 2001) as biotic (from plankton to humans) (de Boer et al., 2000; de Voogt et al., 1990; Jones and de Voogt, 1999).

In contrast to PCBs, the use and production of polybrominated diphenyl ethers (PBDEs) is still being continued, although recently some restrictions have been issued following risk assessment reports (EU, 2003). PBDEs are used as flame retardants to improve fire safety in both commercial and domestic applications, where they are sometimes added in concentrations up to 30% by weight (WHO, 1994). These chemicals have shown a rise in production since they were first introduced in the 1960s, with a substantial increase since the end of the 1970s due to

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the growing popularity of personal computers and other electronic equipment (de Boer et al., 2000). Unfortunately, this has lead to their widespread presence in various environmental compartments (de Boer et al., 2001; de Wit, 2002; Ikonomou et al., 2002; Law et al., 2003).

Sediments, particularly estuarine sediments, are one of the major sinks for these contaminants in the aquatic environment (Allchin et al., 1999; de Boer et al., 2001; Voorspoels et al., 2004). The study of sediments is an important step in mapping possible exposure pathways to various aquatic organisms, since contaminants in the sediments may be bioavailable to sediment dwelling organisms (Pruell et al., 1993). Furthermore, dated sediment profiles have been used to estimate the historical deposition of PCBs and other organic pollutants in the river bed impacted by local and regional sources (Fox et al., 2001).

The drainage basin of the Scheldt river covers a very densely populated and highly industrialised area of northern France, western Belgium and the southwestern Netherlands (Baeyens et al., 1998), resulting in a high pollution level with POPs (Voorspoels et al., 2003, 2004), as well as heavy metals (Coteur et al., 2003). High concentrations of PBDEs (up to 500 ng/g dw for BDE 209) were already measured in sediment samples from the Scheldt river (de Wit, 2002), while de Boer et al. (2003) have reported high levels of BDE 209 (up to 4600 ng/g dry weight) in suspended particulate matter (SPM) from the Western Scheldt and attribute this to spillage during use further upstream.

The scope of this work was (1) to optimise a rapid and simple analytical method for the determination of organochlorinated and organobrominated pollutants in sediments and (2) to measure the concentrations of organic pollutants in two sediment cores (~50 cm) from the Scheldt river, near Antwerp, Belgium and to evaluate temporal trends in pollution of PCBs, OCPs and PBDEs.

2. Materials and methods

2.1. Samples

Sediment cores (~50 cm) were taken in March 2000 with a 2-cm-diameter gravity corer from two locations situated in the Scheldt river (near Kruibeke, south of Antwerp, Belgium) from zones with different flow characteristics. The two locations (A and B) were located within 100 m from each other. Subsamples were obtained by slicing the core in 1–5 cm intervals. Wet sediments were kept at +4 °C until further treatment. Prior to analysis, sediments were dried at room temperature for 48 h.

2.2. Reagents and standards

The following compounds were included: hexachlorobenzene (HCB), α-, β- and γ-hexachlorocyclohexane (HCH) isomers (the sum expressed as HCHs), *p,p'*-DDE, *p,p'*-DDD, and *p,p'*-DDT (the sum expressed as DDTs) and 27 PCB congeners (IUPAC nos.: 18, 28, 31, 44, 52, 74, 95, 99, 101, 105, 110, 118, 128, 132, 138, 149, 153, 156, 163, 170, 177, 180, 183, 187, 194, 196 and 199). PCBs 46 and 143 were used as internal standard (IS) for PCBs and pesticides. Additionally, 10 PBDE congeners were also measured and polybrominated biphenyl (PBB) 103 was used as IS for BDE 28, 47, 66, 85, 99 and 100, BB 155 was used as IS for BDE 138, 153 and 154, while ^{13}C-labeled BDE 209 was used as IS for BDE 209.

All solvents used for the analyses (*n*-hexane, acetone, dichloromethane and iso-octane) were of SupraSolv grade (Merck, Darmstadt, Germany). Individual analytical standards (CIL, Andover, MA, USA; Dr. Ehrenstorfer Laboratories, Augsburg, Germany) were used for identification and quantification. A certified material (CRM 536—PCBs in freshwater sediment—IRMM, Geel, Belgium) was used for the method optimisation.

2.3. Extraction and clean-up

The sediment sample (around 1 g) was placed into a hexane pre-washed extraction thimble. A triple amount of copper powder (<63 μm, Merck) was added and mixed with the sediment, followed by the addition of internal standards. Samples were extracted using a Soxhlet extractor B-811 (Büchi, Switzerland) operated in hot extraction mode. Several solvent mixtures (75 ml), such as hexane/acetone (3:1, v/v), hexane/acetone (1:1, v/v) and hexane/dichloromethane (3:1, v/v) were tested for different extraction times (2, 3 and 4 h). In each case, the extract was concentrated and transferred onto a solid-phase extraction (SPE) cartridge filled (from the bottom) with 8 g acidified silica (40% concentrated sulphuric acid, w/w), 0.5 g anhydrous sodium sulphate and 1 g copper powder. The elution of PCBs, OCPs and PBDEs was done with 15 ml hexane and 10 ml DCM. The eluate was concentrated to near dryness by a rotary evaporator and further by a gentle nitrogen stream. The final extract was re-solubilized in 80 μl iso-octane.

2.4. Instrumental analysis

The determination of PCBs and OCPs was performed by gas chromatography with electron capture detection (GC-ECD). An Agilent (Palo Alto, CA, USA) 6890 GC-μECD was equipped with a 50 m×0.22 mm×0.25 μm HT-8 capillary column (SGE, Zulte, Belgium). Helium was used as carrier gas at a constant flow of 1.0 ml/min and Ar/CH_4 (95:5) was used as make-up gas (40 ml/min). One microliter was injected in the pulsed splitless mode (pulse pressure=40 psi, pulse time=1.2 min) with the split outlet opened after 1.2 min. Injector and detector temperatures were set at 290 and 320 °C, respectively. The temperature program of the HT-8 column was set to 90 °C

for 1.2 min, then increased with 20 °C/min to 180 °C, kept for 1 min, then increased with 3 °C/min to 275 °C, kept 0.5 min and further ramped by 5 °C/min to 290 °C and kept for 18 min.

Additionally, an Agilent 6890GC-5793 quadrupole mass spectrometer (MS) equipped with a 10 m×0.10 mm×0.10 μm HT-8 narrow bore capillary column was tested for its ability of performing fast analysis of PCBs. The oven program started from 90 °C, for 1 min, then increased with 50 °C/min to 200 °C, kept for 0.5 min, then increased with 25 °C/min to 250 °C, kept for 0.2 min and finally ramped by 75 °C/min to 300 °C and kept 5 min. Total analysis time was 11 min. Helium was used as carrier gas at 0.4 ml/min. The MS was operated at 70 eV in electron impact ionisation (EI) mode with ion source, quadrupole and transfer line temperatures of 230, 150 and 300 °C, respectively. Dwell times were set to 10 ms and two ions from the molecular ion cluster of each PCB homologue group (M^+ and $[M+2]^+$) were monitored. The relative retention times to the nearest internal standard and ratios between the monitored ions were used as identification criteria. A deviation of ion ratios of less than ±20% from the theoretical value was considered acceptable for identification.

The determination of PBDEs was performed with an Agilent 6890GC-5973MS equipped with a 25 m×0.22 mm×0.25 μm HT-8 capillary column and operated in electron capture negative ionisation (ECNI) mode. The ion source, quadrupole and interface temperatures were 250, 150 and 300 °C, respectively. Helium was used as carrier gas at constant flow (1.0 ml/min) with an initial pressure of 14.40 psi and with methane as moderating gas. The MS was operated in the selected ion monitoring (SIM) mode and the electron multiplier voltage was set at 2100 V. One microliter of the extract was injected in solvent vent mode (injector temperature at 90 °C, kept for 0.05 min, then increased with 700 °C/min to 280 °C, vent time 0.03 min, vent flow 100 ml/min). The splitless time was 1.50 min. The temperature of the HT-8 column was programmed from 90 °C, kept for 1.5 min, then increased with 30 °C/min to 180 °C, kept for 0.5 min, then increased with 5 °C/min to 270 °C, kept for 0.5 min and finally ramped with 25 °C/min to 290 °C and kept for 15 min. Dwell times were set to 25 ms. Ions *m/z* 79 and 81 were monitored for the entire run.

The analysis of BDE 209 was performed on a 12 m×0.18 mm×0.10 μm AT-5 capillary column (Alltech, Lokeren, Belgium). Helium was used as carrier gas at an initial flow rate of 1.0 ml/min (kept for 5 min) and then raised to 1.5 ml/min at 20 ml/min^2. The oven temperature program started from 90 °C, kept for 1.35 min, and then increased with 25 °C/min to 290 °C, kept for 8 min. Methane was used as moderating gas and the ion source, quadrupole, and interface temperatures were 250, 150 and 300 °C, respectively. Dwell time was set to 50 ms. The ECNI-MS was operated in SIM mode at the *m/z* 484.7, 486.7 and 494.7, 496.7 for BDE 209 and ^{13}C-BDE 209, respectively.

2.5. Quality control

Quality for PCB and OCP analyses was assured through the analysis of procedural blanks, blind duplicate samples and certified material (CRM 536—PCBs in freshwater sediment). The obtained values for PCB congeners in CRM 536 were all within 10% of the certified values.

For PBDEs, an in-house reference material (a sediment sample used for the BROC interlaboratory feasibility study—BROC, 2003) was used for method validation. The concentrations of individual PBDE congeners did not differ with more than 15% from the mean values obtained in the BROC study (BROC, 2003). Recoveries of individual PBDE congeners, including BDE 209, obtained during BROC spiking experiments (BROC, 2003) are between 82% and 93% with a standard deviation less than 16%.

The limit of quantification (LOQ) for PCBs, OCPs and PBDEs was based on GC/ECD and GC/MS performance and on laboratory background levels, which were determined by analysing procedural blanks. Procedural blank levels were consistent (RSD<30%) and therefore the median blank value was used for subtraction. LOQs were established at three times the standard deviation of the procedural blank level, resulting in a certainty of more than 95% for results given for the samples. LOQs for PCBs and OCPs ranged between 0.1 and 0.4 ng/g when 1 g of sediment was analysed. Recoveries of internal standards were between 86% and 92% (RSD<9%). LOQs for tri- to hexa-PBDEs ranged between 0.05 and 0.1 ng/g dw, while for BDE 209 LOQ was 0.2 ng/g dw.

Additionally, the Toxicological Centre (University of Antwerp-UA, Belgium) has participated to several interlaboratory studies for PCBs, OCPs and PBDEs organised through the Quasimeme office. Sediment samples analysed for PCBs and OCPs (Table 1) and PBDEs (Table 2) showed good agreement between values obtained by UA and mean values of participating laboratories.

Table 1
Concentrations (ng/g dw) of PCBs and OCPs in two sediment samples (68MS and 69MS) used in the QUASIMEME 2002 interlaboratory test (UA—results of Toxicological Center, University of Antwerp versus mean of participating laboratories)

	68MS		69MS	
	UA	mean	UA	mean
PCB 28	0.20	0.21	1.30	0.90
PCB 101	0.50	0.56	1.10	1.37
PCB 105	0.18	0.17	0.53	0.44
PCB 118	0.48	0.43	1.30	1.36
PCB 138	1.15	1.23	2.65	2.71
PCB 153	1.28	1.21	2.98	2.71
PCB 180	0.87	0.72	1.59	1.59
p,p'-DDE	0.78	0.65	0.66	0.59
p,p'-DDT	0.37	0.30	0.76	0.41
HCB	0.17	0.11	1.36	0.94

Table 2
Concentrations (ng/g dw) of PBDEs in three sediment samples (1MS, 3MS and BROC) used in the QUASIMEME 2002 and 2003 interlaboratory tests (UA—results of Toxicological Center, University of Antwerp versus the mean of participating laboratories)

	1MS		3MS		BROC	
	UA	Mean	UA	Mean	UA	Mean
BDE 28	0.43	0.54	0.13	0.13	0.75	0.626
BDE 47	9.64	8.93	0.93	0.96	10.20	10.14
BDE 99	12.71	13.18	0.98	1.05	12.29	14.20
BDE 100	3.00	2.65	0.11	0.12	2.90	3.04
BDE 153	2.31	1.80	0.23	0.21	1.85	1.93
BDE 154	1.89	1.64	0.11	0.11	1.59	1.71
BDE 183	1.01	0.32	0.18	0.14	0.75	0.45
BDE 209	920	863	47.0	45.2	1234	1163

2.6. Statistical analysis

Statistical analysis was performed with SPSS 12.0 statistical software (Chicago, IL, US). The influence of extraction time and solvent mixtures on the recovery of PCBs from CRM 536 was analysed with univariate analysis of variance (ANOVA) using general linear model and Scheffe test for post hoc comparison. Values were considered significantly different when $p<0.05$.

Principal components analysis (PCA) was performed with Statistical software Minitab Version 14.10 (Minitab Quality Plaza, 1829 Pine Hall Road, State College, PA 16801-3008, USA) using the correlation matrix. Results have been graphically displayed as score plot and loading plot.

3. Results and discussion

3.1. Method optimisation

The method described in the present paper was adapted from a previously described method for the extraction of organohalogenated pollutants from soils (Covaci et al., 2002).

For method optimisation, a certified material CRM 536 (PCBs in freshwater harbour sediment) was used and concentrations obtained during different method setups were compared with the certified values. For extraction, an automated Soxhlet was used in hot extraction mode, where the sample is permanently in contact with hot, but non-boiling solvent. No significant differences were observed between the PCB concentrations obtained from the certified material CRM 536 for different extraction times (2, 3 and 4 h, respectively), allowing thus a dramatic reduction in the extraction time compared with the 8–16 h usually needed for classical Soxhlet extraction (Smedes and de Boer, 1997).

Several solvent mixtures were tested for the extraction efficiency and it has been found that the mixtures hexane/acetone (3:1, v/v) and hexane/acetone (1:1, v/v) gave similar recoveries for the certified PCBs, while the use of a mixture hexane/dichloromethane (3:1, v/v) resulted in significantly lower values (Fig. 1). In order to increase the extraction efficiency, it is necessary to use a more polar solvent, such as acetone, which can penetrate the more polar core of sediment particles. A similar observation has already been reported for soil (Covaci et al., 2002). The hexane/acetone (3:1, v/v) mixture was preferred because the lower percentage of acetone in the extraction mixture results in a final raw extract free of acetone, which is necessary for clean-up on acidified silica.

The sulphur originates under anaerobic conditions from microbial sulphate reduction (via sulphide) and the degradation of organic sulphur compounds (Riis and Babel, 1999). Smedes and de Boer (1997) have shown that the presence of elemental sulphur in the finale extract prior to GC analysis may have a bad effect on the performance of the ECD detector and on the correct peak assignment. For sulphur elimination, copper powder was added in the extraction thimble and on the top of the acidified silica gel cartridge, thus eliminating a tendentious additional step in the clean-up procedure. The sulphur removal by means of Cu was very efficient for all analysed samples and the

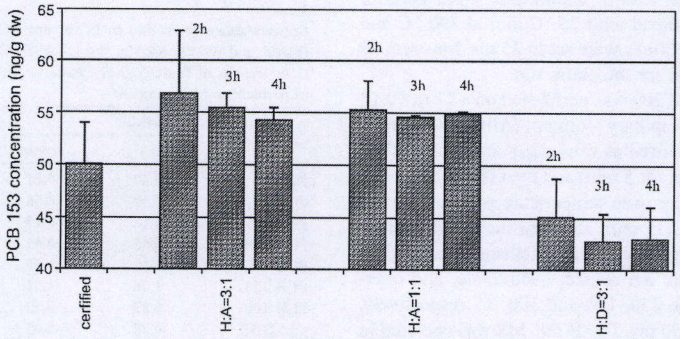

Fig. 1. Influence of extraction time and composition of extraction solvent mixture on the recovery of PCB 153 from CRM 536 (H—hexane, A—acetone, D—dichloromethane). Error bars represent 1.96× standard error.

Table 3
Recoveries of certified PCB congeners from CRM 536

	Certified values (S.D.)	Recovered values (S.D.)	%
PCB 28	44 (5)	48 (3)	110
PCB 52	38 (4)	41 (2)	109
PCB 101	44 (4)	48 (0)	110
PCB 105	3.5 (0.6)	3.8 (0.4)	110
PCB 118	28 (3)	27 (1)	96
PCB 128	5.4 (1.2)	4.6 (0.5)	86
PCB 138	27 (4)	33 (2)	122
PCB 149	49 (4)	52 (2)	118
PCB 153	50 (4)	57 (4)	114
PCB 156	3.0 (0.4)	3.6 (0.2)	120
PCB 163	17 (3)	18 (3)	105
PCB 170	13 (1)	13 (1)	96
PCB 180	22 (2)	22 (1)	101

reduction of elemental sulphur (which may be up to 7–10 mg/g sediment) was done to a level at which it did not interfere with the GC analysis. Riis and Babel (1999) have already demonstrated that Cu powder reacts with elemental sulphur if the contact time is more than 30 min. Furthermore, the use of Cu powder results in a higher reaction surface than in case of Cu granules or Cu wire. They have also shown that the commercially available Cu powder (<63 mesh, Merck) can be used directly, without any additional pretreatment. Furthermore, no adsorption of organohalogenated pollutants has been observed on the Cu powder during the present work, in accordance with observations by Riis and Babel (1999).

The use of acidified silica for clean-up resulted in cleaner extracts than silica or Florisil, but some OCPs, such as dieldrin, heptachlor and heptachlorepoxide, are completely destroyed by the acidic treatment. The use of basic silica (33% KOH, w/w) in combination with acid silica might improve the extract cleanliness, but this should be applied when only PCBs and PBDEs are the target analytes. Several other OCPs, such as HCHs and DDTs, are labile in basic conditions.

Due to some co-elutions on the HT-8 column when ECD was used (such as PCB 110/*o,p'*-DDD, PCB 132/*p,p'*-DDD, PCB 128/PCB 174, PCB 156/PCB 172), the use of a confirmatory analysis by GC/MS was mandatory. It was previously shown (Covaci and Schepens, 2001) that narrow bore capillary columns could be successfully used in the determination of PCBs by GC/MS. Therefore, a narrow bore column with a similar stationary phase as the one used in the GC/ECD was used on GC/MS. Results for the certified material CRM 536 obtained on both columns agreed satisfactory (difference less than 10%) for PCBs which did not present co-elutions on ECD (such as PCB 118, 153, 180). Moreover, these concentrations (Table 3) were in agreement with the certified values (>90% for most congeners).

3.2. Presence of organic pollutants in sediments from the Scheldt river

3.2.1. Levels of pollutants

OCP levels were low, with HCHs being below LOQ, HCB values between 0.5 and 1.3 ng/g dw and with DDTs between 6.6 and 44.4 ng/g dw. *p,p'*-DDE (41%) and *p,p'*-DDD (41%) were the principal contributors to the sum DDTs (Table 4) in all samples, except for A2, where *p,p'*-DDT was the principal component (37%).

The PCB concentrations in the upper layers of the two sediment cores are similar to levels (up to 200 ng/g dw) obtained in 1987–1988 from Western Scheldt estuary (Van Zoest and Van Eck, 1993), showing that the PCB pollution

Table 4
Concentrations (ng/g dry weight) of organochlorinated and organobrominated pollutants in sediment cores from two locations (A and B) from the Scheldt river

	Depth (cm)	PCB 153	Sum PCBs	Tri- to hexa-BDEs	BDE 209	HCB	*p,p'* -DDE	*p,p'* -DDD	*p,p'* -DDT	Sum DDTs	Sum HCHs
A1	0–1	23.5	183	85.1	2842	0.7	4.4	4.4	3.0	11.8	<0.3
A2	2–3	32.3	238	179	7412	1.2	6.7	6.5	7.9	21.0	<0.3
A3	4–5	22.0	167	29.7	NA	1.1	5.4	4.9	1.9	12.2	<0.3
A4	6–7	28.2	201	3.7	1736	1.1	5.5	5.7	3.1	14.3	<0.3
A5	8–9	14.8	105	2.2	653	0.3	3.2	3.2	0.2	6.6	<0.3
A6	10–15	24.3	201	4.8	401	1.2	5.1	5.7	2.4	13.3	<0.3
A7	20–25	28.9	235	32.2	1370	0.8	6.8	8.0	2.8	17.6	<0.3
A8	35–40	46.4	400	11.8	1165	1.1	11.4	10.9	5.2	27.6	<0.3
A9	45–50	23.6	192	1.4	332	0.4	5.2	4.9	2.6	12.7	<0.3
A10	55–58	38.1	310	2.9	913	1.0	10.0	8.4	5.0	23.4	<0.3
B1	0–1	27.9	232	272	8413	0.8	6.6	7.0	2.8	16.4	<0.3
B2	2–3	30.6	232	180	5580	1.3	5.9	5.8	3.1	14.9	<0.3
B3	4–5	25.8	206	171	NA	1.0	6.6	6.4	2.1	15.1	<0.3
B4	6–7	21.2	173	47.5	NA	0.6	4.9	5.9	1.5	12.4	<0.3
B5	10–15	23.4	198	4.3	469	0.7	5.1	6.6	1.8	13.4	<0.3
B6	20–25	19.0	151	2.0	315	0.5	3.8	4.9	0.8	9.4	<0.3
B7	35–40	36.6	311	1.4	515	0.8	8.1	4.1	3.3	15.6	<0.3
B8	45–50	26.5	242	2.5	668	0.7	7.2	7.4	2.3	16.9	<0.3

NA—not available due to insufficient sample.

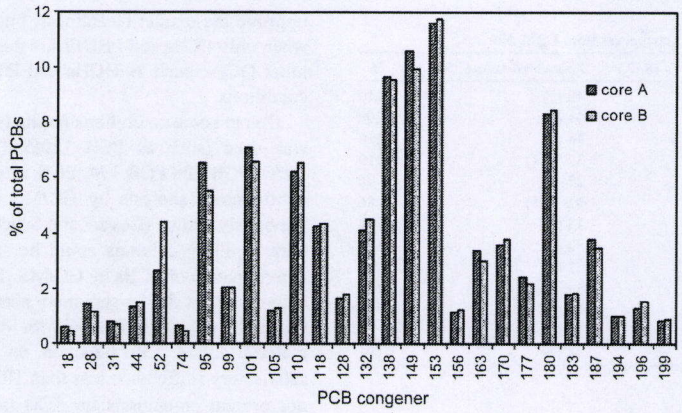

Fig. 2. Profiles of selected tri- to octa-CB congeners in two sediment cores from the Scheldt river.

has only slightly decreased in the last 15 years. This is probably due to the heavy industrialisation of the river's catchement area with continuous input of pollutants. The PCB concentrations found in the surface layers of the sediment cores were also similar to levels up to 120 ng/g dw found in sediments from the Amsterdam and Rotterdam harbour (de Boer et al., 2001). The PCB congener profile was similar between the two sediment cores (Fig. 2). Concentrations of tri- and tetra-CB congeners were low, while the predominant homologues were the penta- to hepta-CBs (Fig. 2), with a profile resembling to a mixture of Aroclor 1254 and 1260.

In the present study, PBDEs were also found in the sediment layers from the Scheldt river (1.4–270 ng/g dw for the sum of tri- to hexa-BDE congeners) (Table 4). The PBDE profile in these samples (Fig. 3) was very similar to the profile of penta-BDE technical mixture (Sjödin et al., 1998). High concentrations of BDE 209 (up to 8400 ng/g dw) were found also in the top layers of the cores from the present study (Table 4).

Previously, lower concentrations of tri- to hexa-BDE congeners (32 ng/g dw) and BDE 209 (1200 ng/g dw) were found in the Western Scheldt, near the Belgian–Dutch border (BROC, 2003). However, this sample was collected with a Van Veen grab sampler which mixes deeper sediment layers with surface layers. This results therefore in a "dilution" of pollutant's concentrations.

3.2.2. Core distribution of pollutants

The depth profiles of PCBs and DDTs were similar in the two sediment cores (depths of 58 and 50 cm, respectively), while the concentrations of PCBs and DDTs showed a slight, but non-significant increase with the depth (Table 4). This suggests that the pollution of the Scheldt river has only slightly decreased in the past years, a phenomenon which was already reported for other polluted rivers, such as the Rhine (de Boer et al., 2001). In contrast, PCB levels in cores from marine or lake sediments show an evident decline in the past years, since their sources are mainly from fall out and atmospheric deposition (Isosaari et al., 2002).

For each sediment layer, the ratios between the levels of p,p'-DDE and p,p'-DDD were close to the unit (1.01 ± 0.10 and 1.04 ± 0.39 for cores A and B, respectively). This indicates that aerobic and anaerobic degradations of p,p'-DDT occurred at equal rates. Similar values for the ratio

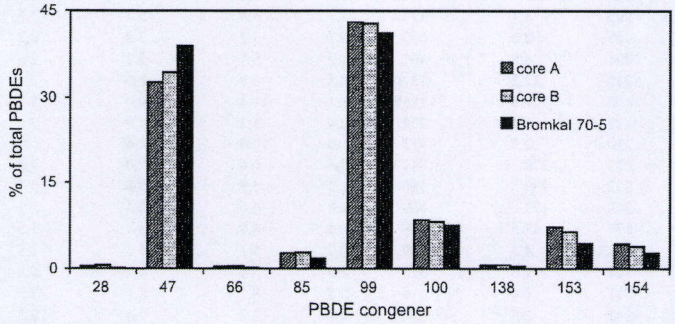

Fig. 3. Profiles of selected tri- to hexa-BDE congeners in two sediment cores from the Scheldt river and in the technical mixture Bromkal 70-5.

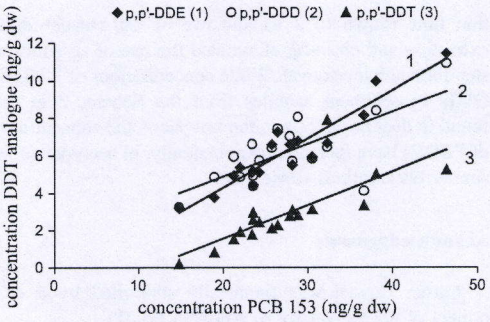

Fig. 4. Correlations between concentrations of DDTs analogues and concentration of PCB 153. (p,p'-DDE: $y=0.259x-0.877$, $R^2=0.894$, $p<0.001$; p,p'-DDD: $y=0.172x+1.451$, $R^2=0.514$, $p<0.001$; p,p'-DDT: $y=0.180x-2.048$, $R^2=0.586$, $p<0.001$).

p,p'-DDE/p,p'-DDD were found in bed sediment from urban sites sampled between 1992 and 2001 from United States river and streams (NAWQA, 2003).

High significant correlation coefficients were found between the concentrations of DDT analogues (p,p'-DDE, p,p'-DDD and p,p'-DDT) and the concentration of PCB 153 (Fig. 4), suggesting a similar trends in pollution changes for the two classes of pollutants.

The depth profiles of PBDEs in the sediment cores were completely different from those of PCBs, showing a steep increase in the surface layers compared with deeper core layers (Table 4). This is in accordance with the increase in the production and use of PBDEs in the last decades and with the high PBDE levels observed in surface sediments from other PBDE-polluted rivers, such as river Tees (Allchin et al., 1999).

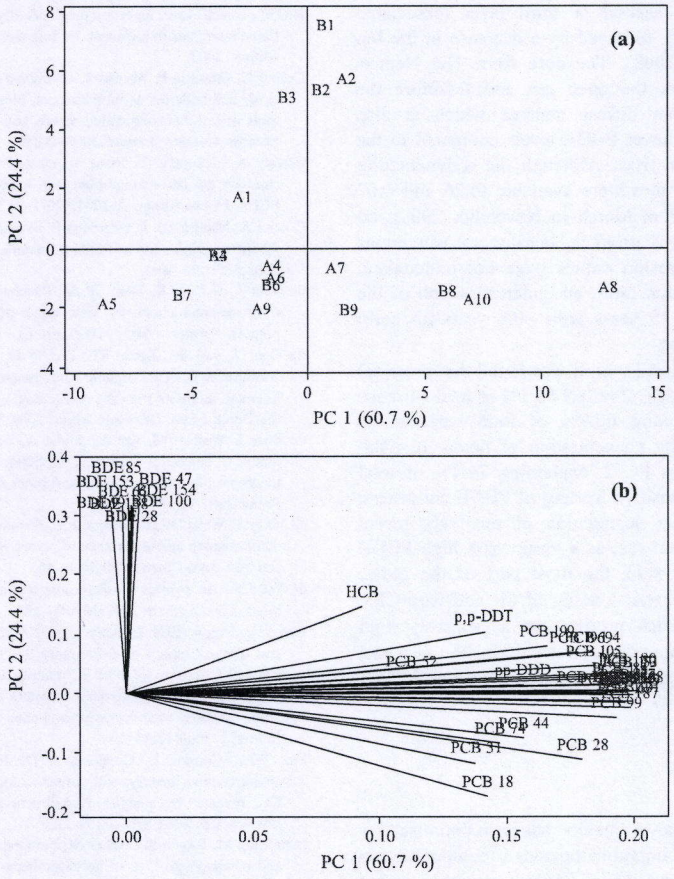

Fig. 5. Score plot (a) and loading plot (b) of PCA analysis based on concentrations of POPs (except of BDE 209) in two sediment cores from the Scheldt river.

Due to high concentrations of BDE 209, an intensive use of technical deca-BDE is evident for the top layers (Table 4), while for tetra- to hexa-BDEs, similarities with the profile of Bromkal 70-5DE is pronounced for medium sediment cores. Compared to the technical penta-BDE mixture, the surface slices from both sediment cores are slightly enriched in BDE 153, which can partly be due to a possible formation of hexa-BDEs by debromination of BDE 209.

Similar to what was observed in sediment cores from the Scheldt river (present study), BDE 209 was also the predominant PBDE congener in sediment cores from Wadden sea, The Netherlands and Drammenfjord, Norway (Zegers et al., 2003). The tri- to hexa-BDE congeners had also a technical penta-BDE mixture profile and their concentrations were 10–15 times lower than concentrations of BDE 209. While the cores from the Scheldt river showed a continuous increase in the PBDE concentrations in the top layers, the sediment cores from The Netherlands and Norway showed a peak in the PBDE concentrations in the second or third layer (associated with years 1994–1998), followed by a decrease in the top layer (Zegers et al., 2003). The core from The Netherlands was collected in the open sea and therefore the PBDE input was from diffuse sources which is also reflected in the much lower PBDE levels compared to the cores from the Scheldt river. Although the sedimentation rates at the collection sites were available (0.26 and 0.67 cm/month measured from March to November 2000), no dating of sediments was possible, because measurements of bioturbation and erosion values were not undertaken. Using only sedimentation rates, an underestimation of the age of the cores (10–15 years with ~0.3 years/cm core) would have been made.

Using PCA as statistical tool, it was found that principal components (PC) 1 and PC 2 reflect 85.1% of total variance (Fig. 5). PC1, explaining 60.7% of total variance, is significantly affected by concentration of penta- to octa-CB congeners, whereas PC 2, explaining 24.4% of total variance, shows high positive loading of PBDE congeners. It can be seen a clear segregation of relatively recent deposed B1, B2, B3 and A2, as a group with high PBDE content. On the other side, the most part of the oldest sediment cores show higher loading of tri- and tetra-CBs. Possible reasons for such profiles are a relatively high contribution of anaerobic microbial dechlorination or a past use of technical mixtures containing lower chlorinated PCBs (Aroclor 1242 and 1254).

4. Conclusions

A simple and fast analysis method has been optimised for the determination of organohalogenated compounds in sediment cores. While the use of Soxhlet extraction in hot extraction mode allowed a substantial reduction of extraction time (down to 2 h), the use of Cu powder during extraction and clean-up eliminated the use of an additional step for sulphur removal. While concentrations of PCBs and DDTs in sediment samples from the Scheldt river were found to decline slightly in the last years, the concentrations of PBDEs have increased exponentially, in accordance with similar observations worldwide.

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3.3 Paper III

PBDEs in marine and freshwater sediments from Belgium: Levels, profiles and relations with biota

Stefan Voorspoels, Adrian Covaci, Johan Maervoet, Paul Schepens

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PAPER

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PBDEs in marine and freshwater sediments from Belgium: levels, profiles and relations with biota

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Sediments from the Belgian North Sea (BNS), the Western Scheldt Estuary (SE) and freshwater watercourses from the Scheldt basin were analysed for eight PBDE congeners, namely BDEs 28, 47, 99, 100, 153, 154, 183 and 209. Previously analysed biological samples from the same locations in the BNS and the SE have been shown to contain large amounts of PBDEs. Surprisingly, PBDE concentrations in the sediments were below the LOQ for samples from the BNS (except BDE 209), while in those from the SE the sum of PBDEs (not including BDE 209) were higher and ranged from 0.20 to 0.41 ng g^{-1} dw. BDE 209 could be detected in 83% of the samples from the BNS and in all the samples from the SE. Concentrations up to 1200 ng g^{-1} were hereby measured in the SE. Compared to the marine and estuarine locations, the sediments from the freshwater watercourses were relatively more polluted with the lower brominated PBDEs (<0.20–19 ng g^{-1} dw). BDE 209 concentrations up to 320 ng g^{-1} dw were measured in those sediments. However, the contribution of BDE 209 to the total amount of PBDEs varied much more at the freshwater locations than in the SE, which suggests a different input of pollutants. PBDE profiles observed in biological samples do not match the profiles of the sediments. BDE 183 and 209 could not be quantified in biota, although these congeners were undoubtedly present in the sediments. This raises questions about the bioavailability of these congeners in the environment.

1. Introduction

Polybrominated diphenyl ethers (PBDEs) are used as flame retardants to improve the fire safety of various items. These chemicals have shown a rise in production since they were first introduced in the 1960s, with a substantial increase since the end of the 1970s due to the growing popularity of computers and other electronic equipment.[1] Of the total worldwide market demand for PBDEs in 2001, more than 80% consisted of the fully brominated decabromodiphenyl ether (BDE 209).[2] This PBDE congener is of special interest because its presence is being increasingly reported worldwide. However, a ban on use or production is not foreseen, because environment and human risk assessment reports have concluded that there is no significant risk.[3]

Both biotic and abiotic compartments are polluted with PBDEs.[1,4] This is why brominated flame retardants (BFRs), and especially PBDEs, have received increasing attention during recent years.[4–6] Their extremely resistant and lipophilic nature is partly responsible for their widespread presence.[4] These properties cause residues of these persistent organic pollutants (POPs) to bioaccumulate in the organic fraction of soils and sediments, and in adipose tissues of biota, which results in an enrichment throughout the food chain.[7]

The water solubility and vapour pressure of PBDEs are very low causing them to be adsorbed rapidly onto solid particles of sediment and soil when released into the environment.[8,9] Sediment and sewage sludge are therefore considered a sink for these pollutants, although pollutants can be still bioavailable once adsorbed to the sediment particles.[10,11] Soil and sediment studies can provide valuable information about the pollution load in a certain area. Additionally, the study of sediments is an important step in mapping possible exposure pathways to various marine and freshwater organisms.

The area studied in this article covers the Belgian North Sea (BNS) and the Western Scheldt Estuary (SE). The drainage basin of the SE covers a very densely populated and highly industrialised region, resulting in a high pollution level with POPs[12–14] as well as heavy metals[15] and non-persistent pollutants.[16] The present study focused on the determination of PBDEs in sediments sampled at predefined locations in both the BNS and the SE. Previous studies have revealed a correlation between distance to Antwerp and degree of pollution in benthic invertebrates and fish.[12,13] Additionally, 14 sediment samples were taken from tributaries of the Scheldt to assess sediment pollution at several inland locations in the Scheldt basin.

Materials and methods

Sampling

Six locations in the BNS (S1–S6), 11 locations in the SE (S7–S17), and 14 freshwater locations in rivers and watercourses of the Scheldt Bassin (T1–T14) were sampled (Fig. 1). Surface sediments from the BNS and SE were taken in October and November 2001 using a Van Veen surface sediment sampler during a cruise with the research vessel *Zeeleeuw*, provided by the Flemish Marine Institute (VLIZ). Because the Scheldt is the main gateway to the Antwerp harbour, the river is highly trafficked and dredged. In order to avoid sampling at sites that were subjected to dredging, a map that showed the dredging activities was used to determine the appropriate sampling locations.

At each location, 4 subsamples of approximately 10 L sediment were taken within a radius of 50 m and combined. The approximate sampling depth was 20–25 cm. The sediment was kept in hexane pre-washed glass receptacles at +4 °C until further processing.

The samples from rivers and watercourses of the Scheldt basin were collected with a 'Petit Ponar' grab sampler during August and September 2001. At each site a mixed sample composed of 5 grab samples was taken. The sediments were sieved with 5 L of the site-water using a 200 μm-mesh sieve to

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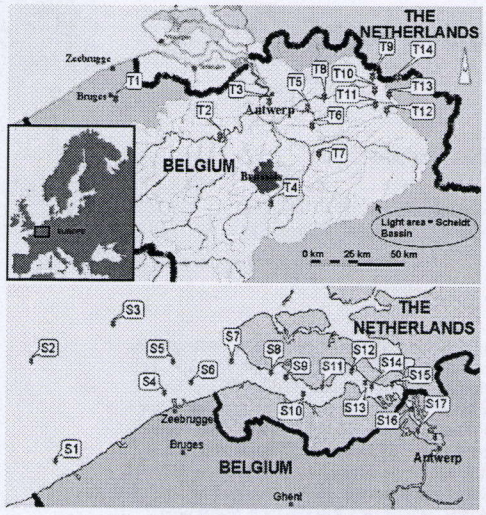

Fig. 1 Sampling locations.

remove the organisms. The sediment was stored in 500 ml polyethylene beakers at +4 °C until further processing.

Targeted compounds

Based on reported abundance[12–17] and toxicity,[4] the following BDE-congeners (IUPAC numbering) were targeted for analysis: 28, 47, 99, 100, 153, 154, 183, and 209. Brominated biphenyl (BB) 103 was used as IS for BDE 28 and 47, and BB 155 was used as IS for BDE 99, 100, 153, 154, and 183. ^{13}C-labeled BDE 209 was used as IS for BDE 209.

Chemicals

All solvents used for the analysis (*n*-hexane, acetone, dichloromethane, and *iso*-octane) were of SupraSolv® grade (Merck, Darmstadt, Germany). Cu powder was >200 Mesh (Merck, Darmstadt, Germany). Individual reference standards for each compound were used for identification and quantification (CIL, Andover, USA; Dr Ehrenstorfer Laboratories, Augsburg, Germany).

Extraction and clean up

The sediments from BNS and SE were dried for 1 week at room temperature until constant weight and consecutively sieved through 1 mm and 180 μm hexane-washed stainless steel sieves. The fine fraction was homogenised and used for analysis. The sediments from the freshwater locations were freeze-dried, sieved through a 180 μm hexane pre-washed stainless steel sieve and the fine fraction was further homogenised.

Between 1 and 5 g of dried homogenised sediment was mixed with Cu powder (triple amount of sample intake) and transferred into a hexane-rinsed extraction thimble and spiked with IS. The samples were extracted for 2.5 h with hexane–acetone (3 : 1; *v*/*v*) by means of an accelerated Soxhlet extractor B-811 (Büchi, Switzerland). The extract was cleaned-up using a multi-layer silica cartridge, containing Cu powder, silica impregnated with concentrated sulfuric acid (44%) and silica impregnated with KOH (33%). PBDEs were eluted with *n*-hexane followed by dichloromethane. The eluate was concentrated to near dryness and the extract was re-solubilised in 80 μl *iso*-octane.

Gas chromatography-mass spectrometry

Details of the instrumental set-up were previously described by Voorspoels *et al.*[12] In short, all PBDE analyses were performed using an Agilent 6890 GC (Palo Alto, USA) connected to an Agilent 5973 mass spectrometer. PBDE congeners 28, 47, 99, 100, 153, 154, and 183 were analysed on a 25 m × 0.22 mm × 0.25 μm HT-8 capillary column (SGE, Zulte, Belgium). For these congeners the mass spectrometer was operated in electron capture negative ionisation (ECNI) in the selected ion-monitoring (SIM) mode at the m/z = 79 and 81. For the analysis of BDE 209, a 12 m × 0.18 mm × 0.10 μm AT-5 capillary column (Alltech, Lokeren, Belgium) was used. The mass spectrometer was operated in ECNI in SIM mode at the m/z ratios of 484.7/486.7 and 494.7/496.7 for BDE 209 and ^{13}C-BDE 209, respectively.

Quality assurance

The quality control (QC) was done by regular injection of solvent blanks and standard solutions. Each batch of 8 samples included a procedural blank, blind duplicate sample and in-house reference material (the sediment sample used for the BROC interlaboratory feasibility study).[18] The individual PBDE-congener concentrations did not differ more than 15% from the mean values obtained in the BROC-study. Recoveries of all congeners varied between 83 and 92% (RSD < 16%).

Procedural blanks for all PBDEs were consistent (RSD around 30%) and therefore the median blank values for these compounds were used for subtraction. Blanks for BDE 209 were one order of magnitude higher than for the other PBDE-congeners. The limit of quantification (LOQ) was determined by laboratory background levels. Two times the standard deviation of the procedural blank level was used as the LOQ, resulting in a certainty of more than 95% of the presented results.

Statistical analysis

Only samples that had measurable amounts (>LOQ) of analytes were used for the statistical calculations. Simple linear regression was used to test for correlations between the total PBDE load and BDE 209 levels. To test for differences between profiles, an ANOVA with Scheffe's post hoc test was performed. Tests were considered significant if p was lower than 0.05. All statistical tests were performed using Statistica® v5.0 software (StatSoft Inc., Tulsa, USA).

Results and discussion

PBDE levels

All results are summarised in Table 1. Levels of PBDEs in the BNS samples were low and the majority of congeners were below the LOQ. The sum of PBDEs (BDE 28, 47, 99, 100, 153, 154 and 183; hereafter referred to as "sum of PBDEs") was never higher than the calculated LOQ for these locations (<0.2 ng g^{-1} dw). BDE 209, however, could be detected in 83% of samples from the BNS, although at rather low concentrations (1.1–24 ng g^{-1} dw). Locations near the industrial harbour of Zeebrugge (S4; S6) were the only locations in the BNS where tri- to hexa-PBDEs could be measured and where BDE 209 showed a tenfold higher concentration compared to the other locations in the BNS (Table 1). Near the harbour of Vlissingen, (locations S7 and S8), slightly but statistically significant elevated BDE 209 levels were seen. However, in sediments taken further upstream in the SE, levels were low and comparable to the concentrations in the BNS until location S15 (near the Dutch–Belgian border).

At locations S7 to S14 (SE) the sum of PBDEs ranged from <0.20 to 0.41 ng g^{-1} dw. Similar to the other PBDEs, concentrations of BDE 209 were relatively low at these

Table 1 Concentrations (ng g^{-1} dw) for individual BDE congeners in sediments from the BNS, SE and freshwater watercourses

	Location	BDE 28	BDE 47	BDE 100	BDE 99	BDE 154	BDE 153	BDE 183	Sum PBDEs[a]	BDE 209
BNS	S1	<0.01	<0.07	<0.02	<0.1	<0.01	<0.01	<0.01	<0.2	1.1
	S2	<0.01	<0.07	<0.02	<0.1	<0.01	<0.01	<0.01	<0.2	1.2
	S3	<0.01	<0.07	<0.02	<0.1	<0.01	<0.01	<0.01	<0.2	1.1
	S4	<0.01	<0.07	<0.02	<0.1	0.03	0.01	<0.01	<0.2	24
	S5	<0.01	<0.07	<0.02	<0.1	<0.01	<0.01	<0.01	<0.2	2.9
	S6	<0.01	0.08	0.02	<0.1	0.03	<0.01	<0.01	<0.2	23
SE	S7	0.01	<0.07	<0.02	<0.1	0.02	<0.01	<0.01	<0.2	17
	S8	0.06	0.24	0.04	<0.1	0.04	0.03	<0.01	<0.2	47
	S9	<0.01	<0.07	<0.02	<0.1	0.01	<0.01	<0.01	<0.2	1.5
	S10	0.02	0.10	0.03	<0.1	0.02	0.02	<0.01	0.41	5.7
	S11	<0.01	<0.07	<0.02	<0.1	0.01	<0.01	<0.01	<0.2	6.1
	S12	<0.01	0.09	0.03	0.10	0.02	0.02	<0.01	0.26	2.8
	S13	0.01	0.07	0.03	0.10	0.02	0.02	<0.01	0.26	1.9
	S14	0.01	0.09	0.03	0.10	0.02	0.02	<0.01	0.27	n.a.
	S15	1.1	6.1	1.9	6.1	1.2	1.0	0.15	18	1200
	S16	0.46	3.7	1.00	3.6	0.79	0.72	0.33	11	550
	S17	0.19	1.9	0.53	2.0	0.29	0.28	0.06	5.3	340
TRIBUTARIES	T1	0.01	3.9	0.04	0.16	0.03	0.03	0.03	4.2	320
	T2	0.05	0.38	0.09	0.40	0.06	0.07	0.10	1.2	8.1
	T3	0.02	0.09	0.04	0.12	0.03	0.04	0.06	0.38	6.0
	T4	0.01	<0.07	<0.02	<0.1	0.03	0.02	0.04	<0.2	1.6
	T5	0.02	0.29	0.07	0.37	0.06	0.07	0.08	0.96	10
	T6	0.22	5.6	1.15	7.4	3.0	1.0	0.78	19	24
	T7	0.02	0.19	0.04	0.25	0.04	0.04	0.04	0.63	2.4
	T8	0.02	<0.07	0.02	<0.1	0.02	0.02	<0.01	<0.2	<0.1
	T9	0.01	0.11	0.03	0.13	0.13	0.02	0.02	0.44	0.5
	T10	0.01	<0.07	0.02	<0.1	0.08	0.02	<0.01	<0.2	<0.1
	T11	0.01	<0.07	<0.02	<0.1	0.02	0.01	<0.01	<0.2	0.5
	T12	0.03	0.09	0.04	0.14	0.16	0.04	0.07	0.57	8.2
	T13	0.01	<0.07	<0.02	<0.1	0.03	0.01	<0.01	<0.2	0.5
	T14	0.01	<0.07	<0.02	<0.1	0.02	0.02	0.05	<0.2	120

[a] BDE 209 is not included in the sum of PBDEs; n.a. = not available.

locations in the SE (1.5–47 ng g^{-1} dw). At locations S15 to S17, concentrations of the lower brominated PBDEs were approximately 10 to 50 times higher than at downstream locations (5.3–18 ng g^{-1} dw). At the same locations, very high levels of BDE 209 could also be measured (up to 1200 ng g^{-1} dw).

The Scheldt discharges into the North Sea, projecting its plume even further than Zeebrugge.[19] Because BDE 209 concentrations in the SE are relatively low until location S15, it is highly unlikely that the elevated levels found near the Zeebrugge harbour are caused by the influence of the SE. The higher levels found near Zeebrugge (S4; S6) and near the Dutch–Belgian border (S15–S17) were probably caused by the industrial harbours of Zeebrugge and Antwerp, respectively. However, the latter is doubtful because location S15 displayed the highest levels and concentrations decrease further upstream. Local input near location S15 may therefore be involved. Higher contamination close to populated areas like harbours and urban centres has been previously reported by others.[20,21]

Compared to the marine and estuarine locations, freshwater sediments seem to be more polluted with PBDEs (<0.20–19 ng g^{-1} dw). The sum of PBDEs at location T6 was the highest one found in this study (19 ng g^{-1} dw). However, BDE 209 was present at rather low concentration at this location (24 ng g^{-1} dw). A point-source for the lower brominated congeners can therefore not be ruled out at this location.

BDE 209 could be detected in 86% of the freshwater samples, with concentrations up to 320 ng g^{-1} dw (at location T1). These elevated concentrations are probably related to the heavy industrialisation and dense population of this region and thus caused by the higher impact of human activity on the inland watercourses. Christensen and Platz have also reported higher PBDE contamination in freshwater than in marine sediments.[20]

De Boer *et al.* reported comparable levels for sediments from The Netherlands.[22] In that study, concentrations up to 7.1 ng g^{-1} dw, 5.5 ng g^{-1} dw and 510 ng g^{-1} dw for BDEs 47, 99 and 209 were reported, respectively. The samples were taken with a Van Veen surface sediment sampler similar to the one used in the present study.

Levels of BDE 47 in sediments from the UK that were taken at or around potential PBDE sources were up to 2 orders of magnitude higher (368 ng g^{-1} dw) than in the present study.[17] In general, PBDE-concentrations in that study were slightly higher than the sediments from the freshwater locations in the present study. The sediment samples were taken with a small hand-held Van Veen surface sediment sampler, which enabled only taking the first centimetres of the sediment. This is in contrast with the larger Van Veen sampler used in the present study, which sampled up to 25 centimetres depth.

Christensen and Platz collected top-layer (0–2 cm) marine sediments in Denmark.[20] Concentrations of the sum of PBDEs ranged from 0.06 to 3.7 ng g^{-1} dw; BDE 209 levels up to 22 ng g^{-1} dw were reported. The sediments that originated from the area of the Copenhagen harbour were the most polluted. These sum of PBDEs concentrations were in the same order of magnitude as the sediments in parts of the SE in the present study. BDE 209 concentrations in the SE in the present study were up to 60 times higher (1200 ng g^{-1}) than those reported from Denmark. Compared to values reported in literature,[20–23] it can be concluded that levels of BDE 209 in the SE (S15–S17) are relatively high.

PBDE profiles

BDE 209 was the most abundant congener, followed by BDE 47 and 99. These profile characteristics have been previously reported by Zegers *et al.* for Western Europe.[24] In the present study, profiles were calculated based only on BDE 28 to 183. As BDE 209 was not included in the profile, it was possible to compare the results with the Bromkal DE70-5 technical penta-mixture and other studies. Including this congener in the profile would also bias the ratios, because the contribution of BDE 209 to the total PBDE-load was 95 ± 4.7% for the SE samples and varied between 52% (T9) and 99% (T1) for the tributaries.

For BNS samples no profile could be established due to the very low PBDE-levels. The profile calculated for the SE was very consistent (RSD <20%, except for BDE 183) (Fig. 2). The profile calculated for the tributaries was less consistent (RSD up to 70%), which can be explained by their geographical distribution; samples T9 and T12 displayed relatively high levels of BDE 154, which influenced the pattern to a rather great extent. The profile at location T1 also deviated, because the levels of BDE 47 were relatively high. Sample T1 was therefore not included in the mean profile calculation.

No significant profile differences were found between the SE and the tributaries. Both sample sets display a similar profile to the Bromkal 70-5DE pentaBDE technical mixture (Fig. 2).

Relations with biota

Although the sum of PBDEs was below the LOQ in the BNS sediments, a similar study performed on mildly migrating benthic invertebrates (such as crab, starfish, shrimp) and fish species (such as dab, plaice, sole, bib, and whiting) reported values for the samples from BNS between 0.02 ng g^{-1} ww and 108 ng g^{-1} ww.[12] In the same study, concentrations in biota from the SE ranged from 0.20 to 980 ng g^{-1} ww.[12]

A significant inverse correlation (r^2 up to 0.77; $p = 0.02$) between PBDE-concentrations in biota from the SE and the distance to Antwerp was also observed in that study.[12] This correlation was not reflected in the data of the sediments, although sediment is the most likely source of contaminants for the biota living above it, since contaminants trapped on the sediments may be bioavailable to sediment dwelling organisms.[10,11]

The sampling locations in the present study were away from any known dredging activity. The low levels found in the sediments could certainly not have been caused by sampling flaw (location). The lack of any correlation between levels and location and the discrepancy between levels in biota and sediments can probably be explained by the sampling methodology. As mentioned previously, sediments of the present study were sampled using a large Van Veen surface sediment sampler. This device however did not only sample the top layer (~1 cm), but went much deeper into the sediment (~20–25 cm). This results in a large dilution of the (more polluted) top layer of the sediment, explaining the relatively low PBDE-levels measured in most samples. Covaci *et al.* analysed two sediment cores from the River Scheldt upstream from Antwerp.[25] Results showed that PBDE concentrations decreased from 270 ng g^{-1} dw in the top layer to 3.7 ng g^{-1} dw at 20–25 cm depth, without significant profile changes for tri- to hexa-BDEs.

To ensure that only the top layer of the sediment is sampled, preferentially a box-corer sampler should be used. In this way dilution is much less pronounced and exposure assessment with regard to biota living above the sediments may be more accurate. Sampling by means of a Van Veen surface sediment sampler can therefore influence the results. Concentrations to which biota are exposed may therefore be higher than reported in this paper. However, PBDE patterns are not altered and can still provide very useful data.[25]

A mean PBDE-profile was calculated for the fish and invertebrates that were previously analysed[12] and is plotted in Fig. 2. The mean profile of the sediments in the present study was identical to the profile of the Bromkal technical mixture. However, the profile found in fish from the same locations differed statistically significantly from the profiles in the sediments. Metabolic processes are most likely related to this observation, because the bioavailability of tetra- to hexa-substituted congeners is assumed to be equal in marine organisms.[26]

However, benthic invertebrates, that are assumed to have lower metabolic capacity than fish,[27] display a profile that was not significantly different from the profile of the technical mixture (Fig. 2). The PBDE-profile in the fishes was extensively altered compared to the sediments. This observation suggests that the limited metabolic capacity of the invertebrates does not seem to enable these organisms to change the pattern to the same extent as can be observed in the fishes. BDE 183 and BDE 209 could not be detected in any invertebrate or fish sample, although they were present in the sediments.

Uptake of higher brominated BDEs was very low in controlled feeding experiments.[28] It was concluded to be related to their high log K_{ow} values and their large molecular size, which hinders them in crossing membranes.[28] Contrarily, in a recent publication by Tomy *et al.*, the uptake of BDE 183 and BDE

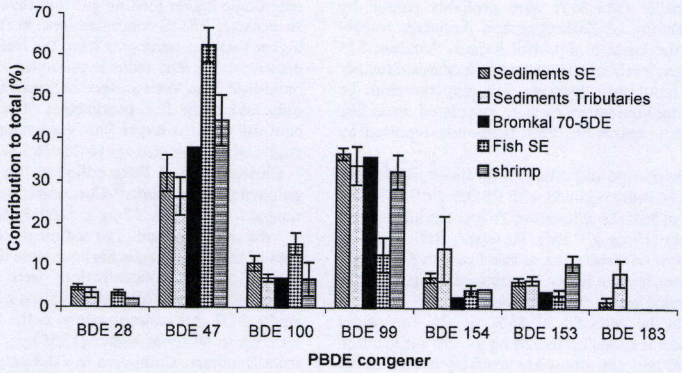

Fig. 2 PBDE profiles in sediments from the SE and freshwater locations, profile of Bromkal 70-5DE and profiles found in biota.[12] Results for Fish indicate the mean profile for dab, plaice, bib, sole and whiting. The error flags indicate 2 × standard error.

209 following oral administration was comparable or even higher, respectively, than the uptake of the other congeners.[29] The absence of BDE 183 and 209 in the biota samples in the study by Voorspoels *et al.* [12] and their presence in the sediments suggests that the aquatic uptake of BDE 183 and 209 may therefore be restricted by the physical, rather than by the biological availability.

Relatively fast metabolism of BDE 183 and 209 in fish has been repeatedly reported,[28–30] which is in agreement with the absence of these congeners in the biota.

To the author's knowledge, no data are available to date concerning metabolism of PBDEs in invertebrates. Possibly a combination of both low uptake and fast metabolism are related to the observation that both BDE 183 and BDE 209 can be found in the sediments, but not in the invertebrates and fish.

Correlations between BDE 209 and other PBDEs

Only locations at which all measurements were above the LOQ were taken into account for calculating the correlation between BDE 209 and the sum of the other congeners. Levels of BDE 209 and the other PBDEs were significantly positively correlated for all samples from the marine locations (BNS and SE). Log-normalised concentrations showed an R^2-value of 0.9372 ($p < 0.05$). The input of these 2 groups of contaminants seems to be related for the SE locations.

For the samples taken at the freshwater locations, the correlation between BDE 209 and other PBDE-congeners was not significant. In particular, location T6 displayed rather high levels of all PBDEs except BDE 209. In general BDE 209 was relatively low and the other PBDEs relatively high in the sediments of the tributaries, which points at a different exposure compared to the SE, where the major PBDE-load consists of BDE 209 (up to 98%). It can therefore be concluded that the exposure to the lower brominated PBDEs and BDE 209 is different in the SE and in the freshwater watercourses of the Scheldt basin.

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3.4 Paper IV

Polybrominated diphenyl ethers in marine species from the Belgian North Sea and the Western Scheldt Estuary: Levels, profiles, and distribution

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Polybrominated Diphenyl Ethers in Marine Species from the Belgian North Sea and the Western Scheldt Estuary: Levels, Profiles, and Distribution

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The Western Scheldt Estuary (SE) is subjected to a variety of suspected PBDE sources, such as a brominated flame retardant manufacturing plant, the Antwerp harbor, and the textile industry located further upstream the river. The Belgian North Sea (BNS) was included in this study to analyze the influence of the SE on the levels found in biota from the BNS locations. Benthic invertebrates, such as shrimp, crab, and starfish, benthic fish, such as goby, dab, plaice, and sole, and gadoid fish, such as bib and whiting, were sampled in the BNS (nonpolluted area) and the SE (polluted area) and analyzed to determine the concentrations and spatial variation of eight polybrominated diphenyl ethers (PBDEs 28, 47, 99, 100, 153, 154, 183, and 209). Levels found in the SE samples were up to 30 times higher than those found in BNS samples, with a gradient increasing toward Antwerp. Levels in BNS ranged from 0.02 to 1.5 ng/g ww in benthic invertebrates and goby, from 0.06 to 0.94 ng/g ww in fish muscle, and from 0.84 to 128 ng/g ww in fish liver. For the SE samples, levels ranged from 0.20 to 29.9 ng/g ww in benthic invertebrates and goby, from 0.08 to 6.9 ng/g ww in fish muscle, and from 15.0 to 984 ng/g ww in fish liver. BDE 209 could only be detected in eight liver samples from the SE and levels ranged between 3.4 and 37.2 ng/g ww. PBDE profiles of the various species at the different locations were compared. Differences in profile were attributed to different exposure and to differences in metabolism among species. Ratios between BDE 99 and 100 were found to be highly location and species dependent, which could be related to differences in metabolism. Some species, such as dab, plaice bib, and whiting, showed preferential accumulation of PBDEs in the liver. Higher brominated congeners in general showed higher affinity for liver than for muscle tissue.

Introduction

Brominated flame retardants (BFRs), and especially polybrominated diphenyl ethers (PBDEs), have received increasing attention during the past years (*1*−*5*). This is due to their massive use to improve fire safety in both commercial and domestic applications, where BFRs are added in concentrations up to 30 wt % (*6*). These chemicals have shown a rise in production since they were first introduced in the 1960s, with a substantial increase since the end of the 1970s due to the growing popularity of personal computers and other electronic equipment (*7*). The worldwide market demand for PBDEs in 2001 was estimated to be 67 390 metric tons, of which 56 100 metric tons consisted of the fully brominated decabromodiphenyl ether (BDE 209) (*8*).

PBDEs can reach the environment through leaching during production and application processes, through volatilization and leaching during use, and through particulate losses during use and disposal (*9*). The current European legislation does not provide for special regulations for bromine recycling, and thus 88% of the plastics containing BFRs end up as landfill, 10% is incinerated, and only less than 3% is recycled or reused in Europe (*10*). This current lack of recycling of BFRs in plastics might also be held partially responsible for the increasing levels in all environmental compartments, biotic (from plankton to humans) (*7*) as well as abiotic (air, water, sediments, soil) (*2*), since plastics account for more than half the worldwide BFR demand.

Most toxicological studies have been performed using commercial PBDEs that are mixtures of congeners and isomers, resulting in a limited amount of data about congener-specific effects (*9*). Acute toxicity was shown to be low in those studies, but serious health consequences, such as thyroidogenic, estrogenic, and hepatic effects, neurodevelopmental disorders, and dioxin-like activity, could result from prolonged exposure (*7*, *9*, *11*, *12*). Cancer has also been described as a possible toxic effect (*12*).

The physicochemical properties of these compounds lead to biomagnification, as they are lipophilic and extremely resistant to degradation (*13*). Humans are at the top of the food chain, which makes them very susceptible to ingestion of large quantities of these compounds through the diet (*9*). The toxicological aspect together with steadily increasing human and environmental levels make it likely that not only humans but also (marine) species might experience adverse effects from these compounds (*14*). Aquatic organisms are especially very efficient in accumulating these compounds (*15*), since in addition to bioaccumulation through the diet, they are also subjected to bioconcentration.

Since benthic organisms are at the base of the marine food chain (e.g. goby, starfish, shrimp, etc.) and are also consumed by humans (e.g. crabs, shrimp, dab, sole, etc.), studies on these organisms can be used to obtain valuable information about the availability of these pollutants to higher trophic levels. Benthic organisms are known to be very sedentary, which makes them very good sentinel species. These data can therefore also be used to establish geographical trends in pollution.

In this work, various benthic species from the Belgian North Sea (BNS) and the Scheldt Estuary (SE) were analyzed for selected PBDEs. The sampling locations were chosen based on the presence of a BFR production plant at Terneuzen (The Netherlands), which is located in the SE, the high level of industrialization of the nearby harbor of Antwerp, and the textile industry further upstream the river. De Boer et al. recently reported high levels of BDE 209 (up to 4600 ng/g dry weight) in suspended particulate matter (SPM) from the Western Scheldt and attribute this to spillage during use further upstream (*16*). Additional interest is generated by the fact that the Scheldt discharges into the North Sea, which is a very extensively exploited fishery region. Pollutants, such as PBDEs, coming from the Scheldt will be dispersed in the narrow southern North Sea basin, where they can accumulate. The BNS was included in the study to analyze the

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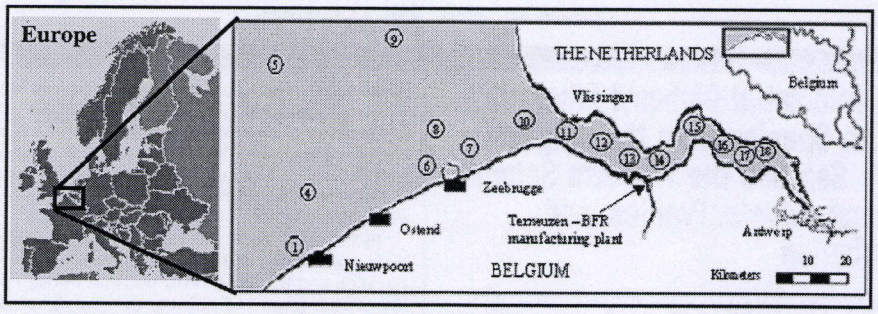

FIGURE 1. Geographical distribution of the sampling locations in BNS and SE.

TABLE 1. Locations of Sampling Points

location	north	east	location	north	east
1 (BNS)	51°09'88"	2°37'87"	11 (SE)	51°25'19"	3°36'91"
4 (BNS)	51°15'78"	2°35'17"	12 (SE)	51°24'29"	3°41'41"
5 (BNS)	51°25'99"	2°31'01"	13 (SE)	51°21'41"	3°46'22"
6 (BNS)	51°21'48"	3°07'58"	14 (SE)	51°20'55"	3°51'29"
7 (BNS)	51°22'89"	3°14'91"	15 (SE)	51°25'78"	4°01'32"
8 (BNS)	51°27'01"	3°10'18"	16 (SE)	51°23'32"	4°03'25"
9 (BNS)	51°32'64"	2°53'34"	17 (SE)	51°22'53"	4°05'19"
10 (SE)	51°26'70"	3°26'36"	18 (SE)	51°23'21"	4°10'53"

TABLE 2. Overview of Sampled Species

class	species name
Benthic invertebrates	*Crangon crangon* (common shrimp) – *Asterias rubens* (red starfish) – *Lyocarcinus holsatus* (flying crab)
Benthic fish	*Pomatoschistus minutus* (sand goby)
Benthic flatfish	*Solea solea* (common sole) – *Limanda limanda* (dab) – *Pleuronectus platessa* (plaice)
Gadoid fish	*Merlangius merlangus* (whiting) – *Trisopterus luscus* (bib)

influence of the SE on the levels found at the different locations. The data presented in this study are complementary to a recently published study by Boon et al., which covers the PBDE pollution in similar marine species from the Northern part of the North Sea and the Tees estuary, U.K. (*17*).

Such detailed PBDE data about this region, which is characterized by possible point sources, and which includes benthic invertebrates and different fish species from both polluted (SE) and nonpolluted (BNS) areas, has not been published to date, according to our knowledge.

Experimental Section

Sampling. Seven locations were selected in the BNS and nine locations in the Scheldt Estuary (SE) (Figure 1 and Table 1). Selection of the species was based upon their occurrence in space and time at the sampling locations. Three species of benthic invertebrate organisms were chosen. These organisms are very suitable as sentinel species, since they tend not to migrate. Crab species have also been extensively used as sentinel organism in monitoring studies of lipophilic contaminants on Canada's West Coast (3). Three flatfish species and two gadoid fish species were also sampled at the same locations. An overview of all sampled species is presented in Table 2.

The number of individuals caught was different for each location and varied between 3 and 10 for starfish, between 30 and 50 for shrimp, crab, and goby, and between 1 and 5 for the other fish species. Sampling campaigns took place during October and November 2001. All organisms were collected with a 3 m beam trawl with fine-meshed nets (6 × 6 mm), at a constant speed of 1.5–2.0 knots for about 30 min., using the research vessel *Zeeleeuw*, provided by the Flemish Marine Institute (VLIZ).

Preliminary sample pretreatment steps were undertaken on board, and they included species determination, recording of fish length, and washing with clean water. Prior to storage in hexane prewashed glass recipients at −20 °C, the flatfishes and gadoids were dissected on board immediately after capture, and only the excised muscle and liver samples were collected. For the invertebrates and goby samples, the entire animals were stored.

Sample Availability. All sampled species were available in large amounts for the BNS locations, unlike for the SE. Crab and shrimp were very abundant and thus available for each location of the BNS and SE. For starfish and goby species, samples could only be taken at one and two locations in the SE, respectively. Dab was also difficult to catch in great quantities for SE locations, resulting in only one pooled sample at one location in the SE. Other flatfish and gadoids were caught in at least five locations on the BNS and three locations in the SE.

Targeted Compounds. Based on reported abundance (*11*, *18–21*) and toxicity (*2*), the following BDE-congeners (IUPAC numbering) were targeted for analysis: 28 (2,4,4′-triBDE), 47 (2,2′,4,4′-tetraBDE), 99 (2,2′,4,4′,5-pentaBDE), 100 (2,2′,4,4′,6-pentaBDE), 153 (2,2′,4,4′,5,5′-hexaBDE), 154 (2,2′,4,4′,5,6′-hexaBDE), 183 (2,2′,3′,4,4′,5,6′-heptaBDE), and 209 (2,2′,3,3′,4,4′,5,5′,6,6′-decaBDE). Polybrominated biphenyl (PBB) 103 was used as internal standard (IS) for BDE 28 to 183. For BDE 209, ^{13}C-BDE 209 was used as IS.

Chemicals. All solvents used for the analysis (*n*-hexane, acetone, isooctane) were of SupraSolv grade (Merck, Darmstadt, Germany). Individual reference standards for each of the eight BDE congeners were used for identification and quantification (CIL, Andover, U.S.A). Sodium sulfate was heated at least 6 h at 600 °C, and silica was prewashed with *n*-hexane before use. Extraction thimbles were pre-extracted for 1 h with the extraction mixture used for the samples and dried at 100 °C for 1 h.

Sample Preparation and Cleanup. Prior to analysis, the samples were defrosted and homogenized, using a high-speed blade-mixing device, except for the shrimp and crab samples, of which only the soft parts were taken. After homogenization, two identical composite samples of each species, location, and tissue were created. Thirty individuals were homogenized for shrimp, goby, and crab samples since these organisms were readily available in large amounts. For starfish samples the pools consisted of 3–8 equally sized individuals. For the fish samples, fish size was taken into account when samples were pooled and the median length

was recorded. Pools of gadoids and flatfish consisted of 3–6 individuals. The only exceptions were pools that consisted only of 1 or 2 individuals for dab from location 14, and for sole from locations 15 and 16, respectively.

The method used for the clean up of the samples has been previously described and validated (22) and is briefly presented below. Between 1 and 10 g of homogenized sample was dried with approximately 15 g anhydrous sodium sulfate, spiked with internal standards PBB 103 and ^{13}C-BDE 209 (between 1 and 20 ng, depending on the pollution load of the sample) and extracted for 2.5 h by hot Soxhlet with 100 mL of hexane/acetone (3/1; v/v). After lipid determination (performed on an aliquot of the extract), the extract was cleaned-up on 8 g of acidified silica. After elution with 15 mL of hexane, the cleaned extract was concentrated to approximately 80 μL.

Chemical Analysis. All analyses were performed using a Hewlett-Packard 6890 GC (Palo Alto, U.S.A.) connected via direct interface with a HP 5973 mass spectrometer. PBDE congeners 28, 47, 99, 100, 153, 154, and 183 in crab, goby, starfish, and fish muscle samples were analyzed with a 10 m × 0.1 mm × 0.1 μm HT-8 (narrow bore) capillary column (SGE, Belgium). Due to residual lipids in the extract (e.g., cholesterol), combined with low column capacity of the 10 m narrow bore column, a 25 m × 0.22 mm × 0.25 μm HT-8 capillary column (SGE, Belgium) was used to analyze these congeners in shrimp and fish liver samples. The carrier gas was helium, and the flow rate was held constant at 0.4 and 1.0 mL/min, for the 10 and 25 m column, respectively. The oven temperature program for the 10 m narrow bore column was starting from 90 °C, kept for 1.5 min, then with 40 °C/min to 220 °C, held 0.5 min, with 25 °C/min to 300 °C, and held 6 min. For the 25 m column, the temperature program was starting from 90 °C, kept for 1.5 min, then with 30 °C/min to 180 °C, held 0.5 min, with 5 °C/min to 270 °C, held 0.5 min, and further with 25 °C/min to 290 °C, and held 15 min.

One microliter of the final cleaned extract was injected into a programmable temperature vaporizer (PTV). The injector was operated in splitless mode for the narrow bore column (injector temperature: 90 °C, held for 0.05 min, then with 700 °C/min to 290 °C, and kept for 7 min; the purge vent opened at 1.5 min). Solvent vent mode was used for the 25 m column (injector temperature: 90 °C, held for 0.05 min, then with 700 °C/min to 280 °C, and kept for 25 min; vent flow was set at 100 mL/min and the purge vent opened at 1.5 min).

The mass spectrometer was operated in electron capture negative ionization (ECNI) in the selected ion-monitoring (SIM) mode at the m/z = 79 and 81.

For the analysis of BDE 209, a 15 m × 0.18 mm × 0.10 μm AT-5 (Alltech, Lokeren, Belgium) capillary column was used. Helium was used as carrier gas at a constant flow rate of 2.0 mL/min. The oven temperature program was starting from 90 °C, kept for 1.35 min, then with 75 °C/min to 305 °C, and was held for 7.50 min. One microliter of the final cleaned extract was injected into a PTV. The injector was operated in solvent vent mode (injector temperature: 90 °C, held for 0.06 min, then with 700 °C/min to 310 °C, and kept for 12 min). The mass spectrometer was operated in ECNI in SIM mode at the m/z ratios of 484.7/486.7 and 494.7/496.7 for BDE 209 and ^{13}C-BDE 209, respectively.

For all columns, methane was used as moderating gas, and the ion source, quadrupole, and interface temperatures were 250, 150, and 300 °C, respectively. Dwell times were set at 10 and 30 for PBDE analysis on the 10 and 25 m column, respectively, and 100 ms for the analysis of BDE 209.

Quality Assurance. The quality control was done by regular analyses of procedural blanks, solvent blanks, spiked samples, blind duplicate samples, in-house reference material, and injection of standards. The two pools that were created for each species, tissue, and location were treated as separate samples. Results for two identical pools did not differ more than 10%, and thus the mean value of the two analyses was taken for further calculations. Analyte identification was based on retention times and peak shape. Recoveries for individual PBDE congeners were established to be between 87 and 104% (RSD ≤ 12%). No recovery differences were found between different spiking levels (0.1 ng/g, 1.0 ng/g, and 5.0 ng/g). Four overlapping 5-point narrow range calibration curves were used to quantify the wide range of sample concentrations found.

Limit of detection (LOD), defined as 3 times the noise level, and limit of quantification (LOQ), based on GC-MS performance, were established at 0.1 pg/μL and 0.8 pg/μL extract injected, respectively, for BDE 28, 47, 99, 100, 153, 154, and 183. Sample intake was adapted to the pollution load of each sample, resulting in LOQs between 0.01 and 0.08 ng/g ww for these congeners. Procedural blank values were found to be consistent (RSD < 10%), and therefore the mean procedural blank value was used for subtraction. Results are reported as "not detected" (ND) when the concentration is lower than 0.01 ng/g ww. LOD and LOQ for BDE 209, established in the same manner, were 0.08 and 0.8 ng/g ww, respectively. Due to relatively high (mean = 0.66 ng/extraction) but consistent (RSD < 30%) procedural blank values for the BDE 209 analyses, mean procedural blank values have been subtracted from the results, and results are only reported if the blank corrected values are higher than 3 times the mean blank value, i.e., reported values are at least 4 times higher than the mean procedural blank. A generally applicable LOQ per gram sample could not be calculated due to different sample intakes related to availability for each sample, but LOQs for the positive samples ranged between 2 and 4 ng/g ww.

No certified reference materials are currently available for PBDEs in biota. The participation of the Toxicological Center in the recent interlaboratory studies for PBDEs showed a very good performance (23, 24), which was repeated in the BROC certification feasibility study, which was organized in 2002–2003 (25).

Statistical Analysis. A number of statistical tests were applied to address all the specific needs of this study. For samples with concentrations below detection limits, which were minor in number, zero was used for the calculations. Simple linear regression coefficient was used to test for correlations between the lipid content and the contaminant load and between the total PBDE load and the distance to Antwerp. Correlations between fish size and contaminant load and between fish size and lipid content were tested with the Kruskal-Wallis test. To compare the mean concentrations in BNS and SE and to compare accumulation factors between species, the Mann Whitney U-test was used. This test was also used to test the differences between PBDE profiles found in liver and muscle and to test differences in the ratio between BDE 99 and 100 in BNS and SE samples. Wilcoxon matched pairs signed rank test was used to compare the levels in muscle and liver, to compare liver accumulation factor for lower brominated congeners with higher brominated congeners. To compare PBDE profiles between species and locations, an unpaired t-test for equal variances was used. Unless stated differently, significance levels for all tests were p = 0.05. All statistical tests were performed using Statistica v5.0 software (StatSoft Inc., Tulsa, U.S.A.).

Results and Discussion

Lipid Content. A lipid determination on a separate sample aliquot was not performed because of the low sample amount for some locations, species, or tissues. Instead the lipid determination was performed on an aliquot of the extract

TABLE 3. Lipid Percentage[a]

	N	mean	SD	median	range
crab	28	1.9	1.2	1.4	0.8–4.8
goby	17	2.0	1.1	1.4	0.8–3.5
starfish	21	2.7	1.1	2.5	1.3–5.3
shrimp	23	0.6	0.1	0.6	0.5–0.8
dab L	7	35.4	7.1	36.8	21.2–42.2
dab M	13	1.8	0.5	1.7	1.2–3.1
plaice L	9	26.7	12.0	22.3	15.0–47.0
plaice M	15	0.8	0.2	0.8	0.5–1.2
bib L	12	55.1	7.8	56.9	41.6–70.1
bib M	23	0.4	0.1	0.5	0.3–0.6
sole L	16	14.8	9.4	13.0	5.1–39.7
sole M	36	1.0	0.5	0.9	0.4–2.2
whiting L	12	34.0	13.7	36.4	9.8–50.4
whiting M	24	0.5	0.1	0.5	0.3–0.7

[a] M = muscle; L = liver.

(1/5th) before clean up. Good lipid recoveries for lean and fatty fish were obtained using our method during QUASIMEME interlaboratory exercises. The basic statistical data of the lipid determinations are shown in Table 3.

The lipid percentage of fish tissue can be influenced by several factors, such as sex, age, and species (*26*). Additionally, an annual cycle in lipid composition, which is related to the fish' nourishment and spawning status, can be observed, with higher lipid percentages in the prespawning period (*27*) and during plankton bloom periods (*28*). This may well affect lipid normalized data presentation, especially in females, since they can dispose of their contaminants during spawning by incorporating large amounts of fat and persistent pollutants in the roe (*26*). In the same study of Larsson et al., male pike showed higher levels of contaminants than females, because elimination in milt is less than in roe (*26*). Moreover, the method used to perform the lipid determination greatly influences the result (*17*), which can introduce substantial errors for lean fishes when results are normalized to lipid weight.

A study in female pike (*Esox lucius*) showed a significant negative correlation between age and levels of the lipophilic contaminants polychlorinated biphenyls (PCBs) and 2,2-bis-(4-chlorophenyl)-1,1-dichloroethylene (p,p'-DDE), which could be explained by yearly elimination of contaminants during spawning (*26*). These effects of spawning on contaminant load are very species dependent, since some species are sexually mature at a very old age (e.g., lake trout), while others only spawn every 3 years or die after spawning (e.g., salmonids) (*26*). Samples in this study were all taken prior to spawning, meaning all lipid percentages are near a theoretical maximum.

Detected PBDE Congeners and Levels. BDE 47 and 100 were found to be the only two congeners present in every sample analyzed. BDE 183 could not be detected in any sample. In the samples from the BNS, PBDE congeners 28, 99, 154, and 153 could be detected in 79%, 83%, 90%, and 71% of the samples, respectively. All congeners could be detected in the samples from the SE, except BDE 28 (96%) and BDE 153 (94%) (Tables SI-1, SI-2, and SI-3). Sum of BDEs refers to the sum of the six consistently detected congeners, namely BDE 28, 47, 99, 100, 153, and 154 (Table 4). BDE 49 was detected in all samples from the SE but not quantified due to the lack of a suitable standard. Polybrominated biphenyl (PBB) 153, which is eluting between BDE 154 and BDE 153 on the HT-8 column, could not be detected in any sample.

The fact that BDE 183 was never detected does not mean that it is not present or that it is not bioavailable. A study of sediments taken from the same locations as the biota samples in this study will give a definitive answer whether this congener is present or not. Results of these analyses are being processed and will be published soon. Following a carp feeding study, Stapleton et al. stated that BDE 183 is not accumulated in carp tissues but is debrominated in the intestine to BDE 154, which does accumulate (*29*).

BDE 209 could be detected in eight pooled liver samples (1 sole, 3 bib, and 4 whiting samples). Due to relatively high but consistent procedural blank values for this congener, special measures have been taken to exclude any false positive results, as described in the section Quality Assurance. Levels of BDE 209 ranged between 3.4 (LOQ = 2.8) and 37.2 ng/g ww (LOQ = 3.5). The latter was found in a bib sample from the SE. A typical chromatogram of both a method blank and a sample is presented in Figure 2.

These low levels, but significantly higher than procedural blanks, of BDE 209 in liver samples are probably related to very recent exposure. Due to a possible very low half-life time of this congener, a very rapid decrease in tissue levels may be observed. Elimination of higher brominated PBDEs occurs more rapidly and half-life times of 7 days have been

TABLE 4. Sum of Six PBDE Congeners for Each Species, Tissue, and Location Expressed in ng/g ww

location	shrimp	crab	starfish	goby	dab liver	dab muscle	plaice liver	plaice muscle	bib liver	bib muscle	sole liver	sole muscle	whiting liver	whiting muscle
							BNS[b]							
1	0.02	0.92	0.14	0.42	6.9	0.26	5.2	0.32	71.6	0.28	4.8	0.33	24.1	0.14
4	*a*	0.85	0.26	*a*	7.1	0.25	3.8	0.06	*a*	*a*	*a*	*a*	19.7	0.22
5	*a*	0.36	0.14	*a*	2.8	0.15	*a*	*a*	*a*	*a*	1.2	0.08	*a*	*a*
6	0.08	1.4	1.5	1.8	*a*	*a*	*a*	*a*	108	0.22	8.2	0.40	89.1	0.92
7	0.04	1.0	1.2	0.80	*a*	*a*	*a*	0.44	97.2	0.38	0.84	0.73	89.9	0.53
8	0.03	0.62	0.98	1.1	6.0	0.40	37.9	0.94	27.8	0.76	2.6	0.55	24.5	0.44
9	0.03	1.2	0.59	0.16	12.0	0.39	3.1	0.09	*a*	*a*	1.0	0.10	*a*	*a*
							SE[c]							
10	0.20	1.2	2.5	3.9	*a*	*a*	*a*	*a*	248	0.86	*a*	*a*	75.2	1.0
11	0.27	14.1	*a*	*a*	*a*	*a*	*a*	*a*	206	0.78	15.0	0.08	*a*	*a*
12	*a*	10.0	*a*	*a*	*a*	*a*	*a*	*a*	174	0.88	*a*	*a*	*a*	*a*
13	0.70	12.8	15.5	*a*	*a*	*a*	94.3	*a*	639	2.2	46.8	6.5	*a*	*a*
14	0.83	27.9	*a*	9.7	18.6	0.66	304	6.1	537	1.9	44.5	5.1	393	2.4
15	2.3	13.6	*a*	*a*	*a*	*a*	*a*	*a*	984	5.6	37.7	6.3	279	1.0
16	8.3	29.9	*a*	*a*	*a*	*a*	74.4	*a*	*a*	*a*	90.1	6.9	236	3.0
17	7.6	*a*	*a*	*a*	*a*	*a*	*a*	*a*	*a*	*a*	93.3	5.3	*a*	*a*
18	7.6	*a*	*a*	*a*	*a*	*a*	*a*	*a*	*a*	*a*	*a*	*a*	*a*	*a*

[a] Not available. [b] BNS = Belgian North Sea. [c] SE = Scheldt Estuary.

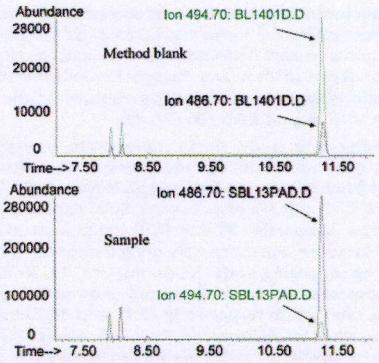

FIGURE 2. GC-ECNI chromatogram of a BDE 209 analysis from a procedural blank and from a sample (37.2 ng/g ww). Equal amounts of internal standard (^{13}C-BDE 209) have been added for both analyses (3.75 ng). Ion *m/z* 486.7 is used to quantify BDE 209; ion *m/z* 494.7 is used to quantify ^{13}C-BDE 209.

calculated for BDE 209 in humans (*30*). Sampling time may become thus very critical in finding BDE 209 in marine biota.

BDE 209 is of special interest because it is believed to be nonbioavailable and to be a virtually harmless compound (*18*). Few studies are published demonstrating that aquatic biota are able to accumulate BDE 209 (*31*). Kierkegaard et al. showed that BDE 209 can be taken up by rainbow trout (*Oncorhynchus* mykiss) in laboratory experiments and that liver was the main target organ (*32*). The first report of BDE 209 being accumulated by freshwater bream in the environment was only recently reported by Lepom et al. (*31*), with concentrations up to 37 ng/g lw. In terrestrial life, Sellström et al. has found BDE 209 in eggs of the top predator peregrine falcon (*Falco peregrinus*) in relatively high concentrations (*33*). This implicates that the birds were able to accumulate the compound and to pass it on to the eggs without prior modification or metabolization. BDE 209 presence in humans working in a recycling factory of electronic household equipment has also been demonstrated (*34*). These findings are of great importance since they show that the assumption that BDE 209 is not bioavailable is not correct. The present study could not clearly confirm the possible bioavailability of BDE 209, but eight samples showed detectable levels above LOQ for this congener. But even if BDE 209 is not taken up, degradation products, i.e., lower brominated BDEs, may still accumulate. However, the degradation pathways of BDE 209 are however poorly understood (*7, 32*).

For each species, levels of PBDEs were statistically higher for all SE locations when compared to the BNS locations, while the lipid percentages for each species were similar for the two areas. This indicates a pollution source in or near the SE. Levels in BNS ranged from 0.02 to 1.5 ng/g ww in benthic invertebrates and goby, from 0.06 to 0.94 ng/g ww in fish muscle, and from 0.84 to 128 ng/g ww in fish liver, respectively. For the SE samples, levels ranged from 0.20 to 29.9 ng/g ww in benthic invertebrates and goby, from 0.08 to 6.9 ng/g ww in fish muscle, and from 15.0 to 984 ng/g ww in fish liver.

Special attention should be attributed to crab and starfish, since levels found in these organisms are significantly higher than the levels found in shrimps. This can be ascribed to their dynamic position in the food web; crabs and starfish are scavengers that also feed on higher organisms once these have died and have dropped to the sea floor (*35*).

PBDE Levels in Fish from Other Studies. Comparison of levels from different studies can be done by using ΣPBDEs or by using individual congeners. The former can have a few drawbacks since the congeners included might vary. Using separate congeners, such as BDE 47, which is most abundant in the marine environment (*2*), can therefore be a solution and will be applied here (*17*).

To be able to compare data with other studies, levels were recalculated to lipid-based results. In the present study, BDE 47 levels ranged from 3 to 108 ng/g lw in species from the BNS, while levels from 8 to 1548 ng/g lw were observed for the SE. A recent study from Boon et al. (*17*) which also included starfish, crab, and whiting, and focused on the northern part of the North Sea, showed comparable levels of PBDEs as the BNS locations in the present study. Comparable BDE 47 levels to the ones found for starfish from the SE in the present study (603 ng/g lw), were also found by Boon et al. in starfish from the polluted Tees estuary with maximum BDE 47 levels of 546 ng/g lw (*17*).

Although the highly polluted Scheldt discharges into the BNS, this does not seem to influence the levels found in biota of that area, since levels of the BNS are comparable to those found in the northern part of the North Sea (*17*).

Similar values as from the BNS samples have been reported for the northern Atlantic Sea, with levels of BDE 47 up to 8 ng/g lw for salmon (*Salmo salar*) (*36*), while for the Baltic Sea 46 ng/g lw (*36*) and 110 ng/g lw (*37*) were observed. For fish from southern Greenland, BDE 47 levels up to 41 ng/g lw were reported, which is comparable to the lower values found in the BNS (*37*). Reported values in flounder liver from locations near the Dutch coastline were comparable with the levels found in the present study for the BNS, whereas BDE 47 levels from the Rotterdam harbor area were as high as 280 ng/g lw, which is comparable to the lower levels in the SE (*38*).

Several studies have reported levels of the same order of magnitude as those found in the SE samples, with concentrations over 1000 ng/g lw in Virginia freshwater fish, which were probably related to textile manufacturing (*15*). Asplund et al. reported values of 1700 ng/g lw for steelhead trout (*Oncorhynchus mykiss*) from Lake Michigan (*39*), while for biota living downstream possible sources in the UK (Tees bay), values up to 9500 ng/g lw were reported in flounder liver (*18*).

Concentrations found in this study are higher than those reported in most other studies, which clearly suggests a source in or near the Scheldt. The presence of a bromine plant and the fact that the upstream area is home to significant textile manufacturing might suggest that these can be contributing factors to the high levels of PBDEs found in the SE.

Observed Profiles. BDE 47 was the most abundant analyte in all analyzed samples with a contribution ranging between 43 and 75%. The general order of decreasing contribution to the total load is BDE 47 > BDE 100 > BDE 99 > BDE 153, BDE 154 > BDE 28, as has been observed previously by others (*17*); BDE 47, BDE 99, and BDE 100 make up about 90% on average of the total PBDE load. The same observations were made by Jansson et al., Christensen et al., and Asplund et al. (*18, 19, 37*).

For some species and locations in this study, this general order was not always observed. Shrimp displayed a deviant profile, with a general order in decreasing concentration of BDE 47 > BDE 99 > BDE 153 > BDE 100 > BDE 154 > BDE 28, for both BNS and SE locations. Similarly the profile in bib tissues was different, namely BDE 47 > BDE 99 > BDE 100 > BDE 153, BDE 154 > BDE 28. Figures 3 and 4 show the different profiles of the different species and tissues for BNS and SE, respectively. The observed profile difference for shrimps can be explained by statistically different BDE 99, BDE 100, and BDE 153 contributions to the total BDE-load, with mean values of 30 ± 1%, 7 ± 1% and 11 ± 1% (n = 13), respectively, while the mean profile contributions of these three congeners for all other species were 10 ± 0%, 16 ± 0%,

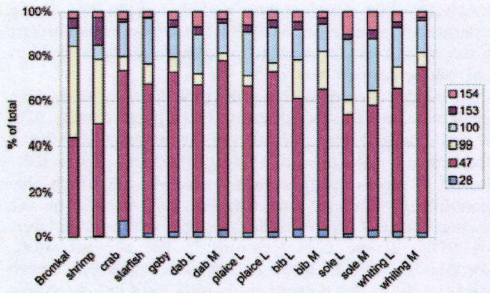

FIGURE 3. PBDE Profiles in BNS samples and profile of Bromkal 70-5DE technical mixture. "L" represents liver samples and "M" represents muscle.

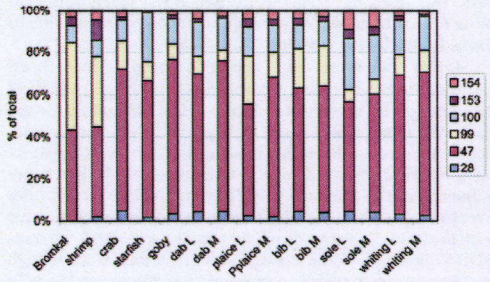

FIGURE 4. PBDE Profiles in SE samples and profile of Bromkal 70-5DE technical mixture. "L" represents liver samples and "M" represents muscle.

and 2 ± 0% (n = 130), respectively. Additionally, a significantly lower relative BDE 47 concentration was noted, which was on average 45 ± 3% (n = 13), while the mean for all other locations was 64 ± 0% (n = 130).

These observations can indicate that metabolization of BDE 99 and 153 is not so well developed in shrimp. These two congeners can both be theoretically metabolized to BDE 47 by debromination processes (*9*), which can also explain the relatively low BDE 47 percentage found in the samples with higher BDE 99 and 153 values.

Stapleton et al., who performed a feeding study on common carp (*Cyprinus carpio*), demonstrated that metabolic debromination from BDE 99 to BDE 47 occurs (*29*). After administration of food pellets spiked with BDE 99, it could be concluded that accumulation of BDE 99 was minimal and that this congener was very quickly transformed to BDE 47 (*40*). Dodder et al. also observed the lower BDE 99 concentrations in carp compared to other fish species collected at the same locations (*41*).

Kierkegaard et al. have also observed extensive metabolization after administration of BDE 209 technical product to rainbow trout (*32*). BDE 209 could hardly be detected in the tissues, but a wide range of nona- to hexa-congeners, as well as BDE 99, appeared. This observation indicates that the uptake and metabolization of BDE 209 in the samples of the present study cannot be ruled out and that metabolization of this congener may well be a partial reason for the high levels of BDEs encountered in the SE samples.

The present study clearly shows though that BDE 99 can accumulate to a rather high extent depending on which species and locations are selected. This absence or accumulation of BDE 99 can be at least partially explained by metabolic differences between species.

Ratio between BDE 99 and BDE 100. In the present study different ratios between BDE 99 and BDE 100 were observed in the different samples and tissues analyzed. When calculating these ratios, the mean was taken of all samples of 1 species or tissue from BNS and SE and the calculated ratios were rounded off to the nearest integer. Several other studies also made interesting observations concerning the ratio between BDE 99 and BDE 100 (*37, 42*).

In the present study, similar ratios were observed for starfish, goby, and whiting; the ratios were statistically different when comparing BNS and SE locations (30:70 and 60:40, for BNS and SE, respectively). Sole, dab, and plaice displayed a comparable BDE 99:BDE 100 ratio of 20:80 for the BNS locations, but ratios were not the same for the three species when looking at the SE samples (20:80, 30:70, and 60:40, respectively). Bib and shrimp showed completely different ratios, with respectively 55:45 and 80:20 for BNS locations, while for SE locations, these ratios were 60:40 and 80:20, respectively. All ratios are presented in Table 5.

The statement made earlier by Christensen et al. that the mean BDE 99:BDE 100 ratio in marine environmental locations which are not excessively polluted, is equal to 30:70, is not a general fact (*37*). In this study, four distinct ratio-groups were found for different species from the same locations (BNS), namely 30:70, 20:80, 55:45, and 80:20. For SE locations, the calculated ratios were 60:40, 40:60, 30:70, 20:80, and 80:20. We hereby conclude that the BDE 99:BDE 100 ratio is clearly species- and location-dependent. Tissue dependence (liver-muscle) could not be demonstrated for this BDE 99:BDE 100 ratio.

A difference in the BDE 99:BDE 100 ratio for a specific species was observed between BNS and SE locations, which is probably related to higher PBDE concentrations in the SE area (10−30-fold increase). This difference was statistically significant for crab and plaice; for the BNS, the ratios were 30:70 and 20:80, respectively, while for the SE, both ratios were 60:40. Minor changes were found for starfish, goby, and whiting (30:70 → 40:60) and for dab (20:80 → 30:70), but these changes were not statistically significant. No profile change could be observed for shrimp and sole samples, which were constant for BNS and SE with 80:20 and 20:80, respectively.

The link between these changes in BDE 99:BDE 100 ratios, which can also be expressed as a relative increase of BDE 99, is most probably due to an increased exposure to this congener. The commercial product Bromkal 70-5DE showed a BDE 99:BDE 100 ratio of 84:16 (*43*). In sediments from the SE, which were analyzed in the BROC feasibility study, this ratio was found to be 80:20 (*25*). *The BDE 99:BDE 100 ratio found for shrimp (80:20) is very similar to the ratio for the Bromkal technical mixture and virtually identical to the ratio found in SE sediment. This ratio does not change between BNS and SE. Shrimp simply reflects the Bromkal and sediment constitution pattern for these congeners, suggesting that the compound is readily bioavailable, but shrimp clearly lacks metabolization capability.*

Profile changes, when comparing a species from a nonpolluted area (BNS) to a polluted area (SE), are probably due to complete or partial lack of metabolization capacity. In the case of a changing ratio, the species' metabolism cannot keep up with the constant input of new pollutants and the ratio BDE 99:BDE 100 increases. If no profile changes can be observed, as for shrimp (ratio 80:20) and sole (ratio 20:80) samples, the species is not able to metabolize and tissue levels reflect the background profile (shrimp), or the metabolic turnover of BDE 99 is of such magnitude that its steady-state BDE profile is maintained (sole). All other species, which present ratios between 80:20 and 20:80, have metabolic abilities somewhere between those of shrimp and sole.

Although sole can probably be classified as a high metabolizer, this is maybe not the case for all congeners.

TABLE 5. BDE 99:BDE 100 Ratios from Other Studies According to the Pollution of the Sampling Site[a]

sample type	low pollution	high pollution	location	reference
Bromkal 70-5DE	na[b]	84:16	na[b]	Sjodin et al. (1998) (*43*)
sediment	na[b]	80:20	Scheldt	Lohman et al. (2003) (*25*)
shrimp	80:20	80:20	BNS – SE	this study
crab	30:70	60:40	BNS – SE	this study
starfish	30:70	40:60	BNS – SE	this study
goby	30:70	40:60	BNS – SE	this study
dab	20:80	30:70	BNS – SE	this study
plaice	20:80	60:40	BNS – SE	this study
bib	55:45	60:40	BNS – SE	this study
sole	20:80	20:80	BNS – SE	this study
whiting	30:70	40:60	BNS – SE	this study
blue mussel	80:20	na[b]	Denmark	Christensen et al. (2001) (*42*)
shorthorn sculpin	30:70	na[b]	Southern Greenland	Christensen et al. (2002) (*37*)
uvak	30:70	na[b]	Southern Greenland	Christensen et al. (2002) (*37*)
starry ray	30:70	na[b]	Southern Greenland	Christensen et al. (2002) (*37*)
steelhead trout	na[b]	60:40	Lake Michigan	Asplund et al. (1999) (*39*)
steelhead trout	na[b]	60:40	Lake Michigan	Asplund et al. (1999) (*39*)
baltic salmon	na[b]	60:40	Baltic Sea	Asplund et al. (1999) (*39*)
baltic salmon	na[b]	60:40	Baltic Sea	Asplund et al. (1999) (*39*)
salmonids	na[b]	50:50	Lake Michigan	Manchester-Neesvig et al. (2001) (*50*)
pike	na[b]	80:20	Sweden	Sellstrom et al. (1998) (*51*)
carp	na[b]	5:95	Hadley Lake (USA)	Dodder et al. (2000) (*40*)
carp	na[b]	5:95	Belgium	unpublished data Covaci et al.
carp	na[b]	0:100	Lab exposure experiments	Stapleton et al. (2003) (*41*)
white crapie	na[b]	60:40	Hadley Lake (USA)	Dodder et al. (2000) (*40*)
smelt	na[b]	80:20	Lake Ontario (USA)	Dodder et al. (2000) (*40*)
whale blubber	70:30	na[b]	Faroe Islands (Atlantic)	Lindstrom et al. (1999) (*52*)
porpoise blubber	60:40	na[b]	England and Wales	Law et al. (2002) (*53*)
porpoise liver	50:50	na[b]	Belgian North Sea coast	Covaci et al. (2002) (*54*)

[a] Division concerning low-high pollution was based on similarity of concentration with levels found in this study (BNS = low; SE = high). [b] na = not available.

BDE 154 contribution to total was found to be statistically higher in sole tissues when compared to all other samples. Relative concentrations of BDE 99 in sole samples are low, as mentioned, which is most probably due to the high metabolic turnover. This lower BDE 99 value can maybe also be related to the higher BDE 154 levels found, since theoretical debromination is possible from BDE 154 to BDE 99. A third possible explanation for this profile observation can be that sole efficiently metabolizes BDE 99 to BDE 47 but can also metabolize BDE 183 to BDE 154, which might explain the high levels of BDE 154. Stapleton et al. has demonstrated the debromination of BDE 183 to BDE 154 by a carp feeding study using food pellets spiked with BDE 183 (*40*). BDE 100 can also accumulate to a higher extent due to its smaller molecular size when compared to BDE 99, since smaller molecular size may allow congeners to bioaccumulate more readily (*44*).

When looking at all the possible explanations for these profiles, it can be concluded that it is very difficult to draw any conclusions just by looking at tissue levels without taking into regard other parameters, such as metabolization, uptake, molecular size, etc.

Ratio of BDE 99:BDE 100 in Other Studies. The use of the BDE 99:BDE 100 ratio cannot give any information about the pollution of the area, unless this ratio of the same species from a nonpolluted location, combined with knowledge about the metabolization ability of the species, is known. In this study, the same species were sampled at different areas (polluted (SE) vs non polluted (BNS)). The exposure of the organisms at the two areas is not equal, and we can assume that the species uptake ability of PBDEs is not location dependent.

For additional data from different locations and background exposures, different BDE 99:BDE 100 ratios were calculated in fish, mussels, and marine mammals from similar studies in Europe, North America, and the Arctic (Table 5). This ratio is dependent on a variety of parameters, such as species, environmental PBDE exposure, environmental profile, and tissue type. The BDE 99:BDE 100 ratio can be useful though, to compare the pollution pattern from different locations.

Marine organisms seem to increase their metabolic capabilities when they climb the evolutionary ladder. The BDE 99:BDE 100 ratios for lower organisms, like mussels and shrimp, tend to be more BDE 99 dominant (80:20), while those for higher organisms, e.g. sole and carp, are more BDE 100 dominant (20:80 and 5:95, respectively, Table 5).

Ikonomou et al. reported that crab was enriched in lower brominated PBDEs (i.e. tri and tetra), whereas sole was enriched in higher brominated PBDEs (i.e. penta and hexa) (*3*). The same observation can be made in this study (Figures 3 and 4): BDE 28 is significantly higher for crab when compared to sole (only for BNS), whereas BDEs 100 and 154 are significantly higher for sole. Following these findings, it might be necessary to include a benthic invertebrate species such as crab in any work attempting to address PBDE profiles in the environmental biota, because the levels of lower brominated congeners might be improperly assessed using only fish. When fish is used, the metabolic differences should be kept well in mind before drawing any conclusions.

Correlations and Relations. Regression coefficients between concentrations found in SE samples and distance to Antwerp were found to be between $R^2 = 0.58$ and $R^2 = 0.77$ ($p < 0.05$; Table 6). These geographical trends were only significant for crab and sole though. This calculation could only be performed on samples for which sufficient analyses had been performed (crab, shrimp, and bib, sole, and whiting as well muscle as liver). Generally, the geographical trend is highly similar for all species, suggesting a rising trend in total PBDE concentration related to one or more point sources situated upstream the Scheldt, as already claimed in other studies (*16*).

TABLE 6. Regression between Total PBDEs and Distance to Antwerp

	R^2	p	N
sole liver	0.77	0.02	6
plaice liver	0.12	0.77	3
bib liver	0.53	0.10	6
whiting liver	0.59	0.23	4
sole muscle	0.68	0.04	6
bib muscle	0.44	0.15	6
whiting muscle	0.36	0.40	4
shrimp	0.52	0.07	7
crab	0.58	0.05	7

The geographical trend in concentrations for the SE is much less pronounced for fish (Table 6), which can be related to their less sedentary character. This is especially the case for the more migrating nature of the gadoids compared to benthic invertebrates (*17*). The large tidal movements on the SE probably also play an important role in this nonsignificant regression coefficients (*45*). For the BNS area, locations 1, 4, 5, and 9 generally show the lowest concentrations. BDE levels of locations 6, 7, and 8, which are located in the vicinity of the Zeebrugge harbor, are more elevated than those of the other BNS locations. This increase is only statistically significant for whiting tissues and for benthic invertebrates. For all other samples the increase in concentration is also present but not statistically significant. This rise in PBDE concentrations can possibly be related to industrial and dredging activities in the harbor of Zeebrugge but is more probably due to influence of the discharge plume of the Scheldt into the North Sea, which can reach as far as Ostend (*45*). From location 10 onward, which can be considered the beginning of the SE, PBDE levels increase very rapidly, going from 1.2 ng/g ww for crabs at location 10 to 29.9 ng/g ww at location 16. For fish liver samples, concentrations up to 984 ng/g ww were observed for bib, but this is probably related to its high lipid content (approximately 55%).

No correlation could be observed between lipid percentage and total PBDE load in the fish samples, nor in starfish or shrimp. This might suggest that for these organisms, the majority of PBDEs is taken up by the diet, and that bioconcentration is of minor importance. For crab samples, however, a regression coefficient of $R^2 = 0.77$ ($p < 0.05$) was calculated between PBDE load and lipid percentage. This might suggest that for crab, bioconcentration plays a rather important role in the accumulation of these compounds. Flying crabs are scavengers, which makes them difficult organisms to draw conclusions from, since their place in the food web is complex: crab feeds on organisms which are located lower in the food chain as well as on dead fish, which occupy a higher position (*35*). This might partly explain the rather high concentrations found in crab tissue.

No relation could be found between fish size and contaminant load for the samples from this study. This observation might be explained by the low sample number, as has been shown in other studies (*26*).

Liver Accumulation. Generally, PBDEs tend to accumulate more in liver than in muscle or adipose tissue (*46*). Lipid normalized concentrations were used to compensate for the influence of the higher lipid concentration in liver tissue. To express the preferential accumulation of PBDEs, the total concentration in liver was divided by the total concentration in liver and the total concentration in muscle. The obtained ratio can be used as an indicator for preferential accumulation in liver; a ratio of 0.5 means that no preferential accumulation is observed; higher or lower ratios indicate preferential liver or muscle accumulation, respectively. For dab, plaice, bib, and whiting, ratios of 0.57 ± 0.02 ($n = 7$), 0.55 ± 0.04 ($n = 8$), 0.64 ± 0.04 ($n = 11$), and 0.67 ± 0.03 ($n = 10$) were found for the sum of PBDEs. A similar ratio was found for whiting by Boon et al., indicating that these species tend to accumulate the targeted PBDE congeners selectively in their liver (*17*). In the present study, a ratio of 0.47 ± 0.03 ($n = 13$) was found for sole, however, suggesting no preferential storage of PBDEs in liver. The previously assumed high metabolic capability of sole, which can result in low tissue concentrations, can also be held responsible for this deviant ratio. Too few samples were available to draw any statistically relevant conclusions though.

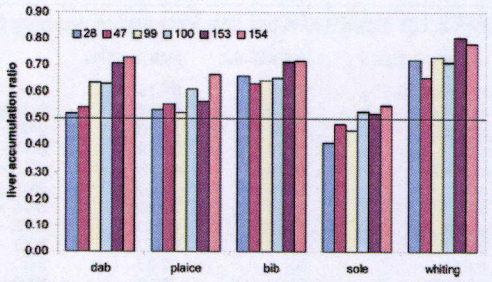

FIGURE 5. Liver accumulation ratio per congener in different fish species; ratios higher than 0.50 indicate preferential liver accumulation. Ratios are higher for higher degree of bromination.

The selective liver accumulation could be governed by passive distribution, by lipid content, or by an active hepatic accumulation process. Also in pike liver, active enrichment for BDE 47 was observed, which was related to the detoxifying role of the liver in xenobiotic metabolization processes and not solely to the passive redistribution governed by lipid content (*46*).

In the present study, liver accumulation could not be related to the liver lipid content, which indicates active enrichment could be more likely. Passive accumulation might be an important element for the higher brominated congeners, whereas it has been proposed by Kierkegaard et al. that higher brominated congeners might display a higher liver accumulation due to a higher log K_{ow} value in combination with their larger molecular size, which hinders their ability to cross membranes (*32*). In the present study, higher brominated congeners also showed higher liver accumulation (Figure 5). This higher liver accumulation was only statistically significant for dab, plaice, and sole; R^2 calculated between degree of bromination and liver accumulation were 0.94, 0.90, and 0.92 ($p > 0.05$), respectively. For bib and whiting, a trend could be observed but was not statistically significant. These observations might suggest that tissue dependent accumulation might well be related to animal species as well as to compound characteristics.

Profile differences could also be observed between muscle and liver, e.g. BDE 47 contribution was significantly lower in liver than in muscle. No possible explanation could be found for this observation.

Bioaccumulation/Biomagnification. PBDEs have been shown to biomagnify in the marine environment (*2, 17, 47*). Some studies showed that biomagnification of BDE 47, 99, and 100 are approximately equal, whereas reduced biomagnification was observed for BDE 154 (*47*). This might offer a partial explanation for the relative lack of BDE 154 and abundance of BDE 47 observed in biota profiles when compared to the expected source composition made up of commercial mixtures (Figures 3 and 4). An explanation should also be looked for in the metabolic potential to reduce BDE 154 levels and to form BDE 47 and other congeners. Results from this study might suggest that profiles observed in tissues are only partially related to uptake and background pollution. A large influence on profile can be due to metabolic processes.

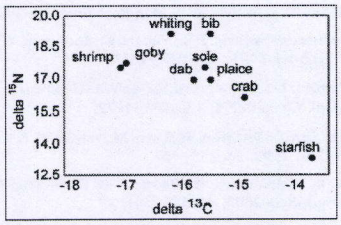

FIGURE 6. Isotopic ratios of ^{13}C and ^{15}N for the different species of the present study (*48*).

The major biomagnification step in the food chain occurs from fish to marine mammals (*17*). Also in lower levels of the food web, from copepods over planktivorous fish to predatory fish, biomagnification was found for tetra- to hexa-PBDEs (*36*). In the present study, no clear trend could be discovered that would suggest any biomagnification from benthic organisms to fish. All fish species in this study feed on benthic invertebrates (*48*) and goby, as the latter could be readily encountered in the fish' stomachs.

The use of naturally occurring stable isotopes of carbon and nitrogen can provide more accurate information on food web positioning of marine organisms (*49*). The carbon and nitrogen isotope ratios (^{13}C/^{12}C and ^{15}N/^{14}N) in an organism reflect those of its diet with a slight selective retention of the heavier isotope and excretion of the lighter one (*48*). These isotope ratio determinations have not been performed on the samples of the present study, but ratios of the same organisms from the same locations were available in the literature and are graphically presented in Figure 6 (*48*). Based on these data, *Asterias rubens* is positioned at the base of the food chain sampled in the present study. The benthic invertebrate species *Crangon crangon* displays a rather high food web positioning based on the isotope ratios, which can be explained by its omnivorous and scavenging feeding pattern. Gadoids display the highest food web position encountered in this study. This is also related to their carnivorous feeding pattern. These food web positioning differences are not reflected in the measured PBDE concentrations in the different species of the present study.

Acknowledgments

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Supporting Information Available

Detailed statistical descriptors (range, median, *N*) of all benthic invertebrate samples and goby from all locations (Table SI-1) and these statistical descriptors for all fish samples from BNS (Table SI-2) and SE (Table SI-3). This material is available free of charge via the Internet at http://pubs.acs.org.

Note Added after ASAP Posting

This paper was released ASAP on 09/06/2003 with an incorrect reference citation in the Ration between BDE 99 and BDE 100 section under Results and Discussion. A sentence was also missing from the Acknowledgments. The correct version was posted 09/10/2003.

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TABLE SI-1. Statistical descriptors from BNS and SE samples: shrimp, crab, starfish, and goby. Concentrations are expressed as ng/g ww

		BDE 28	BDE 47	BDE 100	BDE 99	BDE 154	BDE 153	ΣPBDEs
shrimp BNS	Range	n.d	0.01-0.03	n.d.-0.01	0.01-0.02	n.d.	n.d.-0.01	0.02-0.08
	Median	n.d.	0.02	n.d.	0.01	n.d.	0.01	0.03
	N	5	5	5	5	5	5	5
crab BNS	Range	0.01-0.11	0.25-0.96	0.05-0.24	0.02-0.09	0.02-0.06	0.01-0.02	0.36-1.4
	Median	0.05	0.57	0.12	0.06	0.03	0.01	0.94
	N	7	7	7	7	7	7	7
starfish BNS	Range	n.d.-0.03	0.07-0.94	0.04-0.26	0.01-0.23	0.01-0.02	n.d.	0.14-1.5
	Median	0.01	0.41	0.14	0.03	0.01	n.d.	0.59
	N	7	7	7	7	7	7	7
goby BNS	Range	0.01-0.04	0.11-1.3	0.02-0.23	0.01-0.12	0.01-0.06	0.01-0.06	0.16-1.8
	Median	0.02	0.45	0.08	0.05	0.02	0.02	0.66
	N	6	6	6	6	6	6	6
shrimp SE	Range	n.d.-0.16	0.07-4.0	0.01-0.66	0.06-3.1	0.01-0.40	0.01-0.88	0.20-8.3
	Median	0.03	0.68	0.10	0.50	0.07	0.18	1.6
	N	8	8	8	8	8	8	8
crab SE	Range	0.09-1.4	0.76-20.2	0.20-3.2	0.06-4.9	0.03-1.0	0.01-0.46	1.8-29.9
	Median	0.68	8.9	1.2	1.6	0.37	0.19	13.6
	N	7	7	7	7	7	7	7
starfish SE	Range	0.28	9.6	3.2	2.2	0.19	0.01	1.6-15.5
	Median	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	N	1	1	1	1	1	1	1
goby SE	Range	0.11-0.34	2.8-7.1	0.55-1.2	0.21-0.73	0.12-0.22	0.10-0.19	3.9-9.7
	Median	0.23	4.9	0.86	0.47	0.17	0.14	6.8
	N	2	2	2	2	2	2	2

n.a. = not available; n.d. = not detected; BNS = Belgian North Sea; SE = Scheldt Estuary

TABLE SI-2. Statistical descriptors of fish data from BNS samples. Concentrations are expressed as ng/g ww

		BDE 28	BDE 47	BDE 100	BDE 99	BDE 154	BDE 153	ΣPBDEs
dab L	Range	0.07-0.28	1.9-8.3	0.50-2.1	0.14-0.59	0.18-0.69	0.03-0.37	2.8-12.0
	Median	0.14	4.3	1.1	0.33	0.50	0.27	6.8
	N	6	6	6	6	6	6	6
dab M	Range	n.d.-0.03	0.08-0.30	0.02-0.05	n.d.-0.03	n.d.-0.01	n.d.-0.01	0.11-0.41
	Median	0.01	0.23	0.04	0.01	0.01	0.01	0.32
	N	6	6	6	6	6	6	6
plaice L	Range	0.02-0.15	2.3-3.8	0.50-1.1	0.05-0.40	0.16-0.35	0.06-0.28	3.1-6.0
	Median	0.09	2.9	1.0	0.22	0.27	0.11	4.4
	N	5	5	5	5	5	5	5
plaice M	Range	n.d.-0.01	0.04-0.31	0.01-0.07	n.d.-0.03	n.d.-0.02	n.d.-0.02	0.06-0.47
	Median	0.01	0.12	0.03	0.01	0.01	0.01	0.19
	N	6	6	6	6	6	6	6
bib L	Range	0.81-4.5	10.3-75.6	3.3-17.1	4.1-20.3	1.4-5.0	1.1-3.5	21.0-122
	Median	2.5	55.5	10.9	7.9	3.7	1.7	97.2
	N	5	5	5	5	5	5	5
bib M	Range	n.d.-0.03	0.08-0.49	0.02-0.13	0.02-0.08	0.01-0.03	0.01-0.01	0.13-0.76
	Median	0.01	0.24	0.04	0.06	0.01	0.01	0.38
	N	5	5	5	5	5	5	5
sole L	Range	n.d.-0.22	0.39-3.7	0.26-2.5	0.02-0.77	0.09-0.78	n.d.-0.34	0.84-8.2
	Median	0.02	1.5	0.71	0.11	0.27	0.02	2.6
	N	7	7	7	7	7	7	7
sole M	Range	n.d.-0.02	0.04-0.34	0.02-0.017	n.d.-0.09	0.01-0.07	n.d.-0.03	0.08-0.73
	Median	0.01	0.21	0.11	0.03	0.03	0.02	0.4
	N	7	7	7	7	7	7	7
whiting L	Range	0.42-2.8	11.1-62.8	2.86-16.2	1.6-14.5	1.2-4.1	0.43-2.8	19.7-89.9
	Median	0.64	16.2	4.8	2.9	1.4	0.79	25.7
	N	6	6	6	6	6	6	6
whiting M	Range	n.d.-0.02	0.05-0.68	0.01-0.15	n.d.-0.05	n.d.-0.02	n.d.-0.01	0.07-0.92
	Median	0.01	0.22	0.05	0.02	0.01	n.d.	0.33
	N	6	6	6	6	6	6	6

n.a. = not available; n.d. = not detected; L = liver; M = muscle

TABLE SI-3. Statistical descriptors of fish data from SE samples. Concentrations are expressed as ng/g ww

		BDE 28	BDE 47	BDE 100	BDE 99	BDE 154	BDE 153	ΣPBDEs
dab L	Range	0.80	12.2	3.0	1.5	0.76	0.30	18.6
	Median	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	N	1	1	1	1	1	1	1
dab M	Range	0.03	0.47	0.10	0.03	0.02	0.01	0.66
	Median	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	N	1	1	1	1	1	1	1
plaice L	Range	2.0-6.5	36.5-186	10.6-40.3	20.2-38.5	2.2-17.7	2.9-15.0	74.4-304
	Median	2.4	47.0	13.1	25.7	2.6	3.5	94.3
	N	3	3	3	3	3	3	3
plaice M	Range	0.11	4.1	0.79	0.71	0.26	0.18	6.1
	Median	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	N	1	1	1	1	1	1	1
bib L	Range	8.5-47.7	107-544	17.5-110	30.3-199	5.5-38.5	5.5-45.2	174-984
	Median	16.2	230	46.7	69.3	15.5	13.5	392
	N	6	6	6	6	6	6	6
bib M	Range	0.03-0.27	0.47-3.2	0.09-0.71	0.15-1.1	0.02-0.19	0.02-0.16	0.78-5.6
	Median	0.05	0.88	0.16	0.25	0.04	0.03	1.4
	N	6	6	6	6	6	6	6
sole L	Range	0.63-6.1	8.3-47.7	3.9-26.7	0.61-7.7	1.3-8.7	0.30-7.3	15.0-93.3
	Median	1.9	26.8	11.4	3.0	4.6	1.8	49.8
	N	6	6	6	6	6	6	6
sole M	Range	n.d.-0.42	0.05-3.9	0.01-1.5	n.d.-0.76	0.01-0.58	n.d.-0.42	0.08-6.9
	Median	0.21	3.2	1.2	0.38	0.46	0.19	5.8
	N	6	6	6	6	6	6	6
whiting L	Range	1.8-10.2	50.6-254	15.9-76.6	4.6-31.3	1.8-8.9	0.46-8.6	75.2-393
	Median	8.9	169	39.1	25.4	7.1	5.1	258
	N	4	4	4	4	4	4	4
whiting M	Range	0.02-0.10	0.72-1.93	0.16-0.45	0.03-0.44	0.02-0.06	n.d.-0.05	1.0-3.0
	Median	0.04	1.2	0.31	0.13	0.03	0.01	1.7
	N	4	4	4	4	4	4	4

n.a. = not available; n.d. = not detected; L = liver; M = muscle

4

PBDEs in the Belgian terrestrial environment



4.1 Introduction

The relatively high exposure and the accessibility of aquatic samples (See *3.4* ***Paper IV***) make that the aquatic environment is rather extensively studied; the majority of PBDE related studies covers this medium (invertebrates, fish, marine mammals and aquatic birds). Studies focussing on the terrestrial environment are much less numerous. Most studies in that area cover (predatory) bird eggs (Law et al, 2003). Due to the fact that most bird species are protected, PBDE studies on tissues are still scarce. This is also the case for terrestrial mammals; the few studies that are published deal with a limited number of samples.
Although better controlled since the implementation of the *product stewardship programme* by the BFR industry (See *1.4 Environmental fate and behaviour of BFRs*), the release of PBDEs and other BFRs occurs mainly during their (terrestrial) production, transport, and application and during use and disposal of BFR containing products. This has undoubtedly led to direct exposure of the terrestrial environment.
Terrestrial predatory birds are interesting study objects concerning persistent organic pollutants seeing what has happened in the 1960's. In that period, eggshell thinning related to DDT exposure and the associated endocrine effects had caused severe population declines in a number of predatory bird species in Europe and North America (Vos et al., 2000). Seeing the possible endocrine disrupting activity of PBDEs (Legler and Brouwer, 2003), the biomagnification that is associated with these chemicals can therefore have serious health effects on top-predators, such as birds of prey (Law et al., 2003; Law et al., 2004). Such birds have been used successfully for biomonitoring of organic pollutants in several studies (Ratcliffe 1993; Herzke et al., 2003). Since they are widespread, sensitive to environmental changes and occupy a top position in the food chain, birds of prey are very suitable to study bioaccumulation (Norstrom et al., 2000). Moreover, the physiology, ecology and behaviour of most common birds are well known. Research on predatory birds can be considered high priority and several publications on PBDEs in terrestrial birds have been published (Selström et al., 1993; Lindberg et al., 2004). Since most of the studies were conducted on eggs, very limited information is available on PBDEs in bird tissues (Herzke et al, 2003; Jaspers et al., 2005). In contrast to avian wildlife, terrestrial non-avian wildlife was hardly investigated, until the recent appearance of studies on grizzly bears and hedgehogs (Christensen et al., 2006; d'Havé et al., 2005).

Paper V investigates tissue levels and tissue distribution of PBDEs in terrestrial predatory birds, such as common buzzard (*Buteo buteo*) and sparrowhawk (*Accipiter nisus*). Liver, muscle, brain and adipose tissue were analysed, together with serum samples. A newly developed method for analysis of PBDEs in serum was applied in this study. This method is presented later (See *5.2.* ***Paper IX***). Until the publication of this paper, no such detailed information was available on the fate and distribution of PBDEs in predatory birds.

Additionally, the PBDE congener patterns of the various species and tissues were examined. Special emphasis was hereby put on the analysis of BDE 209.

In general, median ΣPBDE levels (BDEs 28, 47, 99, 100, 153, 154, and 183) in sparrowhawks ranged from 360 to 1900 ng/g lw, depending on the tissue, which was around one order of magnitude higher than in common buzzards (26 - 130 ng/g lw). These differences could be attributed to the species' feeding habits (See *4.5.* ***Paper VIII***). In general, adipose tissue and muscle contained the highest load of PBDEs (30-35 %), followed by liver and serum. In all species, the lowest PBDE load was measured in brain, which is a fairly lipid rich tissue. This indicates that passive (lipid content related) diffusion of pollutants could not completely describe the PBDE tissue distribution. There were no differences in PBDE congener patterns between the various tissues within individuals of a certain species. Inter-species differences in PBDE patterns were present between sparrowhawk and common buzzard.

BDE 209 was detected in nearly all serum and in some liver samples, but not in any other tissues. This observation suggests that exposure to BDE 209 is low or that this congener is poorly accumulated. However, research has shown that BDE 209 can be metabolically transformed to lower brominated congeners (Stapleton et al., 2004a) that are no longer allowed for use in the EU due to their potential toxicity (Darnerud 2003; See *1. Introduction and background information*). Until now, this debromination has not been investigated in predatory birds, but the fact that BDE 209 is bioavailable and bioaccumulating in these birds can be considered alarming.

Because practical and ethical reasons impede the sacrifice of free-living birds and because of the fact that a large number of birds are categorised as "*protected species*", methods for non-destructive biomonitoring using eggs (Norstrom et al., 2000; Herzke et al., 2003; Jaspers et al., 2005), blood (Henriksen et al., 1998) or feathers (Dauwe et al., 2005) have been developed for a wide range of pollutants. PBDEs were never studied in feathers. ***Paper VI*** studies the feasibility of the use of predatory bird feathers as a non-destructive tool for monitoring PBDEs. In previous studies that investigated organic pollutants in bird feathers, sampling could not be considered non-destructive, because a large amount of feathers was necessary. Therefore, the present study was performed on a larger predatory bird (common buzzard), occupying a higher position in the food chain. This allowed measurement of PBDEs in one single tail feather. Furthermore, levels in both the feather and internal tissues were significantly related to each other, showing that predatory feathers give an estimate of the contamination levels. While additional studies need to investigate the influence of external contamination and other confounding factors, such as age, sex, condition factor and season, feathers seem to represent a potential non-destructive biomonitoring tool for organic pollutants in predatory birds.

In ***Paper VII***, we investigated PBDE tissue levels and distribution in red fox (*Vulpes vulpes*), which is a terrestrial top-predator. We have analysed muscle, liver and adipose tissue of 33 red foxes from Belgium for their content of polybrominated diphenyl ethers (PBDEs). Median sums of seven tri- to hepta-BDEs (BDEs 28, 47, 99, 100, 153, 154, and 183) were 2.2, 2.4 and 3.4 ng/g lw in adipose tissue, liver and muscle, respectively, which can be considered relatively low. This is probably related to the high capacity of the foxes to metabolise and eliminate lower brominated congeners. It is remarkable that BDE 209 dominated the PBDE congener profiles in the red fox samples whenever it was present above the LOQ. In samples containing BDE 209, this congener contributed on the average approximately 70% to the total PBDE content. The particular PBDE congener profile observed in the red fox resembles that seen in grizzly bears from Canada, where BDE 209 also dominated the congener profile in several samples, but differs from those previously reported for terrestrial avian species. BDE 209 was measured in concentrations as high as 760 ng/g lw in the liver, but the detection frequency was not more than 40%. In the animals with the highest BDE 209 levels, this congener was detected in muscle, liver as well as in adipose tissue. This was confirmed by an expert laboratory, which eliminated any doubt or scepticism that could arise from these results. Therefore, our data confirms unambiguously that BDE 209 does bioaccumulate in terrestrial top predators, such as the red fox.

The data on levels in terrestrial top-predators obtained in ***Papers V*** and ***VII*** can be very useful to assess the biomagnification potential of PBDEs in terrestrial food chains if data on their prey would be available. Such data is provided in ***Paper VIII***, which investigates PBDEs in small terrestrial prey species, such as great tits (*Parus major*, passerine), wood mice (*Apodemus sylvaticus*) and bank voles (*Clethrionymus glareolus*). Compilation of all data provided more insight in the following food chains: small rodents → buzzards, small rodents → foxes, and passerines → sparrowhawks. Unfortunately, biomagnification of BDE 209 could not be assessed because levels of this congener were below the LOQ in the prey species (See *2.5.4. Limit of Quantification and background concentrations*). All other congeners, except BDE 28, were biomagnified in both predatory bird species. Biomagnification factors (BMFs) were calculated as the ratio between the lipid normalised concentrations in the predator and in the prey. The calculated BMFs ranged from 2 to 34 for the sum of PBDEs in predatory bird food chain.
Remarkably, biomagnification was not observed in the studied food chain of the fox. Although the fox is a top-predator, this is not reflected in the PBDE concentrations that were measured in its tissues. As mentioned before (***Paper VII***), the fox most likely possesses a specific metabolic capacity that enables him to rapidly eliminate (native) PBDEs. This discrepancy between expectation and observation should be taken into account when selecting species for environmental monitoring purposes. Not all top-predators will give a representative reflection of the pollution of their habitat, but confounding factors, such as

metabolism, can influence the results to a great extent and can therefore lead to misinterpretations.

4.2 Paper V

Levels and distribution of polybrominated diphenyl ethers in various tissues of birds of prey

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Levels and distribution of polybrominated diphenyl ethers in various tissues of birds of prey

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Distribution of polybrominated diphenyl ethers in birds of prey is tissue dependent.

Abstract

In the present study, concentrations and tissue distribution of polybrominated diphenyl ethers (PBDEs; IUPAC # 28, 47, 99, 100, 153, 154, 183, and 209) were examined in brain, adipose tissue, liver, muscle, and serum of birds of prey. Median ∑PBDE levels (BDE 28–183) in the tissues of sparrowhawks ranged from 360 to 1900 ng/g lipid weight (lw), which was in general one order of magnitude higher than in the tissues of common buzzards (26–130 ng/g lw). There were no differences in PBDE congener patterns between the various tissues within individuals of a certain species. Inter-species differences in PBDE patterns and in particular the percentage of BDE 99, 100 and 153 were, however, pronounced between sparrowhawk and common buzzard. BDE 209 was detected in nearly all serum and in some liver samples, but not in any other tissues. This observation suggests that exposure to BDE 209 is low or that this congener is poorly accumulated. Passive (lipid content related) diffusion could not completely describe the PBDE tissue distribution, e.g. the lowest PBDE-load was measured in brain, a fairly lipid rich tissue.
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Keywords: Birds of prey; Polybrominated diphenyl ethers; BDE 209; Tissue distribution; Organs

1. Introduction

Polybrominated diphenyl ethers (PBDEs) were first placed on the market in the 1960s as flame retardants and are still used to improve fire safety of consumer and industrial products (WHO, 1994). A substantial increase in production has been seen since the end of the 1970s due to the growing popularity of personal computers and other electronic equipment and due to stricter fire regulations in various countries (de Boer et al., 2000). Environmental levels of PBDEs have been continuously increasing since then (de Wit, 2002). Discharges and emissions during production and use, but also disposal at the end-of-life of flame-retarded products, account for this phenomenon. These chemicals are persistent and lipophilic which results in their bioaccumulation in the fatty tissues of organisms and enrichment throughout food chains (de Boer et al., 2000; Law et al., 2003). Because PBDEs have a toxicological potential (McDonald, 2002; Darnerud, 2003), their biomagnification can have serious health effects on top-predators, such as birds of prey (Law et al., 2003, 2004).

Birds of prey have been used successfully for biomonitoring of organic pollutants in several studies (Ratcliffe, 1993; Herzke et al., 2003). Since they are widespread, sensitive to environmental changes and occupy a top position in the food chain, birds of prey are very suitable to study bioaccumulation (Norstrom et al., 2000). Moreover, the physiology, ecology and behavior of most common birds are well studied. Because practical and ethical reasons impede the sacrifice of free-living birds, methods for non-destructive biomonitoring using eggs (Norstrom et al., 2000; Herzke et al., 2003; Jaspers et al., 2005), blood (Henriksen et al., 1998) or feathers (Dauwe et al., 2005) have been developed. A rapid increase of PBDE concentrations was seen in pooled guillemot (*Uria aalge*) eggs from the Baltic Proper during the late 1970s and

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early 1980s (Sellström et al., 1993). In herring gull (*Larus argentatus*) eggs from the Great Lakes, the PBDE concentrations increased exponentially from 1981 to 2000 (Norstrom et al., 2000). Since most of the studies were conducted on eggs, very limited information is available on PBDEs in bird tissues (Herzke et al., 2003; Jaspers et al., 2005) and in terrestrial organisms in general.

The primary aim of the present study was to investigate the tissue distribution of PBDEs in birds of prey, such as sparrowhawk and buzzard. Until now, no detailed information was available on the faith and mobility of PBDEs in such birds. Additionally, the PBDE concentrations and congener patterns of the various species and tissues were examined. Special emphasis was hereby placed on the analysis of BDE 209, the principal component of the Deca-BDE commercial mixture. This commercial mixture is the only product that is still allowed for use in the EU after passing the risk assessment test (EUR 20402 EN) and therefore it is currently receiving a lot of attention. Research has shown, however, that BDE 209 can be metabolically transformed to lower brominated congeners (Stapleton et al., 2004) that are no longer allowed for use in the EU due to their potential toxicity (Darnerud, 2003).

2. Materials and methods

2.1. Sample collection

Birds of prey belonging to five species were collected by the staff of the Wildlife Rescue Center (WRC) at Opglabbeek (Flanders, Belgium). Some birds were found dead, while others died due to traffic accident related trauma or were euthanized by a veterinarian because of the severity of the sustained injuries. Due to ethical reasons, no bird was killed for the purpose of this study. The WRC collected 44 birds from the eastern part of Flanders (Belgium) between November 2001 and March 2003. Most of the sampled birds were common buzzards (*Buteo buteo*, 29 individuals) and sparrowhawks (*Accipiter nisus*, 7 individuals). In addition four long-eared owls (*Asio otus*), two barn owls (*Tyto alba*), and two tawny owls (*Stryx aluco*) were collected (Table 1).

Liver, brain, pectoral muscle and abdominal adipose tissue were excised directly after collection of dead birds or after euthanasia of birds that could not be rescued. Because not all birds died on the spot after the accident but were left wounded, some showed signs of starvation. Hence, adipose tissue could not be collected from all individuals. Blood samples were only taken from birds that underwent euthanasia. Whole blood samples were centrifuged immediately after collection and the serum fraction was isolated. All samples were stored at −20 °C until further treatment.

2.2. Analysis

Based on reported frequency of occurrence (Voorspoels et al., 2003) and toxicity (de Wit, 2002), the following PBDE congeners (IUPAC numbering) were analyzed: 28, 47, 99, 100, 153, 154, 183, and 209. Brominated biphenyl (BB) 153 was also analyzed. BDE 77 was used as internal standard (IS) for BDEs 28, 47, 99, 100, 153, and 154. BDE 128 was used as IS for BDE 183 and ^{13}C-labeled BDE 209 for BDE 209. For the analysis of BB 153, BB 155 was used as IS.

The method used for analysis of bird tissues (except serum) has previously been described (Voorspoels et al., 2003) and is briefly summarized below. Depending on the type of tissue, 0.2–6 g of homogenized sample were chemically dried using anhydrous Na_2SO_4, transferred into an extraction thimble, spiked with IS and soxhlet extracted with hexane/acetone mixture (3:1, v/v). An aliquot of the extract was used for gravimetric lipid determination. Cleanup was conducted by column chromatography on silica impregnated with concentrated sulfuric acid (48%, w/w). The cleaned extract was concentrated and the solvent was changed to *iso*-octane before injection into the gas chromatographic (GC) system.

Table 1
Sample inventory and feeding habits

English name	Scientific name	No. of samples	Feeding habits (Snow and Perins, 1998)
Common buzzard	*Buteo buteo*	29	Preferably small mammals (e.g. mice, voles, rabbits, squirrels, rats, moles, young hare); the percentage of birds in the diet varies between a few and 25%, occasionally reptiles and amphibians
Sparrowhawk	*Accipiter nisus*	7	Birds, in particular, locally available passerines of appropriate size comprise 95–99% of the prey items; occasionally voles and mice
Long-eared owl	*Asio otus*	4	In the open countryside dominate small rodents, in particular voles; the percentage of birds in the diet is usually low; in urban areas, birds can play a prominent role
Barn owl	*Tyto alba*	2	Small rodents, in particular voles and mice; the percentage of birds in the diet is usually low; occasionally amphibians and insects
Tawny owl	*Strix aluco*	2	Variable; small rodents, in particular voles and mice; the percentage of birds in the diet is quite high and can exceed 50%; occasionally amphibians

The method used for the serum analysis has previously been described (Covaci and Voorspoels, 2005) and is briefly presented below. Approximately 3 ml of serum were left standing overnight with IS added, then mixed with formic acid and extracted using solid-phase extraction cartridges (Oasis® HLB, Waters Corp., Milford, MA, USA). Cleanup was done by column chromatography on silica impregnated with concentrated sulfuric acid (48%, w/w). The cleaned extract was concentrated and the solvent was changed to *iso*-octane before injection into the GC system. Total cholesterol and triglycerides were determined enzymatically in a separate aliquot of serum. Total lipids were calculated from total cholesterol and triglycerides as described by Phillips et al. (1989).

PBDEs were analyzed by gas chromatography-electron capture negative ionization mass spectrometry (GC/ECNI-MS) operated in the selected ion monitoring (SIM) mode. Details of the instrumental conditions are published elsewhere (Voorspoels et al., 2003). For tri- to hepta-BDE congeners and BB 153, a 25 m × 0.22 mm × 0.25 μm HT-8 capillary column (SGE, Zulte, Belgium) was used and ions $m/z = 79$ and 81 were monitored. For the analysis of BDE 209, a 12 m × 0.18 mm × 0.10 μm AT-5 capillary column (Alltech, Lokeren, Belgium) was used and ions $m/z = 484.7/486.7$ and 494.7/496.7 were monitored for BDE 209 and ^{13}C-labeled-BDE 209, respectively.

2.3. Quality assurance and quality control

The method quality control (QC) was done by regular analysis of procedural blanks and blind duplicate samples (RSD < 5%). Instrumental QC was done by regular injection of solvent blanks and standard solutions. The efficiency/capacity of the method was demonstrated by successful participation in an international interlaboratory study on the determination of PBDEs in biota (de Boer et al., 2002). Recoveries for individual PBDE congeners

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were between 87% and 104% (RSD < 12%). Procedural blanks were consistent (RSD < 12%) and therefore the mean procedural blank value was subtracted. After blank subtraction, the limit of quantification (LOQ) was set at 3 times the standard deviation of the blank, which ensures >99% certainty that the reported value is originating from the sample and not from an exceptionally high blank value. This implies varying LOQs depending on the sample intake and the PBDE congener analyzed for. Three overlapping 5-point narrow-range calibration curves were used to quantify the wide range of concentrations found.

2.4. Statistical analysis

Before statistical analysis, samples with levels below LOQ were assigned a value of $(1 - p) \times LOQ$, with 'p' being the proportion of measurements with levels below LOQ (Voorspoels et al., 2002). The tissue lipid content of the different species was compared using ANOVA. The measured PBDE concentrations deviated from Gaussian distribution (Shapiro–Wilks test, $p > 0.05$), therefore the data were log-transformed before conducting statistical analysis. ANOVA with Scheffe's post hoc test was used to compare the overall levels between the species and to detect differences in levels between the different tissues. Differences in the PBDE congener patterns between the different bird species and tissues were analyzed using principal component analysis (PCA) and ANOVA. For BDE 28 and 209 more than 50% of the results were below LOQ, and hence, these congeners were excluded from the PCA. The factor-scores obtained from the PCA were normally distributed, thus parametric statistics (ANOVA with Scheffe's post-hoc test) were applied to test for the significance of the observed differences. Clustering was based on the outcome of these tests. The level of significance was set at $\alpha = 0.05$ throughout this study. Statistical calculations were performed using XLSTAT v.7.5 (Addinsoft), GraphPad Instat v. 3.06 (GraphPad Software Inc.) and SPSS v.11 for Windows (SPSS Inc.).

3. Results and discussion

3.1. Lipids

No significant differences in the average lipid content of brain, adipose tissue, liver, muscle and serum were observed between the species (Table 2). However, within a particular species, the lipid content of the different tissues can be susceptible to great variance caused by nourishment and health status of the animals. In brain, the lipid content was the most consistent throughout all individuals (7.4 ± 0.2%). This is related to the function of the brain; lipids in this tissue do not have an energy reserve function and they are not mobilized whenever the bird is deprived of food. Even under extreme starvation, the lipid content of brain is kept constant. It was shown that lipid levels in the brain of bats are nearly constant irrespective of body fat levels (Clark and Shore, 2001).

Muscle and liver are more susceptible to changes in the lipid content, which result in a much wider variation of this parameter in these tissues (Table 2). The lipid percentage of adipose tissue was very consistent, because it does not alter with nutritional status of the bird. However, the amount of adipose tissue declines when lipids are metabolized. When this happens, the lipophilic pollutants that were stored in the adipose tissue are remobilized and circulate through the body until a new steady state is established. This "remobilization effect" can theoretically result in higher pollutant levels in the other tissues than before starvation. However, this could not be observed in this sample set. Birds for which no adipose tissue was available, did not display significantly higher pollutant levels in the other tissues. The impact of the "remobilization effect" is probably too small to be noticed in this rather small random sample set.

In the present study, the influence of the body condition of the bird on the lipid-normalized results was investigated. A total body burden was calculated based on lipid normalized levels in brain, muscle and liver (tissues that were available for all individuals). Further, the bird's body condition was assessed by assigning the bird to a "fat class" based on whether or not abdominal adipose tissue was available for sampling (fat class 1 and 0, respectively). Statistical analysis of the median PBDE burden for each fat class revealed that birds with no sampled abdominal adipose tissue (median burden 1100 ng/g lipid weight [lw]) had higher lipid normalized PBDE levels than those from fat class 1 (median burden 120 ng/g lw). However, these medians could not be proven statistically different due to the large variation in the burdens calculated from these tissues, but a trend was visible.

Due to the influence of body condition and due to all other factors that may affect lipid levels, wet weight (ww)-based results might be preferred. However, lipid-normalized results are mostly used for tissue profiling and data comparison, since most data presented in literature are expressed in ng/g lw.

3.2. Levels of PBDEs

All PBDE congeners except BDE 28 and BDE 209, were consistently found in muscle, liver, adipose tissue and serum. In brain, PBDE levels were relatively low, and hence, only the most prominent congeners were detected (Table 2). Results of all owl species were combined into one group seeing the similarity of the various owl species and the small sample sizes.

The sum of PBDEs (except BDE 209) in individual birds covered a concentration range of almost 4 orders of magnitude. The lowest levels were generally found in buzzard brain (median 26 ng/g lw), while the highest levels were measured in sparrowhawk fat (median 1 900 ng/g lw). The highest individual concentration of $\sum$PBDEs was 26,000 ng/g lw and was measured in sparrowhawk liver.

Many factors can influence the levels of organic contaminants in birds of prey. Significant differences between species in both tissue and egg PCB concentrations have been reported previously (Hoshi et al., 1998; Herzke et al., 2002; Kenntner et al., 2003) and have been attributed largely to differences in their dietary exposures (Hoshi et al., 1998) and their abilities to metabolize persistent organochlorine compounds (Walker et al., 1987; Ronis and Walker, 1989; Fossi et al., 1995). Other studies, however, have shown that age, sex and body condition are also important in affecting PCB concentrations in eggs and tissues (Newton et al., 1981; Donaldson and Braune, 1999; Kenntner et al., 2003; Wienburg and Shore, 2004). Additionally, tissue residues have also been reported to vary seasonally in association with fluctuations in body fat reserves (Kenntner et al., 2003) and to rise following starvation (Lambeck et al., 1991).

Sparrowhawks feed primarily on locally available small birds, such as passerines, while buzzards prefer small mammals

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Table 2
Statistical descriptors of ∑PBDEs (BDEs 28, 47, 99, 100, 153, 154, 183), BB 153 and BDE 209 levels and lipid content in all samples

Species	Statistic	Brain (ng/g lw)			Fat (ng/g lw)			Liver (ng/g lw)			Muscle (ng/g lw)			Serum (ng/g lw)		
		∑PBDEs	BB 153	BDE 209	∑PBDEs	BB 153	BDE 209	∑PBDEs	BB 153	BDE 209	∑PBDEs	BB 153	BDE 209	∑PBDEs	BB 153	BDE 209
Buzzard	*n*		29			16			29			29			20	
	Det freq	29/29	25/29	0/29	16/16	16/16	0/16	29/29	27/29	3/29	29/29	29/29	0/29	20/20	18/20	16/20
	Mean	130	3	n.a.	110	1	n.a.	480	5	79	380	8	n.a.	140	2	15
	Median	26	1	n.a.	60	1	n.a.	70	1	24	130	2	n.a.	54	1	9
	Min	0.15	<0.14	n.a.	22	0.06	n.a.	12	<0.27	19	17	0.25	n.a.	3	<0.01	2
	Max	1600	38	n.a.	370	3	n.a.	4800	36	190	4400	95	n.a.	780	10	58
	Lipid % (SD)		7.4 (0.8)			89 (4.4)			3.6 (1.3)			4.4 (2.3)			1.1 (0.7)	
Owls	*n*		8			1			8			8			3	
	Det freq	8/8	7/8	0/8	1/1	1/1	0/1	8/8	8/8	0/8	8/8	8/8	0/8	3/3	3/3	1/3
	Mean	87	2	n.a.	200	3	n.a.	250	5	n.a.	300	9	n.a.	230	4	9
	Median	87	1	n.a.	n.a.	n.a.	n.a.	250	2	n.a.	250	3	n.a.	290	1	n.a.
	Min	0.76	<0.14	n.a.	n.a.	n.a.	n.a.	17	0	n.a.	31	0.56	n.a.	8	0.13	n.a.
	Max	174	6	n.a.	n.a.	n.a.	n.a.	480	16	n.a.	740	28	n.a.	380	12	n.a.
	Lipid % (SD)		7.0 (0.7)			80 (n.a.)			3.8 (0.5)			3.9 (2.2)			0.9 (n.a.)	
Sparrowhawk	*n*	7	2	7	7	2										
	Det freq	7/7	7/7	0/7	2/2	2/2	0/2	7/7	7/7	2/7	7/7	7/7	0/7	2/2	2/2	2/2
	Mean	1200	10	n.a.	1900	3	n.a.	4900	46	17	4100	75	n.a.	840	7	26
	Median	360	2	n.a.	n.a.	n.a.	n.a.	1300	12	n.a.	1600	18	n.a.	n.a.	n.a.	n.a.
	Min	140	1.1	n.a.	540	1.04	n.a.	280	2.1	15	79	0.73	n.a.	800	6	16
	Max	5800	38	n.a.	3300	5	n.a.	26000	200	19	18000	310	n.a.	900	8	36
	Lipid % (SD)		7.7 (0.6)			91 (3.8)			3.6 (1.1)			2.7 (2.4)			n.a.	

All concentrations in the organs and adipose tissue are expressed in ng/g lw. When only one sample was available, the value is given as "Mean", when two samples were available, the median was considered "not available" (n.a.).

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such as mice, voles, rabbits, squirrels, rats, moles, and young hare (Table 1) (Snow and Perrins, 1998). Owls also prey mainly on small rodents, in particular voles (Snow and Perrins, 1998). Depending on the owl species and the habitat they live in, the percentage of birds in their diet can exceed 50% (Table 1) (Snow and Perrins, 1998). These differences in diet composition, in particular between sparrowhawk and buzzards/owls, might partly explain why the highest concentrations of PBDEs were seen in sparrowhawks (Snow and Perrins, 1998). Total PBDE concentrations in buzzards and owls were not statistically different, which is consistent with their similar dietary pattern. In the present study, levels of total PBDEs in sparrowhawk were approximately one order of magnitude higher (Table 2) than those in buzzards and owls. This also explains why BDE 28, which represents only a small fraction of the total PBDE burden, was detected in most sparrowhawk samples, but not in the other species.

Furthermore, the bird's habitat can also be considered a confounding factor. The use of birds of prey as geographical indicators for local pollution is very complex. All bird species analyzed in this study are sedentary throughout the year in Belgium, although it cannot be excluded with certainty that among common buzzards and sparrowhawks found between September and April were migrants from Scandinavia.

Finally, age was shown to affect PBDE levels to a great extent in sparrowhawk (Law et al., 2003). Serum concentrations in adults were found to be around 10-100 times higher than in nestlings (Law et al., 2003). Furthermore, females can reduce their body burden due to transfer of contaminants into eggs. Female sparrowhawks are thought to lose up to one third of their organochlorine body burden in this way (Newton et al., 1981). This highlights some difficulties that can confound data interpretation. In the present study, confounding factors were not further investigated due to lack of data (e.g. sex was only determined for sparrowhawks; $n = 7$).

3.3. Comparison with other studies

In general, PBDE related studies on terrestrial bird tissues are scarce. Most research was done on eggs (mostly of fish-eating birds), although some data on tissues are available. Lepom et al. (unpublished data) have analyzed $\sum$PBDEs in sparrowhawk serum of both nestlings (1–30 ng/ml) and adults (200–400 ng/ml). The highest serum level in sparrowhawks of the present study was 7.7 ng/ml, but the sample set was very limited ($n = 2$). Muscle tissue of starlings (*Sturnus vulgaris*) was analyzed for PBDEs by Sellström (1999) and results for these passerines were between 5.7 and 13 ng/g lw ($\sum$PBDEs). These results are lower than in the present study (0.66–133 ng/g lw), but can be allocated to the species difference and the trophic level. Jaspers et al. (in press) recently reported about PBDEs in muscle and liver of sparrowhawk and common buzzard. Concentrations were in the same range as those of the present study; the median $\sum$PBDEs in buzzard liver and muscle were 180 and 280 ng/g lw, respectively, and in sparrowhawk liver and muscle 3100 and 2200 ng/g lw, respectively. Recently, concentrations of PBDEs in eggs of little owls (Jaspers et al., 2005) were found to be similar (median 108 ng/g lw) to those seen in owl tissues in the present study (median 87–290 ng/g lw).

Mean $\sum$PBDE levels in peregrine falcon eggs from Sweden were around 4000 ng/g lw, with individual values up to 39,000 ng/g lw (Lindberg et al., 2004). Median summed PBDE concentration was 1900 ng/g lw in peregrine falcon eggs from Greenland (Sørensen et al., 2004). In the present study, the median sum of PBDEs was also 1900 ng/g lw in sparrowhawk liver, while the highest individual concentration was 26,000 ng/g lw in sparrowhawk liver, therefore these levels can be considered similar to the levels observed in the peregrine falcons.

3.4. BDE 209

Even after the recent completion of the European Union Risk Assessment Report for BDE 209, there is a need for further information and/or testing (EUR 20402 EN, 2002). It is possible that the current Predicted Environmental Concentration/Predicted No-Effect Concentration (PEC/PNEC) approach for secondary poisoning may not be appropriate in terms of both the PEC and the PNEC, and could underestimate the risk. Hence, an expanded monitoring program to determine whether the finding of BDE 209 in top predators is a widespread or localized phenomenon, and further toxicity testing are necessary. A second aspect of the concern for secondary poisoning is that although the substance is persistent, there is evidence that it can degrade under some conditions to more toxic and bioaccumulating compounds. BDE 209 was found in a variety of species, mainly those living in terrestrial ecosystems (de Boer et al., 2004; Jaspers et al., in press) Although in the present study BDE 209 could only be quantified in 6 out of 44 liver samples (up to 190 ng/g lw) (Table 2), it was present above LOQ in 19 out of 25 serum samples (up to 0.51 ng/ml or 58 ng/g lw). The observed difference in the detection frequencies seen in tissues and serum can, however, be partially explained by the methodology. Procedural blanks were around 10–100 times lower for the serum analysis, resulting in much lower LOQs, that facilitated quantifying levels as low as 4 pg/ml.

The relatively low levels of BDE 209 in serum and liver suggest that exposure to this congener is low or that it is poorly accumulated due to a lower bioavailability than other PBDE congeners and/or a short half-life in birds. The half-life of BDE 209 was estimated at around 7 days in humans (Hagmar et al., 2000) and around 2.5 days in rats (Mörck et al., 2003). In rats orally exposed to BDE 209, the highest lipid normalized concentrations were found in serum and liver, and the lowest concentrations were found in adipose tissue (Mörck et al., 2003). The relatively higher levels in serum and liver are also in accordance with the nature of these tissues, since serum, and to a lesser extent liver, are good indicators for recent exposure (Hobson and Clark, 1992). Furthermore, the birds of the present study that had measurable amounts of BDE 209 in liver also had the highest serum levels. However, this result could not be statistically supported due to the low number of birds in which BDE 209 was determined in both liver and serum

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($n = 3$, due to the limited availability of serum samples). However, these results provide further evidence that BDE 209 is bioavailable to birds of prey and accumulating in their tissues.

BDE 209 has already been found in 18 out of 21 eggs of Swedish peregrine falcon (*Falco peregrinus*) (up to 430 ng/g lw), and the authors concluded that this congener is taken up by the bird via its diet and then transferred to the egg (Lindberg et al., 2004). Jaspers et al. (2005) found BDE 209 in only 1 out of 40 eggs of little owl (*Athena noctua*) (17 ng/g lw), which was related to the high LOQ of the procedure involving soxhlet extraction. Recently, Jaspers et al. (in press) also found BDE 209 in liver of sparrowhawk, buzzard and long-eared owl (median 52–66 ng/g lw). Although in the present study BDE 209 could only be detected in some liver samples, it has been found in muscle of various bird species, such as peregrine falcons and sparrowhawks (de Boer et al., 2004). de Boer et al. (2004) also stated that BDE 209 bioaccumulates in terrestrial birds to only a limited extent and that this accumulation is species-dependent. This may explain the low detection frequency of this congener in the liver samples from species included in the present study.

BDE 209 has previously been found in human serum. Strikingly, levels in humans are comparable or lower than those found in the raptors. Occupationally exposed Swedish electronics recycling workers had BDE 209 serum concentrations up to 5.1 ng/g lw (median 1.8 ng/g lw) (Thuresson et al., 2004). BDE 209 in Mexican blood samples (Lopez et al., 2004) were as high as 15 ng/g lw (median 9.5 ng/g lw), which is comparable to the present study.

3.5. BB 153

Although PBBs have not been extensively used in Europe (de Boer et al., 2000), BB 153, a major compound of Hexa-BB technical mixture, was found in most bird samples analyzed (Table 2). Similar as for PBDEs, the lowest BB 153 levels were generally seen in muscle and brain and the highest in sparrowhawk liver, in which a median concentration of 13 ng/g lw was found, what can be considered as relatively low. The presence of this compound in terrestrial wildlife from this geographical region (North Belgium) contrasts with findings in marine biota from the North Sea and Scheldt Estuary, where this compound was never detected (Voorspoels et al., 2003). Whether this is related to exposure or metabolism cannot be concluded from these data. Other studies also report the presence of BB 153 in e.g. eggs of peregrine falcons from Sweden at concentrations up to 370 ng/g lw (Lindberg et al., 2004), which is comparable to the levels found in sparrowhawk liver in the present study (up to 200 ng/g lw). Median levels in owl livers from the present study were around 1.7 ng/g lw, and thus in the same order of magnitude as those found in little owl eggs (median 1.3 ng/g lw) (Jaspers et al., 2005).

3.6. PBDE patterns in tissues

PBDE profile differences between tissues were investigated using ANOVA-analysis. No differences in the congener profile between the tissues were found. Therefore an overall profile, not accounting for the tissues, was created for buzzard, sparrowhawk and owls, and is presented in Fig. 1.

Congeners can be ordered according to their relative contribution to the total PBDE content as follows for buzzard: 153 ~47 > 99 > 183 > 100 > 154; for sparrowhawk 99 > 47 > 153 > 100 ~ 183 > 154; for owls 153 > 99 > 47 > 183 > 100 > 154. BDEs 47, 99, and 153 were the most abundant congeners and contributed each between 15% and 35% to the total sum of PBDEs. BDEs 100, 154, and 183 represented between 3% and 13% of total PBDEs (Fig. 1). Apart from BDE 209, tribrominated diphenyl ether 28 was the least frequently detected congener and it could mainly be found in adipose tissue and

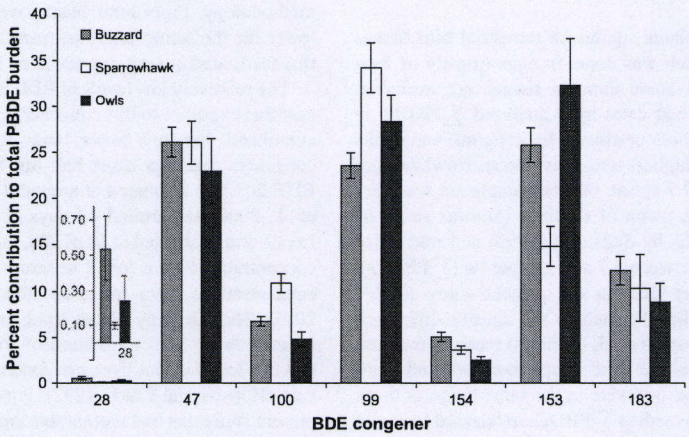

Fig. 1. Percentage contribution of individual PBDE congeners to the total PBDE burden per species (group). Because there were no differences between the profiles in the various tissues, all tissues were used to calculate the profiles. The profile of BDE 28 is enlarged to highlight the differences. Error bars represent 1.96 × standard error of the mean.

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liver. Only 30% and 15% of buzzard and owl muscle samples, respectively, showed measurable amounts of BDE 28. Slightly higher detection frequencies were seen in buzzard and owl brain (55% and 25%, respectively). Sparrowhawk muscle and brain showed higher concentrations of BDE 28 than buzzard and owls, with a detection frequency of 75%. This is most probably related to the overall higher levels found in this species.

BDE 183 was found in nearly all tissues, except in some brain samples. The presence of this congener can be considered as indicative that birds were exposed to the technical octa-mix (Law et al., 2003). For the time being, there is no experimental evidence for metabolic breakdown of BDE 209 to BDE 183, another (theoretically) possible explanation for the presence of BDE 183 in tissues.

Similar to the present study, the congener profile in eggs from Belgian little owls was also dominated by BDE 99 and BDE 153, followed by BDE 47; BDE 183 was detected in all samples and contributed around 9% of the total PBDE load (Jaspers et al., 2005). BDE 183 also contributed around 10% of the total PBDEs in the samples of the present study, and this confirms the observation that terrestrial birds are more exposed to the higher brominated diphenyl ethers than fish-eating species, such as guillemots and cormorants, which generally have BDE 47 as dominating congener, while BDE 183 is not present or at very low levels (Law et al., 2002; Jaspers et al., in press). Also in glaucous gull liver from Svalbard, only BDE 47 and 99 were detected (Herzke et al., 2003). In heron adipose tissue and eggs, BDE 47 accounted for 40–50% of the total PBDE load (D'Silva et al., 2004).

In other studies on eggs (Herzke et al., 2002) or serum (Law et al., 2003) of sparrowhawk, a predominance of BDE 47 was observed, while in the sparrowhawk tissues from the present study the dominating congener was BDE 99, followed by BDE 47. The same profile was observed in sparrowhawk liver by Jaspers et al. (in press). It seems that even within one species, external or other factors can influence the profile to a rather large extent. Some data indicate that profile differences between species could more likely be attributed to different metabolic pathways than to differences in prey pattern (Law et al., 2003), while others have suggested that, at least for PCBs, the patterns are influenced more by individual dietary factors than by interspecies differences in metabolic capacity (Bachour et al., 1998).

In the present study, the overall profiles of PBDEs were statistically investigated using PCA. PCA is a statistical tool that can project multidimensional data onto two dimensions, which are easier to view. PCA uses the full data sets and does not require averaging by categories. It is an elegant way to compare the congener profiles between the different species and tissues. In this case, only samples with measurements above LOQ of all major PBDE congeners (BDE 47, 99, 100, 153, and 183) and BB 153 were used. The application of the PCA to the data (163 observations and 7 variables) indicated that the first two principal components (PC) accounted for 63% of the total variability. The extraction of the PCs was based upon the Kaiser criterion.

The average factor score of PC 1 of each species and tissue is plotted against the average factor score of PC 2, together with the factor loadings (Fig. 2). The factor loadings for each compound, indicating the influence of that factor on the observed difference, are plotted as lines, while the factor scores are plotted as points. In this biplot, the impact of each compound on the variability of the PC is also indicated. In such plots, one is usually looking for clustering of the data. To test the significance of the difference of the clusters, ANOVA with Scheffe's post hoc test was carried out and clusters were created based on the outcome of this test.

All sparrowhawk samples are located on the left side of the graph, separated from all other species (sparrowhawk cluster). Buzzard samples belong statistically to another cluster (buzzard cluster), which is well separated from the sparrowhawk cluster, indicating a clear profile difference between these two species. No significant differences could be found between the three owl species, which statistically belong to the same cluster (owl cluster). However, this might be related to the small sample sizes, since the mean factor scores for the three owl species are not well clustered, which points at profile differences. We can therefore conclude that there are statistically significant differences in the profiles of PBDEs between sparrowhawk, buzzard and owls included in the present study. The observed profile differences might indicate that these three raptor species have a different metabolic capacity. It is also possible that PBDE patterns in the prey of these raptors were different, since they belong to different food chains. Voles, the primary prey of buzzards and owls, are predominantly herbivorous, while a considerable part of small passerines, the prey of sparrowhawk, mainly feed on insects and worms (Table 1). However, as shown previously by Law et al. (2003), the influence of habitat and food

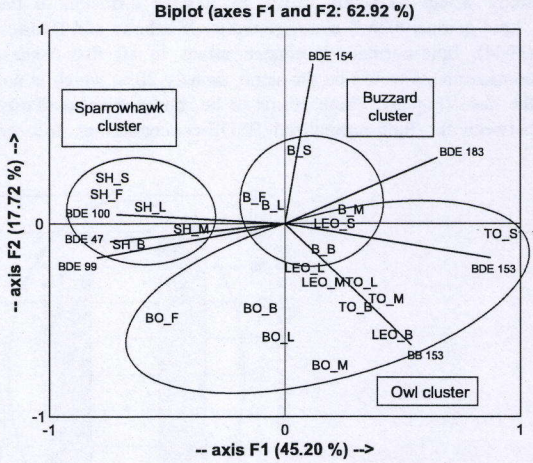

Fig. 2. Principal component biplot of the factor loadings (full line originating from the center of the plot) and the average factor scores for all matrices analyzed (codes). Species are marked by their specific code (B, buzzard; SH, sparrowhawk; BO, barn owl; LEO, long-eared owl; TO, tawny owl). Tissue identification: _B, brain; _F, fat; _L, liver; _M, muscle; _S, serum. The three clusters on the plot represent statistically different groups and are named accordingly.

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speciation cannot adequately explain the pattern differences of the PBDE contamination. We can therefore hypothesize that metabolic action has a contribution.

3.7. Tissue distribution

To calculate the tissue distribution of PBDEs in individual birds, lipid normalized concentrations were used to compensate for the variation in tissue lipid content. The relative distribution of PBDEs in brain, adipose tissue, muscle, liver and serum was calculated for those individuals of whom all five tissues were available ($n = 12$). Tissue distribution could not be calculated for BB 153 due to the low levels found. Lipid content was not determined in all serum samples due to the low sample amount that was sometimes available. Those samples in which the lipid content was not measured were included in the data set and it was assumed that their lipid percentage was equal to the mean of all analyzed samples. The tissue distribution was statistically analyzed using ANOVA with Scheffe's post hoc test. No differences in PBDE tissue distribution could be observed between the different species and therefore all birds are treated as one population.

Statistical analysis revealed that the relative PBDE burdens in adipose tissue and muscle were not different (30–35% of the total load for each), but higher than in the other tissues (Fig. 3). Also, lipid-normalized PBDE-concentrations in liver and serum were neither statistically different (15–20% of the total load), but brain showed significantly lower levels (<5%) than all other tissues. To the authors' best knowledge, this is the first time that PBDEs are simultaneously investigated in five different tissues of birds of prey.

If the PBDE-tissue distribution in the birds of the present study would be solely driven by passive diffusion to the "lipid-compartment" as suggested by Matthews and Dedrick (1984), lipid-normalized concentrations in all five tissues/compartments would be the same, namely 20%, which is not the case (Fig. 3). There seems to be, however, a similarity between the lipid-normalized PBDE-concentrations and the metabolic turn-over-time of each specific tissue/compartment. Adipose tissue has an energy reserve function, consisting almost exclusively of lipids (~90%). The fat that is found in muscle tissue has a similar function. This can explain the similar lipid normalized concentrations in these two tissues. Both fat and muscle can be considered tissues with a low metabolic turnover. Since the liver is the main detoxifying organ of the body, it can be considered a fairly active organ, resulting in lower PBDE levels than in fat and muscle. The lipid content of serum is fairly low (~1%), which might explain the low burden of lipophilic pollutants in this body compartment. Strikingly, only approximately 5% of the PBDE load in the investigated tissues could be found in the brain and this is significantly lower than in the other tissues. This indicates that lipophilic PBDEs accumulate to a lesser degree in the brain than in muscle, liver or serum, although the lipid content of brain (~7.4%) is higher than that of muscle (~4.0%), liver (~3.6%) or serum (~1.0%). If only passive distribution were involved, PBDEs would be expected to distribute from the blood and accumulate in different body compartments in proportion to their lipid content (Matthews and Dedrick, 1984). The observations in the present study can only partially be explained by this hypothesis, but similar observations have been documented for POPs. Bachour et al. (1998) suggested that the efficiency of the blood–brain barrier is probably protecting the bird's brain. We can therefore assume that, in spite of its relatively high lipid content, the brain is better protected against accumulation of PBDEs than the other tissues.

Compounds may enter the brain by passive diffusion or by transporter mediated uptake. Being small, neutral and lipophilic molecules, PBDEs theoretically have ideal physicochemical properties to diffuse freely across biological membranes, such as the blood–brain barrier (Camenisch et al., 1996). However, a recent study on developing chicken embryos indicated that tissue distribution of PCBs was related primarily to the lipid content and composition of these tissues and that the composition of the cerebral lipids may play a more important role than previously suspected (Maervoet

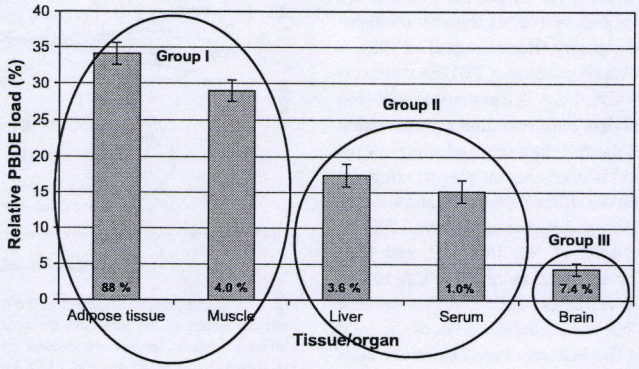

Fig. 3. Tissue distribution of total (lipid normalized) PBDEs (not including BDE 209). Three statistically different distribution groups can be distinguished: adipose tissue and muscle (Group I) have relatively higher loads than liver and serum (Group II), that have on their turn relatively higher loads than brain tissue (Group III). Mean lipid content of each tissue is printed on the bars. Error bars represent 1.96 × standard error of the mean.

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et al., 2005). We can assume that the same hypothesis can be used for PBDEs. Recently, partition coefficients between trioleoylglycerol and phosphatidylcholine were established for 20 xenobiotics in vitro, and it was revealed that PCBs have a much higher affinity for triglycerides than for phospholipids (Sandermann, 2003). Furthermore, it is known that the liver of chicken embryos mainly contains neutral lipids, such as triglycerides and cholesterol esters, while brain lipids are primarily composed of more polar lipids, such as phospholipids, and free cholesterol (Noble and Cocchi, 1990; Maldjian et al., 1996). Conclusions regarding any repercussions of these relatively low PBDE levels in brain on the suspected neuro (developmental) toxicity of these chemicals cannot be drawn.

4. Conclusion

PBDE congeners, including BDE 209, were detected in several tissues of birds of prey, with the highest levels measured in sparrowhawk fat. No differences could be seen in the PBDE congener patterns between the various tissues within individuals belonging to the same species, while interspecies differences in the PBDE patterns were pronounced between sparrowhawk and common buzzard. BDE 209 was detected in nearly all serum and in some liver samples, but not in any other tissue. This suggests that exposure to BDE 209 is low or that this congener is poorly accumulated, which might be related to a lesser bioavailability compared to other PBDE congeners and/or a short half-life time of this compound once absorbed by the birds. Adipose tissue and muscle were found to contain the highest load of PBDEs (30–35%), followed by liver, serum and brain.

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4.3 Paper VI

Can predatory bird feathers be used as a non-destructive biomonitoring tool of organic pollutants?

Veerle L.B. Jaspers, Stefan Voorspoels, Adrian Covaci, Marcel Eens

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Can predatory bird feathers be used as a non-destructive biomonitoring tool of organic pollutants?

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The monitoring of different types of pollutants that are released into the environment and that present risks for both humans and wildlife has become increasingly important. In this study, we examined whether feathers of predatory birds can be used as a non-destructive biomonitor of organic pollutants. We demonstrate that polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT) and polybrominated diphenyl ethers (PBDEs) are measurable in one single tail feather of common buzzards (*Buteo buteo*) and that levels in this feather and internal tissues are significantly related to each other ($0.35<r<0.76$ for all 43 buzzards; $0.46<r<0.84$ when excluding 17 starved birds). Our findings provide the first indication that feathers of predatory birds could be useful in non-destructive biomonitoring of organic pollutants, although further validation may be necessary.

Keywords: biomonitor; feathers; polybrominated diphenyl ethers; polychlorinated biphenyls; organochlorine pesticides; birds of prey

1. INTRODUCTION

Worldwide, many pollutants, such as heavy metals and persistent organic pollutants (POPs), are released into the environment. POPs, which include polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs; e.g. dichlorodiphenyltrichloroethane (DDT)), along with polybrominated diphenyl ethers (PBDEs) are lipophilic pollutants that accumulate within biological tissues and biomagnify through food chains. PCBs and DDT have been shown to disrupt endocrine systems, to cause population declines and to present risks for both humans and wildlife (Jones & De Voogt 1999; Ross *et al.* 2000). Although concentrations have declined since the 1970s, their presence in the environment still remains of concern. PBDEs are intensely used as flame-retardants and have therefore recently emerged as ubiquitous environmental pollutants (Hites 2004). PBDEs have also been associated with endocrine disruption (Darnerud 2003).

Predatory birds have been used extensively in the past as biomonitors of environmental contamination, because they are sensitive to environmental changes and are situated high on the food chain, thus accumulating high levels of POPs (Furness 1993). Moreover, several pollutants with health risks for humans, such as DDT, have been identified following reports of adverse effects in wild bird populations (Ratcliffe 1967). While many studies have measured heavy metals in feathers (Burger 1993), reports on concentrations of organic pollutants in feathers of predatory birds are lacking in literature. Recently, PCBs and DDTs could be quantified in feathers of small passerines, such as the great tit (*Parus major*) (Dauwe *et al.* 2005). However, concentrations were very low and feather sampling could not be considered non-destructive, because a large amount of feather tissue was necessary. Therefore, the present study was performed on a larger predatory bird, occupying a higher position in the food chain.

Here, we report on levels of organic pollutants (PCBs, DDTs and PBDEs) in feathers, muscle and liver tissue of the common buzzard (*Buteo buteo*). We further investigate if there is a correlation between levels of organic pollutants in liver and muscle and levels in the corresponding feathers. The existence of such a relationship would allow future use of feathers of predatory birds in biomonitoring of contamination with organic pollutants. The use of hair, also a keratinous tissue, has recently been established as a successful method for the analysis of POPs (Zupancic-Kralj *et al.* 1992; Dauberschmidt & Wennig 1998; Covaci *et al.* 2002; Altshul *et al.* 2004; D'Havé *et al.* 2005). In contrast to hair, which is continuously growing, feathers grow only for a certain period of time and only during this limited time period are they connected to the blood stream (and its circulating pollutants).

2. MATERIAL AND METHODS

Between October 2003 and June 2004, 43 cadavers of common buzzards (including only two juveniles) were collected in collaboration with Wildlife Rescue Centres in Flanders (Vogelbescherming Vlaanderen vzw, Belgium). The birds had died due to traffic accident, natural causes or starvation ($n=17$). No birds were killed for the purpose of this study. Liver and pectoral muscle were excised and stored at −20 °C until sample preparation. One outermost tail feather of each bird was removed, stored in a paper envelope and used for further analysis.

Analytical procedures for feathers were similar to the method described by Covaci & Schepens (2001), while procedures for internal tissues are described in detail by Voorspoels *et al.* (2003) and Dauwe *et al.* (2005). Briefly, feathers were washed with distilled water and cut into pieces of approximately 1 mm. Feathers were weighed (~200 mg) and left standing overnight at 40 °C with hydrochloric acid and a mixture of hexane and dichloromethane (4 : 1, v/v). After liquid extraction, clean-up was performed on acidified silica. Approximately 1.5 g of liver or muscle was weighed, mixed with anhydrous Na_2SO_4 and Soxhlet extracted with a mixture of hexane and acetone (3 : 1, v/v). After gravimetric lipid determination on an extract aliquot, the remaining extract was cleaned-up on acidified silica. Analysis was done using a gas chromatograph coupled with a mass spectrometer (GC/MS) operated in electron capture negative ionization mode for PBDEs and OCPs, and using a GC/MS in electron ionization mode for PCBs. In all samples, seven PBDE congeners (28, 47, 99, 100, 153, 154 and 183), bromo biphenyl 153, 25 PCB congeners (18, 28, 52, 74, 95, 99, 101, 105, 110, 118, 128, 132, 138, 149, 153, 156, 167, 170, 177, 180, 183, 187, 194, 196 and 199) and DDT and metabolites (*p,p'*-DDT, *p,p'*-DDE and *p,p'*-DDD, expressed here as DDTs) were analysed.

All statistical analyses were performed using STATISTICA v. 5.5 for Windows (Statsoft 2000, Tulsa, OK, USA) and GRAPHPAD INSTAT v. 3.06 for Windows (GraphPad Software Inc., San Diego, CA, USA). Samples with levels below the limit of quantification (LOQ) were assigned a value of $(1-p)\times$LOQ, with p the proportion of measurements with levels below the LOQ (Voorspoels

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Table 1. Median concentrations and range of organic pollutants in feathers, muscle and liver of buzzards (*Buteo buteo*) from Belgium ($n=43$). (n.a, not applicable.)

	feather (ng g^{-1})	muscle (ng g^{-1} ww)	liver (ng g^{-1} ww)
mean lipid (%)	n.a.	3.87	4.04
sum of 18 PCBs	26 (5.1–200)	220 (18–2400)	145 (9.0–10 400)
sum of three PBDEs	1.4 (0.5–10)	5.7 (0.4–104)	2.8 (<0.1–310)
sum of DDT and DDE	4.9 (1.6–62)	73 (2.6–820)	34 (2.2–1300)

Table 2. Pearson's correlation coefficients (r) calculated between log concentrations in feathers (ng g^{-1}), muscle (ng g^{-1} ww) and liver (ng g^{-1} ww) of buzzards (*Buteo buteo*). ($^{*}p<0.05$; $^{**}p<0.01$.)

	feather–muscle		feather–liver	
sample size (n)	all buzzards (43)	excl. starved (26)	all buzzards (43)	excl. starved (26)
sum of 18 PCBs	$r=0.76^{**}$	$r=0.84^{**}$	$r=0.60^{**}$	$r=0.62^{**}$
sum of three PBDEs	$r=0.73^{**}$	$r=0.74^{**}$	$r=0.43^{**}$	$r=0.40^{*}$
sum of DDT and DDE	$r=0.53^{**}$	$r=0.56^{**}$	$r=0.35^{*}$	$r=0.46^{*}$

et al. 2002). Compounds with over 50% of the measurements below the LOQ were excluded from the statistical analysis. Data were not normally distributed (Shapiro–Wilk's test) and were log-transformed ($\log(x+1)$) to meet normal distribution requirements. Parametric Pearson correlations were calculated between concentrations of sum PCBs, PBDEs and DDTs in feathers, liver and muscle.

3. RESULTS

In feathers, 18 PCB congeners (range 5.1–200 ng g^{-1}), *p,p'*-DDT and its metabolite *p,p'*-DDE (range 0.2–6.4 and 1.1–60 ng g^{-1}, respectively), plus the environmentally predominant PBDE congeners 47, 99 and 153 (range 0.5–10 ng g^{-1}) could be quantified with more than 50% of all samples above LOQ. Median concentrations and range of sum PCBs, sum PBDEs and sum DDTs in feathers, muscle and liver, respectively, are listed in table 1, while correlations between concentrations in feathers and muscle or liver are shown in table 2. All correlations were found to be significant (table 2). The highest correlation was found for PCBs between levels in feathers and muscle ($r=0.76$, $p<0.01$; figure 1). When starved birds ($n=17$) were excluded even higher correlations were found ($r=0.84$, $p<0.01$; table 2). In general, higher correlation coefficients were found between feathers and muscle samples than between feathers and liver samples (table 2).

4. DISCUSSION

Our results show that organic pollutants can be measured in a single tail feather of predatory birds and that concentrations in feathers reflect to a certain extent concentrations in internal tissues (table 2). The strongest correlation was found between levels of PCBs in feathers and muscle. Future (experimental) studies should examine whether correlations can improve when potentially confounding variables such as age, sex, condition of birds and season (which may affect external contamination) are being adequately controlled for. When the starved birds were excluded, correlations between concentrations in

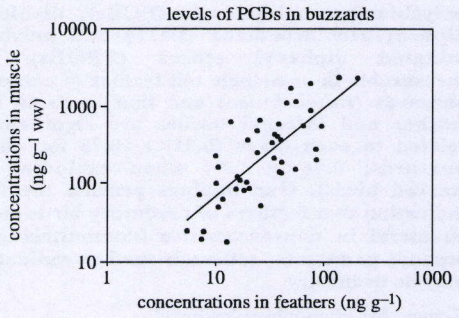

Figure 1. Relation between sum PCB concentrations in feathers and muscle samples of common buzzards ($n=43$) from Belgium ($\log y=1.15 \log x+0.68$; $r=0.76$).

feathers and internal tissues improved, indicating the importance of controlling for the condition of the birds. It is interesting to note that similar correlation coefficients have been reported for heavy metals between levels in feathers and internal tissues (r ranging from 0.51 to 0.84 between feathers and liver, and from 0.31 to 0.74 between feathers and muscle; Burger 1993). Recently, D'Havé *et al.* (2005) evaluated the use of hair of hedgehogs (*Erinaceus europaeus*) as a non-destructive biomonitor for PBDEs. They observed positive relationships between hair and internal tissues that, after removal of two outliers in their dataset, were in the lower range of correlations found in our study ($0.43<r<0.53$; D'Havé *et al.* 2005).

Higher correlation coefficients were found between feathers and muscle samples than between feathers and liver samples. Assuming that concentrations in feathers reflect circulating concentrations in the body at the time of their formation, feathers may not reflect recent changes in contamination. Since the turnover rate in liver (a highly metabolically active tissue; Voet & Voet 1995) is higher than in muscle, concentrations in liver probably reflect more recent

exposure, which may explain the lower correlation coefficients found between feather and liver samples.

The lower correlation coefficients observed in this study for PBDEs and DDTs in comparison to PCBs could possibly be explained by different degrees of external deposition onto the feather surface or by different metabolization rates. Although for heavy metals, external contamination of feathers has been observed (Burger 1993), Dauwe *et al.* (2005) found no increase of PCBs concentrations with the age of the feather, suggesting little influence of exogenous contamination. Clearly, however, the influence of external contamination needs to be evaluated in more detail.

Given that feathers can be easily preserved and that bird collections in museums and private collections date from the late 1700s, feathers could be useful for retrospective biomonitoring of POPs, as has been successfully done for heavy metals (Burger 1993), and to study regional and temporal trends (Rocque 2005). However, external contamination cannot be completely excluded and should be investigated. While many POP biomonitoring studies have previously focused on bird eggs, feathers have the advantage that they can be collected in any season and from each age or sex class. Moreover, since one feather can easily be removed from a living bird without causing severe damage, non-destructive biomonitoring may be of valuable use with regard to endangered species.

In conclusion, we show for the first time that levels of some organic pollutants in predatory bird feathers and internal tissues are related to each other. At present, our results cannot explain all the variation observed between levels in feathers and internal tissues, but instead show that feathers of predatory birds give a good estimate of contamination levels. While additional studies need to investigate the influence of external contamination and other confounding factors, feathers seem to represent a potential non-destructive biomonitoring tool for organic pollutants in predatory birds.

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4.4 Paper VII

Remarkable findings concerning PBDEs in the terrestrial top-predator red fox (*Vulpes vulpes*)

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Remarkable Findings Concerning PBDEs in the Terrestrial Top-Predator Red Fox (*Vulpes vulpes*)

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In the present study, we have analyzed muscle, liver, and adipose tissue of 33 red foxes from Belgium for their content of polybrominated diphenyl ethers (PBDEs). Median sums of seven tri- to hepta-BDEs (BDE 28, BDE 47, BDE 99, BDE 100, BDE 153, BDE 154, and BDE 183) were 2.2, 2.4, and 3.4 ng/g lipid weight in adipose tissue, liver, and muscle, respectively. These levels were lower than those found in various species of voles and mice, the main prey species of the red fox. This is probably related to the high capacity of the foxes to metabolize and eliminate lower brominated congeners. BDE 209 generally dominated the PBDE congener profiles in the red fox samples. In samples containing BDE 209, this congener contributed, on the average, approximately 70% to the total PBDE content. BDE 209 was measured in concentrations as high as 760 ng/g lipid weight in the liver, but the detection frequency was not more than 40%. In animals with the highest BDE 209 levels, this congener was detected in muscle, liver, as well as in adipose tissue. Other abundant congeners were BDE 153 and BDE 47, which prevail in other terrestrial species. The particular PBDE congener profile observed in the red fox resembles that seen in grizzly bears from Canada, but differs from those previously reported for terrestrial avian species. Our data confirms unambiguously that BDE 209 does bioaccumulate in terrestrial top predators, such as the red fox.

Introduction

Polybrominated diphenyl ethers (PBDEs) were first introduced onto the market in the 1960s and were used since then as flame retardants to improve fire safety in applications, where they are added in concentrations up to 30 wt % (*1*). A substantial increase in production was seen since the end of the 1970s due to the growing popularity of personal computers and other electronic equipment, and due to stricter fire regulations (*2*). Since then, environmental levels of PBDEs have been continuously increasing (*3, 4*). Spillage and emission during production and use, but also improper product disposal, account for this phenomenon. These chemicals are shown to be persistent and lipophilic, which results in bioaccumulation in fatty tissues of organisms and enrichment throughout the food chain (*4*). PBDEs have already been identified in tissues of invertebrates and fish (*5*), aquatic and terrestrial birds (*6*), marine and terrestrial mammals (*4, 7*), and humans (*8*). Several reviews have been dedicated to the toxicological effects of PBDEs in exposed organisms (*9–11*), while others focused mainly on their endocrine disrupting properties in humans and wildlife (*12, 13*). The current knowledge on the potential health risks associated with PBDE exposure ascribe these chemicals the potential to disrupt normal thyroid homeostasis, to cause neurological and developmental effects, and to possibly cause cancer in laboratory animals. The observed effects on the thyroid system (*12*) and the estrogen-mediated gene expression (*14*) can also be related to the presence of OH–PBDEs, which are biologically formed. It is, therefore, clear that prolonged exposure to these pollutants can interfere with normal physiology and biochemistry. The high toxicological potential of PBDEs can have health consequences for top-predators, such as red foxes, which can theoretically build up substantial amounts of these persistent chemicals in their body following biomagnification through the food chain.

Data on PBDE concentrations in terrestrial biota are scarce. Most available data are on birds of prey (*4, 15, 16*), while limited data are available on rabbits (*Oryctolagus cuniculus*), moose (*Alces alces*), reindeer (*Rangifer tarandus*) (*17*), on hedgehogs (*Erinaceus europaeus*) (*18*), and on grizzly bears (*Ursus arctos*) (*7*). This lack of data hampers the assessment of PBDE exposure and highlights the need for more research related to terrestrial species.

In the past, fox species have already been proposed as a bioindicator for organochlorine pollution (*19, 20*). They are widely distributed omnivores capable of adapting easily to local food sources, which makes them ideal for monitoring local exposure. However, foxes possess highly developed metabolic systems, as do humans, which can complicate data interpretation through formation of toxicologically potent metabolites that are not always considered in toxicological assessments.

Red fox (*Vulpes vulpes*) are major predators of small mammals including voles, mice, squirrels, hares, and rabbits, which represent 90% of their diet (*21*). The relevance for the foxes of the present study was confirmed by a detailed investigation on the stomach content of foxes from the same geographical region (*22*). Additionally, dead animals and domestic garbage, particularly in urban environments, represent additional food resources (*23*). The red fox used in the present study are, therefore, considered as opportunistic omnivores.

In the present study, muscle, liver, and adipose tissue of red fox (*Vulpes vulpes*) were analyzed for their PBDE, polychlorinated biphenyl (PCB), and organochlorine pesticide (OCP) content. Data on PCBs and OCP were included to support the discussion on PBDEs. Hereby, special emphasis was placed on BDE 209 since the bromine industry and its lobby group Bromine Science and Environmental Forum (BSEF) claims that this fully brominated congener is not bioavailable and, therefore, harmless. This study is the first to report on PBDEs in the red fox and contributes to a better understanding of levels, metabolism, and tissue distribution of PBDEs in terrestrial mammalian wildlife.

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Materials and Methods

Sample Collection. Although Belgium was officially declared rabies-free in 2001, the epidemiological surveillance of rabies is still maintained. Therefore, this study was based on foxes processed for a diagnostic screening of rabies infection. Between October 2003 and March 2004, the staff of the Pasteur Institute (Brussels, Belgium) collected thirty-three red foxes (*Vulpes vulpes*) from the south of Belgium. Data on gender and habitat (urban–rural) were recorded. All 33 foxes included in this study were found dead due to traffic accident traumas or provided by hunters. Liver (right lobe), muscle (left thigh), and abdominal adipose tissue were collected. Due to food deprivation related to trauma or pathology, adipose tissue could be collected only from 27 individuals. In three cases, abdominal damage prevented the collection of the liver (30 livers collected), which was only collected if it was undamaged. All samples were stored at −20 °C until further treatment.

Chemicals. All solvents used for the analysis (*n*-hexane, acetone, dichloromethane, and iso-octane) were of SupraSolv grade (Merck, Darmstadt, Germany). Individual reference standards for each analyte were used for identification and quantification (Wellington, Guelph, ON, Canada; Dr. Ehrenstorfer Laboratories, Augsburg, Germany). Sodium sulfate was heated for at least 6 h at 600 °C, and silica was pre-washed with *n*-hexane and dried overnight at 60 °C before use. Extraction thimbles were pre-extracted with hexane.

Sample Preparation and Analysis. The following PBDE congeners (IUPAC numbering 28, 47, 99, 100, 153, 154, 183, and 209) and brominated biphenyl (BB) 153 were targeted for analysis. BDE 77, BDE 128, BB 155, and ^{13}C–BDE 209 were used as internal standards (IS). Details can be found elsewhere (*5*, *16*). The PCB congeners analyzed were the following: 28/31, 52, 74, 95, 99, 101, 105, 110, 118, 128, 138/163, 149, 153, 156, 170, 180, 183, 187, 194, 196/203, and 199. The following OCPs were also determined: α-, β-, and γ-hexachlorocyclohexane (hereafter referred to as "HCHs"), *trans*-chlordane (TC), *cis*-chlordane (CC), *trans*-nonachlor (TN), oxychlordane (OxC) (hereafter referred to as "CHLs"), 2,2-bis(4-chlorophenyl)-1,1,1-trichloroethane (*p,p'*-DDT), 2,2-bis(4-chlorophenyl)-1,1-dichloroethylene (*p,p'*-DDE), 2,2-bis(4-chlorophenyl)-1,1-dichloroethane (*p,p'*-DDD), (hereafter referred to as "DDTs"), and hexachlorobenzene (HCB).

The method used for analysis of fox tissues has been previously described (*5*) and is briefly summarized below. Depending on the type of tissue, 0.2 g (adipose tissue), 2 g (liver), or 6 g (muscle) of homogenized sample were mixed with anhydrous Na_2SO_4, spiked with IS, and Soxhlet extracted with hexane/acetone (3:1, *v/v*). An aliquot of the extract was used for gravimetrical lipid determination. The extracts were cleaned-up on silica impregnated with concentrated sulfuric acid (48%, *w/w*) and analytes were eluted with hexane and dichloromethane. The cleaned extract was evaporated to dryness and reconstituted in iso-octane.

PBDEs were analyzed by gas chromatography–electron capture negative ionization mass spectrometry (GC/ECNI–MS) operated in selected ion monitoring (SIM) mode. Details of the instrumental conditions were published elsewhere (*5*). For tri- to hepta-BDE congeners and BB 153, a 25 m 0.22 mm × 0.25 μm HT-8 capillary column (SGE, Zulte, Belgium) was used and ions $m/z = 79$ and 81 were monitored. For the analysis of BDE 209, a 12 m × 0.18 mm × 0.10 μm AT-5 capillary column (Alltech, Lokeren, Belgium) was used and ions $m/z = 484.7/486.7$ and 494.7/496.7 were monitored for BDE 209 and ^{13}C–BDE 209, respectively.

PCBs were analyzed by GC-electron impact MS (GC/EI–MS) operated in SIM-mode using a 30 m × 0.25 mm × 0.25 μm DB-1 capillary column (J&W, Folsom, CA). OCPs were analyzed by GC/ECNI–MS operated in SIM-mode on a 25m × 0.22 mm × 0.25μm HT-8 capillary column (SGE, Zulte, Belgium). Instrumental conditions for PCB and OCP analysis were published elsewhere (*24*).

A subset of 10 samples were sent to the Laboratory for Water Analysis of the German Environment Agency (Umweltbundesamt, Berlin, Germany) for confirmatory analysis of BDE 209. These samples were extracted by accelerated solvent extraction with toluene followed by a multilayer silica gel column chromatography and gel permeation chromatography cleanup (*25*). Analyses were conducted using a short 2 m × 0.25 mm × 0.25 μm RTX-CLPesticides column (Restek, Germany), which provides sufficient selectivity and high sensitivity (Lepom and Sawal, unpublished results). The carrier gas was helium at constant flow (4.7 mL/min), and the oven was programmed from 150 °C to 300 °C at 25 °C/min. Ions $m/z = 484.7/486.7$ and 494.7/496.7 were monitored for BDE 209 and ^{13}C–BDE 209, respectively.

Quality Assurance and Quality Control. Instrumental QC was done by regular injection of solvent blanks and standard solutions, while the analyst and method QC was ensured through replicate sample analyses (RSD < 5%) and procedural blanks (RSD < 12%). Therefore, the mean procedural blank value was used for subtraction. After blank subtraction, the limit of quantification (LOQ) was set at 3 times the standard deviation of the blank, which ensures >99% certainty that the reported value is originating from the sample. This implies varying LOQs (between 2 and 500 pg/g ww) depending on the sample intake and on the PBDE congener. Absolute recoveries for individual PBDE congeners were between 87 and 104% (RSD < 12%). The efficiency of the method was demonstrated by successful participation in several international interlaboratory studies on the determination of PBDEs in biota (QUASIMEME and NIST).

Due to analytical difficulties and low concentrations which have to be measured, reports on the presence of BDE 209 in biological tissues often provoke some skepticism. Therefore, confirmatory analysis of a subset of 10 samples covering a wide range of BDE 209 concentrations ranging from not detected to several hundred ng/g lw was done in the Laboratory for Water Analysis of the German Environment Agency. The agreement of results in this small interlaboratory exercise was excellent (differences were <10% for levels higher than 10 ng/g lipid weight (lw) and <20% for levels lower than 10 ng/g lw), in particular when taking into account the high interlaboratory variation for BDE 209 seen in recent international studies.

Statistical Analysis. Before statistical analysis, samples with levels below LOQ were assigned a value of p × LOQ, with "p" being the proportion of measurements with levels above LOQ (*26*). As expected for environmental samples, the data was not-normally distributed (Shapiro–Wilk's test), and therefore, nonparametric statistics were further used. Spearman rank correlations were calculated between the PBDE levels in the various tissues and between the levels of BDE 209 and the lower brominated BDEs. A Kruskal–Wallis test was used to compare the PBDE levels in the different tissues and to test for differences related to gender and habitat (rural–urban). Statistical calculations were performed using SPSS v.11 for Windows (SPSS Inc.).

Results and Discussion

Lipids. The lipid percentages of muscle and liver tissue can be susceptible to great variation caused by nourishment and health status of the animals (Table 1). The lipid percentages of adipose tissue on the other hand was more consistent (RSD = 28%) and does not alter so much with the nutritional status of the animal, while the amount of adipose tissue declines when lipids are mobilized and depleted. In the present study, adipose tissue was readily available in 27 out

TABLE 1. Lipid Content, Percentage of Results above Limit of Quantification, Median, Minimum, and Maximum Concentrations of PBDEs and BB 153 in All Muscle, Liver, and Adipose Tissues (ng/g lipid weight) of Red Foxes from Belgium

		BDE 28	BDE 47	BDE 100	BDE 99	BDE 154	BDE 153	BDE 183	sum PBDEs	BDE 209	BB 153
adipose tissue	median	<0.03	0.23	0.05	0.33	0.04	0.76	0.37	**2.2**	<3.7	0.18
($n = 27$)	minimum	<0.03	< 0.68	<0.18	< 0.90	< 0.07	<0.08	<0.03	**0.73**	<3.7	<0.03
	maximum	<0.03	23	9.5	31	3.7	33	4.9	**82**	200	20
	% > LOQ	0	33	26	37	48	96	67		15	96
	lipid % (SD)						72 (22)				
liver	median	<0.04	0.33	0.05	0.11	0.02	1.2	0.61	**2.4**	<9.1	0.57
($n = 30$)	minimum	<0.04	< 0.81	<0.21	< 1.1	<0.09	<0.09	<0.04	**0.61**	<9.1	<0.04
	maximum	< 0.04	62	35	6.9	1.0	25	7.4	**115**	760	38
	% > LOQ	0	40	23	10	23	93	67		40	90
	lipid % (SD)						5.8 (3.5)				
muscle	median	<0.05	1.4	0.02	0.74	0.02	0.83	0.14	**3.4**	<3.9	0.19
($n = 33$)	minimum	<0.05	0.54	<0.05	<0.28	<0.07	<0.16	<0.01	**1.0**	<3.9	<0.01
	maximum	< 0.05	11	8.4	12	2.9	20	4.8	**44**	290	9.3
	% > LOQ	0	100	39	97	27	100	55		21	52
	lipid % (SD)						3.4 (1.7)				

of 33 foxes, which indicates that these animals were probably not deprived of food before dying.

PBDEs. Sum of PBDEs (BDE 28−183) ranged from 0.73 to 82 ng/g lw (0.53−34 ng/g ww) in adipose tissue, from 0.61 to 115 ng/g lw (0.04−4.3 ng/g ww) in liver and from 1.0 to 44 ng/g lw (0.05−1.8 ng/g ww) in muscle (Table 1). Large inter-individual differences were seen in the sample set. Since diet is assumed to represent the major contributor to lipophilic persistent contaminant burdens in mammals, the variance likely reflects individual differences in diet, which in his turn, is dependent on habitat.

Red foxes are opportunistic feeders and their diet is diverse and depends on the environment, the period of the year, hunting habits, and special nutritional requirements such as feeding of the litter. Red foxes are mainly predators of small mammals including voles, mice, squirrels, hares, and rabbits (*21*). In a Belgian study, common voles (*Microtus arvalis*), water voles (*Arvicola terrestris*), wood mice (*Apodemus sylvaticus*), brown rats (*Rattus norvegicus*), and bank voles (*Clethrionomys glareolus*) constituted 90% of mammals retrieved in the stomach of red foxes (*22*). In spring and summer, reptiles, insects, and berries may supplement the diet. If accessible, red foxes may also invade poultry yards and feed on eggs and birds. Dead animals and domestic garbage, particularly in urban environments, represent additional food resources (*23*). Red foxes prefer the borders of forested areas and adjacent open lands. Although mostly encountered in rural environments, red foxes have also extended their habitat to cities and suburban areas (*21, 23*). In the present study, both rural and urban foxes were included.

Owing to the high trophic position of the red fox, PBDE tissue levels were assumed to be of the same order as those found in other terrestrial top predators. However, the PBDE levels seen in the present sample set did not confirm this hypothesis. For example, other terrestrial top-predators from Belgium, such as common buzzards (*Buteo buteo*), which feed on a similar prey as foxes, showed tissue levels that were approximately 30 times higher (median sum of the seven tri- to hepta-BDEs in liver = 70 ng/g lw) (*16*). However, birds and mammals phylogenetically differ to a great extent, which has to be taken into account. Further, in the foxes of the present study, median sum of the same seven BDE congeners were even lower than those found in various species of voles and mice, the main prey species of the red fox (*27*).

Age, gender, and habitat can influence the persistent pollutant load in animals to a rather great extent. Unfortunately, the age of the animals of the present study was not available and could, therefore, not be assessed. The individuals of the present study were, therefore, grouped according to gender and habitat (male, female, rural, urban), resulting in 4 subclasses (Figure 1). The concentrations and concentration ranges of PBDEs in urban foxes tended to be higher than in rural ones, suggesting differences in exposure between the two populations (Figure 1). However, these visually observed differences were not statistically supported (Kruskal−Wallis test). No differences were observed between males and females. Unfortunately, no data was available on the age of the animals.

BDE 209. Although, in general, BDE 209 is rarely measured in terrestrial mammalian wildlife, it could be detected in the present study in 40%, 21%, and 15% of the liver, muscle, and adipose samples, respectively (Table 1). Levels of BDE 209 varied between <3.7 and 760 ng/g lw. BDE 209 was previously found in humans (*28*), in terrestrial avian wildlife species (*6, 16*), and in terrestrial mammals (*7*). In these studies, the highest BDE 209 levels were approximately 190 ng/g lw in liver (*6, 16*). Median values were not calculated because the detection frequency was less than 50%, which would yield a value below LOQ. The median BDE 209 level calculated only for samples with concentrations above LOQ was 27 ng/g lw. The relatively high detection frequency of BDE 209 conflicts with the presumed high metabolic capacity of the red fox (*29, 30*) regarding PBDEs and with the expected short half-life of BDE 209 in mammals, which was estimated to be about 7 days in humans (*31*) and 2.5 days in rats (*32*). Mörck et al. (*32*) also concluded that BDE 209 was extensively and much more readily metabolized than the lower brominated congeners in the rat. In the present study, BDE 209 was the predominant PBDE congener in red foxes whenever it was measured above LOQ, pointing at the opposite (Figure 2). Mörck et al. (*32*) also found that intestinal uptake in rats was only 10%. The assumption of a similar low uptake combined with a high turn-over would imply a relatively high and/or recent exposure to BDE 209 in order to reach the tissue levels found in the present study.

However, no BDE 209 could be detected in a parallel study involving mice and voles from Belgium (*27*), which can constitute up to 90% of the fox's diet. BDE 209 might be ingested via external contamination of food with soil or atmospheric particulate matter, which is typical for voles, mice, earthworms, and human garbage, but also grasses, grains, fungi, berries, and fruit. This hypothesis is, in part, supported by the fact that BDE 209 has been found in soil, as well as dust particles (*33*). A similar assumption was made by Christensen et al. (*7*) to explain the observed prevalence of BDE 209 in grizzly bear tissue. In that study, BDE 209 contributed up to 83% of the total PBDE load (*7*). The authors

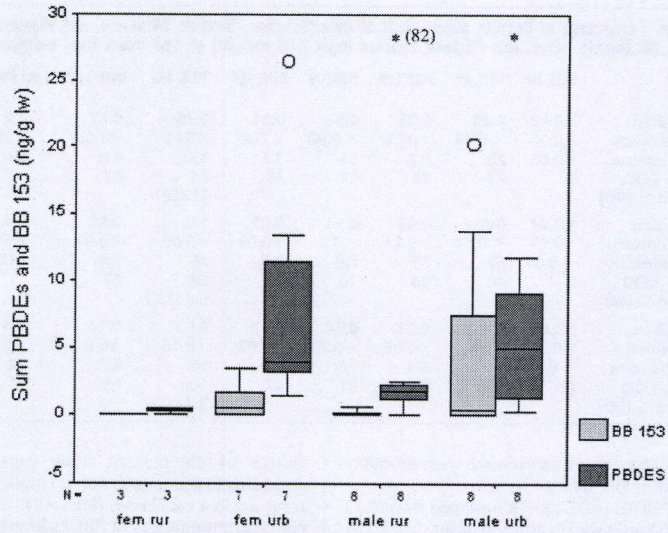

FIGURE 1. Box plot of sum PBDEs (seven tri- to hepta-BDEs) and BB 153 concentrations in red foxes grouped according to sex and habitat (female urban, female rural, male urban, male rural). Concentrations and range in rural samples tend to be lower than in urban samples. These trends were however not confirmed by statistical analysis. ° represents outliers, * represents extremes.

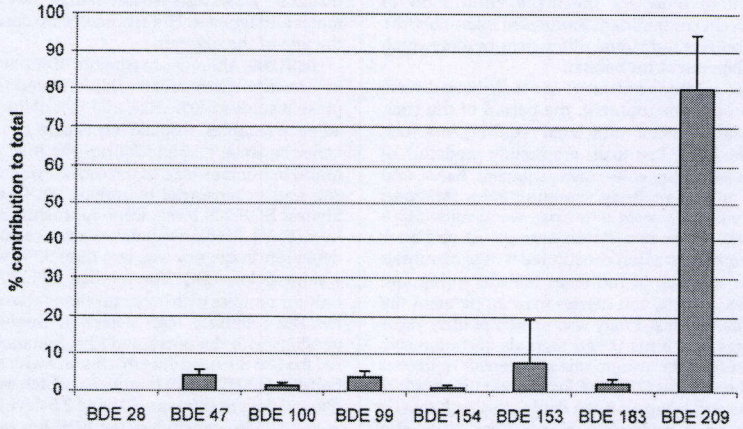

FIGURE 2. General median PBDE-profile in the red fox. The profile was based only on those samples of which all congeners could be measured ($n = 13$). BDE 209 was the dominant congener. Error bars represent 1.96 × se.

suggested that atmospheric transport of BDE 209 might be higher than expected and that air-to-plant partitioning may play an important role in the exposure (*7*). Additionally, the feeding upon human garbage (primarily urban foxes) can represent a substantial source of BDE 209 exposure for foxes. In the present study, BDE 209 levels were slightly higher in urban foxes, but this observation could not be statistically confirmed.

For a high intake of BDE 209, the formation of debromination products can be expected, resulting in an increase in the levels of lower-brominated BDEs (*34*). However, since levels of lower-brominated BDEs are very low compared to the levels of BDE 209, this hypothesis seems unlikely. Mörck et al. (*32*) reported that in rats exposed to BDE 209, only small amounts of nona-BDEs were formed (<0.5% of initial dose). They concluded, therefore, that debromination did not seem obvious as a first step metabolism in rats. In the present study, no peaks corresponding to octa- and nona-BDEs could be seen during the qualitative investigation of chromatograms for samples with the highest BDE 209 levels.

The highest lipid-normalized levels of BDE 209 were found in liver, followed by muscle and adipose tissue. Further, this is one of the first reported findings of BDE 209 in adipose tissue of terrestrial wildlife. In accordance with the present study, the highest BDE 209 levels in orally exposed rats were seen in liver and serum, while the lowest concentrations were seen in adipose tissue (*32*).

PBDE Patterns. The PBDE profile was calculated using only those individuals that had measurable levels of both BDE 209 and lower-brominated BDEs ($n = 13$). PBDE profiles were not significantly different between the various tissues. Therefore, a mean profile was calculated (Figure 2). BDE 28 was always <LOQ, while BDE 183 could be found in the majority of the samples, but at rather low levels (median = 0.61 ng/g lw in liver). This congener is assumed to be representative for exposure to the octa−technical mixture

TABLE 2. Lipid Content, Median, Minimum, and Maximum Concentrations of the Five Major PCB Congeners (ng/g lipid weight) in Muscle, Liver, and Adipose Tissue of Red Foxes from Belgium. The Other Congeners Contributed <2% to the Total PCB-burden

		CB 153	CB 138/163	CB 180	CB 170	CB 194	sum all PCBs
adipose tissue ($n = 27$)	median	95	25	127	66	20	**440**
	minimum	4.7	1.6	8.2	4.4	2.5	**31**
	maximum	1070	310	1770	840	440	**4650**
	lipid % (SD)			72 (22)			
liver ($n = 30$)	median	95	68	170	59	17	**510**
	minimum	9.8	5.6	23	6.4	<0.35	**81**
	maximum	1480	780	5350	1370	550	**9500**
	lipid % (SD)			5.8 (3.5)			
muscle ($n = 33$)	median	63	17	100	51	19	**350**
	minimum	<6	<2	< 4	<1.5	<1	**<15**
	maximum	880	240	1610	940	640	**4340**
	lipid % (SD)			3.4 (1.7)			

(*35*). BDE 209 was the major congener (≈70%) in all tissues (when above LOQ), followed by BDE 153 (≈19%), BDE 47 (≈5%), and BDE 183 (≈3%) (Figure 2). Christensen et al. (*7*) reported a similar profile in grizzly bears from Canada. The high contribution of BDE 209 to the total PBDE burden is quite unique and, until now, the exact reason for this still remains unknown.

Can Metabolism be Related to These Low Levels? Low levels of persistent lipophilic pollutants, such as PBDEs, are not uncommon in top predator mammalian wildlife. A recent study on grizzly bears from British Columbia (Canada) that investigated differences in pollutant loads in bears that feed mainly on salmon and in "interior" bears that feed primarily on plants, also revealed relatively low levels of pollutants (*7*). No differences in PBDE concentrations could be observed between the two groups of bears. Further, the mean PBDE levels in all bears were relatively low (11 ng/g lw), although one group of bears preyed mainly on salmon, a fish species often heavily contaminated with PBDEs (*36*). Similar to the foxes in the present study, PBDE levels were lower than what would be expected from the feeding habits. However, Christensen et al. (*7*) did not discuss any possible metabolic action in the bears.

Letcher et al. (*37*) reported on the high metabolic activity in polar bears. Extensive metabolism of PCBs was observed on the basis of PCB metabolite presence in the polar bear tissues. A study on polar fox, Hoekstra et al. (*38*) also showed a disparity between the levels of contaminants in fox and its position in the food chain, hypothesizing intensive metabolism in the fox. This hypothesis is strengthened by the report of low levels of organochlorine pollutants (e.g., PCBs) in bear and fox from Japan (*39*).

Studies on PBDEs in foxes are not published to date, but some research has been done on PCBs and PCB-metabolism in dogs, which belong to the same family (*Canidae*) as the fox. High metabolic action in dogs has been shown with exposure experiments using beagle dogs (*30*). After administration of CB 153, metabolites were formed quickly and were easily eliminated through the feces. Cytochrome P450 CYP2B and 2C play a primary role in the metabolism of CB 153 in dogs. Sipes et al. (*30*) also indicated that the reason dogs can readily metabolize CB 153 may be attributed to the extraordinary ability to form an unusual metabolic intermediate (namely 2,3-arene oxide) (*40, 41*). Further, a study on the metabolism of CB 153 in dogs and foxes concluded that foxes might have that specific CYP2B subfamily (*29*). It has been shown that foxes, like other *Canidae* species, possess a very high metabolic capacity to transform PCBs (*29, 38*).

Although no exposure experiments with PBDEs have been conducted on foxes, the data on metabolism available in the literature and the structural similarity between PCBs and PBDEs may suggest a high metabolic capacity also for the latter compounds. This would, at least partially, explain the low PBDE-levels in the present study. However, this hypothesis seems in contradiction with the sometimes relatively high levels of BDE 209, which can only be due to a specific resistance of this molecule to metabolism or to a very high intake by the foxes.

Tissue Distribution. The sum of PBDEs (BDE 28 to BDE 183) was significantly correlated in the various tissues ($p < 0.01$). The Spearman correlation coefficients were 0.759, 0.769, and 0.877 between fat–liver, muscle–liver, and fat–muscle, respectively. No correlation could be found between levels of BDE 209 in the different tissues. This can be due to the low number of observations where all BDE 209 could be measured in more than one tissue of the same animal ($n = 5$). The lipid-normalized PBDE burdens (sum BDE 28 to BDE 183) in the different tissues did not show any statistically significant differences (Kruskal–Wallis test, $p < 0.05$). However, a higher average load in muscle ($37 \pm 4\%$) than in liver ($29 \pm 5\%$) was observed, but this could not be statistically confirmed. A similar tissue distribution has been previously seen in sparrowhawk and buzzards (*16*), which in that case was statistically significant. For this reason and because it is a relatively clean matrix, muscle seems a suitable tissue for analysis.

BB 153. Although PBBs have never been extensively used in Europe (*2*), BB 153 could be found in nearly all individuals, although the detection frequency was tissue-dependent (Table 1). The highest concentrations were seen in liver (38 ng/g lw or 2.8 ng/g ww) and can be considered as relatively low. BB 153 levels observed in sparrowhawk liver from the same geographical region were as high as 200 ng/g lw (4.6 ng/g ww) (*16*).

PCBs. Only five out of the 24 PCB congeners that were determined, namely CBs 138/163, 153, 170, 180, and 194, contributed each for more than 5% to the total PCB-burden. All other measured congeners contributed less than 2% to the total PCB-burden. This resulted in a very specific congener profile in the fox tissues (Figure SI-1, Supporting Information), which is related to the previously mentioned high metabolic activity of fox. Similar PCB congener profiles, with CB 180 being the dominating congener, have been previously reported for fox (*29*).

Furthermore, due to metabolism, PCB-levels (Table 2) in fox are relatively low compared to other terrestrial top predators, such as sparrowhawks and buzzards (Table 3). This is in accordance with previous publications on organic pollutants in the fox (*29, 38*). In the present study, median PCB levels were around 100–200 times higher than those of PBDEs (Table 3). In buzzards and sparrowhawks from the same geographical region (*16*), the PBDE-burden was higher, resulting in a ratio sum PCBs/sum PBDEs between 20 and 60 (Table 3). The higher ratio (sum PCBs/sum PBDEs) in fox

TABLE 3. Comparison of Median Values Form Sum PCBs and Sum PBDEs (ng/g Lipid Weight) in Three Terrestrial Top Predators (Sparrowhawk, Common Buzzard, and Red Fox) from Belgium

species	tissue	n	sum PBDEs	sum PCBs	ratio
sparrow hawk	**liver**	7	2100	38 600	18
	muscle	7	2000	46 700	23
	adipose	2	1900	64 400	34
buzzard	**liver**	29	75	4450	59
	muscle	29	150	6340	42
	adipose	16	60	2680	45
fox	**liver**	30	2.4	510	***213***
	muscle	33	3.4	350	***103***
	adipose	27	2.2	435	***198***

might be an indication that PBDEs are even more readily metabolized than PCBs in fox.

Pesticides. The major contributor to the total OCP burden in all tissues was OxC (median between 14 and 211 ng/g lw). The other abundant OCPs were TN and HCB (median between 1.7 and 28 ng/g lw and between 2.2 and 5.5 ng/g lw, respectively). In the vast majority of terrestrial species, *p,p'*-DDE, a breakdown-product of *p,p'*-DDT, is a metabolic end-point and the major organochlorine contaminant. This metabolite could, however, only be ranked as the third most abundant OCP in the fox (Figure SI-2, Supporting Information). No differences could be observed in the distribution of OCPs between muscle and adipose tissue. This was not unexpected, seeing that the lipids in both tissues are similar in both composition and scope. However, pesticide concentrations in liver were higher than in both other tissues, e.g., OxC levels were approximately 10 times higher in liver (Figure SI-2). Almost 80% of the total OCP burden in the three tissues could be recovered in liver. This observation is in accordance with the assumed high metabolic capacity of the fox, and furthermore, it has been seen earlier in the terrestrial mammalian predator hedgehog (*42*). The liver is the main detoxifying organ, which makes it more likely to present higher levels of metabolites.

Conclusion

In general, the PBDE levels measured in the foxes can be considered low, with median sum of PBDEs between 2.2 and 3.4 ng/g lipid weight in adipose tissue, liver, and muscle. These levels were lower than those found in various prey species of the red fox. This is probably related to the high capacity of the foxes to metabolize and eliminate lower brominated congeners. BDE 209 generally dominated the PBDE congener profiles in the red fox samples whenever it was measured. In samples containing BDE 209 (around 40%), this congener contributed, on the average, approximately 70% to the total PBDE content. BDE 209 was measured in concentrations as high as 760 ng/g lipid weight in the liver. In animals with the highest BDE 209 levels, this congener was detected in all tissues. Our data confirms unambiguously that BDE 209 does bioaccumulate in terrestrial top predators, such as the red fox.

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Supporting Information Available

Median levels (ng/g lipid weight) of PCB congeners and organochlorine pesticides in adipose, muscle, and liver of red foxes from Belgium are presented in Figures SI-1 and SI-2, respectively. This material is available free of charge via the Internet at http://pubs.acs.org.

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SUPPORTING INFORMATION

Title: Remarkable findings concerning PBDEs in the terrestrial top-predator red fox (*Vulpes vulpes*)

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Median levels (ng/g lipid weight) of PCB congeners and organochlorine pesticides in adipose, muscle and liver of red foxes from Belgium are presented in Figures SI-1 and SI-2, respectively.

Figure SI-1. PCB-profiles in adipose tissue, muscle and liver of red foxes from Belgium. Only 5 congeners contribute each more than 5 % to the total load. All other congeners contributed less than 2 % each.

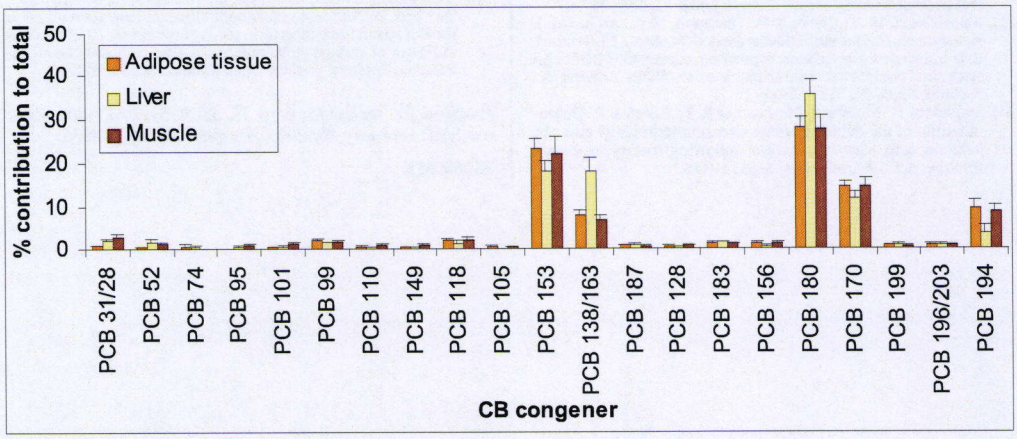
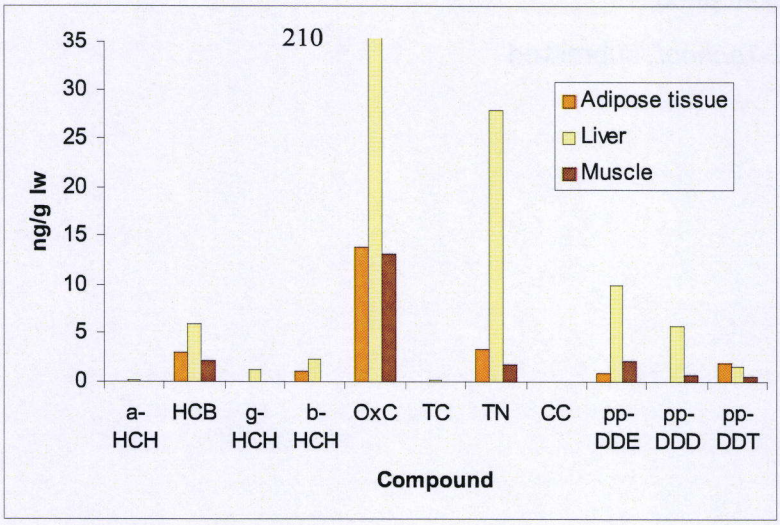


Figure SI-2. Median levels of organochlorine pesticides in adipose, muscle and liver tissues of red foxes from Belgium. The metabolic end-product oxychlordane (OxC) is the dominant product in all tissues. Highest lipid-normalized levels were seen in liver.



4.5 Paper VIII

Biomagnification potential of PBDEs in terrestrial food chains

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Biomagnification potential of PBDEs in terrestrial food chains

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Abstract

Eight polybrominated diphenyl ethers (PBDE) congeners (IUPAC # 28, 47, 99, 100, 153, 154, 183 and 209) were measured in great tits (*Parus major*), wood mice (*Apodemus sylvaticus*) and bank voles (*Clethrionomys glareolus*). These data were combined with previously obtained data on PBDEs in common buzzards (*Buteo buteo*), sparrowhawks (*Accipiter nisus*) and red fox (*Vulpes vulpes*). This enabled estimation of the biomagnification potential of PBDEs in the following three terrestrial food chains: great tit – sparrowhawk, small rodents – buzzard, and small rodents – fox. Biomagnification of BDE 209 could not be assessed because levels of this congener were below the LOQ in the prey species. All other congeners, except BDE 28, were biomagnified in both predatory bird species. Biomagnification of BDE 28 could not be observed from rodents to buzzard. Biomagnification factors (BMFs) were calculated as the ratio between the lipid normalized concentrations in the predator and in the prey. BMFs ranged from 2 to 34 for the sum of PBDEs in predatory bird food chain. Although the fox is a top-predator, this is not reflected in the PBDE concentrations that were measured in its tissues. In the small rodent – fox food chain, no biomagnification could be observed. This observation is most likely related to the high metabolic capacity of the fox with regard to organohalogens and should be taken into account when selecting species for environmental monitoring purposes. Not all top-predators will give a representative reflection of the pollution of their habitat, but confounding factors, such as metabolism, can influence the results to a great extent and can therefore lead to misinterpretations.

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INTRODUCTION

Polybrominated diphenyl ethers (PBDEs) were first introduced on the market in the 1960s and were used since then as flame retardants to improve fire safety in both commercial and domestic applications. A substantial increase in production has been seen since the end of the 1970s due to the growing popularity of personal computers and other electronic equipment and due to stricter fire regulations (1). Concurrent with the increasing use, environmental levels of PBDEs have risen since their first application (2). Spillage and emission during production and use, release from the consumer products in which they are used and also disposal at the end-of-life of the consumer products, account for this phenomenon. These compounds are chemically and biologically persistent and furthermore lipophilic, which results in their bioaccumulation in fatty tissues of organisms and enrichment throughout food chains (3). As a consequence of biomagnification, increasing concentrations of POPs can be found with increasing trophic level, leading to highest concentrations in top predators. Because PBDEs can interfere with normal physiology and biochemistry (4-6), their biomagnification may have health consequences for top-predators, such as birds of prey and foxes.

Compared to marine species, reports on PBDE concentrations in terrestrial species are generally scarce, with most data being available for birds of prey (3,7-9). Limited information is available for other terrestrial mammals, i.e. rabbits (*Oryctolagus cuniculus*), moose (*Alces alces*), reindeers (*Rangifer tarandus*) (10), hedgehogs (*Erinaceus europaeus*) (11), grizzly bears (*Ursus arctus*) (12), polar bears (*Ursus maritimus*) (13) and red foxes (*Vulpes vulpes*) (14). Until this date, only few studies have discussed biomagnification of PBDEs and they were oriented towards aquatic biota. Boon et al. (15) found that the lipid-normalized levels of the six major PBDE congeners in North Sea fish were similar to the levels in the invertebrates, which was confirmed by Voorspoels et al. (16). However, a biomagnification in concentrations of generally more than an order of magnitude could be observed from gadoid fish to marine mammals (15). Additionally, Muir et al. (17) demonstrated that PBDEs show substantial biomagnification from seals to polar bears, with biomagnification factors (BMFs) for PBDEs ranging from 3.9 to 71. Unfortunately, to the best of the authors' knowledge, biomagnification of PBDEs was not yet studied in terrestrial species.

The objective of this study was to extend the database on PBDE concentrations in small avian and mammalian terrestrial prey species (passerines and rodents). To assess the biomagnification potential of the most commonly reported PBDE-congeners in terrestrial food chains, these data were combined with previously published data on terrestrial predators from the same area, i.e. birds of prey (8,9) and red fox (14). For comparative means, the most persistent polychlorinated biphenyl (PCB) congeners were also investigated in all samples.

MATERIALS AND METHODS

Samples

Small rodents of two different species (wood mouse (*Apodemus sylvaticus*; n = 21) and bank vole (*Clethrionomys glareolus*; n = 42)) were trapped at 8 different locations around Antwerp (Belgium) during February and March 2001 (total n = 63). The animals were killed and liver, muscle and brain were excised. In order to obtain sufficient amount for analysis, 12 pooled samples (on average 5 individuals) were prepared for each tissue based on species and location. The homogenized pooled samples were stored at -20°C until further treatment. Great tits (*Parus major*) were obtained from different sampling campaigns around Antwerp conducted between 2001 and 2003. Individual fat samples (n = 25) together with individual (n = 6) and pooled (n = 9) egg samples were available from these passerines.

The predators of the present study (buzzard (*Buteo buteo*), sparrowhawk (*Accipiter nisus*) and fox (*Vulpes vulpes*)) were collected in Flanders, Belgium from November 2001 until September 2003. Liver, muscle and adipose tissue were analyzed earlier and details are given elsewhere (8,9,14).

The samples of the present study were selected based upon their importance in 3 small terrestrial food chains. Small rodents are the main prey species of common buzzards and also contribute around 40 % to the diet of the red fox. Passerines, such as the great tits, form the vast majority of the sparrowhawks (*Accipiter nisus*) diet.

Chemicals

All solvents used for the analysis (*n*-hexane, acetone, dichloromethane, and *iso*-octane) were of SupraSolv® grade (Merck, Darmstadt, Germany). Individual reference standards for each analyte were used for identification and quantification (Wellington, Guelph, ON, Canada; Dr. Ehrenstorfer Laboratories, Augsburg, Germany). Sodium sulfate was heated for at least 6 h at 600°C and silica was pre-washed with n-hexane and dried overnight at 60°C before use. Extraction thimbles were pre-extracted with hexane.

Sample preparation and analysis

The following PBDE congeners (IUPAC numbering 28, 47, 99, 100, 153, 154, 183, and 209) were targeted for analysis. The PCB congeners analyzed were the following: 28, 52, 74, 95, 99, 101, 105, 110, 118, 128, 138/163, 149, 153, 156, 170, 180, 183, 187, 194, 196/203, and 199. BDE 77, BDE 128, CB 46, CB 143 and ^{13}C-BDE 209 were used as internal standards (IS).

The method used for analysis of the tissues of the small rodents and passerines has been previously described (16), as well as the method used for the analysis of the eggs (18). Methods are briefly summarized below. Depending on the type of tissue, 0.2 g to 10 g of homogenized sample was mixed with anhydrous Na_2SO_4, spiked with IS and Soxhlet extracted

with hexane/acetone (3:1, *v/v*). An aliquot of the extract was used for gravimetrical lipid determination. The extracts were cleaned-up on silica impregnated with concentrated sulfuric acid (48%, *w/w*) and analytes were eluted with hexane and dichloromethane. The cleaned extract was evaporated to dryness and reconstituted in 100 µl *iso*-octane.

PBDEs were analyzed by gas chromatography-electron capture negative ionization mass spectrometry (GC/ECNI-MS) operated in selected ion monitoring (SIM) mode. Details of the instrumental conditions were published elsewhere (16). For tri- to hepta-BDE congeners and BB 153, a 25 m × 0.22 mm × 0.25 µm HT-8 capillary column (SGE, Zulte, Belgium) was used and ions m/z = 79 and 81 were monitored. For the analysis of BDE 209, a 12 m × 0.18 mm × 0.10 µm AT-5 capillary column (Alltech, Lokeren, Belgium) was used and ions m/z = 484.7/486.7 and 494.7/496.7 were monitored for BDE 209 and ^{13}C-BDE 209, respectively.

PCBs were analyzed by GC-electron impact MS (GC/EI-MS) operated in SIM-mode using a 30 m × 0.25 mm × 0.25 µm DB-1 capillary column (J&W, Folsom, CA, USA). OCPs were analyzed by GC/ECNI-MS operated in SIM-mode on a 25m × 0.22mm × 0.25µm HT-8 capillary column (SGE, Zulte, Belgium). Instrumental conditions for PCB analysis are published elsewhere (19).

The quality control was done by regular analyses of procedural blanks, blind duplicate samples, and random injection of solvent blanks and standards. The quality of the methods used, was verified by regular participation in interlaboratory exercises for PBDEs (Quasimeme, NIST and AMAP). Procedural blanks were consistent and therefore the mean blank values were used for subtraction. Limit of quantification (LOQ) for PBDEs was based on the procedural blanks and was set at 2 times the standard deviation (SD) of the procedural blanks for all congeners except BDE 209, for which 3 × SD was used. LOQs varied with the sample intake. In order to facilitate statistical data treatment, measurements below LOQ were replaced by p × LOQ, where "p" is the fraction of measurements above LOQ (14). All calculations related to biomagnification were based upon median concentrations.

All statistical calculations were done using SPSS v.11 for Windows (SPSS Inc.). To test for concentration differences among the two small rodent species, a t-test was used. One-way ANOVA was used to test for tissue-dependence of measured concentrations. Biomagnification factors were compared by means of a t-test and a spearman correlation was used to test the correlation between the biomagnification and the log K_{ow} of the PBDE congeners.

RESULTS AND DISCUSSION

PBDE levels in rodents and passerines

No significant differences (t-test, $p < 0.05$) were observed between PBDE levels measured in the 2 rodent species, and therefore data were combined and the species were further treated as one population. Sum of PBDEs (BDE 28 to 183) ranged from 2.4 to 23

ng/g lw in liver, from 2.2 to 30 ng/g lw in muscle and from 0.23 to 4.9 ng/g lw in brain (Table 1). BDE 209 could not be detected above the LOQ in any tissue of rodents. Although concentrations of BDE 209 tended to be higher in liver than in the other tissues, results were not reported due to the strict quality assurance measures. It is however noteworthy to mention that, due to the size of the rodents, the amount of tissue that was available for analysis was low, which caused elevated the LOQs (ranging from 7.3 to 17 ng/g lw for BDE 209).

The highest lipid normalized PBDE concentrations in the small rodents could be measured in liver and muscle. Levels in brain were significantly lower (One-way ANOVA, $p > 0.05$). This was also observed in the previously investigated birds of prey (8) that are also included in the present study. In these birds, the PBDE load in the brain was also significantly lower than in liver and muscle. Further, this phenomenon was also demonstrated for PCBs in rat (20), pork (21) and chicken (22). Current results indicate that the previous observations made for birds can be extrapolated to mammals, such as rodents, and that the brain is to some extent protected against xenobiotics, such as PBDEs.

The low PBDE concentrations measured in the rodents are most likely related to their granivorous character, although voles may also to some extent feed on insects. To the authors' knowledge, the current data on PBDEs in small terrestrial mammals is limited to rabbit muscle, in which levels of BDE 47, 99 and 100 were all < 1.8 ng/ lw (10) and to hedgehogs (11). For the latter, median sum of PBDEs (BDEs 28, 47, 99, 100, 153, 154, 183; n = 43) ranged from 1.2 to 9.5 ng/g lw, depending on the tissue (11).

PBDE levels found in the passerines of the present study were higher compared to the rodents, probably due to their insectivorous diet (Table 1). Sum of PBDEs (BDE 28 to 183) ranged from 50 to 500 ng/g lw in body fat and from 87 to 540 ng/g lw in eggs (Table 1). All congeners, except BDE 28, could be measured in 100 % of the egg and fat samples. BDE 209 was not determined in the passerine samples.

PBDE levels in birds of prey and fox

PBDE concentrations in the predatory species used in the present study were already extensively discussed (8,9,14). Results are summarized in Table 2. Levels in sparrowhawk were approximately one order of magnitude higher than in buzzard. This also explains why BDE 28, which represents only a small fraction of the total PBDE burden, was detected in most sparrowhawk samples, but not in the buzzard. Such significant species-specific differences in the concentration of lipophilic contaminants have been reported previously and have been largely attributed to differences in their dietary exposures (23,24,25). Sparrowhawks feed primarily on locally available small birds, such as passerines, while buzzards prefer small mammals such as mice, voles, rabbits, squirrels, rats, moles, and young hare (26). These differences in diet composition (26) might partly explain why highest concentrations of PBDEs were seen in sparrowhawks. However, when assessing levels in

birds of prey, one has to take into account that a vast amount of confounding factors can be present, such as abilities to metabolize persistent compounds (27,28,29), age, sex, body condition and habitat (25,30-32).

PBDE levels in fox can be considered low for an omnivorous terrestrial top-predator. Levels for the sum of PBDEs (BDE 28 to 183) ranged from 0.61 to 120 ng/g lw (0.04 to 4.3 ng/g ww) in liver and from 1.0 to 44 ng/g lw (0.05 to 1.8 ng/g ww) in muscle (Table 2). Similar as for birds of prey, several confounding factors are related to the pollutant levels in the fox. Red foxes are opportunistic feeders, resulting in a diverse diet that depends on the habitat, the period of the year, hunting habits and specific dietary habits, such as feeding on litter. Red foxes are mainly predators of small mammals (33). In a Belgian study, wood mice and bank voles accounted for the majority of mammals retrieved in the stomach of red foxes (34). In spring and summer, reptiles, insects and berries may supplement the diet. If accessible, red foxes may also invade poultry yards and feed on eggs and birds. Dead animals and domestic garbage, particularly in urban environment, represent additional food resources (35).

BDE 209 was present in both the birds of prey and the fox (8,14). This pollutant could however not undoubtedly be determined in small rodents and was not investigated in the passerine samples (see higher). Therefore it was not possible to assess its biomagnification in the investigated food chains. It has been argued that highly brominated PBDE congeners, especially BDE 209, would have a negligible bioavailability due to large molecular size, low water solubility and low vapor pressure (36). This does not seem to be the case, since BDE 209 could be measured in these top-predators (8,14). The source of BDE 209 in these animals remains unclear though, since the levels in the prey were < LOQ. It is possible that routes of exposure to BDE 209 are not directed through the common prey, but that uptake occurs through other (dietary) factors (14).

Biomagnification potential of PBDEs

Because the 3 predatory species occupy top positions in the food chain, it was most likely that biomagnification of persistent and lipophilic PBDEs through their preys would be substantial. Therefore, BMFs (the ratio of lipid normalized concentrations in predator and prey) were calculated (Table 3). The BMF values for PBDEs in buzzard and sparrowhawk ranged from 2 to 34, depending on the congener, thus evidencing biomagnification. All PBDE congeners that could be determined in both prey and predator were biomagnified (BMF > 1) (Table 3; Figure 1).

Surprisingly, no biomagnification could be observed from rodents to foxes (Table 3; Figure 1). The median levels of sum PBDEs measured in fox were even lower than those in rodents (Tables 1 and 3; Figure 1). This was not in accordance with the biomagnification hypothesis and the observations made for BMFs in birds (Table 3).

In addition, BMFs were also calculated for the most persistent PCBs, such as PCBs 153, 138/163, 170, 180 and 194, in the three food chains (Table 3). More details on PCB concentrations in the studied species can be found as Supporting Information (Tables SI-1, SI-2 and SI-3). In contrast to PBDEs, the BMFs for PCBs in foxes were > 1 and ranged from 1 to 8, depending on the congener, meaning that biomagnification has occurred. However, the BMFs for PCBs in foxes were approximately 10 times less than for birds of prey, for which BMFs for PCBs ranged from 18 to 79 (Table 3).

Biomagnification of PBDEs was previously shown for some aquatic food chains. An increase in concentrations of generally more than an order of magnitude occurred from gadoid fish to marine mammals (15). Additionally, Muir et al. (17) have shown that all four major PBDE congeners (BDEs 47, 99, 100 an 153) were biomagnified from ringed seals to polar bears. In that study, BDE 153 showed the highest BMF (71), indicating that it is a highly bioaccumulative compound (17). Although the highest BMF in the present study was calculated for BDE 153 in buzzard (BMF = 34), the BMFs of the different congeners did not show any significant difference. The BMFs for both PBDEs and PCBs of the present study are highly food chain dependent. Except for the passerine-sparrowhawk food chain, BMFs for PCBs were significantly higher than those of PBDEs.

The average BMF for the avian food chains is plotted against the log K_{ow} of the PBDE congeners (Figure 2). The BMFs for PBDEs were to a certain degree dependent on the log K_{ow} of the congeners. For sparrowhawk, a significant correlation between the BMF and the log K_{ow} was observed ($\rho = 0.750$; $p = 0.05$), while for buzzard a correlation was only observed for BDEs 28 to 154 (Figure 2). This congener is less biomagnified in buzzard than what was expected based on its lipophilicity. Burreau et al. (38) have seen the same for higher brominated PBDEs in fish. The authors concluded that hydrophobicity does not restrict the biomagnification of the higher brominated PBDEs, but probably rather molecular size or weight, leading to a lack of correlation with the trophic position (38). This would also explain the lack of correlation between BDE 209 in prey and predator in the present study. Further, this is in accordance with the observations concerning buzzard in the present study, but raises questions about the observations in the sparrowhawk (Figure 2). BMFs are most likely dependent both on the species and on the log Kow of the congener. This is strengthened further by the observations by Ramu et al. (39), who could not find any relation between log K_{ow} and BMF in finless porpoises (*Neophocaena phocaenoides*), which contradicts the results of the present study.

It appears that the lack of biomagnification of PBDEs and the relatively low biomagnification of PCBs in fox is most likely related to a high metabolic ability of fox to transform specific xenobiotics, such as PCBs (23,40,41). PCB metabolism has been previously investigated in dogs, which belong to the same family (Canidae) as the fox. After dogs were administered CB 153, metabolites were formed quickly, which was attributed to the dog's ability to form an unusual metabolic intermediate (namely 2,3-arene oxide) (42,43).

Further, a study on the metabolism of CB 153 in foxes concluded that they also might possess the above-mentioned specific metabolic ability [40]. Recently, Kunisue et al. [44] investigated PCB levels in both food and dog tissues and concluded that PCBs did not show any bioaccumulation. This is in accordance with what is seen in the foxes of the present study. Although no exposure experiments with PBDEs on foxes have been conducted, the data on metabolism available in literature and the structural similarity between PCBs and PBDEs, may suggest a high metabolic capacity for the latter compounds as well. This would, at least partially, explain the low PBDE levels and BMF values calculated for fox in the present study.

Highly developed metabolism of xenobiotics in mammals has been reported earlier. Letcher et al. [45] reported on the high metabolic activity in polar bears. Extensive metabolism of PCBs was observable on the basis of PCB metabolites that could be determined in the polar bear's tissues [45]. In a study on polar fox (*Alopex lagopus*), Hoekstra et al. [41] also showed a disparity between the levels of contaminants in fox and its position in the food chain, hypothesizing intensive metabolism in the fox. This hypothesis is further strengthened by the report of low levels of organochlorine pollutants (e.g. PCBs) in bear and fox from Japan [23] and by the results of the present study.

When the results of the present study are combined with previous research on biomagnification and metabolism of PBDEs, we can conclude that the biomagnifying effect is food chain dependent. Some species, such as the red fox, are probably able to degrade or excrete some of the native PBDE congeners. Therefore, not all top-predators will give a representative reflection of their habitat, but confounding factors, such as metabolism, can influence the results to a great extent and can therefore lead to misinterpretations.

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Supporting Information Available

Details on the PCB concentrations in all studied species are presented in Tables SI-1, SI-2 and SI-3. This material is available free of charge via the Internet at http://pubs.acs.org.

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Figure 1. PBDE concentrations (ng/g lw; median) in rodent and fox (both muscle and liver), in rodent and buzzard (both muscle and liver) and in passerine (fat) and sparrowhawk (muscle and liver). Transfer from rodents to fox did not result in biomagnification; levels in fox or similar are lower than in the prey. In both birds of prey under study, biomagnification is obvious, with levels in the predators being an order of magnitude higher than in their prey.

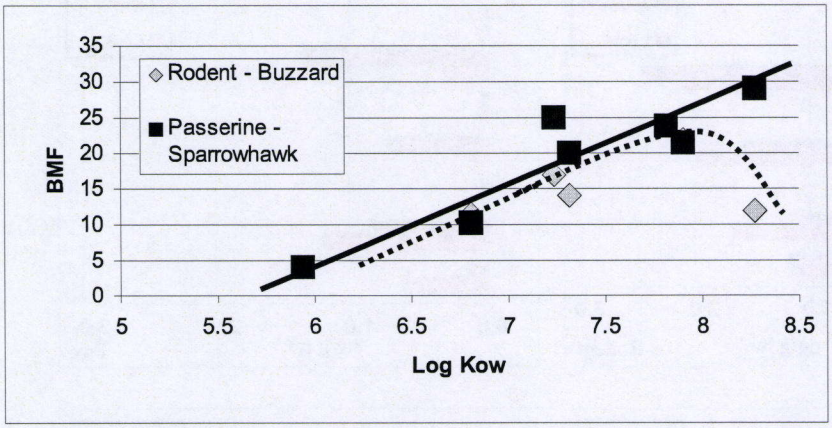

Figure 2. The biomagnification factor (BMF) of PBDEs plotted versus the log K_{ow}. The mean BMF is used in the plot. The log Kow of the PBDEs increases in the following order: BDE 28, 47, 100, 99, 154, 153 and 183. In the passerine-sparrowhawk food chain, the BMF increases with increasing log K_{ow}, while this is not the case for the rodent-buzzard food chain, where BDE 183 biomagnifies to a lesser extent. Log K_{ow}-values are taken from Braekevelt et al., 2003 (37).

Table 1. PBDE levels (ng/g lw) and mean lipid % (SD) in the various tissues of both prey species.

			BDE 28	BDE 47	BDE 100	BDE 99	BDE 154	BDE 153	BDE 183	Sum PBDEs
Rodents (n = 63; 12 pools)	Liver	Mean	< 0.09	3.0	0.39	2.3	< 0.12	1.9	1.5	9.2
		Median	< 0.09	2.2	0.32	2.0	< 0.12	1.7	1.1	7.7
		Min	< 0.09	0.89	0.11	< 0.51	< 0.12	0.63	0.18	2.4
		Max	0.16	8.0	1.1	7.0	0.25	3.6	5.5	23
		Lipid % (SD)					6.5 (1.7)			
	Muscle	Mean	< 0.15	3.3	0.58	2.2	< 0.20	1.4	1.3	9.0
		Median	< 0.15	2.5	0.37	1.6	< 0.20	1.1	1.2	7.1
		Min	< 0.15	< 1.4	< 0.16	< 0.88	< 0.20	0.52	< 0.16	2.2
		Max	0.34	12	2.3	8.5	0.29	3.3	2.6	30
		Lipid % (SD)					2.2 (0.69)			
	Brain	Mean	< 0.21	< 2.0	< 0.22	< 1.2	< 0.27	< 0.57	0.15	1.2
		Median	< 0.21	< 2.0	< 0.22	< 1.2	< 0.27	< 0.57	< 0.21	0.43
		Min	< 0.21	< 2.0	< 0.22	< 1.2	< 0.27	< 0.57	< 0.21	0.23
		Max	< 0.21	2.2	0.49	1.8	< 0.27	< 0.57	0.60	4.9
		Lipid % (SD)					8.2 (0.64)			
Passerines	Fat (n = 25)	Mean	1.8	56	13.0	50	5.2	22	10	160
		Median	0.77	44	10.9	37	3.3	16	6.3	120
		Minimum	< 0.60	18	4.8	16	1.6	6.2	1.2	50
		Maximum	9.0	170	35	160	19	81	45	500
		Lipid % (SD)					73 (7.7)			
	Eggs (n = 15)	Mean	0.54	90	13	64	6.1	31	19	220
		Median	< 0.20	83	12	52	5.8	26	16	210
		Minimum	< 0.20	37	3.4	24	1.2	10	4.4	87
		Maximum	1.9	170	27	180	16	83	60	540
		Lipid % (SD)					10 (2.1)			

Table 2. BFR levels (ng/g lw) and mean lipid% (SD) in the relevant tissues of the predators

			BDE 28	BDE 47	BDE 100	BDE 99	BDE 154	BDE 153	BDE 183	sum PBDEs
Buzzard (n = 44)	Liver	Mean	0.63	160	45	200	37	220	64	720
		Median	< 0.04	21	5.4	16	4.6	17	7.4	76
		Minimum	< 0.04	< 0.40	< 0.20	< 1.0	< 0.10	< 0.10	< 0.04	< 1.7
		Maximum	7.4	2450	770	3650	440	3050	1120	10600
		Lipid % (SD)					3.8 (1.3)			
	Muscle	Mean	1.1	120	34	150	46	210	80	640
		Median	< 0.05	32	6.4	32	8.3	37	20	150
		Minimum	< 0.05	2.9	< 0.05	2.1	0.6	2.3	1.0	9.1
		Maximum	10	1190	450	2020	790	3670	890	8830
		Lipid % (SD)					4.2 (2.3)			
Sparrowhawk (n = 12)	Liver	Mean	9.5	2180	1370	3480	270	1280	920	9500
		Median	1.9	500	280	760	73	340	170	2060
		Minimum	< 0.04	88	39	84	12	35	12	280
		Maximum	74	15100	10700	26600	1590	8490	6990	64000
		Lipid % (SD)					3.0 (1.3)			
	Muscle	Mean	17	1520	990	2130	260	1040	440	6390
		Median	4.3	400	270	710	82	340	200	1980
		Minimum	< 0.05	10	2.6	11	2.9	19	33	79
		Maximum	140	6010	6410	9320	1780	6900	1900	32500
		Lipid % (SD)					2.2 (2.0)			
Fox	Liver (n = 30)	Mean	< 0.04	3.0	1.4	0.43	0.10	3.3	0.74	8.9
		Median	< 0.04	0.33	0.05	0.11	0.02	1.2	0.61	2.4
		Minimum	< 0.04	< 0.8	< 0.20	< 1.0	< 0.10	< 0.10	< 0.04	0.61
		Maximum	< 0.04	62	35	6.9	1.0	25	7.4	120
		Lipid % (SD)					5.8 (3.5)			
	Muscle (n = 33)	Mean	< 0.05	2.2	0.44	1.5	0.29	2.7	0.50	7.6
		Median	< 0.05	1.4	0.02	0.74	0.02	0.83	0.14	3.4
		Minimum	< 0.05	0.54	< 0.05	< 0.30	< 0.10	< 0.20	< 0.01	1.0
		Maximum	< 0.05	11	8.4	< 0.40	2.9	20	4.8	44
		Lipid % (SD)					3.4 (1.7)			

Table 3. Biomagnification factors (BMFs: the ratio of concentration of pollutant between predator and prey) of individual PBDE congeners in the three food chains under study.

	Passerine - Sparrowhawk			Rodent - Buzzard			Rodent - Fox		
	Fat-Liver	Fat-Muscle	*Mean*	Liver	Muscle	*Mean*	Liver	Muscle	*Mean*
BDE 28	2	6	***4***	n.a.	n.a.	***n.a.***	n.a.	n.a.	***n.a.***
BDE 47	11	9	***10***	10	13	***12***	< 1	< 1	***< 1***
BDE 100	26	24	***25***	17	17	***17***	< 1	< 1	***< 1***
BDE 99	21	19	***20***	8	20	***14***	< 1	< 1	***< 1***
BDE 154	22	25	***24***	n.a.	n.a.	***n.a.***	n.a.	n.a.	***n.a.***
BDE 153	21	21	***21***	10	34	***22***	< 1	< 1	***< 1***
BDE 183	27	32	***29***	7	17	***12***	< 1	< 1	***< 1***
Sum PBDEs	18	17	***17***	9	18	***14***	< 1	< 1	***< 1***
PCB 153	20	18	***19***	25	65	***45***	2	2	***2***
PCB 138/163	23	20	***21***	18	79	***49***	2	1	***2***
PCB 180	21	19	***20***	18	55	***36***	5	5	***5***
PCB 170	26	23	***24***	26	n.a.	***26***	5	n.a.	***5***
PCB 194	25	21	***23***	57	n.a.	***57***	8	n.a.	***8***
Sum PCBs[$]	21	19	***20***	20	60	***40***	2	3	***3***

n.a.: not available because the median concentration in the prey was below the LOQ
[$]: Including CBs 153, 138/163, 180, 170 and 194

SUPPORTING INFORMATION

Title: Biomagnification potential of PBDEs in terrestrial food chains

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Tables SI-1, SI-2 and SI-3 present details on levels of selected PCBs in both prey species and in the predators that were analyzed in the present study. The SI consists of 4 pages.

Table SI-1. Statistical descriptors of the concentrations of the most persistent CB congeners (ng/g lw), together with the sum of all measured PCBs and mean lipid % (SD) in the prey species.

			CB 153	CB 138/163	CB 170	CB 180	CB 194	Sum all PCBs *
Rodents (n = 63; 12 pools)	Liver	Mean	78	55	16	42	3.1	300
		Median	46	34	11	38	2.3	220
		Min	15	8.4	< 3.4	6.0	< 0.25	61
		Max	270	210	39	83	12	840
		Lipid % (SD)			6.5 (1.7)			
	Muscle	Mean	52	23	7.0	27	2.1	170
		Median	27	12	< 5.9	21	< 0.50	110
		Min	9.3	< 6.3	< 5.9	< 10	< 0.50	24
		Max	190	100	27	81	11	630
		Lipid % (SD)			2.2 (0.69)			
	Brain	Mean	11	< 4.8	< 4.5	< 7.9	< 0.35	25
		Median	3.2	< 4.8	< 4.5	< 7.9	< 0.35	6.3
		Min	3.2	< 4.8	< 4.5	< 7.9	< 0.35	4.3
		Max	28	15	< 4.5	< 7.9	< 0.35	72
		Lipid % (SD)			8.2 (0.64)			
Passerines	Fat (n = 25)	Mean	970	610	200	660	93	3890
		Median	780	490	140	450	67	2960
		Minimum	341	240	31	190	23	1510
		Maximum	2530	1390	680	2360	335	10100
		Lipid % (SD)			73 (7.7)			
	Eggs (n = 15)	Mean	980	700	170	560	80	4110
		Median	850	550	150	520	73	3560
		Minimum	470	360	94	290	31	2020
		Maximum	1960	1770	380	990	150	9670
		Lipid % (SD)			10 (2.1)			

* Sum all PCBs includes the following congeners: PCB 28, 52, 74, 95, 99, 101, 105, 110, 118, 128, 138/163, 149, 153, 156, 170, 180, 183, 187, 194, 196/203, and 199.

Table SI-2. Statistical descriptors of the concentrations of the most persistent CB congeners (ng/g lw), together with the sum of all measured PCBs and mean lipid % (SD) in birds of prey.

			CB 153	CB 138/163	CB 170	CB 180	CB 194	Sum all PCBs *
Buzzard (n = 44)	Liver	Mean	7580	3920	2400	6020	1300	30800
		Median	1160	610	290	700	140	4450
		Minimum	64	42	15	34	7	260
		Maximum	77400	41200	24900	70800	17600	316000
		Lipid % (SD)	3.8 (1.3)					
	Muscle	Mean	5740	3020	1670	4310	770	23400
		Median	1750	920	460	1130	210	6780
		Minimum	2	2	2	2	2	385
		Maximum	57800	31500	15600	47600	8270	238000
		Lipid % (SD)	4.2 (2.3)					
Sparrowhawk (n = 12)	Liver	Mean	37900	26100	9580	26600	4400	166000
		Median	15500	11000	3670	9370	1660	65500
		Minimum	2430	1770	540	1300	200	10900
		Maximum	179000	119000	43600	128000	19100	755000
		Lipid % (SD)	3.0 (1.3)					
	Muscle	Mean	41000	27200	10200	29700	4780	178000
		Median	14300	9520	3210	8710	1380	57700
		Minimum	360	190	87	240	1380	1350
		Maximum	211000	136000	50300	152000	21000	884000
		Lipid % (SD)	2.2 (2.0)					

* Sum all PCBs includes the following congeners: PCB 28, 52, 74, 95, 99, 101, 105, 110, 118, 128, 138/163, 149, 153, 156, 170, 180, 183, 187, 194, 196/203, and 199.

Table SI-3. Statistical descriptors of the concentrations of the most persistent CB congeners (ng/g lw), together with the sum of all measured PCBs and mean lipid % (SD) in fox.

			CB 153	CB 138/163	CB 170	CB 180	CB 194	Sum all PCBs *
Fox	Liver (n = 30)	Mean	230	170	180	560	66	1300
		Median	95	68	60	170	17	510
		Minimum	10	6	6	23	< 0.35	82
		Maximum	1480	780	1370	5350	550	9500
		Lipid % (SD)	5.8 (3.5)					
	Muscle (n = 33)	Mean	150	40	120	230	70	690
		Median	63	17	51	100	19	350
		Minimum	< 6	< 2	< 1.5	< 4	< 1	< 15
		Maximum	880	240	940	1610	640	4340
		Lipid % (SD)	3.4 (1.7)					

* Sum all PCBs includes the following congeners: PCB 28, 52, 74, 95, 99, 101, 105, 110, 118, 128, 138/163, 149, 153, 156, 170, 180, 183, 187, 194, 196/203, and 199.

5

PBDEs and Man



5.1 Introduction

Since PBDEs can be found in every environmental compartment that was investigated (See above), it is obvious that also humans are exposed to these chemicals. The most commonly investigated human samples, are serum and milk, since these are relatively easy to obtain (Sjödin et al., 2003). All other human tissues, such as muscle, liver and adipose tissue, require invasive techniques that are ethically unacceptable. Limited data is available on PBDEs in human adipose tissue, sampled during autopsies (Covaci et al., 2002). Another matrix that might be of interest in regard to PBDEs but that was not thoroughly studied until now is hair. Seeing the low levels that were measured in bird feather (See *3.2.* ***Paper II***), the levels in hair are also expected to be very low and will probably not facilitate any measurements above LOQ with the current methodology.

In a non-exposed population, levels of PBDEs in serum are expected to be low because they are lipophilic compounds that, to a certain extent, are distributed to different tissues throughout the body in correlation with their lipid content and composition. The lipid percentage of serum is around 0.6 %, which is very low compared to other tissues. These low PBDE levels in serum are quite demanding for the analytical methodology. Additionally, laboratory background levels can be sometimes relatively high in PBDE analysis (See *2. Analytical methodology*), which can hamper the necessary low LOQ needed for PBDE determination in serum samples. Hence, measurement of PBDEs in human serum is a challenging analysis.

Paper IX describes the optimisation of a simple, fast and sensitive method for the measurement of 12 PBDEs, including BDE 209, in human serum. This method is based on solid-phase extraction. Several solid-phase sorbents were tested, such as Empore™ C_{18}, Isolute Phenyl, Isolute ENV+ and OASIS™ HLB. OASIS HLB was found to facilitate the analysis with the highest absolute recoveries (between 64 and 95%, depending on the congener). Removal of co-extracted biogenic materials was performed using a 6 ml disposable cartridge containing (from bottom to top) silica impregnated with sulphuric acid, activated silica and anhydrous sodium sulphate. Analytes were quantified using a gas chromatograph coupled with a mass spectrometer operated in ECNI mode. The LOQ ranged between 0.2 and 25 pg/ml serum (0.1 and 4 ng/g lipid weight) and was dependent on the analyte levels in procedural blanks. This procedure resulted in the highest LOQ for PBDE congeners found in higher concentrations in blanks (e.g. BDE 47, 99 and 209; See *2.5.4. Limit of quantification and background concentrations*). The use of OASIS™ HLB SPE cartridges allowed a good method precision. The intermediate precision was less than 12 % for all congeners, except for BDE 209, for which it was less than 17 %. The method variation can be considered excellent, considering the low levels that are found in the serum samples (typically < 10 pg/ml serum). The accuracy of the method was tested in international inter-laboratory

exercises, already discussed earlier (See *2.5.6. International inter-laboratory exercises*). The method yielded results that deviated less than 30 % from the assigned value for all congeners (typically between 5 and 10 %), which was considered as "excellent". In ***Paper IX***, this method was applied to a set of serum samples from a random Belgian population. The obtained results were within the range of PBDE levels in other non-exposed population from Europe. Next to this small human sample set, the method described in ***Paper IX*** was also applied to the serum samples of the predatory birds discussed earlier (See *4.2.* ***Paper V***).

As shown in ***Paper IX***, the human exposure to persistent organic pollutants is conveniently assessed through analysis of blood serum, and the concentrations are traditionally expressed on a lipid weight basis. This normalisation is done to compensate for the inter- and intra-individual lipid content of different tissues and organs, which is important to study tissue distribution (See *3.4.* ***Paper IV***, *4.2.* ***Paper V***, and *4.4.* ***Paper VII***) or biomagnification (See *4.5.* ***Paper VIII***). Lipid determination is a crucial step in the analytical procedure and is a determining factor for the final result. It can be considered equally important as the chemical analysis of PBDEs itself.

The majority of the lipid determinations that were performed in this thesis are done gravimetrically. For serum, enzymatic lipid determination methods are commonly used because they are more precise, fast and less laborious. Enzymatic determinations of total lipid (TL) content in serum implies the measurement of individual lipid classes, such as triglycerides (TG), cholesterol (CHOL) and phospholipids (PL). In addition, enzymatic measurements are nowadays done on small amounts of serum (< 100 µl). However, PL are not routinely measured in most clinical laboratories and therefore, TL values are often predicted from formulas based on measurements of only TG and CHOL. A formula for TL computed in 1989 based upon 81 individual lipid measurements was extensively used in the past (Philipps et al., 1989).

Paper X re-assesses the commonly used formula of Philipps. A larger data set (n=483) from Belgian, Swedish and Norwegian populations (with data on TG, CHOL, and PL) was used to compute a new formula for TL. A very good correlation ($r = 0.949$, $p < 0.001$) was found between TL values calculated with the proposed formula and TL values obtained by summation of individually measured TG, CHOL and PL. Statistically different values of TL were computed by using different previously published formulas. This highlights the importance of using an appropriate formula in order to obtain accurate lipid-normalized concentrations of POPs. Nevertheless, TL values derived using a mathematical formula that is based only on TG and CHOL are only an estimate and they will not be as accurate as TL values obtained by summation of individually measured TG, CHOL and PL. Individual measurements of TG, CHOL and PL should be the analyses of choice if they are available.

Paper XI studied human exposure to BFRs through the diet, which is regarded as a primary route of exposure to these chemicals (Harrad et al., 2004). Human dietary exposure was never assessed before in Belgium. A market-basket, containing various meat, fish and dairy food products was purchased at Belgian supermarkets and analysed for their PBDE content. The market-basket was assembled based on the average Belgian food consumption (KB 1992) and on questionnaires from the supermarket's sales profile. Additionally, fast food samples were also investigated.

Not unexpected, fish had the highest overall PBDE levels, followed by dairy products, fast food and meat products. One fresh salmon filet had the highest total concentration of PBDEs of all foods, whereas levels in steak and chicken breast were the lowest of all foods analyzed. BB 153 could not be determined above the LOQ in any sample. This was expected since levels of this compound are known to be rather low in European samples in general.

A limited number of studies of PBDEs in food for human consumption is available from other parts of the world (Schecter et al., 2004; Ohta et al., 2002; Bocio et al. 2003; Kiviranta et al., 2004). Also in those studies, the highest levels were seen in fish. Compared to those studies, levels in food on the Belgian market is comparable. Nevertheless it should be emphasised that these chemicals are antropogenic xenobiotics and thus they do not belong in our food.

Based on measured PBDE levels and on the mean daily food consumption in Belgium (KB 1992), an average daily dietary intake estimate of PBDEs was calculated. It should be mentioned that intake calculations did not include any fruit or vegetables, which (should) contribute to a great extent to the total daily diet. Levels in these foods are expected to be very low, seeing the low fat content of these products and the lipophilicity of the pollutants under investigation. Nevertheless, some studies reported the presence of PBDEs in fruits and vegetables, although at lower levels than in food of animal origin (Ohta et al., 2002; Kiviranta et al., 2004).

Total average dietary PBDE intake in the present study was estimated between 23 and 48 ng/day. This value is in accordance with what was previously reported by others in other countries. A limited number of publications is currently available on the issue of dietary PBDE intake and levels range from 41 to 91 ng/day (Darnerud et al., 2001; Ryan and Patry, 2001; Lind et al., 2002; Bocio et al., 2003; Harrad et al., 2004; Kiviranta et al., 2004). Although these intake estimations were based on diets from geographical distinct areas, such as Canada, Finland, Spain, Sweden and the UK, the daily intake estimations are fairly comparable.

Although fish only is a minor constituent of the Belgian diet, this food can be considered a major contributor to the total daily PBDE intake (around 40 %) due to the high PBDE levels in fish, while meat products (although low contaminated), account for around 30 % of the total dietary intake of PBDEs. Dairy products only contribute to a minor degree. Also other studies have reported a similar uptake distribution. Sjödin and co-workers (2003) found that around 50% of the total dietary PBDE uptake in Sweden originated from fish, while the rest was from

meat and dairy products. The fish contribution was slightly lower in the present study, but this may be due to the dietary habit of Belgians, which tend to be more meat than fish consumers.

Although PBDE levels in food can be considered relatively low, it appeared obvious that one of the main routes of exposure of the general human population to PBDEs is through the diet (Watanabe and Sakai, 2003). Later, more insight in the environmental behaviour of PBDEs was generated, which has led to recognition of dust as an important route of (terrestrial) exposure (Tamade et al., 2002; Harrad et al., 2004).

5.2 Paper IX

Optimisation of the determination of polybrominated diphenyl ethers in human serum using solid-phase extraction and gas chromatography-electron capture negative ionisation mass spectrometry

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Optimization of the determination of polybrominated diphenyl ethers in human serum using solid-phase extraction and gas chromatography-electron capture negative ionization mass spectrometry

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Abstract

A simple, rapid, sensitive and reproducible method based on solid-phase extraction (SPE) and acidified silica clean-up was developed for the measurement of 12 polybrominated diphenyl ethers (PBDEs), including BDE 209, and 2,2′,4,4′,5,5′-hexabromobiphenyl (BB 153) in human serum. Several solid-phase sorbents (Empore™ C_{18}, Isolute Phenyl, Isolute ENV+ and OASIS™ HLB) were tested and it was found that OASIS™ HLB (500 mg) gives the highest absolute recoveries (between 64% and 95%, R.S.D. < 17%, $n = 3$) for all tested analytes and internal standards. Removal of co-extracted biogenic materials was performed using a 6 ml disposable cartridge containing (from bottom to top) silica impregnated with sulphuric acid, activated silica and anhydrous sodium sulphate. PBDEs and BB 153 were quantified using a gas chromatograph coupled with a mass spectrometer (MS) operated in electron-capture negative ionization mode. The method limits of quantification (LOQ) ranged between 0.2 and 25 pg/ml serum (0.1 and 4 ng/g lipid weight). LOQs were dependent on the analyte levels in procedural blanks which resulted in the highest LOQs for PBDE congeners found in higher concentrations in blanks (e.g. BDE 47, 99 and 209). The use of OASIS™ HLB SPE cartridge allowed a good method repeatability (within- and between-day precision < 12% for all congeners, except for BDE 209 < 17%, $n = 3$). The method was applied to serum samples from a random Belgian population. The obtained results were within the range of PBDE levels in other non-exposed population from Europe.
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Keywords: Polybrominated diphenyl ethers; Human serum; Solid-phase extraction

1. Introduction

Brominated flame retardants (BFRs) and, in particular polybrominated diphenyl ethers (PBDEs), have been identified as new environmental contaminants with global distribution as shown by their identification in both aquatic and terrestrial compartments in Europe [1,2], North America [1,3] and Asia [4]. Although banned for use for several decades, polybrominated biphenyls (PBBs) may still be found in various environmental compartments [5]. PBDEs have been shown to act as hormone disruptors, neuro-developmental toxicants and, in some cases, carcinogenic agents. This has raised high concerns about exposure to even low environmental concentrations [6]. Although increasing levels in the last decades have been found in human milk from Sweden [7] and human serum from Norway [8], human exposure to PBDEs in Europe is still at low levels [9]. Therefore, there is a need for sensitive, yet simple methods, ready to be applied in epidemiological studies involving PBDEs.

Most methods developed in the past for the determination of PBDEs in human serum employed liquid–liquid extraction (LLE) [10–12] which has the disadvantages of being laborious. Recent methods take advantage of solid-phase extraction (SPE) techniques and several sorbents have been already tested [13–15]. The SPE techniques have several advantages over the LLE procedures, such as reduced solvent consumption and processing time, possibility of miniaturization, high reproducibility and parallel sample preparation. However, none of the previous

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articles offer a comparison between the efficiencies of various sorbents.

The aim of this study is to set up a simple, rapid, sensitive and reproducible method using SPE for the determination of 12 major PBDE congeners and BB 153 in human serum. The list of PBDE congeners includes BDE 209, the principal component of the sole PBDE technical mixture presently used in Europe [16]. Due to relatively high analytical background of BDE 209 [13,14], this congener was not measured or not reported in most studies on the PBDE levels in human serum. Additionally, different SPE sorbents were compared with regard to their ability to yield high recoveries of PBDEs.

2. Experimental

2.1. Reagents, materials and solutions

PBDE congeners 28, 47, 49, 66, 85, 99, 100, 138, 153, 154, 183 and 209 (nomenclature and structure in Ref. [17] and Table 1) were purchased from Wellington Laboratories (Guelph, ON, Canada). ^{13}C-BDE 209 was also purchased from Wellington Laboratories and had ten bromine atoms and twelve ^{13}C atoms, with an isotopic purity of 99%. Brominated biphenyl (BB) 153 was from Accustandard (New Haven, CT, USA). BDE 104 and BDE 140 were used as internal standards (IS), while BDE 139 was used as recovery standard (RS) and were also obtained from Accustandard. They were shown not to co-elute with the analytes of interest and has similar degree of bromination to the predominant analytes found in human serum. All standard solutions were prepared in *iso*-octane. Acetone, *n*-hexane (Hex), dichloromethane (DCM), methanol (MeOH) and *iso*-octane were of pesticide grade (Merck, Darmstadt, Germany). Concentrated sulphuric acid (98%) and formic acid (99%) were from Merck. Anhydrous sodium sulphate (Na_2SO_4) and silica gel (Merck) were washed with Hex and used after heating overnight at 150 °C. The acidified silica gel (44% acid, w/w) was prepared as previously described [18]. All glassware was washed with detergent, rinsed with water and dried at 150 °C. Prior to use, all glassware was rinsed with Hex.

A Positive Pressure Manifold (3 M Company, St. Paul, MN, USA) was used for the extraction and clean-up. The following SPE cartridges were tested: 10 mm/6 ml C_{18} Empore^{TM} extraction disk cartridges (3 M Company), 500 mg/6 ml Oasis^{TM} HLB cartridges (Waters, Milford, MA, USA), 500 mg/10 ml Isolute Phenyl cartridges and 500 mg/6 ml Isolute Env+ cartridges (International Sorbent Technology, Hengoed, UK). Empty polypropylene columns for clean-up (6 ml) were from Supelco (Bellefonte, PA, USA).

2.2. Serum samples and lipid determination

Pooled serum used for the development and validation of the analytical method was obtained in 2002 from the Blood Transfusion Centre of the University Hospital of Antwerp (Belgium) and it was a composite from approximately 100 individual serum samples. Individual serum and pooled umbilical cord serum samples were available from other projects which took place between 1999 and 2004 in Belgium. Total cholesterol (CHOL) and triglycerides (TG) were determined enzymatically in a separate aliquot of serum at a clinical laboratory. Total lipids (TL) were calculated as described by Covaci et al. [19] using the following formula: $TL\,(g/l) = 1.12 \times CHOL + 1.33 \times TG + 1.48$. Therefore, the concentrations of PBDEs in individual samples were also reported as ng/g lipid weight.

2.3. Extraction and clean-up

The procedure for extraction and clean-up of PBDEs from human serum was used with major modifications from the method described by Covaci and Schepens [18] for the determination of polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) in human serum. The modifications consisted in the use of higher volumes for elution from the SPE cartridges

Table 1
Absolute recoveries and relative standard deviation (in parentheses) of target analytes and internal standards using different SPE cartridges

Analyte	Structure	C_{18} Empore^{TM}	Isolute-phenyl	Isolute-ENV+	OASIS^{TM} HLB
BDE 28	2,4,4′-Tribromodiphenyl ether	47 (4)	20 (17)	49 (13)	84 (10)
BDE 47	2,2′,4,4′-Tetrabromodiphenyl ether	38 (4)	15 (16)	54 (22)	88 (9)
BDE 49	2,2′,4,5′-Tetrabromodiphenyl ether	41 (4)	16 (16)	56 (18)	84 (6)
BDE 66	2,3′,4,4′-Tetrabromodiphenyl ether	38 (4)	13 (15)	53 (20)	85 (7)
BDE 85	2,2′,3,4,4′-Pentabromodiphenyl ether	35 (2)	12 (14)	58 (12)	92 (5)
BDE 99	2,2′,4,4′,5-Pentabromodiphenyl ether	31 (5)	11 (13)	50 (2)	88 (6)
BDE 100	2,2′,4,4′,6-Pentabromodiphenyl ether	31 (3)	11 (15)	46 (7)	84 (5)
BDE 138	2,2′,3,4,4′,5′-Hexabromodiphenyl ether	27 (2)	10 (13)	54 (12)	86 (6)
BDE 153	2,2′,4,4′,5,5′-Hexabromodiphenyl ether	22 (4)	8 (16)	44 (9)	82 (4)
BDE 154	2,2′,4,4′,5,6′-Hexabromodiphenyl ether	24 (4)	8 (17)	43 (14)	74 (9)
BDE 183	2,2′,3,4,4′,5′,6-Heptabromodiphenyl ether	18 (4)	7 (16)	38 (11)	66 (12)
BB 153	2,2′,4,4′,5,5′-Hexabromobiphenyl	24 (4)	10 (15)	46 (11)	81 (3)
BDE 104 (IS1)	2,2′,4,6,6′-Pentabromodiphenyl ether	38 (6)	17 (18)	46 (19)	84 (8)
BDE 140 (IS2)	2,2′,3,4,4′,6′-Hexabromodiphenyl ether	29 (8)	10 (19)	45 (18)	95 (5)
^{13}C-BDE 209 (IS3)	2,2′,3,3′,4,4′,5,5′,6,6′-Decabromodiphenyl ether ($^{13}C_{12}$)	22 (28)	8 (50)	28 (13)	64 (17)

Recoveries are given as percentages (%). Each experiment was done in triplicate.

(other than Empore™ C_{18} disks) and an additional layer of activated silica in the clean-up cartridge.

All samples were first thawed and then homogenised by shaking the serum for 2 min. Internal standards (500 pg BDE 104, BDE 140 and ^{13}C-BDE 209) were added to a glass tube and the solvent was evaporated. The ISs were redissolved in 100 μl acetone, vortexed, after which 5 ml serum was added, then vortexed and finally sonicated for 20 min. The spiked serum was kept overnight at +4 °C.

The next day, the serum was mixed with 2 ml formic acid and 3 ml water and sonicated for 20 min. Prior to sample application, the SPE cartridges were washed with 5 ml DCM and activated with 5 ml MeOH and 5 ml water. After sample loading at a low positive pressure of 2–4 psi, the SPE cartridges were rinsed with 3 ml water. The sorbent bed was dried thoroughly under a nitrogen stream at 20 psi positive pressure (10 min) and by centrifugation (15 min, 4000 rpm). The SPE cartridges were eluted with 3 × 3 ml DCM in a separate tube and the eluate was concentrated to ~1 ml under nitrogen.

An empty cartridge (6 ml) filled (from bottom to top) with 2 g of acid silica, 200 mg activated silica (freshly activated for 2 h at 200 °C) and 500 mg Na_2SO_4 was pre-washed with 5 ml DCM. The concentrated eluate obtained from the SPE cartridge was then loaded on the clean-up cartridge and the analytes were eluted with 8 ml DCM. The final eluate was concentrated under a gentle nitrogen stream at room temperature until dryness and resolubilised in 60 μl *iso*-octane. Recovery standard (40 μl BDE 139 with a concentration of 10 pg/μl) was added, the mixture was vortexed and transferred to a vial for GC analysis.

As procedural blank, 5 ml of water in place of serum sample was subjected to the same procedure. The value of each analyte in the procedural blank was subtracted from values found in the serum samples.

2.4. Instrumentation

An 6890 Agilent (Palo Alto, CA, USA) gas chromatograph (GC) coupled to a 5973 mass spectrometer operated in electron-capture negative ionization (ECNI) was equipped with a 12 m × 0.18 mm × 0.10 μm AT-5 (5% phenyl dimethylpolysiloxane) capillary column (Alltech, Lokeren, Belgium). The ion source, quadrupole and interface temperatures were 250, 150 and 300 °C, respectively. Helium was used as carrier gas at constant flow (1.0 ml/min). One μl extract was injected in solvent vent mode (injector initial temperature 90 °C, kept for 0.05 min, then raised with 700 °C/min to 295 °C, kept for 18 min, vent flow 100 ml/min, vent time 0.03 min, purge time 1.5 min). The temperature of the AT-5 column was programmed from 90 °C, kept for 1.50 min, then raised with 30 °C/min to 200 °C, then with 5 °C/min to 275 °C and finally with 40 °C/min to 300 °C, and kept for 5 min. The following ions were monitored during the entire run: $m/z = 79$ (bromine trace) and 81, $m/z = 485/487$ and 495/497, corresponding to the ion $[C_6Br_5O]^-$ obtained by fragmentation of BDE 209 and ^{13}C-BDE 209, respectively [20]. Dwell times were set at 40 ms.

For the determination of BB 153, the GC/ECNI-MS was equipped with a 25 m × 0.22 mm × 0.25 μm HT-8 (1,7-dicarbacloso-dodecarborane 8% phenyl methyl siloxane) capillary column (SGE, Zulte, Belgium). The ion source, quadrupole and interface temperatures, together with injection parameters were as described above. The temperature of the HT-8 column was programmed from 90 °C, kept for 1.50 min, then raised with 30 °C/min to 200 °C, then with 5 °C/min to 300 °C, and kept for 20 min. Bromine ions $m/z = 79$ and 81 were monitored during the entire run. Dwell times were set at 40 ms.

2.5. Recovery experiments

For adsorbent selection, four different cartridges were tested (see Section 2.1). For each type of adsorbent, three spiked and two non-spiked pooled serum samples, together with two procedural blanks, were used to assess the recoveries of analytes and internal standards. The spiking level was 80 pg/ml serum for all PBDEs except BDE 209 spiked at 250 ng/ml serum. The extraction and clean-up procedures were as described in Section 2.3, except for experiments conducted on C_{18} Empore™ extraction disk cartridges for which the procedure described by Covaci and Schepens [18] was used. To assess between-day precision and accuracy, experiments on the OASIS™ HLB cartridge were performed in three replicates during 3 days. The absolute recovery was determined using external calibration with BDE 139 as RS, while the recovery relative to IS (BDE 104 and BDE 140) was calculated using internal standard calibration. The tri- (BDE 28), tetra- (BDE 47, 49, 66) to penta- (BDE 85, 99, 100) congeners were calculated based on BDE 104, while hexa- (BDE 138, 153, 154), hepta- (BDE 183) congeners and BB 153 were calculated based on BDE 140. BDE 209 was calculated based on ^{13}C-BDE 209.

2.6. Method validation

Five-points calibration curves were created for the quantification and high correlation coefficients ($R^2 > 0.998$) were obtained for the tested interval (0.5–1000 pg/ml). The ratios between peak areas of analytes and the corresponding ISs or RS were plotted against the corresponding concentration ratios using inverse square of concentration-weighted linear regressions. Quantification was based on the sum of ions 79 and 81 for all analytes and IS, except for BDE 209 and ^{13}C-BDE 209 for which ions m/z 487 and 495 were used. The identification of PBDEs was based on their relative retention times to the IS used for quantification, ion chromatograms and intensity ratios of the monitored ions (for BDE 209). At least two procedural blanks were included with each sample batch and values obtained for the serum samples were blank-corrected. All individual samples were processed in one batch together with two procedural blanks and the pooled serum used for method validation.

2.7. Quality assurance

While our laboratory regularly participates with good results to the Arctic Monitoring and Assessment Programme (AMAP) ring test for PCBs and OCPs in humans serum, organised by the Toxicological Centre of Québec (Canada), there are no such

programs for the determination of PBDEs in human matrices. However, regular participation to the QUASIMEME proficiency exercises for PBDEs in environmental samples ensures a sufficient knowledge on the analysis of PBDEs. A variation of less than 15% from mean values obtained from 42 participating laboratories was obtained during the second interlaboratory study on PBDEs, where a human milk sample processed through a similar SPE method, was included [21].

3. Results and discussion

3.1. General considerations

The use of the SPE procedure replaced the LLE step and implicitly allowed a reduced solvent consumption together with parallel processing of a higher number of samples. The tested SPE adsorbents had a high hydrophobic character needed for the successful retention of highly lipophilic compounds, such as PBDEs. In the present method, the hydrophilic co-extractables present in serum were removed by SPE, followed by the elimination of hydrophobic compounds (mainly lipids) by normal phase clean-up on acidified silica gel.

The activated silica layer on the top of the clean-up cartridge was essential for a good performance of the clean-up step and allowed an efficient retention of polar biogenic material. The activated silica acted as trapping layer for cholesterol and prevented the conversion of cholesterol to the acid-resistant cholestene in the acidified silica layer [14,22].

The short AT-5 capillary column offered sufficient resolution for the baseline separation of the investigated PBDE congeners (Fig. 1). Furthermore, no degradation of BDE 209 was observed due to a short run time (<18 min). However, this type of stationary phase does not allow a separation between BDE 154 and BB 153, which is achievable on a HT-8 stationary phase (Fig. 2).

3.2. Recovery

The absolute recoveries of the analytes and ISs calculated based on the RS are shown in Table 1. For BDE 209, to exclude variations from inconsistent values in procedural blanks and non-spiked serum samples, only the recovery of ^{13}C-BDE 209 was evaluated. The lowest recoveries were observed on the Isolute Phenyl, followed by C_{18} Empore^{TM} and Isolute-ENV+, while the highest recoveries were obtained on the OASIS^{TM} HLB cartridge (Table 1). This is probably due to a higher hydrophobic character associated with an increased retention capability through hydrophilic interactions between the sorbent and analytes. On the OASIS^{TM} HLB cartridge, the absolute recoveries of most PBDE congeners and ISs were in acceptable range (Table 1), while the lowest recoveries (between 64% and 74%) were observed for ^{13}C-BDE 209 and BDE 183, followed by BDE 154. The lower recoveries observed for higher brominated PBDE congeners are probably due to poor desorption from the SPE cartridge because of high lipophilicity of these compounds and/or strong π–π interactions with the sorbent. More polar solvents (e.g. acetone or methanol) could not be used for the elution of PBDEs from the SPE cartridge due to their incompatibility with the subsequent acid silica clean-up. Similar recoveries on OASIS^{TM} HLB with those observed in the present study were obtained by Sjödin et al. [14] for which mean recoveries of the ^{13}C-labelled ISs ranged from 69% to 95% for the PBDEs (except BDE 209) and BB 153.

The recoveries of analytes relative to the ISs used for their quantification on the four tested sorbents are given in Table 2. For C_{18} Empore^{TM}, Isolute Phenyl, and Isolute-ENV+, a strong

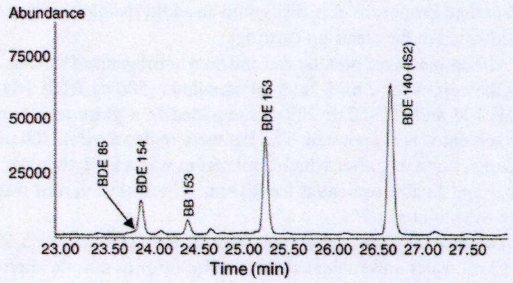

Fig. 2. Partial chromatogram (ions *m/z* 79 and 81 monitored) of a spiked serum on HT-8 (25 m × 0.22 mm × 0.25 μm) showing baseline separation of BDE 154 (1.5 pg/ml) and BB 153 (0.8 pg/ml).

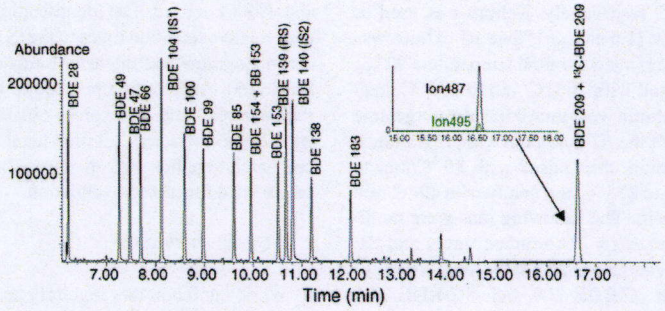

Fig. 1. Total ion chromatogram (ions *m/z* 79 and 81 together) on AT-5 (12 m × 0.18 mm × 0.10 μm) of a spiked serum sample after SPE and clean-up. The serum sample was spiked with 80 pg/ml serum of all analytes except BDE 209, which was spiked with 250 pg/ml. Ions *m/z* 487 and 495 were monitored for BDE 209 and ^{13}C-BDE 209, respectively.

Table 2
Relative recoveries and relative standard deviation (in parentheses) of target analytes based on internal standard calibration using different SPE cartridges

Analyte	C_{18} Empore™	Isolute-phenyl	Isolute-ENV+	OASIS™ HLB
BDE 28	153(4)	156(5)	114(7)	96(9)
BDE 47	141(1)	143(10)	140(15)	109(3)
BDE 49	130(3)	124(3)	131(12)	95(4)
BDE 66	121(2)	101(5)	123(15)	95(3)
BDE 85	111(3)	100(6)	135(6)	106(11)
BDE 99	103(1)	91(12)	119(6)	102(4)
BDE 100	98(1)	84(8)	105(1)	95(5)
BDE 138	106(6)	99(11)	130(3)	94(5)
BDE 153	99(5)	90(12)	115(1)	88(6)
BDE 154	100(8)	97(9)	108(4)	81(5)
BDE 183	67(3)	65(15)	88(2)	68(8)
BDE 209	104(5)	110(10)	97(6)	98(6)
BB 153	95(5)	94(9)	112(3)	90(5)

BDE 28, 47, 49, 66, 85, 99 and 100 were calculated based on BDE 104, while BDE 138, 154, 153, 183, together with BB 153 were calculated based on BDE 140. Recoveries are given as percentages (%). Each experiment was done in triplicate.

tendency of decreasing recoveries with the increasing number of bromine atoms was observed (Tables 1 and 2). Such trend was less evident for the OASIS™ HLB, for which only BDE 153, BDE 154 and BDE 183 presented lower recoveries than its corresponding IS (Table 2). To compensate for the difference in the absolute recoveries of each congener and its corresponding IS (BDE 140), the concentrations of BDE 153, BDE 154 and BDE 183 measured in individual samples were corrected with 10%, 20% and 30%, respectively.

3.3. Procedural blanks and limits of quantification

A low level of contamination of procedural blanks has been obtained by minimizing the number of steps and glassware used for the analysis. Only BDE 47, 99 and 209 were detected at higher, but consistent (R.S.D. < 25%) levels in the procedural blanks. For these PBDE congeners, the limit of quantification (LOQ) was dependent on the procedural blanks and was calculated as 3 × S.D. of the blank value [23]. For the remaining PBDE congeners and BB 153, which were not detected or at very low levels in the procedural blanks, the LOQs were calculated in the spiked serum samples on the basis of a signal-to-noise ratio (S/N) of 10. For calculation of concentrations in the samples, the value of each PBDE congener in the procedural blank was subtracted from the corresponding value in the sample and the resulting value was compared to the LOQ calculated for each congener. LOQs ranged between 0.2 and 1.5 pg/ml for BB 153 and all PBDEs except BDE 209, for which LOQ was 25 pg/ml, and were in the range of LOQ values previously reported for PBDEs [13]. Expressed on a lipid weight basis, the LOQs ranged between 0.1 and 4 ng/g lipid weight (the latter corresponding to BDE 209).

Table 3
Within- and between-day precision and accuracy of the method developed for the determination of PBDEs and BB 153 in human serum which includes OASIS™ HLB SPE and multilayer clean-up cartridge

Analyte	Within-day precision (%)	Between-day precision (%)	Accuracy (%)
BDE 28	5	10	−4
BDE 47	5	9	+9
BDE 49	2	6	−5
BDE 66	3	7	−5
BDE 85	2	5	+6
BDE 99	4	6	+2
BDE 100	3	5	−5
BDE 138	6	6	+6
BDE 153	4	4	−11
BDE 154	4	9	−18
BDE 183	10	12	−32
BB 153	3	3	−10
BDE 104 (IS1)	7	8	−16
BDE 140 (IS2)	5	5	−5
^{13}C-BDE 209 (IS3)	15	17	−36

Validation was done at single fortification level 80 pg/ml serum for all PBDEs, except BDE 209 spiked at 250 ng/ml serum. Each experiment was done in triplicate.

Table 4
Linear regression equations and correlation coefficients for calibration curves of each PBDE congener

Analyte	Linear regression equation	Correlation coefficient (R^2)
BDE 28	$y = 0.90 \times x - 9.7 \times 10^{-4}$	1.000
BDE 47	$y = 0.87 \times x + 1.0 \times 10^{-3}$	0.999
BDE 49	$y = 1.22 \times x + 2.5 \times 10^{-4}$	0.998
BDE 66	$y = 1.00 \times x - 5.4 \times 10^{-4}$	0.998
BDE 85	$y = 0.82 \times x + 1.9 \times 10^{-3}$	0.999
BDE 99	$y = 0.89 \times x + 9.8 \times 10^{-4}$	0.998
BDE 100	$y = 1.05 \times x + 2.3 \times 10^{-3}$	0.999
BDE 138	$y = 0.90 \times x + 6.4 \times 10^{-4}$	0.999
BDE 153	$y = 1.11 \times x - 1.4 \times 10^{-3}$	0.999
BDE 154	$y = 1.24 \times x - 1.0 \times 10^{-3}$	0.999
BDE 183	$y = 0.92 \times x + 1.1 \times 10^{-3}$	0.998
BDE 209	$y = 1.24 \times x + 5.2 \times 10^{-3}$	0.999
BB 153	$y = 1.20 \times x + 2.9 \times 10^{-3}$	0.998

Five-points calibration curves (0.5–1000 pg/ml) were created as the ratios between peak areas of analytes and the corresponding internal standards plotted against the corresponding concentration ratios.

Table 5
Concentrations of PBDE congeners and BB 153 in 4 pooled cord serum and 11 individual serum samples from Belgium (1999–2004)

	Cord 1	Cord 2	Cord 3	Cord 4	Serum 1	Serum 2	Serum 3	Serum 4	Serum 5	Serum 6	Serum 7	Serum 8	Serum 9	Serum 10	Serum 11
Lipids (g/l)	2.63	2.62	2.60	2.60	4.19	6.45	5.11	6.11	7.02	7.24	5.60	7.80	6.11	6.34	5.77
Serum (pg/ml)															
BDE 28	<0.2	<0.2	<0.2	<0.2	0.5	<0.2	0.8	0.3	<0.2	0.5	0.3	2.5	0.8	0.4	0.4
BDE 47	3.6	3.3	2.9	3.2	12.9	3.2	10.4	3.4	1.7	8.5	7.7	5.1	5.2	8.6	10.6
BDE 100	0.3	0.3	0.4	0.5	1.5	1.7	1.7	1.1	0.5	1.3	1.0	1.9	11.6	1.7	1.9
BDE 99	1.7	<0.9	1.7	<0.9	4.3	<1.3	2.8	<1.3	1.3	2.1	1.6	<1.3	1.5	2.6	3.2
BDE 153	1.6	1.2	2.2	1.6	6.5	9.1	10.7	17.3	6.9	10.0	7.6	8.9	18.8	10.6	9.1
BDE 183	<0.3	<0.3	<0.3	<0.3	0.9	<0.5	1.1	<0.5	<0.5	1.3	1.3	0.9	1.9	2.6	1.5
Sum PBDEs[a]	7.5	5.5	7.4	6.0	35.5	15.1	27.5	23.1	10.8	23.7	19.6	20.0	39.8	26.5	26.6
BDE 209	56	122	72	74	64	55	135	58	28	26	62	61	94	107	191
BB 153	<0.3	<0.3	<0.3	<0.3	1.6	3.9	5.0	1.5	4.3	3.0	3.9	5.8	10.0	7.5	4.2
Lipid weight (ng/g)															
BDE 28	<0.10	<0.10	<0.10	<0.10	0.12	<0.10	0.15	<0.10	<0.10	<0.10	<0.10	0.32	0.13	<0.10	<0.10
BDE 47	1.38	1.26	1.11	1.22	3.07	0.50	2.03	0.56	<0.40	1.17	1.38	0.65	0.85	1.36	1.83
BDE 100	0.13	0.10	0.15	0.19	0.35	0.27	0.34	0.18	<0.10	0.18	0.18	0.25	1.90	0.27	0.33
BDE 99	0.64	<0.40	0.65	<0.40	1.02	<0.40	0.56	<0.40	<0.40	<0.40	<0.40	<0.40	<0.40	0.40	0.55
BDE 153	0.62	0.46	0.84	0.61	1.55	1.42	2.09	2.83	0.99	1.38	1.36	1.15	3.07	1.68	1.57
BDE 183	<0.20	<0.20	<0.20	<0.20	0.21	<0.20	0.21	<0.20	<0.20	<0.20	0.22	<0.20	0.30	0.41	0.27
Sum PBDEs[a]	2.88	2.10	2.87	2.31	6.32	2.35	5.37	3.78	1.55	3.27	3.49	2.56	6.50	4.19	4.61
BDE 209	22.5	46.5	27.5	28.6	15.4	8.6	26.5	9.5	4.0	3.6	11.1	7.8	15.4	16.9	33.1
BB 153	<0.15	<0.15	<0.15	<0.15	0.38	0.60	0.97	0.25	0.61	0.41	0.69	0.75	1.63	1.18	0.73

To facilitate comparison with other studies, the results were expressed in pg/ml and ng/g lipid weight. For calculation of the sum, concentrations below LOQ were set to 1/2LOQ.

[a] All congeners except BDE 209.

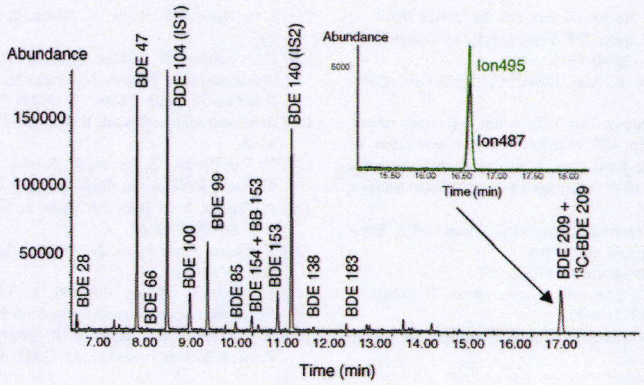

Fig. 3. Total ion chromatogram (ions *m/z* 79 and 81 together) on AT-5 (12 m × 0.18 mm × 0.10 μm) of a non-spiked pooled serum sample after SPE and clean-up. Note that BDE 154 and BB 153 co-elute on AT-5. Ions *m/z* 487 and 495 were monitored for BDE 209 and ^{13}C-BDE 209, respectively.

3.4. Validation

The precision was established by repeated determinations ($n=3$) using serum spiked with 80 pg/ml for all PBDE congeners except BDE 209 spiked at 250 ng/ml serum. The precision was expressed as the relative standard deviation (R.S.D.). The use of OASIS^{TM} HLB SPE cartridge allowed a good repeatability with a within- and between-day precision of less than 10% and 12%, respectively, except for BDE 209 for which precision was 15% and 17%, respectively (Table 3). The accuracy was estimated by analysing the pooled serum samples ($n=3$) spiked at a concentration level of 80 pg/ml serum. The values of PBDE congeners in the non-fortified serum sample were subtracted in each case. The highest deviations from were observed for ^{13}C-BDE 209, followed by BDE 183, BDE 154 and BDE 153 (Table 3). High correlation coefficients ($R^2 > 0.998$) were obtained for the inverse square of concentration-weighted linear regressions constructed for each analyte in the concentration range expected in human serum samples (0.5–1000 pg/ml). The regression equations for individual PBDE congeners are presented in Table 4 and it can be seen that the variation interval of the slopes had a very narrow range (between 0.82 and 1.24).

3.5. Analysis of individual human serum samples

The method has been used for the determination of PBDEs and BB 153 in serum samples collected from Belgian population. The results expressed in pg/ml serum as well as ng/g lipid weight are presented in Table 5 and a typical chromatogram obtained for sample no. 1 is given in Fig. 3. In all samples, some PBDE congeners were under LOQ (BDE 49 and 66 < 0.2 pg/ml, BDE 85, 154 and 138 < 0.3 pg/ml.

The measured levels of PBDEs (except BDE 209) in the Belgian serum specimens were in the same range with levels measured in Belgian human serum [24], human adipose tissue [25] or human milk [26]. They are also in the same range as previously reported PBDE levels in serum samples from Norway [8], Sweden [7,10], The Netherlands [27], but lower than levels reported from USA [28].

The presented method for the determination of PBDEs in human serum using SPE and GC/ECNI-MS is fast, simple, consumes lower amounts of solvents, demands less equipment and presents a low risk of sample contamination compared to traditional methods based on LLE. Up to 24 samples can be prepared within a day by one analyst. The method shows sufficient sensitivity and provides repeatable quantification in a wide concentration range for all investigated analytes.

Acknowledgment

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5.3 Paper X

Evaluation of total lipids using enzymatic methods for the normalisation of persistent organic pollutants in serum

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Evaluation of total lipids using enzymatic methods for the normalization of persistent organic pollutant levels in serum

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Abstract

The human exposure to persistent organic pollutants (POPs) is conveniently assessed through analysis of blood serum or plasma and the POPs concentrations are traditionally expressed on a lipid weight basis. Enzymatic determinations of total lipid (TL) content in serum, which imply the measurement of individual lipid classes, such as triglycerides (TG), cholesterol (CHOL) and phospholipid (PL), use small amounts of serum/plasma (<100 μl). These have also become a more precise and less laborious alternative for the gravimetric lipid determinations which were preferred in the past. However, PL are not routinely measured in most clinical laboratories and therefore, TL values are often predicted from formulas based on measurements of only TG and CHOL. In the present study, a large data set (n=483) from Belgian, Swedish and Norwegian populations was used to compute a new formula for TL [TL=1.33*TG+1.12*CHOL+1.48 (g/l)] by means of multiple linear regression. A very good correlation (r=0.949, p<0.001) was found between TL values calculated with the proposed formula and TL values obtained by summation of TG, CHOL and PL. Statistically different values of TL were computed when different previously published formulas were compared, which can have a strong influence on the calculation of lipid-normalized concentrations of POPs. Nevertheless, TL values derived using a mathematical formula which includes only TG and CHOL will be less accurate than TL values obtained by summation of TG, CHOL and PL, which should be the calculation method of choice if PL measurements are available.
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Keywords: Total lipids; Enzymatic measurements; Blood; Computed formula; Persistent organic pollutants

1. Introduction

The concentration of lipid-soluble persistent organic pollutants (POPs), such as polychlorinated biphenyls (PCBs), polybrominated diphenylethers (PBDEs), chlorinated pesticides and polychlorinated dioxins and furans (PCDD/Fs), is usually expressed relative to the total lipid (TL) content rather than to the sample weight. The lipid-normalized POPs concentrations depend both on the lipid percentage of the tissue and on the method used to determine the lipid content.

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Historically, gravimetrical methods (Folch et al., 1957; Bligh and Dyer, 1959; Smedes, 1999) have been used for the measurement of the TL content in human serum. The gravimetrically measured TL strongly depends on the solvent mixture used for extraction and on the analyst's skills and experience. Recently, enzymatic methods have become increasingly popular for the determination of lipids in very small amounts of human serum or plasma (Phillips et al., 1989; Grimvall et al., 1997; Pauwels et al., 2000). Through the measurement of individual classes of lipids (triglycerides (TG), cholesterol (CHOL) and phospholipids (PL)), enzymatic methods offer a more accurate way of expressing the TL content by summation of TG, CHOL and PL. However, PL measurements are not routinely performed in most clinical laboratories and therefore, the TL often needs to be predicted from formulas based on measurements of only TG and CHOL. The most used formula has been derived in 1989 from a relatively low number of subjects (n=81; Phillips et al., 1989). Independently from the present study, another formula based on several Swedish populations with a large number of subjects (n=617) has recently been proposed (Rylander et al., 2006). However, differences between TL values predicted by various formulas and their influences on the lipid-normalized levels of POPs have not yet been assessed in the study of Rylander et al. (2006).

The main aim of the present work was to compute a formula for predicting TL using a data set (n= 483) consisting of individual measurements of TG, CHOL and PL from independent Belgian, Swedish and Norwegian populations. Additionally, we compared the resulting TL values calculated with the different available formulas, evaluated differences between gravimetric and enzymatic lipid determinations and estimated differences in lipid-normalized POPs levels arising from the use of different formulas for TL prediction.

2. Materials and methods

2.1. Description of data sets

Enzymatic measurements of individual classes of lipids (TG, PL, and CHOL) in human serum were available from several populations from Belgium (G1–G2), Sweden (G3) and Norway (G4). Detailed information for these groups has been provided in previous studies and is briefly given in Table 1. In each case, whole blood was centrifuged (15 min, 2000×g) within 24 h after collection. Serum was separated and stored at −20 °C until analyzed. Concentrations of TG, PL and CHOL (sum of free and esterified cholesterol) were determined by enzymatic methods using reagents from Boehringer–Mannheim (TG and CHOL; Mannheim, Germany) and Waco Chemicals (PL; Neuss, Germany), as described in the corresponding studies (Table 1). The TL concentration was calculated by summation of the amounts of TG, CHOL and PL. In these calculations, the average molecular weights of TG and PL were assumed to be 807 and 714. For CHOL we used an average molecular weight of 571, assuming that the proportion of free and esterified CHOL serum was 1:2.

Groups G1–G4 were used to compute a new formula for the calculation of TL values, while groups G5–G7 from Belgium (Table 1) were used to compare the TL values obtained by applying different formulas. For the latter groups, only individual measurements of TG and CHOL were available. Levels of PCBs and p,p'-DDE were also available for group G5 (Voorspoels et al., 2002), which allowed the comparison of lipid-normalized pollutant levels based on various formulas.

2.2. Statistics

A formula for predicting TL from independent measurements of TG and CHOL was derived by multiple linear regression analysis conducted on data sets

Table 1
Characteristics of populations used in the present study

Code	Country	Year of sampling	Women		Men		Available lipid data	References
			N	Age [mean (range)]	N	Age [mean (range)]		
G1	Belgium	1996–1998	98	32 (24–42)			TG, CHOL, PL	Pauwels et al., 2000
G2	Belgium	1999	47[a]	58 (50–65)			TG, CHOL, PL	Koppen et al., 2002
G3	Sweden	2001	141	40 (19–56)			TG, CHOL, PL	Lindström et al., 2004
G4	Norway	2003	105	53 (21–80)	92	56 (24–88)	TG, CHOL, PL	Kvalem et al., 2005
G5	Belgium	2001	19	37 (18–49)	113	40 (20–53)	TG, CHOL	Voorspoels et al., 2002
G6	Belgium	1999	271	39 (20–60)	228	39 (18–61)	TG, CHOL	Van den Bosch et al., 2001
G7	Belgium	1999	120	17 (17–18)	80	17 (17–18)	TG, CHOL	Nawrot et al., 2002

[a] Pooled samples from 200 individuals.

Table 2
Formulas for prediction of TL (g/l) from measurements of TG and CHOL

No.	Formula	Reference
F1	TL (g/l)=2.27*CHOL+TG+0.62	Phillips et al. (1989)
F2	TL (g/l)=1.31*CHOL+1.31*TG+0.92	Rylander et al. (2006)
F3	TL (g/l)=1.12*CHOL+1.33*TG+1.48	Present study

containing measurements of TG, CHOL and PL. Correlations were calculated using Pearson correlation coefficients. A finding was considered statistically significant if $p<0.05$. ANOVA with Scheffe's post-hoc test was used to compare TL values obtained with different formulas. All statistical calculations were performed with Statistica v.5 for Windows (Statsoft, Tulsa, OK, USA).

3. Results and discussion

3.1. Prediction of TL from enzymatic measurements of TG and CHOL

The use of enzymatic techniques for the measurement of lipid classes in serum offers a good standardized way of calculating the TL content. Considerably lower volumes of serum are needed compared to gravimetric techniques. In addition, the assays for determination of individual classes of lipids are automated, offer a good reproducibility, and do not rely on the analyst's skills or the character of extraction solvents. The assays for each lipid class follow the same principle and are based on the spectrophotometric detection of one of the products formed after specific enzymatic hydrolysis of lipids. Ideally, TL should be calculated by summation of TG, CHOL and PL. However, PL measurements are often not performed in most clinical laboratories and therefore, several formulas (F1 and F2; Table 2) for the prediction of TL from measurements of only TG and CHOL have been proposed in the literature (Phillips et al., 1989; Rylander et al., 2006).

Using 4 population groups (G1–G4) from Belgium, Sweden and Norway ($n=483$) for which measurements of TG, CHOL and PL were available, a new formula (F3) for predicting TL was computed using multiple linear regression (Table 2). A good correlation ($r=0.949$, $p<0.001$) was found between the TL values obtained by summation of TG, CHOL and PL and the TL values predicted by F3. A similar correlation was found between the summation-obtained TL values and the TL values predicted with F2 ($r=0.947$, $p<0.001$), while the corresponding correlation using F1 was weaker ($r=0.897$, $p<0.001$). Although not tested in the present study due to missing age information for some groups, age and gender have been shown to be co-variables of minor importance in the prediction of TL from TG and CHOL (Rylander et al., 2006).

TL values predicted for different groups of individuals (G1–G4, G5, G6 and G7) using formulas F1 to F3 were compared using ANOVA (Fig. 1). For each studied group, TL values predicted with F2 and F3 were statistically identical in most cases and were significantly lower than TL values predicted with F1 (Fig. 1). The obtained TL mean values always increased in the order: F2<F3<F1. The statistically higher results obtained

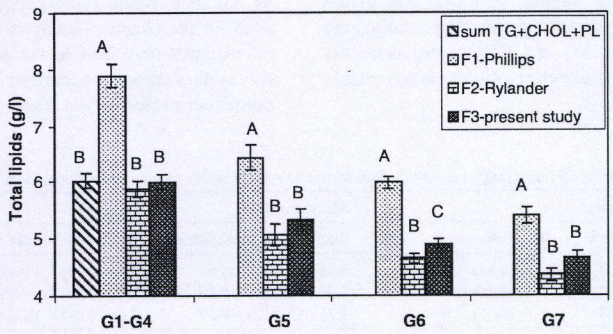

Fig. 1. Mean values and 2 S.E. values for total lipids (g/l) predicted by applying F1–F3 to data sets for which TG and CHOL measurements were available. Within the same group, values marked by different letters are significantly different (ANOVA, $p<0.01$).

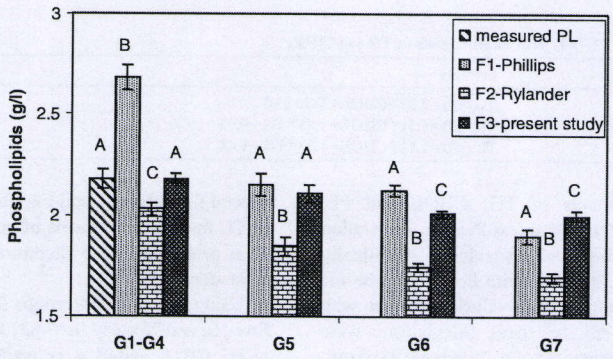

Fig. 2. Mean and 2 S.E. values for phospholipids (g/l) predicted by applying F1–F3 to data sets for which only TG and CHOL measurements were available. Values marked by different letters are significantly different (ANOVA, $p<0.01$).

using F1 which was derived in 1989 from a group of 81 US adults (Phillips et al., 1989) suggest that its use should be revised.

The observed differences in results are exclusively due to the prediction of PL from TG and CHOL measurements using PL=TL−CHOL−TG. PL are only based on CHOL measurements using F1 (PL=0.766*CHOL+0.62), while using F2 and F3, PL values are based upon both CHOL and TG (PL=0.31*(TG+CHOL)+0.92 and PL=0.33*TG+0.12*CHOL+1.48 (all units in g/l), respectively). These formulas were used for calculating PL for the selected populations and results are presented in Fig. 2.

PL values are highly dependent on the dietary habits of the studied population and are therefore subject to high variation between individuals. Furthermore, they are not in strict relation with TG and CHOL ($r<0.48$ for F1–F3 applied to Group G1–G4). PL predicted with different formulas were statistically different in most cases, suggesting differences in the formulas used to compute them. Apparently, the prediction of PL from TG and CHOL measurements is not very precise and therefore, direct measurements of PL are preferred.

3.2. Gravimetric vs. enzymatic measurements of total lipids

Several studies (Akins et al., 1989; Grimvall et al., 1997; Pauwels et al., 2000) have shown moderate to good correlations between TL calculated by summation of enzymatically measured TG, CHOL and PL and gravimetrically measured TL ($r=0.98$, 0.82, and 0.75, respectively). For groups G1, G2 and G7, for which gravimetrically measured TL were also available, correlations between the predicted TL and gravimetric TL values are presented in Table 3.

Gravimetric methods are more prone to errors than enzymatic methods due to the low lipid weights available and strongly depend on the analyst's skills and on the character of the solvent extraction mixtures used. For serum, which contains lipid classes of different polarity, the solvent mixtures used for extraction will largely be responsible for the measured TL content. While often being part of the procedure used for the chemical analysis of POPs, the gravimetric methods may lead to the loss of volatile analytes during the extract evaporation (when lipid determination is performed on the whole extract) and may affect

Table 3
Mean (S.E.) for TL (g/l) predicted by applying F1 to F3 to independent data sets for which gravimetric and enzymatic measurements were available

Group	N	TL measured		TL predicted		TL summed
		Gravimetric	F1 (Phillips et al., 1989)	F2 (Rylander et al., 2006)	F3 (Present study)	
G1	98	7.24 (0.22) ^{A}	5.40 (0.12) ^{B}, $r=0.68$	4.36 (0.11) ^{C}, $r=0.63$	4.66 (0.10) ^{C}, $r=0.62$	5.09 (0.12) ^{BC}, $r=0.69$
G2	47	6.62 (0.10) ^{A}	7.84 (0.10) ^{B}, $r=0.85$	6.28 (0.09) ^{A}, $r=0.85$	6.50 (0.09) ^{A}, $r=0.85$	7.00 (0.10) ^{A}, $r=0.87$
G7	200	5.11 (0.08) ^{A}	5.39 (0.07) ^{B}, $r=0.69$	4.35 (0.06) ^{C}, $r=0.69$	4.65 (0.06) ^{D}, $r=0.68$	n.a.

n.a. – not applicable.
Pearson's correlation coefficients are calculated between the gravimetrically measured TL and the predicted TL. Within the same group (row-wise), values followed by different letters are significantly different (ANOVA, $p<0.05$).

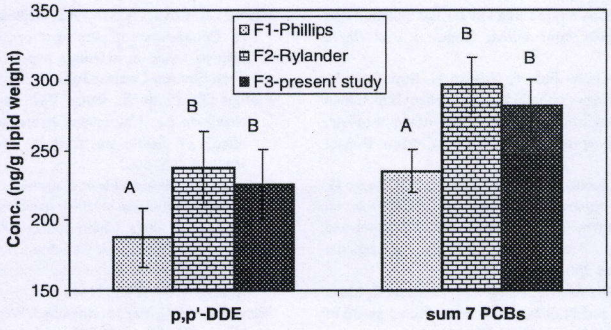

Fig. 3. Mean concentrations and 2 S.E. of PCBs and *p,p'*-DDE (ng/g lipid weight) obtained after applying F1–F3 to group G5 for which only TG and CHOL measurements were available. Values marked by the different letters are significantly different (ANOVA, $p<0.01$).

the detection limit of certain analytes (when an aliquot of extract is used for lipid determination) (Sandau, 2000). Mixtures consisting of a polar and a non-polar solvent (methanol/chloroform (Folch et al., 1957; Bligh and Dyer, 1959) or isopropanol/cyclohexane (Smedes, 1999) have been shown to give the most accurate results. The Folch method was shown to yield 15–40% higher lipid amounts than a mixture of hexane/acetone probably due to a better extraction of polar lipids, e.g. PL (Sandau, 2000). However, long experience is usually required to obtain high reproducibility with gravimetric methods. This was shown also in intercalibration studies for POPs where the TL content was requested to be measured (AMAP, 2004).

3.3. Errors in determination of POPs

The quantitative determination of POPs in serum usually requires accuracy at below nanogram range and precision within ±10%. If serum concentrations are lipid-normalized, additional uncertainty derives from the lack of consistency in gravimetric lipid measurements (AMAP, 2004) and in TL values predicted with different formulas. Since the concentration of lipids in serum is low, an error in the TL values can dramatically change the lipid-normalized POPs concentrations.

Lipid-normalized concentrations of PCBs based upon the newly computed formula (F3) were significantly higher with 16% that the ones calculated with F1. A similar difference (17% was also observed for *p,p'*-DDE. However, for both PCBs and *p,p'*-DDE, results obtained using F2 and F3 were statistically not different (Fig. 3). This means that the results obtained using TL computed with F2 and F3 are comparable, while the results obtained with F1 are underestimating the lipid-normalized concentrations of POPs.

4. Conclusions

A new formula, TL=1.33*TG+1.12*CHOL+1.48 (g/l), based on a large data set (n=483) from Belgium, Sweden and Norway was computed for the calculation of TL based on measurements of TG and CHOL. TL values calculated using this formula were found to be similar to TL values obtained using the formula of Rylander et al. (2006), but significantly lower than TL values obtained using the formula of Phillips et al. (1989). Nonetheless, the TL levels derived using a mathematical formula which includes TG and CHOL will not be as precise as the TL levels summed for measurements of TG, CHOL and PL and the inclusion of PL measurements is therefore recommended.

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5.4 Paper XI

Dietary PBDE intake: A market-basket study in Belgium

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Dietary PBDE intake: A market-basket study in Belgium

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Abstract

A food market-basket, representative for the general Belgian population, containing various meat, fish and dairy food products, was assembled and analysed for its polybrominated diphenyl ether (PBDE) content. Additionally, fast food samples were also investigated. Based on the measured PBDE levels, an average daily dietary intake estimate of PBDEs was calculated. Of all foods analysed, fish had the highest average sum of PBDE levels (BDEs 28, 47, 99, 100, 153, 154, and 183; 460 pg/g ww), followed by dairy products and eggs (260 pg/g ww), fast food (86 pg/g ww) and meat products (70 pg/g ww). One fresh salmon filet had the highest total concentration of PBDEs (2360 pg/g ww), whereas levels in steak and chicken breast were the lowest of all foods analysed. BDE 209 was never found above LOQ in any food.

PBDE intake calculations were based on the average daily food consumption in Belgium and were estimated between 23 and 48 ng/day of total PBDEs (lower and upper bound). This value is in accordance with what was previously reported for diets from geographical distinct areas, such as Canada, Finland, Spain, Sweden and the UK. Although it is only a minor constituent of the Belgian diet, fish is the major contributor to the total daily PBDE-intake (around 40 %) due to the high PBDE levels in this type of food. Although low contaminated, meat products account for around 30 % of the total dietary intake of PBDEs. Dairy products and eggs contribute to a lesser degree (less than 30 %).

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1. Introduction

Polybrominated diphenyl ethers (PBDEs) were first introduced on the market in the 1960s and were used since then as flame retardants to improve fire safety in our modern society, where they are commonly added to consumer products in concentrations up to 10 % by weight (EHC-162, 1994). A substantial increase in production has been seen since the end of the 1970s due to the growing popularity of personal computers and other electronic equipment and due to stricter fire regulations (de Boer et al., 2000). Environmental levels of PBDEs have been continuously increasing since then (de Wit, 2002). Spillage and emission during production and use, but also disposal at the end-of-life of the products they are used in, account for their widespread environmental presence. These chemicals are persistent and lipophilic, which results in their bioaccumulation in the fatty tissues of organisms and enrichment throughout food chains (Law et al., 2003). PBDEs were also found in human tissues in Europe (Meironyté et al., 1999; Thomsen et al., 2002; Schroeter-Kermani et al., 2000), Canada (Ryan et al., 2002), and the United States (Betts, 2002; Petreas et al., 2003; Schecter et al., 2005). The presence of PBDEs in human tissues is of particular concern because of their toxicological potential regarding neurodevelopment and the endocrine system (Darnerud, 2003; Bergman, 2000; McDonald, 2002).

Due to the lipophilic nature of these man-made chemicals, PBDEs are mainly found in lipid-rich food of animal origin, such as meat, fish and dairy products, which are a part of our daily diet. It has been shown that food, and more in particular food of animal origin, is responsible for more than 90% of the average human intake of PCBs (Liem et al., 2000). Because PBDEs are lipophilic synthetic halogenated chemicals that resemble PCBs to a great extent, it is expected that also exposure to PBDEs will occur mainly through the diet. The importance of the different routes of human exposure to PBDEs (diet and inhalation/ingestion) is not completely understood at present and opinions about the contribution of the dietary intake vary among publications. In general, the proportion of the total intake that is diet related, was estimated between 73 (Wijesekera et al., 2002) and 93% (Harrad et al., 2004). The rest of the intake originates from inhalation and ingestion of dust, which is known for attracting large quantities of PBDEs (Stapleton et al., 2005; Wilford et al., 2005; Jones-Otazo et al., 2005). However, the contribution of dust inhalation/ingestion to the total PBDE exposure is susceptible to great variance depending on several factors, such as life style (indoor – outdoor), age (toddler – adult), home environment, and work place exposure (Jones-Otazo et al., 2005).

In the present study, various meat, fish, eggs and dairy food products were analysed for their PBDE content. Additionally, fast food samples were also investigated. Based on the PBDE levels measured, an average daily dietary intake estimate of PBDEs was calculated.

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2. Materials and Methods

2.1. Sample collection.

Sample selection was based on average food (of animal origin) consumption estimates of the Belgian population (KB 03.03.1992). Selected products were purchased in April 2005 at 2 large-chain supermarkets and 1 "biological/organic" supermarket. Not all products were available at all stores. Fish and meat products were also purchased at a local Antwerp fish store and a local butcher's. Additionally, most popular fast food was sampled from 3 different restaurants. The samples were homogenised immediately after collection and stored at -20°C until further treatment.

2.2. Chemicals.

All solvents used for the analysis (*n*-hexane, acetone, dichloromethane, and *iso*-octane) were of SupraSolv® grade (Merck, Darmstadt, Germany). Individual reference standards for each of the compounds were used for identification and quantification (Cambridge Isotope Laboratories, Andover, MA, USA; AccuStandard, New Haven, CT, USA). Sodium sulphate was heated for 6h at 600°C and silica was pre-washed with *n*-hexane and dried overnight at 60°C before use. Extraction thimbles were pre-extracted for 1h and dried at 100°C for 1h.

2.3. Analysis.

The following PBDE congeners (IUPAC numbering) were targeted for analysis: 28, 47, 99, 100, 153, 154, 183, and 209. BDE 77 was used as IS for all congeners, except 183 and 209. BDE 128 was used as IS for BDE 183 and ^{13}C-BDE 209 was used as IS for BDE 209. In addition, brominated biphenyl (BB) 153 was also analysed, using BB 155 as internal standard (IS).

The method used for the analyses has previously been described (Voorspoels et al., 2003) and is briefly summarised below. Depending on the type of tissue, 0.2 to 10 g of homogenised sample were chemically dried using anhydrous sodium sulphate, transferred to an extraction thimble, spiked with IS and Soxhlet extracted with *n*-hexane/acetone mixture (3:1, *v/v*). An aliquot (~1/5th) of the extract was used for gravimetrical lipid determination. Clean-up was achieved by column chromatography on silica impregnated with concentrated sulphuric acid (48 %, *w/w*). The cleaned extract was concentrated and the solvent was changed to *iso*-octane before injection into the gas chromatographic (GC) system.

Instrumental analysis of PBDEs was done by gas chromatography-electron capture negative ionisation mass spectrometry (GC/ECNI-MS) operated in selected ion monitoring (SIM) mode. Details of the instrumental conditions are published elsewhere (Voorspoels et al., 2003). For tri- to hepta-BDE congeners and BB 153, a 25 m 0.22 mm x 0.25 µm HT-8 capillary column (SGE, Zulte, Belgium) was used and ions m/z = 79 and 81 were monitored. For the analysis of BDE 209, a 12 m x 0.18 mm x 0.10 µm AT-5 capillary column (Alltech,

Lokeren, Belgium) was used and ions m/z = 484.7/486.7 and 494.7/496.7 were monitored for BDE 209 and ^{13}C-BDE 209, respectively.

2.4. Quality Assurance and Quality Control.

The method quality control (QC) was done by regular analysis of procedural blanks (1 in each batch of 8 samples) and blind duplicate samples (1 in each batch of 8 samples), of which an RSD < 10 % was considered acceptable. Instrumental QC was done by regular injection of solvent blanks and standard solutions. The efficiency and capacity of the method was demonstrated by successful participation in international inter-laboratory exercises on the determination of PBDEs and PCBs in biota (de Boer and Cofino, 2002). Recoveries for individual PBDE congeners were between 80 and 104 % (RSD < 12 %).

Procedural blanks of both PBDEs and PCBs were consistent (RSD < 12 %) and therefore the mean procedural blank value was used for subtraction. After blank subtraction, the limit of quantification (LOQ) was set at 3 times the standard deviation of the blank, which ensures > 99 % certainty that the reported value is originating from the sample. This, however, implies varying LOQs depending on the sample intake and on the PBDE congener.

3. Results

This study is the first to report the levels of PBDEs in Belgian foods in a market-basket survey. We could not observe any differences in PBDE concentrations between the foods from the different supermarkets and vendors, therefore averages were calculated using all analyses regardless of the origin. This was not unexpected since Belgium is a small country and foods are not transported over large distances. However, we have found a wide variation in sum of PBDE congeners concentrations across the food groups sampled.

Results are presented as the average sum of PBDEs (tri- to hepta-BDEs) for each food item (wet weight normalised) in Table 1. Also the fat content of each food item is given in Table 1. Because the number of congeners with levels below the LOQ in certain foods was sometimes substantial, such as for meat products, estimations were essential in order to be able to assess the intake. Sum of PBDE levels are therefore classified in low, medium and upper bound, according to whether levels below LOQ for individual congeners were replaced by 0, ½LOQ or the LOQ, respectively. All further discussion on levels is based upon the medium bound sum of PBDE levels. BDE 209 could never be detected above the LOQ and therefore this congener is not reported nor discussed. BB 153 could not be determined neither above the LOQ in any sample. This was not unexpected since levels of this compound are known to be rather low in European samples in general (Voorspoels et al., 2003; Voorspoels et al., 2006).

The results of the present study are presented as the sum of PBDEs (BDEs 28, 47, 99, 100, 153, 154, and 183). Fish and sea food had the highest average PBDE levels (460 pg/g ww, 10 – 90 percentiles 37 – 1060 pg/g ww), followed by dairy products and eggs (260 pg/g

ww, 10 - 90 percentiles 100 - 450 pg/g ww), fast food (86 pg/g ww, 10 - 90 percentiles 52 - 130 pg/g ww), and meat products (70 pg/g ww, 10 - 90 percentiles 17 - 130 pg/g ww). One fresh salmon filet had the highest total concentration of PBDEs (2360 pg/g ww), whereas cod had the lowest levels in fish (29 pg/g ww) and levels in steak and chicken breast were the lowest of all foods analysed (< 20 pg/g ww).

Table 1. Average PBDE-levels (pg/g ww) and lipid content of the different foods.

	Food	Source[§]	Lipid %	Lower bound[#]	Medium bound[#]	Upper bound[#]
Fish and seafood	Cod	SM1,FS	0.3	40	48	56
	Shrimp	SM1,SM2,FS	1.3	51	61	70
	Sardines	SM1	7.2	1	52	110
	Mackerel	SM1,SM2	16	170	200	220
	Trout	SM1,FS	3.1	270	270	280
	Smoked salmon	SM1,SM2,FS	13	1020	1020	1030
	Fresh salmon	SM1,FS	13	1570	1580	1580
	Mean of Means			***450***	***460***	***480***
Meat products	Beef steak	SM1,SM2,B	0.5	0	17	31
	Chicken breast	SM1,SM2,B,BI O	1.3	1	18	31
	Minced meat	SM1,SM2,B	18	3	54	110
	Pork sausage	SM1,SM2,B	24	32	75	120
	Hamburger	SM1,SM2,B	9.6	39	80	120
	Pork chop	SM1,SM2,B	2.8	62	91	120
	Salami	SM1,SM2,B,BI O	34	63	92	120
	Meatloaf	SM1,SM2,B	27	110	140	160
	Mean of Means			***40***	***70***	***100***
Eggs and dairy products	Eggs	SM1,SM2,BIO	10	35	100	170
	Cheese	SM1,SM2,BIO	34	18	120	220
	Butter	SM1,SM2,BIO	83	280	550	810
	Mean of Means			***110***	***260***	***400***
Fast food	Quick King Fish	FF1	18	0	52	110
	Mc Donalds Filet-O-Fish	FF2	11	5	55	110
	Quick Double Chicken	FF1	18	48	78	110
	Mc Donalds Mc Chicken	FF2	12	0	52	110
	Quick Gaint	FF1	18	76	100	130
	Pizza Hut Super Supreme	FF3	13	73	100	130
	Mc Donalds Big Mac	FF2	12	160	160	160
	Mean of Means			***52***	***86***	***120***

[§] SM = Supermarket; FS = Fish shop; B = Butcher; BIO = Bioshop; FF = Fasfood restaurant

[#] Lower Bound = N.D. (not detected) was replaced by zero; Medium bound = N.D. was replaced by 1/2 LOQ; Upper bound = N.D. was replaced by LOQ

4. Discussion

4.1. Levels.

Extensive studies on PBDEs in food are rather scarce. Furthermore, comparison of results in other studies is complicated by the fact that there is no standardised manner of reporting results; the congeners included in the summation of PBDEs sometimes differ between studies. Schecter and coworkers (2004) performed a similar market basket investigation in the U.S. In that study, the highest PBDE-levels were also seen in fish in general and in salmon in particular and levels in fish were in the same range as in the present study (range 8.5 - 3078 pg/g ww vs. 22 – 2364 pg/g ww in the present study) (Schecter et al., 2004). It was shown before that salmon can be an important source of pollutants (Hites et al., 2004). Levels in dairy products, such as butter, and eggs were also comparable to the levels in the present study. The levels in meat however, were significantly lower in the present study when compared to the results of Schecter and co-workers (2004). Comparison of the results obtained for meat is rather difficult, since different foods were analysed in that study, such as duck, turkey, and bacon. A study from Japan also reported similar levels in salmon (970 pg/g ww), beef (16 pg/g ww) and chicken (6 pg/g ww) as in the present study (Ohta et al., 2002).

In a study by Bocio and co-workers (2003) various foods from Spain were analysed for PBDEs. Levels in meat (steak and hamburger) and eggs were comparable to those of the present study. The highest levels in that study were also seen in fish. Tittlemier and co-workers (2004) focused on PBDEs fish and shellfish that were available on the market for consumption. Levels measured in salmon and shrimp in that study are comparable to the levels of the present study. From the studies that are available in the literature, it can be concluded that fish bears the highest PBDE load and that levels in other food are similar, regardless of the origin.

An interesting observation on the lipid content of the fast food samples is noteworthy. Table 1 shows the lipid content of the fast foods that were analysed (beef-, chicken- and fish-based). It appears from the present results that the fast food's lipid content is rather related to the restaurant than to the type of food. The lipid content for all samples for restaurant 1 (FF1) was 18 %, while for restaurant 2 (FF2) this was 12 %. One would expect that chicken- or fish-based food would be less fat. Our results however do not confirm this assumption and show that beef-, chicken- or fish-based fast foods contain equal amounts of fat.

4.2. Intake estimation

The intake calculations were based on a theoretical estimate of the average daily food consumption (KB 03.03.1992). The fast food intake estimate was based upon 1 restaurant visit each 2 weeks. Intake calculations did not include any fruit or vegetables, which (should) contribute to a great extent to the total daily diet. Levels in these foods however, are expected to be very low, seeing the low fat content of these products and the lipophilicity of

the pollutants under investigation. However, some studies reported the presence of PBDEs in fruits and vegetables (Ohta et al., 2002; Kiviranta et al., 2004). Those studies indeed revealed a low PBDE content in these foods and thus a low contribution to the total dietary PBDE intake (< 20 %). It should be mentioned that the market-basket in the present study was not comprehensive, but rather indicative. Next to the items mentioned above, also other products, such as milk and vegetable oils, were not analysed.

For the intake calculations of the present study, the average theoretical daily consumption of each food category or individual food (g) was multiplied with the corresponding concentrations (pg/g ww), which resulted in a total daily intake (pg/day) (Table 2). The data used to estimate the dietary exposure to PBDEs might slightly overestimate the actual dietary exposure because most analyses were done on fresh unprocessed foods, which were not boiled, cooked, baked or fried prior to analysis (except the smoked salmon, shrimp and fast food samples). Cooking processes have been shown to lead to losses of PCBs and other organochlorines in trout, via the loss of fat (Zabik et al., 1996). Other processes that occur during food preparation can also reduce the pollutant load of foods, such as volatilisation and extraction into the cooking oil. Several studies reported reductions of PCBs and depending on the applied cooking process, these reductions varied between 15 and 65 % (Zabik et al., 1992; Salama et al., 1998; Schecter et al., 1998; Wilson et al., 1998). No data is currently available on the loss of PBDEs following food preparation processes, but it can be assumed that, based on the similar physical properties to PCBs, similar losses will also occur following cooking. Additionally, the uptake of PBDEs in the GI tract is not well studied and varies with the degree of bromination (Kierkegaard et al., 1999). PBDE uptake estimates vary among authors. Stapleton and co-workers (2005) assumed a value of 90 % in their calculation of potential doses to children as a result of ingesting PBDE-contaminated house dust in homes in Washington, D.C. Also studies on systemic absorption of BDE 47 in mice indicated that absorbtion of this congener exceeded 80 % (Staskal et al., 2005). The relevance of these data to humans or other mammals is unknown. Therefore, the discussion is limited solely to the PBDE intake.

Total average dietary PBDE intake in the present study was estimated between 23 and 48 ng/day (Table 2). This value is in accordance with what was previously reported by others in other countries. A limited number of publications is currently available on the issue of dietary PBDE intake and levels range from 41 to 91 ng/day (Darnerud et al., 2001; Ryan and Patry, 2001; Bocio et al., 2003; Harrad et al., 2004; Kiviranta et al., 2004, Huwe and Larsen, 2005). Although these intake estimations were based on diets from geographical distinct areas, such as Canada, Finland, Spain, Sweden and the UK, the averaged daily intake estimations are fairly comparable. Furthermore, food items that were included in the various studies were not always the same, as some authors only analysed meat (Huwe and Larsen, 2005), while others also included cereals, fruits, vegetables and beverages (Kiviranta et al.,

2004). However, contributions of these types of food to the total PBDE intake were rather low, not affecting the total estimated intake to a great extent (< 20 %).

Table 2. Estimated dietary PBDE intake (ng) in Belgium.

	EDC* (g)	Lower bound intake	Medium bound intake	Upper bound intake	% of total intake§
Fish and seafood	30	13	14	14	39
Meat products	150	5.8	11	15	30
Cheese	30	0.6	3.6	6.5	10
Eggs	30	1.1	3.1	5.1	9
Butter	5	1.4	2.7	4.1	8
Fast food	20	1.0	1.6	2.4	5
Daily total		23	35	48	100

* EDC = Estimated daily consumption (g) (KB 03.03.1992)

§ Based on the medium bound intake estimate

Although it is only a minor constituent of the Belgian diet, fish is a major contributor to the total daily PBDE-intake (around 40 %) due to the high PBDE levels, while meat products account for around 30 % of the total dietary intake of PBDEs (Table 2). Dairy products and eggs contribute to a lesser extent (< 30 %). Also other studies have reported a similar intake distribution. Sjödin and co-workers (2003) found that around 50% of the total dietary PBDE intake in Sweden originated from fish, while the rest was from meat and dairy products. The fish contribution is slightly lower in the present study, but this can be related to the dietary habit of Belgians, which tend to be more meat than fish consumers.

5. Conclusion

Except for fish, levels of PBDEs in the Belgian diet are low. The total intake was estimated between 23 and 48 ng/day in this market-basket. Over time, this leads to ingestion of vast amounts of these man-made chemicals. Unlike for PCBs, food contamination with PBDEs is currently not monitored in Belgium.

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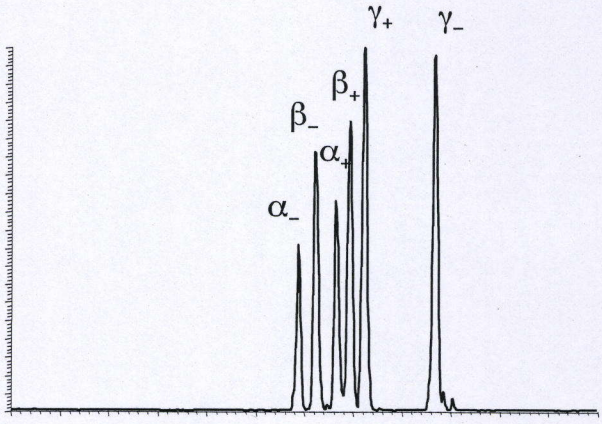
6

HBCD



6.1 Introduction

Next to PBDEs, several other BFRs are used in large quantities (See *1. Introduction and background information*). After TBBP-A, which is less environmentally relevant because it is used as an covalently bound FR, commercial HBCD-mixtures are mostly used. This BFR is commonly applied to flame retard extruded and expanded polystyrene that is used as thermal insulation in buildings. World market demand for HBCD in 2001 was 16,700 tonnes (BSEF, 2006). ***Paper XII*** summarises the current knowledge and state-of-the-art concerning HBCD.

HBCD was first detected in environmental samples during the middle to late-1990s, initially in ambient air, sewage sludge and river sediments in Sweden (Sellström et al., 1998; Sellström et al., 1999; de Wit, 2000). Subsequent studies in many countries have confirmed the widespread distribution of HBCD in the environment (See ***Paper XXVII***, not included in this thesis). The EU has issued a risk assessment study for HBCD that started in 1997 and that hasn't finished to this date. The main issues currently under review are the biodegradability of HBCD and its toxicity in water and sediment. A draft version of the report is currently under discussion (See *1.6. Legislation and policy*), but additional testing of the degradability of the individual isomers will be necessary before a definitive conclusion on the persistence of HBCD can be drawn.

Until recently, only three HBCD isomers, α-, β-, and γ-HBCD, had been reported in technical mixtures, and several contradictory chemical structures have been reported (Becher, 2005). It seems that more stereoisomers can be isolated from the technical mixtures, although at much lower concentrations than α-, β-, and γ-HBCD (Heeb et al., 2005); in total, five individual HBCD diastereoisomers, named as α-, β-, γ-, δ- and ε-HBCD were isolated and identified. The α-, β- and γ-HBCD diastereomers, which account for more than 99% in technical mixtures, could each be further resolved into two peaks on a chiral, permethylated β-cyclodextrin stationary phase, whereas δ- and ε-HBCD both eluted as single peaks from the chiral column (Heeb et al., 2005). It is clear that HBCD technical mixtures consist of several different HBCD molecules. Therefore, the use of the terminology "HBCDs" instead of "HBCD" would be preferable (See *1.3.2.2. Hexabromocyclododecane*; ***Paper XXVII***, not included in this thesis).

Seeing that diastereo- and enantioselective processes occur in the environment, the stereochemistry of HBCD is of importance. This can lead to relative enrichment of different stereoisomers of HBCD, which was confirmed by research: (+/-) α-HBCD is enriched in aquatic organisms, while (+/-) γ-HBCD is the major congener in technical mixtures. At present, the knowledge on the stereochemical implications of HBCD remains unclear due to lack of research.

Because of the complex stereochemistry, isomer-specific data are needed to elucidate sources, distribution and fate of HBCD for an assessment of the risks related to this

bioaccumulative compound. All environmental scientists should adopt a diastereoisomer- and enantiomer-specific approach to HBCD analysis using LC-MS (See *2.4.2. LC/MS*). This will improve the quality and breadth of analytical data that is available, and assist regulators and risk assessors to evaluate the risks associated with its continued use.

The visions, opinions and overview presented in ***Paper XII*** were not appreciated by the BFR-industry. In a "Letter to the Editor", entitled "*HBCD: Facts and insinuations*", Dieter Drohmann, spokesman of the *HBCD Industry Working Group*, states that "*he disagrees with some fundamental statements in the feature article*" and he tries to cast doubt on the data that was presented. His major point of discussion was the fact that HBCD is presented as a persistent organic pollutant. He is convinced that some of the requirements for this classification, such as persistence, long-range atmospheric transport (LRAT) and toxicity are not met.

Paper XIII is a reply to the letter of Dieter Drohmann. The first point of discussion was "persistence". The evidence that supports the classification of HBCD as persistent organic pollutant is presented in ***Paper XII***. The risk assessment report also states that all requirements are met to classify HBCD as a persistent compound. Furthermore, the fact that α-HBCD was measured in blubber of harbour porpoises at concentrations in the ppm-range, argues very strongly for the persistence of this congener (Zegers et al., 2005). In that case it has been transferred from the source of discharge and bioaccumulated through an entire food chain to a marine top-predator without degrading.

The second point of discussion was LRAT. Drohman's arguments were based on an industry-sponsored study that involved modelling activities. Although such modelling activities provide useful information, they are no substitute for real environmental measurements. We suggested that HBCD undergoes LRAT on the basis that it has been detected in air samples from very remote sites in northern Sweden and Finland, and the finding of HBCD in polar bears from Greenland and Svalbard in the Arctic Ocean further supports this hypothesis. As far as the detection of HBCD in fish from Swiss mountain lakes is concerned, local sources can be entirely ruled out (Schmid et al., 2004). It is clear from these studies that LRAT of HBCD is occurring, but its importance relative to other sources remains to be established.

Although ***Paper XII*** states differently, Drohmann also argues "*relevant and high-quality studies on [the toxicity of] HBCD are in the public domain*". In our view, the lack on toxicological data is manifested in two ways. Firstly, all studies to date have been focused on the technical product, dominated by the γ-HBCD isomer, whereas in biota the dominant congener is the α-HBCD isomer. Secondly, studies to date have been primarily focused on acute toxicity, hence the database on non-lethal effects is incomplete. It is therefore fair to say that the toxicological database needs elaboration.

It seems that the major concern of the *HBCD Industry Working Group* relates to the possible future classification of HBCD as POP according to the definition of the Stockholm convention.

Whilst from the information currently available, we consider that it is clear that HBCD is a persistent organic pollutant ("*When I see a bird that walks like a duck and swims like a duck and quacks like a duck, I call that bird a duck.*"— James Whitcomb Riley), it remains to be seen whether it will be categorised as a POP within the more restrictive definitions of the Stockholm Convention.

Paper XIV reports on the diastereomeric and, for the first time ever, on the enantiomeric composition of HBCDs in muscle and liver of several fish species sampled in the SE. This study was possible through collaboration with the Norwegian Institute of Public Health. These samples were analysed earlier for PBDEs in another study (See *3.4.* ***Paper IV***).

All three diastereoisomers were separated by HPLC on a Waters' Symmetry™ C_{18} using methanol/acetonitrile as mobile phase. Baseline separation of HBCDs enantiomers was achieved on a chiral permethylated β-cyclodextrin column (Machery-Nagel NUCLEODEX™ beta-PM). A tandem mass spectrometer was used and operated in multiple reaction monitoring.

The total HBCD content (sum of α-, β- and γ-diastereoisomers), as well as the distribution of diastereoisomers and enantiomers varied between the species. The levels of total HBCD found in fish tissues were higher than those measured in fish from European rivers with no known point sources of HBCD, but lower than in fish samples collected near factories producing or using HBCD. The lipid normalised concentrations of total HBCD, were higher in liver than in muscle for bib and whiting, while in sole, HBCD had no preferential distribution between the tissues. A similar pattern for liver and muscle distribution was already observed for polybrominated diphenyl ethers (PBDEs) in these species (See *3.4.* ***Paper IV***). The α-HBCD diastereoisomer was most abundant in all fish samples with a higher contribution to the total HBCD levels in liver compared to muscle for bib and whiting. The γ-HBCD diastereoisomer accumulated less in liver than in muscle of sole, bib and whiting. For the first time, enantiomer fractions were determined for HBCD diastereoisomers in liver of three fish species and in muscle of two fish species. A significant enrichment of the (+) α-HBCD enantiomer was found in whiting and bib liver samples. A high enantioselectivity has also been seen for the γ-HBCD diastereoisomer in whiting liver. The implications of these enantioselective processes have great opportunities for future research.

At present, HBCD is found as a ubiquitous environmental pollutant. It possesses all physico-chemical properties that characterise POP. Although they are currently not classified as such, it is sound to say that HBCD is a persistent organic pollutant. The discovery of the complex stereochemistry of HBCD has however raised more questions than it has solved. To enable correct assessment of the environmental and toxicological impact of HBCD, future research should adopt an isomeric and enantiomeric approach.

6.2 Paper XII

Hexabromocyclododecane challenges scientists and regulators

Robin J. Law, Martin Kohler, Norbert V. Heeb, Andreas C. Gerecke, Peter Schmid, Stefan Voorspoels, Adrian Covaci, Georg Becher, Karel Janák, Cathrine Thomsen

Environ. Sci. Technol. 39 (2005), 281A-287A

Hexabromocyclododecane CHALLENGES Scientists *and* Regulators

With 16 potential isomers, this brominated flame retardant is an analytical challenge for environmental scientists and a conundrum for regulators.

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Brominated flame retardants (BFRs) reduce the flammability of a broad range of consumer products. The annual global demand for these high-production-volume chemicals rose to 204,000 tonnes (t) in 2001 (*1*). Hexabromocyclododecane (HBCD), a technical mixture of several isomers, is primarily used in extruded and expanded polystyrene for thermal insulation in buildings. World production of HBCD in 2001 totalled 16,700 t.

Other common BFRs include tetrabromobisphenol-A (TBBP-A) and decabromodiphenyl ether (DecaBDE). Their annual world market demands are 119,700 and 56,100 t, respectively (*2*). TBBP-A is used primarily in electronic circuit boards and is chemically bound within the resin. In contrast, DecaBDE and HBCD are simply mixed with the material that is to be flame-retarded. Therefore, these compounds are more likely to leach out during both product use and disposal.

In the mid- to late 1990s, HBCD was first detected in ambient air, sewage sludge, and river sediments in Sweden (*3–5*). Subsequent studies in many coun-

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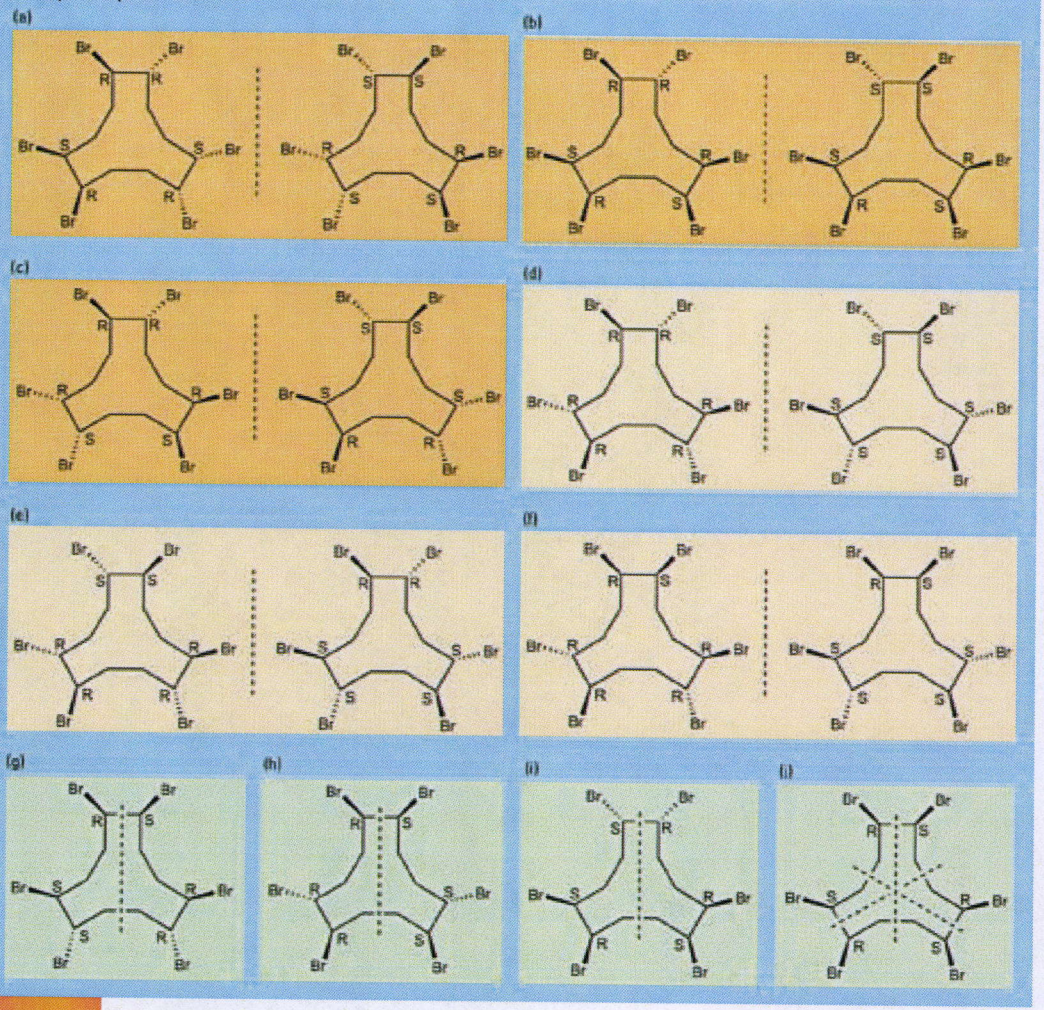
FIGURE 1

Possible HBCD stereoisomers

Bromination of cyclododeca-1,5,9-trienes theoretically results in 16 stereoisomers—6 enantiomeric pairs (a–f) and 4 meso forms (g–j). The first three pairs of enantiomers (a–c) are assigned as the α-, β-, and γ-HBCD stereoisomers, respectively, which dominate in technical products. So far, structures (d–f) have not been found, whereas two meso forms, whose stereochemistries are not yet assigned, have been detected in a technical product. This graphical representation has been chosen to display all relevant symmetry elements.



tries around the world have confirmed the widespread distribution of HBCD in the environment. As a result, HBCD is currently undergoing a risk assessment within the EU. The main issues outstanding within this assessment are the biodegradability of HBCD and its toxicity in water and sediment.

HBCD's complex stereochemistry

Technical-grade HBCD mixtures are obtained via bromination of cyclododeca-1,5,9-triene (CDT) isomers. Depending on the purity of the starting material and the conditions of the industrial processes, a range of technical products with various isomer compositions and melting points are formed.

Figure 1 represents all possible HBCD stereoisomers. Bromination of CDT results in six stereocenters at positions 1, 2, 5, 6, 9, and 10. From all 4 possible CDT isomers, 16 HBCD stereoisomers may be formed, including 6 diastereomeric pairs of enantiomers and 4 meso forms. As a consequence, 10 diastereomers are distinguishable in an achiral environment and 16 stereoisomers in a chiral system.

Structural elucidation of stereoisomers. To date, only three HBCD isomers—labeled α-, β-, and γ-HBCD—have been characterized in technical mixtures (*6*; Figure 2a), and several contradictory chemical structures have been published. However, eight stereoisomers have been isolated from a low-melting-point technical HBCD mixture (*7*). The unambiguous identification relied on chromatographic retention, mass spectrometry (MS), X-ray crystal structure analysis, and optical rotation measurements. Five individual HBCD diastereomers—named α-, β-, γ-, δ-, and ε-HBCD—were isolated via normal- and reversed-phase (RP) liquid chromatography (LC). The α-, β-, and γ-HBCD diastereomers could each be further resolved into two peaks on a chiral, permethylated β-cyclodextrin stationary phase, whereas δ- and ε-HBCD both eluted as single peaks from the chiral column (*7*).

Optical rotation measurements revealed the presence of three pairs of enantiomers: (–) α- and (–) β-HBCD eluting before the (+) α- and (+) β-HBCD enantiomers, and (+) γ-HBCD eluting ahead of (–) γ-HBCD (Figure 2b). No optical rotation was detected for δ- and ε-HBCD. Therefore, these two stereoisomers were tentatively assigned as meso forms. In one low-melting-point technical-product investigation, (+/–) γ-HBCD was the most abundant diastereomer (81.6%). Lower contributions to the total HBCD content of 11.8, 5.8, 0.5, and 0.3% were found for (+/–) α-, (+/–) β-, δ-, and ε-HBCD, respectively.

Structural analogy to HCH. The insecticide hexachlorocyclohexane (HCH) has become a textbook example of the relevance of stereospecific processes in the environment. Stereoselective partitioning of HCH in different environmental matrices and relative enrichment of certain stereoisomers during long-range transport have been studied via separation of diastereomers and enantiomers (*8*, *9*). The distribution of HCH stereoisomers in biota is clearly different than in technical mixtures (*10*). For example, (+/–) α-HCH are the major stereoisomers in technical HCH, but β-HCH is the most abundant diastereomer in biota (*11*). Furthermore, enantioselective processes must be involved because enantiomeric ratios of (–) and (+) α-HCH differ in biological samples (*9*, *11*, *12*). These findings clearly indicate the differing environmental fates of individual HCH stereoisomers. Nowadays, HCH stereoisomers are ubiquitous in the environment and recognized as persistent and bioaccumulating compounds.

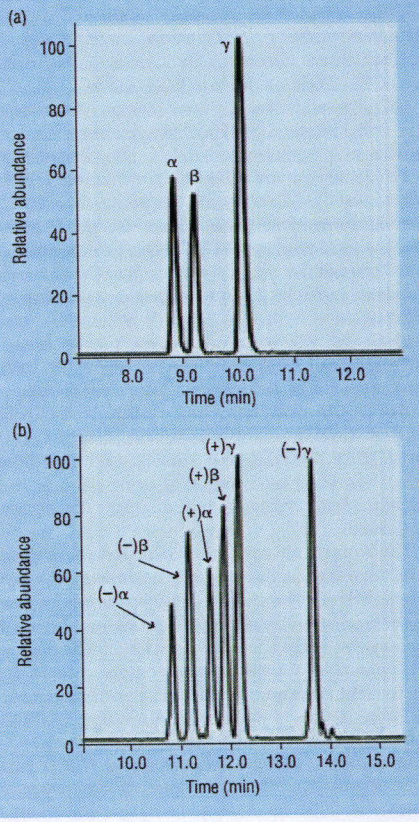
HCH and HBCD have clear parallels. Both have been produced in quantities on the order of 10,000 t per year, and, at first glance, the stereochemistry of HBCD seems to be similar to that of the 1,2,3,4,5,6-HCHs. In both cases, six stereocenters are formed upon complete halogenation. Although 16 stereoisomers, 4 meso forms, and 6 pairs of enantiomers are possible for HBCD, only 9 stereoisomers, 7 meso forms, and 1 pair of enantiomers are expected for HCH. Crystal structure analysis revealed that α-HCH is the optically active form and hence can be resolved as two enantiomers, whereas all other stereoisomers are meso forms.

Implications of stereochemistry. It thus seems likely that HBCD, just like HCH, undergoes diastereo- and enantioselective processes in the environment, which induce relative enrichment of different stereoisomers. The latest data confirm this hypothesis. Variations in solubility and partitioning behavior, as well as uptake and metabolism of individual diastereomers, are thought to explain the enrichment of (+/–) α-HBCD in aquatic organisms. As observed with HCH, enantiospecific interaction with biota, different metabolic pathways, and variable biodegradation rates may alter the enantiomeric ratios of (+/–) α-, (+/–) β-, and (+/–) γ-HBCD (*13*).

FIGURE 2

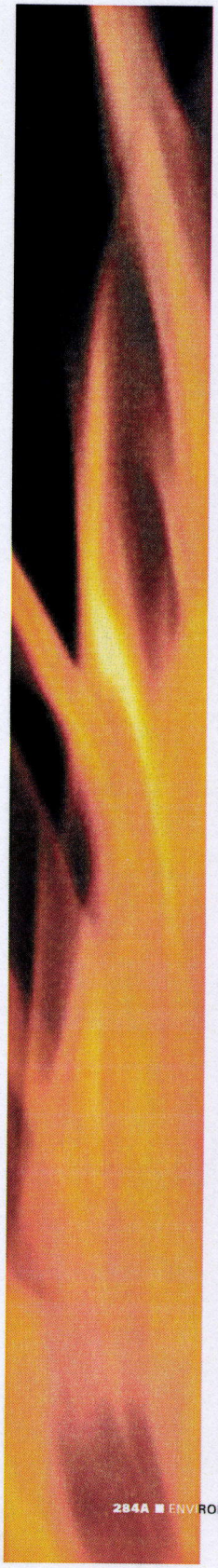
Separation of α-, β-, and γ-isomers

These HPLC/MS/MS chromatograms show the major hexabromocyclododecane diastereomers on (a) a C_{18} reversed-phase column and (b) a chiral permethylated β-cyclodextrin column.



Analyzing HBCD

LC/MS/MS is the preferred analytical technique, because it allows both diastereo- and enantioselective determination of HBCD in environmental samples. Gas chromatography (GC) or high-performance LC (HPLC) MS are commonly used for



the quantitative determination of HBCD. However, both of these well-established methods have limitations.

As with many other halogenated persistent organic pollutants (POPs), HBCD has been analyzed via GC/MS, with detection in both the positive and negative ion modes. Because of the higher sensitivity, the mass spectrometer is usually operated in the electron-capture negative-ion (ECNI) mode, with only the bromide ion typically monitored during chromatographic runs. However, larger fragment ions, which are necessary for structural confirmation of HBCD, do not form in ECNI mode.

GC separation has its own serious limitations. It is well known that HBCD diastereomers interconvert at temperatures >160 °C (*14*). Because of this thermal isomerization and the fact that HBCD elutes from the GC column at temperatures >160 °C, a broad, unresolved peak is observed (*15*). Therefore, the total amount of HBCD can be determined by GC but not the quantities of each of the isomers. Moreover, pure HBCD undergoes decomposition by elimination of hydrogen bromide at temperatures >240 °C (*16, 17*). Not surprisingly, partial breakdown (*15*) and even complete loss of HBCD have been reported in GC systems. The thermal exposure of HBCD must therefore be minimized during analysis. Cold on-column injection, short GC columns, thin-film stationary phases, and high flow rates are several measures that can minimize the risk of thermal degradation and reduce the elution temperature.

The brominated compounds that have been used as internal standards, such as brominated diphenyl ethers (BDEs) or brominated biphenyls, have a better thermal stability and therefore cannot be used to compensate for the breakdown of HBCD during GC separation. Furthermore, because isotopically labeled HBCD standards cannot be used when only the bromide ion is monitored, quantifying HBCD by GC/ECNIMS is problematic.

In contrast, RPLC easily separates HBCD isomers (Figure 2a). HPLC, coupled to electrospray ionization (ESI) or atmospheric-pressure chemical ionization MS, is a versatile tool for the isomer-specific determination of HBCD in environmental samples. However, the sensitivity of LC/MS is lower than that of GC/ECNIMS, so HBCD could only be detected in environmental samples with relatively high contamination levels. However, a recently described sensitive and selective method that uses LC/ESI-MS/MS and single reaction monitoring for the transition $[M–H]^-$ (*m/z* 640.6) → Br^- (*m/z* 79 and 81) yielded a limit of detection (LOD) of 4–6 pg γ-HBCD for a standard solution injected on-column (*18*). Janák et al. further optimized both the LC and MS conditions to separate the diastereomers and recorded a LOD of 0.5 pg for γ-HBCD in standard solutions and 5–10 pg for γ-HBCD in fish extracts injected on-column (*13*).

HPLC with chiral, permethylated β-cyclodextrin columns has been successfully used to separate the enantiomers of the (+/–) α-, (+/–) β-, and (+/–) γ-HBCD diastereomers (*7, 13*). With chiral LC/MS/MS, Janák et al. fully separated the three pairs of enantiomers of HBCD in a single analysis, with LODs for the different isomers between 10 and 20 pg injected on-column (*13*; Figure 2b). However, matrix components coeluting with the analytes may lead to serious ion suppression of the primary ion $[M–H^-]$, resulting in much lower sensitivity. This problem might be avoided with thorough sample cleanup to remove interfering components before HPLC analysis. Furthermore, using isotopically labeled internal standards is essential to compensate for potential variations in sensitivity during and between sample runs. Both ^{13}C-labeled and ^{2}H-labeled standards of α-, β-, and γ-HBCD are now commercially available. LC/MS results obtained with other internal standards must be regarded as questionable.

In the environment

HBCD has been detected in practically all environmental media and is now considered to be a ubiquitous contaminant. The flame retardant has been detected in both urban and rural air across Sweden and at very remote sites in northern Sweden and Finland, suggesting that it undergoes long-range atmospheric transport (*19, 20*). This has been confirmed by the detection of HBCD, after atmospheric transport from Western Europe and Eastern North America, in polar bears from Greenland and Svalbard in the Arctic Ocean (*20*).

The widespread occurrence of HBCD in sewage sludge is a result of diffuse leaching from flame-retarded products into wastewater streams (*21*). Applying these sludges to agricultural or other land may redistribute the contained HBCD to the soil–sediment compartment and further into aquatic or terrestrial food chains, as demonstrated by BDEs (*21*).

HBCD has been found in river sediments downstream of urban centers or known industrial sources and in marine sediments at substantially higher concentrations than BDEs (*3, 22–24*). HBCD has also been detected in both freshwater and marine biota (*15, 23, 25*). Fish that lived downstream from an HBCD manufacturing plant had very high concentrations of HBCD (>10 mg/kg wet weight) (*23*). Food-chain studies have shown that HBCD is bioaccumulative and can be transferred from sediment via invertebrates and predatory fish to fish-eating top predators, such as birds and seals (*23, 24*). A Swedish study of the changes over time in HBCD concentrations in archived Baltic Sea guillemot eggs indicated an increase in concentrations since 1969. However, during the past decade, concentrations in those eggs have stabilized (*25*).

The major intake of HBCD for humans is from food and indoor air or dust. According to the few data available on levels of HBCD in food, fish appear to be a major source (*19*). Furthermore, HBCD has been found in house dust at very high concentrations (*26–28*), an indication that indoor exposure through inhalation or ingestion is highly likely and could contribute significantly to human exposure. However, the levels of HBCD observed so far in hu-

man milk (*29*) and blood (*30*) are low and in the same range as those measured for hexa-BDE congeners.

A few studies have reported on the environmental levels of individual HBCD diastereomers. The diastereomeric profile of HBCD in most sediment samples is similar to that of commercial formulations: γ-HBCD is the most abundant isomer (*23, 31*). However, in some locations, a considerable contribution from α-HBCD has been observed (*22, 23, 32*). Temperatures reach >160 °C during the production of certain polymers, and this may lead to the thermal conversion of γ- to α-HBCD (*22, 23*). In those cases, the HBCD source is likely the production of heat-treated polymer materials or textiles and not an HBCD technical product itself.

In contrast to sediments, the α-isomer seems to dominate the HBCD profile in the vast majority of biota (*24, 31–34*). Several factors may cause this change. First, the elution order in RPLC indicates that α-HBCD is more hydrophilic than β- or γ-HBCD; this is in accordance with the water solubility determined for the individual HBCD diastereomers (*35*). Second, it has been shown that liver microsomes from marine mammals metabolize β- and γ-HBCD more rapidly than α-HBCD and thereby form hydroxy-metabolites (*34*). Finally, evidence for the bio-isomerization of HBCD diastereomers in fish has been recently reported (*36*). Significant concentrations of the other isomers were detected in fish experimentally exposed only to α- or γ-HBCD. In both cases, the β-isomer was formed least often. All these observations are in accordance with the HBCD profile generally found in fish, which shows a dominance of the α- over the γ- isomer, and little or no β-isomer. The differing isomer profiles are shown in Figure 3. To date, the γ- and ε-HBCD isomers have not been found in environmental samples.

HBCD enantiomers can exhibit different biotransformation and biological activities, so researchers must also evaluate this possibility. Janák et al. have already used chiral LC/MS/MS to demonstrate the selective accumulation of the HBCD enantiomers in marine fish (*13*).

Toxicity

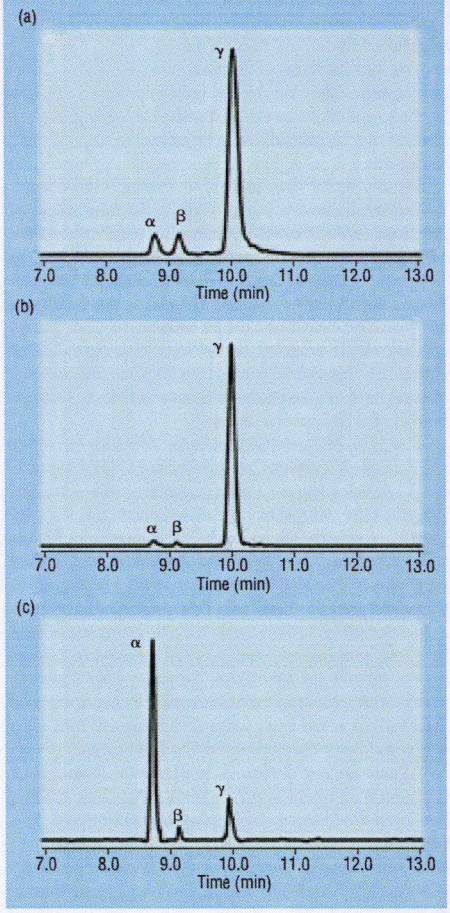
The toxicological database for HBCD lacks relevant, high-quality studies (*37*). The direct acute and chronic toxicity of HBCD appears to be low, but sublethal effects can't be ruled out. HBCD has an antagonistic effect on detoxification enzymes and thus may also increase the toxicity of other compounds (*38*). It has also been suggested that HBCD may induce cancer by a nonmutagenic mechanism (*38, 39*) and may disrupt the thyroid hormone system (*37, 40, 41*).

The endocrine-disrupting activity of HBCD is currently under investigation within the EU-funded project Flame Retardants Integrated Risk Assessment for Endocrine Effects (FIRE; *42*). Neonatal exposure to HBCD can induce developmental neurotoxic effects, such as aberrations in spontaneous behavior, learning, and memory function (*43, 44*). HBCD can also alter the normal uptake of the neurotransmitters in rat brains (*45*). Last year, Birnbaum and Staskal concluded that further studies are needed on developmental effects, endocrine disruption, and longer-term effects such as carcinogenesis (*46*). To date, no information exists on the relative toxicity of the different HBCD diastereomers and enantiomers. Also, until very recently, toxicological testing has been conducted solely with technical HBCD products rich in γ-HBCD, whereas internal exposure may be dominated by other isomers.

FIGURE 3

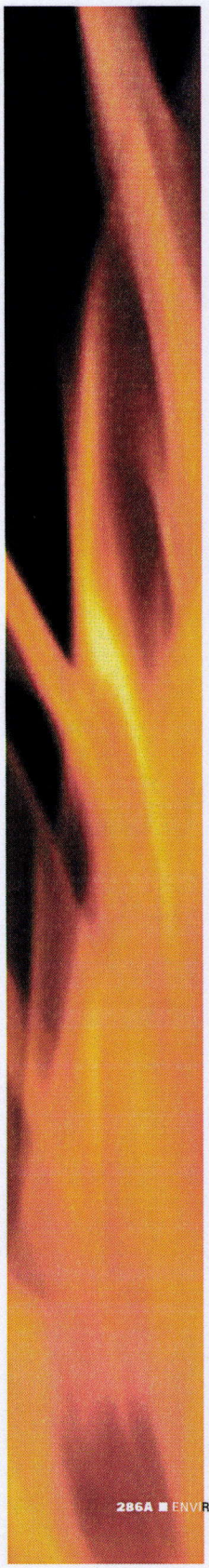
α-HBCD isomer dominates biota

These chromatograms were obtained by HPLC/MS/MS for (a) a technical mixture, (b) a sediment sample from the Western Scheldt estuary in The Netherlands, and (c) plaice (fish) liver from the same area.

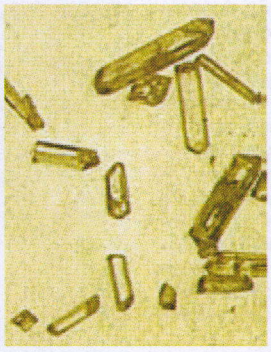


Regulation and risk assessment

Different countries have various strategies and legal frameworks to assess the safety of HBCD in the environment. Within the EU, HBCD is covered by



the Council Regulation 793/93 on the Evaluation and Control of the Risks of Existing Substances. The EU risk assessment for HBCD began in 1997, with Sweden as the lead country. A draft risk assessment report is currently under discussion within the EU. Additional testing of the degradability of the individual isomers will be necessary before a definitive conclusion on the persistence of HBCD can be drawn. The bromine industry has agreed to conduct further studies on degradation products of HBCD.



According to the European Brominated Flame Retardant Industry Panel, data from new toxicity, fate, and emission studies have been submitted recently. Bert-Ove Lund, head of the New and Existing Substances Unit at the Swedish Chemicals Inspectorate, anticipates that the EU risk assessment process for HBCD will be completed during 2005. In its current draft version, the HBCD risk assessment states that further information and testing are required; a need also exists to limit the risks to human health and the environment.

The U.S. EPA addresses flame retardants under the Toxic Substances Control Act of 1976. EPA is currently developing a rule to complement a national flammability standard for residential upholstered furniture, which is under consideration by the Consumer Product Safety Commission (CPSC). This rule would require notification and review of 16 flame-retardant chemicals or categories of chemicals, including HBCD, which have been identified by CPSC and industry as likely to be used to flame-retard fabrics on furniture. According to Kenneth Moss from EPA's Office of Pollution Prevention and Toxics, this is the only formal mention of HBCD in any regulatory context in the United States.

Canadian regulators identified HBCD and four halogenated cyclohexanes for assessment during the next few years. In Japan, manufactured and imported amounts of HBCD must be reported to the government. Further steps include preliminary toxicity evaluation and, if necessary, guidance and advice for risk reduction, release minimization measures, and long-term toxicity investigation. In Australia, the National Industrial Chemicals Notification and Assessment Scheme is currently updating information on use, importation, and production of BFRs, including HBCD, to determine the need for a full risk assessment and further regulatory activities.

Environmental monitoring and quantification of HBCD emissions under the voluntary emissions reduction program of the Bromine Science and Environmental Forum have shown that emission control from both HBCD production and usage is feasible for even small- and medium-sized enterprises, according to the Organisation for Economic Co-operation and Development. The Commission for the Protection of the Marine Environment of the North-East Atlantic has included HBCD on its list of chemicals for priority action to protect the marine environment. And as yet, no official position exists on HBCD as a POP candidate, according to Heidelore Fiedler, a scientific affairs officer with the UN Environment Programme. At present, HBCD is included in neither the list of 27 chemicals under the Rotterdam Convention on Prior Informed Consent (PIC) nor the initial list of 12 POPs named within the Stockholm Convention on POPs. But both conventions have a mechanism to add new chemicals: New PIC chemicals or new POPs can be added following proposals from individual member countries, evaluation in the review committee of the respective convention, and acceptance by the Conference of the Parties.

Challenges ahead

The risk assessment of HBCD is not yet complete. Because of HBCD's complex stereochemistry, isomer-specific data are needed to elucidate its sources, distribution, and fate for an assessment of the risks related to this bioaccumulative compound. We urge all environmental scientists to use LC/MS in all applicable studies and adopt a diastereomer- and enantiomer-specific approach to HBCD analysis. Improving the quality and breadth of available analytical data will help regulators and risk assessors evaluate the risks associated with its continued use.

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6.3 Paper XIII

Letter: Response to "HBCD: Facts and insinuations"

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Environ. Sci. Technol. 40 (2006), 1-2

Letters

HBCD: Facts and insinuations

The Hexabromocyclododecane (HBCD) Industry Working Group—producers and users of the flame retardant HBCD—disagrees with some fundamental statements in a July 1, 2005, *ES&T* feature article by Law et al. (*1*). The article is a comprehensive and helpful review of recent work on HBCD from academic research groups. Unfortunately, the relevant information is prejudiced by some flawed facts and unsubstantiated conclusions. The following examples of these are not intended as a comprehensive list but aim to provide the reader with the scientific background to draw better-informed conclusions.

The article insinuates that HBCD is persistent or similar to a persistent organic pollutant, without providing a solid assessment. For instance, the authors write: "As with many other halogenated persistent organic pollutants (POPs), HBCD has been analyzed via GC/MS . . ." (*1*). This casual assertion of persistency is supported by neither the authors' own data (*2*) nor other work (*3–4*). All three studies demonstrate a rapid degradation in the relevant compartments for all three diastereomers of the technical product. A thorough discussion of the available evidence would be more helpful for supporting a sound decision by regulators rather than an attempt to foreclose their conclusion.

The article also states: "HBCD has been detected . . . at very remote sites . . . suggesting . . . long-range atmospheric transport. This has been confirmed by the detection of HBCD, after atmospheric transport from Western Europe and Eastern North America, in polar bears from Greenland and Svalbard in the Arctic Ocean" (*1*). However, the original, referenced publication states: "There are large regional variations in concentrations in top predators with highest . . . HBCD in biota from regions influenced by atmospheric transport from Western Europe and eastern North America. . . . Results for [polydiphenyl ethers] indicated that there are local sources . . . in the arctic. Further work is needed to evaluate the relevance of local sources" (*5*). A thorough assessment of the long-range transport potential of HBCD commissioned by industry concludes that HBCD has a very low potential to reach remote areas (*6*).

The HBCD Industry Working Group is concerned about monitoring data reporting HBCD in biota living in urban and remote areas. We are committed to elucidating the causes for the pattern, and an extensive monitoring program is under development. However, we will only be effective in our counteraction if we work closely along the lines of facts.

Another example is the constructed link between HBCD and hexachlorocyclohexane (HCH). Both substances, together with well over 300 others of the ~75,000 organic substances in the Syracuse database, are halogenated cycloaliphatics; an intrinsic property of an aliphatic compound is that it exists as diastereomers. Enantioselectivity is characteristic of biological processes, but it does not contribute to "clear parallels" between the two substances. In fact, there are essential discrepancies: HBCD is used mainly as a flame retardant in polystyrene insulation boards and the back coating of upholstery fabric, whereas HCH, an agricultural insecticide, has been used widely and at volumes that are an order of magnitude higher than HBCD production volumes. The authors claim that the production volume for HCH is 10,000 t/yr, whereas the International Agency for Research on Cancer reports 53,000 t/yr for the U.S. (1951); 54,500 t/yr for France, West Germany, and Spain (1973); 18,000 t/yr for the USSR (no year); and additional undisclosed volumes in Italy, Poland, Yugoslavia, Romania, and the former East Germany (*7*).

The article claims that according to Darnerud, (*8*) the "toxicological database for HBCD lacks relevant, high-quality studies". The authors refer to conference presentations on results generated with nonvalidated methods, uncertain reliability, and doubtful relevance. However, relevant and high-quality studies on HBCD are in the public domain. Industry has volunteered to have HBCD evaluated in the frame of the U.S. EPA's High Production Volume Challenge Program. Study summaries are publicly available (*9*), and the data have been reviewed by EPA (*10*). In the meantime, a revised study summary that includes more recent studies has been submitted (*11*). The reported exposures of fish and mammals are within the preliminarily predicted no-effect levels derived from these data for the respective species (*12*). All of these studies have been performed with the technical product. We disagree with the authors that these data do not sufficiently reflect "internal exposure", which "may be dominated by other isomers" (*1*). Enantioselectivity is expected to also occur in tested animals, and the diastereomer selectivity has been confirmed in a series of studies (*9*).

In parallel to continuous emission control, the HBCD Industry Working Group prioritizes the understanding of both the environmental fate of HBCD and its degradation products and the contribution of potential emission sources in the life cycle of HBCD. A similar approach of conclusions and actions based on facts would have better served the original article.

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References for this letter are available as Supporting Information at http://pubs.acs.org/est.

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Supporting Information for Drohmann, D. HBCD: Facts and insinuations. *Environ. Sci. Technol.* **2006**, *40*, 1.

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Response

We appreciate the comments from Drohmann. Our article (*1*) was, of necessity, focused on the stereochemistry of HBCD and its implications for environmental analysis and assessment. It was not intended to be a comprehensive review of all aspects of the environmental chemistry and toxicology of HBCD. We are grateful, therefore, for the opportunity to present additional information on these aspects.

Drohmann argues that our statements regarding the persistence of HBCD are supported "by neither the authors' own data nor other work." In the Supporting Information for this letter (available on the web), Table S1 presents the available information on the degradability of HBCD. As Drohmann states, it is apparent that HBCD degrades much faster under anaerobic conditions than the persistence criteria currently in use, but under aerobic conditions degradation is much slower. In aerobic soils, one study found only 10% degradation over 112 days, whereas in another study the half-life was 63 days. However, in the latter study, activated sludge was mixed with the soil sample in order to "simulate conditions in which sludge is added to agricultural fields" (*2*). Such an addition makes this study inappropriate for drawing general conclusions about degradability in soils, and we regard the first study as more conclusive.

Usually a half-life of >6 months in soil is regarded as the cut-off point for rapid degradability, and so, on the basis of these data, HBCD is persistent. Further, the rapporteur of the draft EU risk assessment document, from KEMI (the Swedish Chemicals Inspectorate), argues that HBCD meets "the P-criterion [for persistence] due to slow transformation (sequential debromination) leading to an end-product (cyclododecatriene), which itself is a PBT [persistent, bioaccumulative, and toxic] candidate. The ubiquitous presence of HBCD in the environment further supports the conclusion of HBCD being a P-substance" (*3*).

More data on the persistence and fate of the individual stereoisomers are needed. However, the fact that α-HBCD has been detected in the blubber of porpoises at concentrations up to 9000 μg/kg lipid weight argues very strongly for the persistence of this isomer, reflecting that in this case it has transferred from the source of discharge and bioaccumulated through an entire food chain to a marine top predator without degrading (*4*).

Drohmann's letter also questions HBCD's potential for long-range transport, concluding that a thorough assessment undertaken by industry finds that the compounds have "a very low potential to reach remote areas." Although such modeling activities provide useful information, they are no substitute for real environmental measurements. We suggested that HBCD undergoes long-range atmospheric transport because of its detection in air samples from very remote sites in northern Sweden and Finland as well as the discovery of HBCD in polar bears from Greenland and Svalbard in the Arctic Ocean. Although there may be some local sources, the human population levels in these areas are probably too small to explain the environmental levels observed. In addition, HBCD has been detected in other remote areas (see Table S2). Amongst these, local sources can be entirely ruled out for the HBCD detected in fish from Swiss mountain lakes. It is clear from these studies that long-range transport of HBCD via the atmosphere occurs, but its importance relative to other sources and transport routes remains to be established.

Drohmann's letter also tries to take the comparison of HBCD and HCH far beyond our original intentions. Our point was that both are or were high-production-volume chemicals, with a clear parallel in their stereochemistry. The differing behavior of HCH stereoisomers was found to contribute to their environmental occurrence and significance, and we were keen to ensure that this aspect was not neglected in studies on HBCD. For this reason, we urged "all environmental scientists to use LC/MS in all applicable studies and to adopt a diastereoisomer- and enantiomer-specific approach to HBCD analysis" (*1*).

Drohmann also says that "relevant and high-quality studies on [the toxicity of] HBCD are in the public domain," whereas our article indicated that the "toxicological database for HBCD lacks relevant, high-quality studies," citing a paper by Darnerud (*5*). In fact, Darnerud stated that "there is a lack of relevant studies of high quality that could form a basis for risk assessment of this compound" (*5*).

In our view, this lack is manifested in two ways. First, all studies to date have focused on the technical product, which is dominated by the γ-HBCD isomer, whereas in biota the dominant congener (often >90% of the total) is the α-HBCD isomer. Moreover, studies to date have been primarily focused on acute toxicity; hence, the database on non-lethal effects is incomplete.

The major concern of the industry working group seems to be the possible future consideration of HBCD as a potential Persistent Organic Pollutant (POP). From the information currently available, we consider that it is clear that HBCD is a persistent organic pollutant (all in lowercase), but it remains to be seen whether it will be categorized as a POP within the more restrictive definitions of the relevant conventions.

As requirements for a comprehensive risk assessment of the continued use of HBCD, we should highlight the need for more information on neurotoxicology, developmental toxicity, and the potential for endocrine disruption. We are pleased that the industry group is committed to further elucidating the environmental fate of all HBCD diastereoisomers, and we look forward to working in parallel with them toward the goal of an effective assessment of the risks associated with the continued production and use of HBCD.

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References are available as Supporting Information at http://pubs.acs.org/est.

Supporting information for Response to HBCD: Facts and insinuations. *Environ. Sci. Technol. 2006,40*, 1.

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Table S1 Data on the degradation kinetics of HBCDs in soil, freshwater sediments, activated sludge, and digested sewage sludge under aerobic and anaerobic conditions.

Study	Compartment	Redox conditions	Treatment	Degradation data	Remarks
Davis et al. (2004) as cited in: National Chemicals Inspectorate (2005) also partially cited in Albemarle Corporation (2005)	soil	aerobic	viable	% removed on day 112: ΣHBCDs: 10% (±)-α-HBCDs: 5% (±)-β-HBCDs: 10% (±)-γ-HBCDs: 10%	
	"	"	sterile	% removed on day 112: ΣHBCDs: 6% (±)-α-HBCDs: 14% (±)-β-HBCDs: 8% (±)-γ-HBCDs: 5%	
	activated sludge	aerobic	viable	% removed on day 56: ΣHBCDs: 22% (±)-α-HBCDs: 7% (±)-β-HBCDs: 27% (±)-γ-HBCDs: 23%	
	"	"	sterile	% removed on day 56: ΣHBCDs: 60% (±)-α-HBCDs: 53% (±)-β-HBCDs: >80% (±)-γ-HBCDs: 56%	The addition of $HgCl_2$ may have caused degradation.
	digester sludge	anaerobic	viable	% removed on day 60: ΣHBCDs: 87% (±)-α-HBCDs: 88% (±)-β-HBCDs:>90% (±)-γ-HBCD: 87%	
	"	"	sterile	% removed on day 60: ΣHBCDs: 93% (±)-α-HBCDs: >93% (±)-β-HBCDs: >93% (±)-γ-HBCDs: 93%	
	freshwater sediment	aerobic	viable	% removed on day 112: ΣHBCDs: 44% (±)-α-HBCDs: 43% (±)-β-HBCDs: 50% (±)-γ-HBCDs: 44%%	
	"	"	sterile	% removed on day 112: ΣHBCDs: 16% (±)-α-HBCDs: 8% (±)-β-HBCDs: 24% (±)-γ-HBCDs: 16%	
	freshwater sediment	anaerobic	viable	% removed on day 113: ΣHBCDs: 33% (±)-α-HBCDs: 14% (±)-β-HBCDs: 51% (±)-γ-HBCDs: 33%	
	"	"	sterile	% removed on day 113: ΣHBCDs: 61% (±)-α-HBCDs: 51% (±)-β-HBCDs: 80% (±)-γ-HBCDs: 60%	$HgCl_2$ was added to inhibit biology. This may have caused degradation

					of HBCDs.
Davis et al. (2005) cited in: American Chemistry Council (2001), Albemarle Corporation (2005), and National Chemicals Inspectorate (2005)	soil	aerobic	viable	$t_{½}$: 63 d	Activated sludge was added - The results are based on concentrations of (±)-γ-HBCDs.
	"	"	sterile	$t_{½}$: 6900 d	The results are based on concentrations of (±)-γ-HBCDs.
	soil	anaerobic	viable	$t_{½}$: 6.9 d	"
	"	"	sterile	$t_{½}$: 82 d	"
	freshwater sediment	aerobic	viable	$t_{½}$: 11 and 32 d	"
	"	aerobic	sterile	$t_{½}$: 190 and 30 d	"
	freshwater sediment	anaerobic	viable	$t_{½}$: 1.5 and 1.1 d	"
	"	"	sterile	$t_{½}$: 10 and 9.9 d	"
Schaefer et al. (1996) as cited in: National Chemicals Inspectorate (2005) and Albemarle Corporation (2005)	activated sludge	aerobic	viable	no degradation observed on day 28	No HBCD measurements - oxygen uptake was measured as a proxy for HBCD degradation.
Gerecke et al. (2006)	digested sewage sludge	anaerobic	viable	$t_{½}$: 0.7 d	(±)-α-HBCDs degraded slightly slower than (±)-β-HBCDs and (±)-γ-HBCDs. No indication for enantioselective degradation was obtained.
			sterile	$t_{½}$: >30 d	

Table S2 Occurrence of HBCDs in remote areas.

Study	Location	Sample	Concentration ΣHBCDs range (min - max)	Units	Remarks
Remberger et al. (2004)	Pallas (Northern Finland)	air	2 - 3	pg m^{-3}	
Remberger et al. (2004)	Pallas (Northern Finland)	deposition sample (wet and dry)	5.1 - 13	ng m^{-2} d^{-1}	
Jenssen et al. (2004)	Svalbard	polar cod	5 - 25	ng g^{-1} (lipid weight)	
Jenssen et al. (2004)	Svalbard	ringed seal	15 - 35	ng g^{-1} (lipid weight)	
Jenssen et al. (2004)	Svalbard	polar bear	5 - 15	ng g^{-1} (lipid weight)	
Bytingsvik et al. (2004)	Bear Island	polar cod (liver)	7.67 - 23.4	ng g^{-1} (lipid weight)	
Vorkamp et al. (2005)	South Greenland	peregrine falcon eggs (homogenized eggs)	<0.1 - 230	ng g^{-1} (lipid weight)	
Ueno et al. (2005)	North North Atlantic	skipjack tuna (muscle tissue)	25	ng g^{-1} (lipid weight)	
Verreault et al. (2005)	Svalbard and Bear Island	glaucuous gulls (plasma)	0.07 - 1.24	ng g^{-1} (wet weight)	
Verreault et al. (2005)	Svalbard and Bear Island	polar bear (plasma)	<0.03 - 0.85	ng g^{-1} (wet weight)	12 out of 14 samples were below 0.03 ng g^{-1} wet weight.
Schmid et al. (2004)	high altitude lakes in Swiss alps	brown trout, brook trout, alpine char and lake trout (muscle tissue)	<10 - 36	ng g^{-1} (wet weight)	For some of the lakes, in which HBCDs have been detected, local sources in the catchment can be ruled out. These fish reproduced naturally.
Bergander et al. (1995) as cited in de Wit (2002)	Ammarnäs (north Sweden)	air	6.1	pg m^{-3}	
Fjeld et al. (2004) as cited in de Wit et al. (2004)	Lofoten	blue mussels	9 - 11	ng g^{-1} (lipid weight)	
Fjeld et al. (2004) as cited in de Wit et al. (2004)	Varanger (northern Norway)	blue mussels	3.6	ng g^{-1} (lipid weight)	
Fjeld et al.	Lofoten	atlantic cod	6.6	ng g^{-1} (lipid	

(2004) as cited in de Wit et al. (2004)		(liver)		weight)
Fjeld et al. (2004) as cited in de Wit et al. (2004)	Varanger (northern Norway)	atlantic cod (liver)	7.7	ng g^{-1} (lipid weight)

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6.4 Paper XIV

Hexabromocyclododecane in marine species from the Western Scheldt estuary: diastereoisomer- and enantiomer-specific accumulation

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Envrion. Sci. Technol. 39 (2005), 1987-1994

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Hexabromocyclododecane in Marine Species from the Western Scheldt Estuary: Diastereoisomer- and Enantiomer-Specific Accumulation

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Hexabromocyclododecane (HBCD) is a widely used brominated flame retardant, which is increasingly reported in the environment. Here, we report on the diastereomeric and, for the first time, on the enantiomeric composition of HBCD in muscle and liver of several fish species caught in the Western Scheldt Estuary (The Netherlands). The total HBCD content (sum of α-, β-, and γ-diastereoisomers), as well as the distribution of diastereoisomers and enantiomers, varied between the species. The levels of total HBCD (9−1110 ng/g lipid weight) found in fish tissues were higher than those measured in fish from European rivers with no known point sources of HBCD but lower than in fish samples collected near factories producing or using HBCD. The concentrations of total HBCD expressed on a lipid weight basis were higher in liver than in muscle for bib and whiting, while in sole, HBCD had no preferential distribution between the tissues. A similar pattern for liver and muscle distribution was already observed for polybrominated diphenyl ethers (PBDEs) in these species. The α-HBCD diastereoisomer was most abundant in all fish samples with a higher contribution to the total HBCD levels in liver compared to muscle for bib and whiting. The γ-HBCD diastereoisomer accumulated less in liver than in muscle of sole, bib, and whiting. For the first time, enantiomer fractions were determined for HBCD diastereoisomers in liver of three fish species and in muscle of two fish species. A significant enrichment of the (+) α-HBCD enantiomer was found in whiting and bib liver samples. A high enantioselectivity has also been seen for the γ-HBCD diastereoisomer in whiting liver.

Introduction

Hexabromocyclododecane (HBCD) is an additive brominated flame retardant (BFR) used in polystyrene foams and upholstery textiles in percentages varying between 0.8 and 4% (*1*). In 2001, the world market demand for HBCD was at about 16 700 tons from which about 9500 tons were consumed in Europe. These figures make HBCD the second most used BFR in Europe, after tetrabromobisphenol A (*1*). Recently, the use of penta- and octamixtures of polybrominated diphenyl ethers (PBDEs) has been restricted in Europe (*2*). There are indications that HBCD is being used as a replacement for these BFRs (*3*).

Technical 1,2,5,6,9,10-HBCD is produced by bromination of cyclododecatriene (CDT) (*4*, *5*). This process leads theoretically to a mixture of 16 stereoisomers (six pairs of enantiomers and four mesoforms). Bromination of cis,trans,-trans-CDT yields three diastereomeric pairs of enantiomers as racemic mixtures (*5*), α-, β-, and γ-HBCD (Figure 1). Further, two mesoforms have been isolated from a low-melting, technical-grade HBCD mixture (*4*). The commercial mixtures consist mainly of the γ-HBCD diastereoisomer (ranging between 75 and 89%), while the α- and β-HBCD are present in considerably lower amounts (10−13% and 1−12%, respectively) (*6*). HBCD is subject to thermal rearrangement at temperatures above 160 °C, resulting in a specific mixture of the three diastereoisomers (78% α-HBCD, 13% β-HBCD, and 9% γ-HBCD), while decomposition becomes significant at temperatures above 200 °C (*7*). This rearrangement is independent of the starting isomer composition (*6*).

Substantial dissimilarities in the structure of the α-, β-, and γ-HBCD diastereoisomers might raise differences in polarity, dipole moment, and as already found, in solubility in water. Solubility of α-, β-, and γ-HBCD in water was recently measured to be 48.8, 14.7, and 2.1 μg/L, respectively (*8*). These different properties may result in distinctive rates of biological uptake and metabolism and could possibly explain the observed differences in their environmental behavior (*9*, *10*). Once released into the environment and because of their low water solubility, it is likely that HBCD isomers will adsorb onto solid particles of sediment and soil (*1*). Its resistance to degradation together with a low estimated octanol−water partitioning coefficient (log K_{ow} = 5.6) makes HBCD potentially bioaccumulative in fatty tissues (*11*, *12*).

Although little is known about the metabolism of HBCD diastereoisomers, there are indications of a relatively short half-life (*13*) and of a different biotransformation of HBCD isomers (*14*). However, the preliminary estimated half-lives for α- and β-HBCD diastereoisomers in juvenile rainbow trout (*Oncorhynchus mykiss*) were 136 and 53 days, respectively, suggesting that the metabolizing capacity of fish for HBCD is limited and that there are differences in the absorption and detoxification rate between different diastereoisomers (*9*). There is also evidence for bioisomerization of HBCD diastereoisomers with a preferential formation of the α-HBCD isomer; lower brominated cyclododecane metabolities could not be detected in fish from this study (*10*). In another experiment, α-HBCD was not significantly biotransformed after 90 min during the incubation of rat and harbor seal liver microsomes, while β- and γ-HBCD diastereoisomers decreased to about one-third of the original value (*14*).

To get a better understanding of environmental fate and behavior of HBCD in biota, it is essential to get isomer specific data on HBCD levels. While the determination of BFRs, and in particular of PBDEs, was recently reviewed (*15*, *16*), methods for analysis of HBCD have received much less attention. Most of the HBCD data generated in the past were obtained by gas chromatography/mass spectrometry (GC/MS) and thus restricted to total HBCD levels. So far, GC/MS could not provide a separation of the individual HBCD diastereoisomers and a mixture of diastereoisomers elutes as a broad peak. As also follows from interlaboratory studies, the precision and accuracy ranges in the HBCD determination

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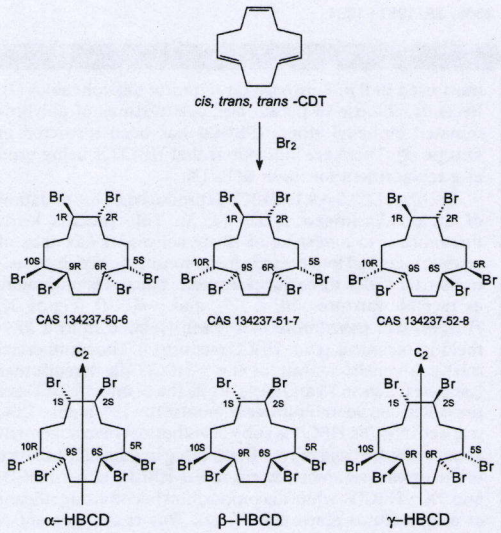

FIGURE 1. Stereochemical structures of the major HBCD isomers.

by GC/MS are larger compared to those obtained for PBDEs (*16*, *17*). Recently, individual HBCD diastereoisomers could be analyzed in biota using reversed-phase liquid chromatography coupled with electrospray tandem mass spectrometric detection (LC/MS-MS) (*17*, *18*).

Moreover, the α-, β-, and γ-HBCD diastereoisomers are chiral and thus may be present in the environment and biota as enantiomeric pairs. In general, a chiral pollutant is produced and released into the environment as racemate and remains as a racemic mixture as long as it is subjected only to achiral interactions, such as hydrolysis, photolysis, leaching, volatilization, and atmospheric deposition (*18*). However, alterations in the enantiomeric composition might occur if the chiral compound is subjected to biochemical processes that in most cases are stereospecific, for example, metabolization (*19*). The enantiomer fraction (EF) that is used to express the enantiomeric composition of pollutants (*20*) may differ between species and tissues and within populations (*21*). Consequently, the investigation of enantiomeric composition of HBCD in biota might be of great importance in understanding metabolization and degradation processes.

Recently, we have established methods based on reversed-phase LC/MS-MS for the determination of HBCD diastereoisomers as well as for estimation of EFs for enantiomeric pairs of α-, β-, and γ-HBCD using chiral permethylated β-cyclodextrin stationary phase (*22*). In the present paper, we report on the levels of HBCD diastereoisomers and their enantiomer fractions in shrimp and in muscle and liver of various fish species from the Western Scheldt Estuary (WSE). The WSE is highly contaminated with BFRs, possibly related to the presence of a BFR manufacturing plant, textile industries, and harbor activities located in the Scheldt Basin (*18*, *22*, *23*).

Experimental Section

Sampling. The sampling of different marine species from the Western Scheldt Estuary (WSE) has been previously described (*23*). The selected sampling sites in the WSE (Figure 2) included two locations in the proximity of a HBCD production plant at Terneuzen, The Netherlands (locations 1 and 2), and three locations toward Antwerp, Belgium (locations 3–5). The collected samples have previously been analyzed for PBDEs (*23*). Unfortunately, not all samples used in the previous study were available for this study. Muscle and liver of gadoids (whiting, *Merlangius merlangus*; bib, *Trisopterus luscus*) and flatfish (sole, *Solea solea*; plaice, *Pleuronectus platessa*), available from at least three locations, were pooled according to species and location using three to six individuals per pool. Muscle of individual eel (*Anguilla anguilla*) and pools of shrimps (*Crangon crangon*) were available from two locations (Table 1). Additionally, a cleaned sediment extract, which originated from the WSE (between Terneuzen and Antwerp), was used to assess the qualitative composition of HBCD diastereoisomers.

Chemicals. All solvents used for the sample preparation and cleanup (*n*-hexane, acetone, *iso*-octane) were of SupraSolv grade (Merck, Darmstadt, Germany). Solvents used for LC/MS-MS (acetonitrile, methanol, water) were of HPLC grade (Merck). Individual HBCD diastereoisomers standards (CIL, Andover, USA) were used for identification and quantification. A HBCD technical mixture (melting point 188–191 °C, Fluka, Buchs, Switzerland) and the technical mixture which was thermally equilibrated at 180 °C for 2 h were used as in-house standards. Pure HBCD diastereoisomers isolated from a technical mixture were obtained from Andreas Gerecke (EMPA, Dübendorf, Switzerland). 2,5-Dibromotoluene (Sigma-Aldrich, Milwaukee, WI) was used as an external standard.

Sample Preparation and Cleanup. The same extracts as those previously prepared for the determination of PBDEs (*23*) were used in this work. Briefly, the homogenized sample was dried with anhydrous sodium sulfate and Soxhlet extracted with hexane/acetone (3:1; *v*/*v*). After lipid determination, the extract was cleaned up on acidified silica and eluted with a mixture of hexane/dichloromethane. After solvent evaporation, the extract was dissolved in *iso*-octane and analyzed by GC/MS for PBDEs (*23*). After analysis, the vials were recapped and stored at −20 °C. For the determination of HBCD diastereoisomers and enantiomers, the solvent was exchanged to acetonitrile (140 μL) and 10 μL of a solution of 2,5-dibromotoluene in acetonitrile (160 μg/mL) was added as external standard.

Separation of HBCD Diastereoisomers. Liquid chromatography was performed using a HTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) and a Surveyor liquid chromatographic pump (ThermoFinnigan, San Jose, CA). Separations were performed on a Symmetry C_{18} (2.1 × 150 mm, 5 μm) column (Waters, Milford, MA). Samples (20 μL) were injected using a mobile phase containing water/methanol/acetonitrile (60/30/10) at a flow rate of 250 μL/min. A linear gradient was used to methanol/acetonitrile (50/50) in 5 min and then the final composition was held for 6 min.

Separation of HBCD Enantiomers. A chiral LC column (4.0 × 200 mm, 5 μm) containing permethylated β-cyclodextrin stationary phase NUCLEODEX beta-PM (Macherey-Nagel GmbH & Co, Düren, Germany) was used. Samples (20 μL) were injected using a mobile phase containing water/methanol/acetonitrile (40/30/30) at a flow rate of 500 μL/min. Initial mobile phase composition was held for 0.5 min followed by a linear gradient to methanol/acetonitrile (30/70) in 8 min and then the final composition was held for 14 min.

MS Detection. A triple-stage quadrupole mass spectrometer TSQ Quantum (Thermo Finnigan) was operated in electrospray ionization negative ion mode using multiple reaction monitoring for $[M-H]^-$ (m/z 640.6) → Br^- (m/z 79.0 and 81.0) with a scan width of 0.8 mass units and scan time of 0.2 s. The first and third quadrupoles were set to unit resolution. Argon of 99.999% purity was used as collision gas at a pressure of 1.2×10^{-3} Torr and the collision energy was set to −17 eV and −21 eV for bromine ions at m/z 79.0 and

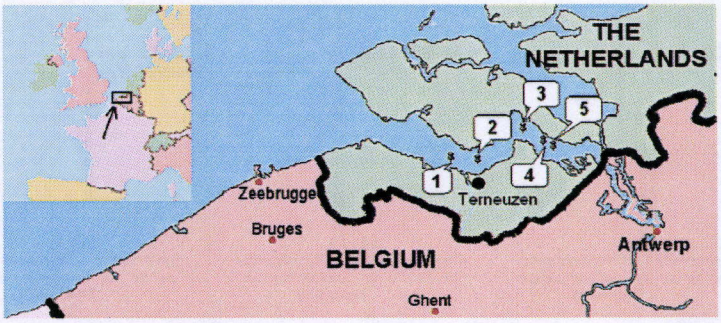

FIGURE 2. Map of the Western Scheldt Estuary showing the sampling locations.

TABLE 1. Concentrations of α-HBCD and γ-HBCD Diastereoisomers in Shrimp and Fish Samples from the Western Scheldt Estuary (ng/g Lipid Weight)

	shrimp		eel		sole				plaice				bib				whiting			
location	whole		muscle		muscle		liver		muscle		liver		muscle		liver		muscle		liver	
lipids (%)	0. 7		26		1. 0		1 3		0.8		34		0.5		54		0.4		30	
	α	γ	α	γ	α	γ	α	γ	α	γ	α	γ	α	γ	α	γ	α	γ	α	γ
1					130	6	150	<1			23	4	97	39	73	5				
2					1100	13	680	<1	38	<2	26	6	53	43	150	5	75	38	170	9
3	28	18	27	3	360	17	110	8					53	<3	100	10	45	<3	16	<3
4	38	<2			110	11	100	<1			21	8					65	51	240	35
5			7	2																

81.0, respectively. Optimization of the MS/MS conditions for γ-HBCD resulted in a spray voltage of 4000 V, sheath gas flow of 60 psi, auxiliary gas flow of 5 psi, capillary temperature of 340 °C, tube lens offset of −69 V, lens zero offset of 1.3 V, and an ion source collision-induced dissociation of 10 V.

Quality Assurance. The quality control was done by repeated injections of solvent blanks (acetonitrile), in-house reference standards (such as a technical HBCD from Fluka, technical HBCD equilibrated at 180 °C), and of standards. The internal standards used for determination of PBDEs by GC (polybrominated biphenyl 103 and ^{13}C-BDE 209) were not used and furthermore were not suitable for the LC conditions. Therefore, for the determination of HBCD diastereoisomers, an external standard was used. This procedure did not allow to correct for losses of HBCD diastereoisomers during the sample preparation. A mean recovery of 90% similar to what has been previously found for hexa-BDEs (*23*) was used for the calculation of HBCD levels. Analytes were positively identified if the absolute retention time agreed with the retention time of the corresponding individual HBCD standard within 2.5% for diastereoisomeric determination and within 1.8% for enantiomer determination. Calibration curves were made from standard solutions at seven (2, 5, 10, 20, 50, 100, and 200 ng diastereomer/mL; $R^2 = 0.993-0.998$) and six (2.5, 5, 10, 20, 50, and 100 ng enantiomer/mL; $R^2 = 0.993-0.995$) concentration levels analyzed in triplicate for the determination of diastereoisomers and enantiomers, respectively. All samples were analyzed at least in duplicate by both reversed-phase LC and chiral LC. The instrumental limit of detection (LOD), defined as 3 times the noise level and based on the LC/MS-MS performance, was 0.5, 1, and 5 pg for γ-HBCD, α-HBCD, and β-HBCD, respectively, while the method limits of quantification of diastereoisomers (LOQ, S/N = 10) were 15, 20, and 75 pg/g wet weight (ww) for γ-HBCD, α-HBCD, and β-HBCD, respectively. None of the analytes were detected in solvent blanks.

Enantiomer Fraction. The enantiomeric composition was expressed as enantiomer fractions (EFs) (*20*) calculated from the peak areas of the enantiomeric pairs by the following formula:

$$EF = \frac{(+)A}{(-)A + (+)A}$$

According to Heeb et al. (*4*), (−) α, (−) β, and (+) γ are first eluting peaks from each corresponding enantiomer pair. Enantiomer fractions were reported as not estimated (n.e.) when the concentration of corresponding diastereoisomers was lower than the LOQ.

Results and Discussion

Despite the increasing interest in HBCD, data on environmental occurrence of HBCD are still scarce. There is a need for more environmental and human monitoring, together with the investigation of exposure pathways and levels and with an imperious demand for data on the individual HBCD isomers. In the present work, we stress an extra dimension that it might be necessary to estimate the exposure and effects based not only on HBCD diastereoisomers but also on HBCD enantiomers.

Separation of Isomers. Several stationary phases were tested for the separation of the three HBCD diastereoisomers, C18 (three types), C8, phenyl, amide, and ether modified silica. A baseline separation was achieved using Waters' Symmetry C_{18} (Figure 3). All three diastereoisomers may be separated using any ratio of methanol/acetonitrile as mobile phase. An increase in the percentage of acetonitrile in the mobile phase resulted in a slightly better separation of HBCD diastereoisomers, mainly between β-HBCD and γ-HBCD. However, in this case, a lower sensitivity for α-HBCD was observed while maintaining a similar sensitivity for γ-HBCD. In contrast, baseline separation of HBCD enantiomers (Figure 4) on the chiral permethylated cyclodextrin column could be achieved only with the mobile phase composition given in the Experimental Section.

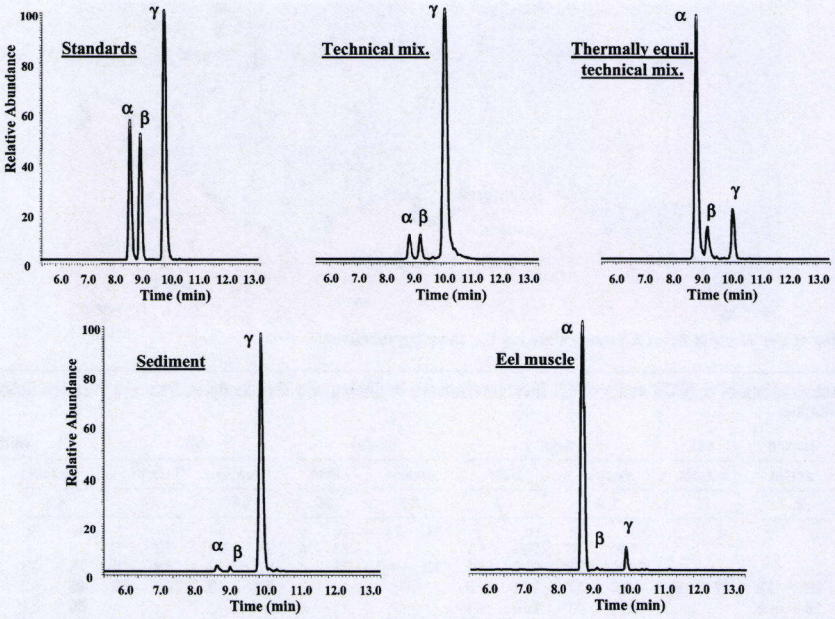

FIGURE 3. Chromatographic separation of HBCD diastereoisomers in standard mixture and biotic and abiotic samples.

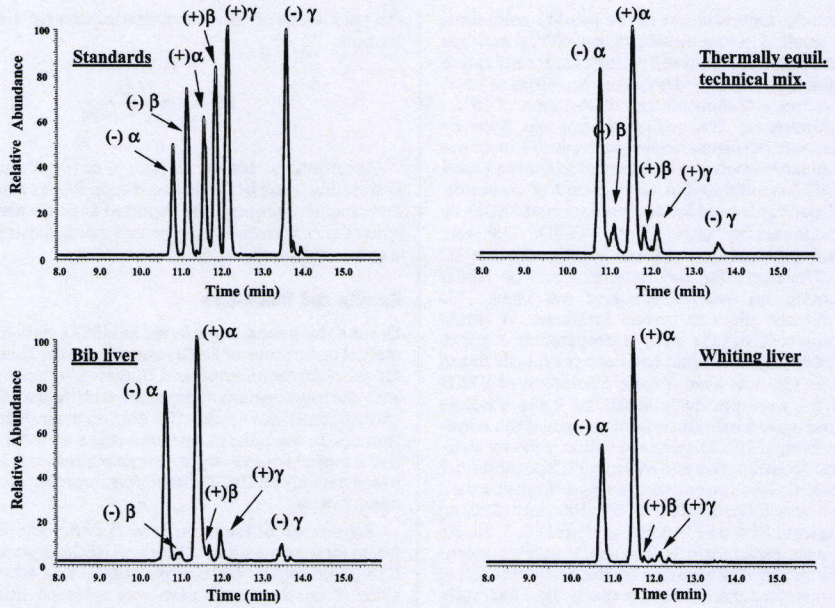

FIGURE 4. Chromatographic separation of HBCD enantiomers in a standard mixture and biotic and abiotic samples. Assignment of optical rotation according to Heeb et al. (4).

Levels of HBCD Diastereoisomers. α-HBCD could be quantified in all 26 samples, while γ-HBCD was detected in 73% and quantified in 35% of all samples. β-HBCD could be detected at low levels in only 25% of the samples and could be quantified only in two bib liver samples (1.6 and 2.2 ng/g lipid weight) and one eel muscle sample (3.4 ng/g lw). The concentrations of individual HBCD diastereoisomers in marine biota from the WSE are summarized in Table 1. Previously, high concentrations of PBDEs have been measured in fish samples and sediments from the WSE (*23*, *24*) and were related to uses of PBDEs in various industries located in the Scheldt Basin. Because of the presence of a HBCD production plant (Broomchemie; 7500 tons HBCD/year) in Terneuzen, The Netherlands (Figure 2), it was suspected that HBCD may be present in fish samples at relatively high levels. In fact, HBCD levels found in fish from

TABLE 2. HBCD Concentrations in Marine Biota as Reported in Literature

species	location	α-HBCD (ng/g)	γ-HBCD (ng/g)	total-HBCD (ng/g)	reference
rainbow trout	Lake Ontario	0.4–3.8[a,b]	0.1–0.8[a,b]	0.5–4.6[a,b]	25
whitefish	Swiss lakes	54–210[b,c]	<15[b,c]	25–210[c,d]	26
whitefish	Lake Winnipeg	1.7[a,b]	1.3[a,b]	3[a,b]	27
fish	Lake Winnipeg			92[b,c]	18
barbel	Cinca River, Spain			nd–1172[a,d]	28
trout	River Skerne, U.K.			20–26[a,b]	29
herring	Baltic sea			34–180[c,d]	30
herring	North sea			21–58[c,d]	30
Baltic salmon	Baltic sea			51[c,d]	30
eel, cod, hake	The Netherlands			<0.6–93[b,c]	31
seastar	Western Scheldt			<30–84[b,c]	31
marine fish	Western Scheldt	6.6–1096[b,c]	<1–51[b,c]	9–1110[b,c]	present study

[a] Wet weight. [b] Determined by LC/MS. [c] Lipid weight. [d] Determined by GC/MS.

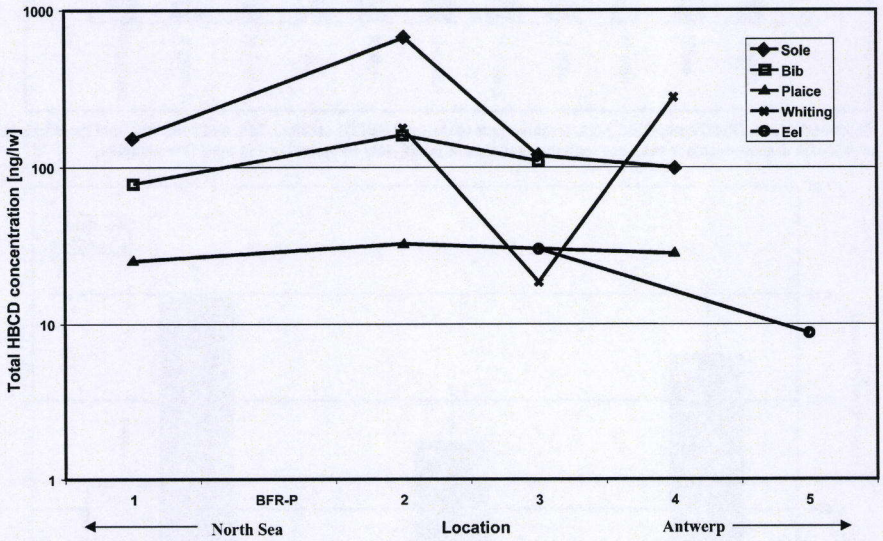

FIGURE 5. Total HBCD levels in liver of sole, bib, plaice, and whiting and in muscle of eel sampled at different locations of the Western Scheldt. BFR-P denotes the location of the BFR production plant (Terneuzen, The Netherlands).

the WSE were higher than levels found in fish samples from locations with no potential sources of HBCD, representing mainly background contamination (Table 2). Much higher HBCD levels (up to 10 000 ng/g ww) have been found in fish sampled near point sources of pollution in the United Kingdom (*29*) and in Sweden (*32*). However, because of the high water volume of the WSE and the high tidal flow, the actual HBCD levels in fish decreased with distance from Terneuzen (Figure 5), with the exception of whiting probably because of its more migratory character. Location 2 situated near the potential HBCD point source exhibited the highest HBCD levels in three out of four fish species. For fish species caught at the same location, the highest levels of total HBCD were found in sole (Table 1). In general, concentrations of HBCD in fish were about 5–10 times lower than those of PBDEs. Similar differences between the HBCD and PBDEs levels have been found by Gerecke et al. in whitefish from six Swiss lakes (*26*). No statistically significant correlations between total HBCD and PBDE concentrations were found at any location with the exception of location 4, for which a high positive correlation ($r^2 = 0.97$, $p < 0.01$) was obtained. Location 4 is situated far enough from the potential point source, so that the HBCD levels in fish from this location are not affected by potential point source and may be considered as background level.

Since the first diastereoisomer-specific analysis of HBCD in biota (*26, 29*), it was recognized that the pattern of HBCD diastereoisomers found in environmental samples may differ from those found in technical products (*6*). The diastereoisomer profile of HBCD in sediment samples was reported to be similar to that of commercial formulations (*17, 33, 34*). Typically, as in Detroit River suspended sediments, the γ-isomer was the dominant diastereoisomer in most samples (*33*). While in sediments from the Drammen River, Norway, α-HBCD had comparable or higher levels than γ-HBCD (*34*), in fish (brown trout, perch, cod, flounder, eel) caught in the same area, only α-HBCD (5–22 ng/g lw) could be measured (*34*). Similarly, we have found γ-HBCD to be the dominant isomer in sediment from the WSE (Figure 6), while α-HBCD and β-HBCD were present at much lower levels.

Recently, it was found that the ratio among the HBCD diastereoisomers might differ for the same species in different environments. While levels of α-HBCD were consistently higher than γ-HBCD in whitefish from Lake Ontario (*25*), concentrations of α-HBCD and γ-HBCD were similar in the same fish species from Lake Winnipeg (*27*). Moreover, in other species (walleye and burbot), γ-HBCD was at least twice as high as α-HBCD, while β-HBCD was consistently detected in all analyzed fishes (*27*). In whitefish and sauger, the levels of α-HBCD and γ-HBCD diastereoisomers were comparable,

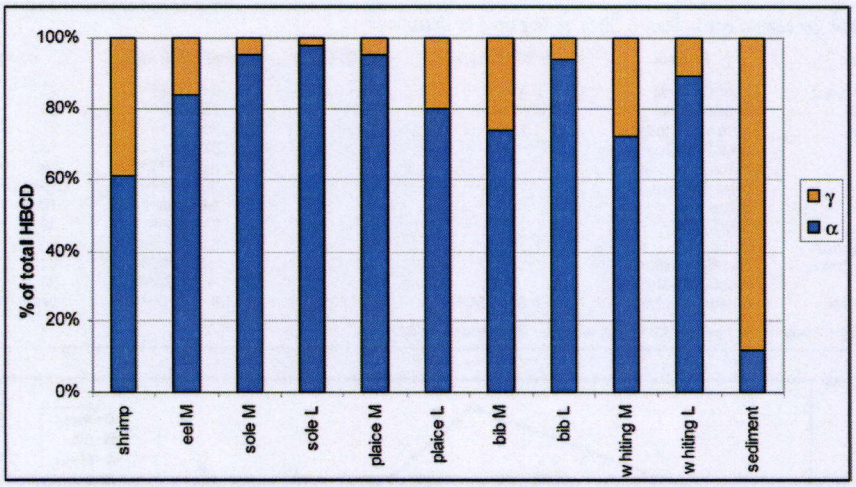

FIGURE 6. Contribution of α-HBCD and γ-HBCD diastereoisomers to the total HBCD in shrimp, fish, and sediment from the Western Scheldt Estuary. The β-HBCD diastereoisomer was not included because it could only be quantified in very few samples.

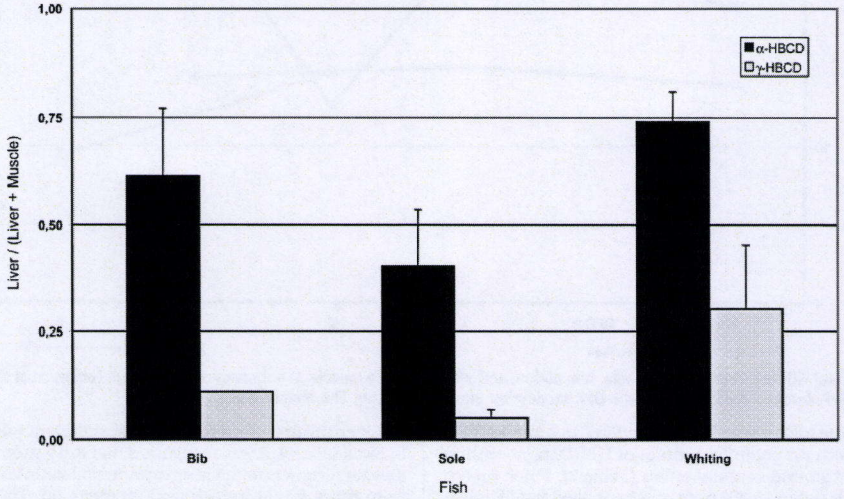

FIGURE 7. Liver accumulation ratio for α-HBCD and γ-HBCD in different fish species; ratios higher than 0.50 indicate preferential liver accumulation.

while in plankton and goldeye, α-HBCD was the dominant isomer, similar to what was observed in the present study. The present knowledge is too limited to point out the reason for such differences in the same fish species and in similar environments.

Accumulation of HBCD Diastereoisomers in Different Fish Tissues. The dominance of α-HBCD over γ-HBCD in all fish species, in both muscle and liver, is demonstrated in Figure 6. Except for plaice, for which we had just one pair of liver and muscle, the ratio of α- to γ-HBCD is larger in liver than in muscle, with sole liver exhibiting total dominance of α-HBCD over γ-HBCD. It seems that α-HBCD is less metabolized in these fish than γ-HBCD.

To express the preferential tissue accumulation of a particular HBCD diastereoisomer, the concentration per lipid weight in liver was divided by the sum of concentrations in liver and muscle. For all species, γ-HBCD accumulated much less in liver than in muscle, that is, the ratio was below 0.5. For bib and whiting, a preferential accumulation in liver was observed for α-HBCD, while sole accumulated slightly more α-HBCD in muscle (Figure 7). Plaice samples were too limited in number to evaluate this species. It can therefore be presumed that γ-HBCD is more easily metabolized in liver and that the metabolizing capacity for HBCD decreases from sole to bib and whiting. This is supported by a recent report which suggests that α-HBCD is much more resistant to biotransformation by liver microsomes than β- and γ-HBCD (*14*). However, a possible bioisomerization of γ-HBCD to α-HBCD in liver cannot be excluded (*10*). A similar preferential liver accumulation as found here for α-HBCD in bib and whiting and preferential muscle accumulation for α-HBCD in sole has also been observed for PBDEs (*23*).

Enantiomer Fractions of HBCD Diastereoisomers. By definition, the EF value for a racemic mixture is 0.50. However, EFs of individual HBCD diastereoisomers isolated from a HBCD technical mixture were different from the EF of a

TABLE 3. Enantiomer Fractions for HBCD Diastereoisomers in Extracts from Different Fish Species from the Western Scheldt Estuary

species	location	ΣHBCD (ng/g lw)	α-HBCD		β-HBCD* [a]		γ-HBCD*	
			EF	RSD	EF	RSD	EF	RSD
bib liver pool 1	1	78	0.59	0.02	0.38		0.63	0.01
bib liver pool 2	2	158	0.56	0.01	0.41	0.07	0.60	0.09
bib liver pool 3	3	112	0.59	0.01	n.e.[b]		0.77	0.02
bib liver			**0.58**	**0.02**	**0.39**		**0.67**	**0.09**
sole liver pool 1	1	153	0.46	0.03	n.e.		n.e.	
sole liver pool 2	2	679	0.46	0.04	n.e.		n.e.	
sole liver pool 3	3	119	0.38*	0.06	n.e.		n.e.	
sole liver			**0.43**	**0.05**	**n.e.**		**n.e.**	
sole muscle pool 1	1	139	0.40	0.03	n.e.		n.e.	
sole muscle pool 2	2	1110	0.43	0.01	n.e.		n.e.	
sole muscle			**0.42**	**0.03**	**n.e.**		**n.e.**	
whiting liver pool	2	178	0.65	0.01	n.e.		0.95	0.02
whiting liver indiv	3	19	0.76*	0.19	n.e.		n.e.	
whiting liver indiv	4	279	0.68*	0.06	n.e.		n.e.	
whiting liver			**0.70**	**0.06**	**n.e.**		**0.95**	**0.02**
eel muscle	3	30	**0.54**	**0.01**	**n.e.**		**n.e.**	

[a] Asterisk (*) denotes indicative values. [b] Not estimated because of low response.

racemate, for example, EF = 0.54 ± 0.06 for α-HBCD. Similarly, the analysis of commercially available individual HBCD diastereoisomers resulted in EFs higher than 0.50. A concentration dependence of the deviation from the racemic value was observed for α-HBCD (EF = 0.51 ± 0.02, 0.54 ± 0.02, and 0.55 ± 0.02 for concentrations of 100, 50, and 25 ng/mL, respectively), but this was less obvious for β-HBCD and could not be seen for γ-HBCD. The deviation of the EFs from 0.50 could be due to a nonracemic composition of standards deriving from potentially enantioselective treatments during preparation and purification. On the other hand, the enantiomers eluting under slightly different conditions may show differential mass spectrometric response.

Because the analysis of purified racemic HBCD diastereoisomers from the different sources (EMPA, CIL) resulted in similar deviations of EFs, it was concluded that the deviations in EFs are most likely due to unequal response of the enantiomers. It is known that LC/MS is sensitive to matrix effects and that ionization may vary with respect to the sample composition. Further, ionization conditions may be different for the same compound peaks eluting sequentially out of the column into the ion source. Although EFs of standards were found slightly different from racemic composition, the observed deviation was not statistically significant and the RSDs observed for the triplicate analysis of each extract were higher than the apparent shift. Therefore, we present the EFs as they were calculated from peak areas without any correction. We have also found that the precision of EF determination dropped with decreasing analyte concentration (RSD > 10% for 10 ng/mL, 0.2 ng injected) for all diastereoisomers. At concentrations below 5 ng/mL (0.1 ng injected), the high RSDs observed (>25%) resulted in a false average of EFs. Therefore, EFs are reported only for samples for which the injected amount of HBCD diastereoisomers was higher than 0.5 ng.

Because of a lower sensitivity of the enantiomer separation method and the fact that the same diastereoisomer is divided into two peaks, EFs could be calculated only for samples with concentrations of HBCD diastereoisomers above the LOQ. To reduce the uncertainty of the results, only peak areas above 7000 units were included in the calculation of EFs. The EFs for fish samples are presented in Table 3. Excluding plaice, EFs for α-HBCD could be determined in most liver samples, in two sole muscle composites, and in one eel muscle. For bib and especially for whiting liver, a strong enrichment of the (+) α-enantiomer was observed. Sole was the only species for which we were able to determine the enantiomeric accumulation in both liver and muscle. It seems that enantiomer fractions are similar for liver and muscle, with a slight enrichment of (−) α- enantiomer (Table 3). For eel muscle, no enantioselective accumulation of α-HBCD was observed. Both β-HBCD and γ-HBCD concentrations were too low to calculate EFs with sufficient precision. EFs given for β-HBCD and γ-HBCD in bib liver and γ-HBCD in whiting liver are indicative values and subjected to relatively large uncertainty. Nevertheless, it seems consistent that EFs were <0.5 for β-HBCD and >0.5 for γ-HBCD. The toxicological significance for the enantioselective enrichment of HBCD diastereoisomers is so far not known. The EF often depends on the pollutant level in the organism; a higher pollutant level will often result in elevated biotransformation rates and thus in EFs different from 0.50 (*21*). However, a slightly opposite trend is indicated by our data (Table 3). An explanation could lie in the rather low metabolic capacity of fish (*9, 10*). At a high HBCD concentration, it is possible that fish are not able to adequately metabolize the HBCD intake and the enantiomeric composition might approach racemic values.

In fish samples with high HBCD levels, a small additional peak eluting just after the first (+) γ-enantiomer was frequently detected, while in some samples, an additional peak eluted just before (−) γ-enantiomer from which it was often not fully separated. Recently, Heeb et al. (*4*) reported on two formerly unknown HBCD stereoisomers isolated from the technical product and assigned them as δ- and ε-HBCD. However, lacking the corresponding standards, it was not possible to confirm the identity of these additional peaks.

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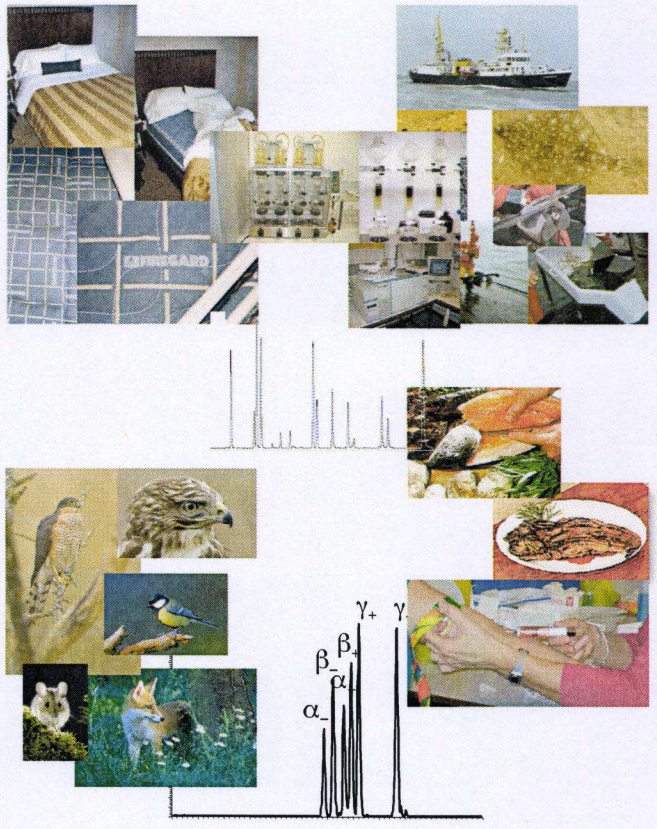
Summary and conclusions



BFRs are a diverse group of chemical compounds, which are used to slow down or prevent the development of fires. For this purpose, BFRs are incorporated into a wide range of consumer products that are considered to imply potential fire hazards, such as TV-sets, computers, household appliances, textiles and upholstery. Because of their extensive production and use, BFRs are classified as "high volume chemicals". Some of them, such as PBDEs, HBCD, and TBBP-A, have led to both scientific and public concern since they have been found to accumulate in Man and wildlife. Exposure to these compounds occurs daily, mainly through the use of flame-retarded materials (from which they are released), through inhalation of dust (to which they are adsorbed) and via the food chain (in which they have accumulated). BFRs have been linked to adverse physiological effects both *in vitro* and *in vivo*. Interference in normal biological processes, such as neurobehavioural development, foetal development (toxicity/teratogenicity), and hormone homeostasis, is suspected. Adequate data on biological effects in man are currently still insufficient to fully understand the toxicology of these chemicals and possible effects can only be deducted from results obtained with laboratory animals.

This thesis presents analytical method development and measurements of various BFRs in a wide range of environmental samples. Since concentrations of BFRs are low and since environmental pollution is not only restricted to these compounds, analysing these chemicals in complex environmental samples is a challenging task. The environmental study consists of several steps such as a correct definition of the environmental and analytical problems, a well-designed sample selection and sampling strategy, selection of analytes, and reproducible and accurate chemical analysis, followed by data evaluation.

At the start of this PhD-work, environmental distribution of BFRs was not yet studied in Belgium. This thesis aimed to provide more insight into the environmental distribution of BFRs and into the analytical aspects involved. For this purpose, analytical methods were developed, starting from existing methodology that was already in use for other persistent organic pollutants, such as PCBs. Principal analysis steps, such as extraction, clean-up and instrumental analysis were optimised for PBDEs in each sample matrix. Since QA/QC of (chemical) analysis has become increasingly important, all methods were validated through successful participation in internationally organised inter-laboratory exercises. Thereafter, these methods were applied to a wide variety of environmental samples that are environmentally relevant and representative for specific ecosystems.

In the first part of this thesis, the presence of PBDEs was investigated in aquatic environments. Sediments and biological samples were investigated from the BNS, the SE and selected Flemish inland freshwater watercourses. The selection of these locations was based upon the presence of suspected point sources of PBDEs pollution, such as: a brominated flame retardant manufacturing plant (Terneuzen), the Antwerp harbour, and the textile industry located in Flanders. The BNS was included in this study to analyse the influence of

the SE on the levels found in sediments and biota from the BNS locations. Sediments from the BNS, SE and freshwater watercourses from the Scheldt basin were analysed for PBDEs. Results of the PBDE analyses in the sediments were below LOQ for nearly all samples from the BNS (except for BDE 209), while in those from the SE, PBDE concentrations were higher but could still be considered low. BDE 209, a controversial BFR due to the producer's claim of being non-bioavailable and therefore biologically harmless, could be detected in more than 80 % of the samples from the BNS and in all the samples from the SE. Compared to the marine and estuarine locations, the sediments from the freshwater watercourses were relatively more polluted with the lower brominated PBDEs. Also in these sediments, BDE 209 was present. However, the contribution of BDE 209 to the total amount of PBDEs was varying much more at the freshwater locations than in the SE, which suggests a different input of pollutants. Another study, based on sediment cores, revealed that the majority of PBDEs was present in the top layer of the sediment. This showed that environmental levels of these pollutants were on the rise in the Belgian environment only during the last decade(s). Further, this means that the commonly applied sediment sampling technique, done by a Van Veen grabber, which samples up to 20 cm depth, results in an underestimation of concentrations due to dilution of the top-layer. This also means that this technique may therefore not be the most suited for the assessment of PBDE concentrations in sediments.

Benthic invertebrates, such as shrimp, crab and starfish, benthic fish, such as goby, dab, plaice, and sole, and gadoid fish, such as bib and whiting, were sampled in the BNS and the SE. The locations that were sampled in the BNS are commercial fishing grounds and all sampled species, except starfish and goby, are suited for human consumption. PBDEs were analysed to determine the concentrations and spatial variation. Levels found in the SE samples were up to 30 times higher than those found in BNS samples, with a gradient increasing towards Antwerp. BDE 209 could be detected in 8 liver samples, which contradicts the industry's claim of non-bioavailability. Some species, such as dab, plaice, bib and whiting, showed preferential accumulation of PBDEs in the liver. Furthermore, higher brominated congeners in general showed higher affinity for liver than for muscle tissue. This tissue dependent distribution may have implications for toxicity.

PBDE profiles observed in biological samples were not matching the profiles of the sediments. Although present in the sediments, BDE 183 could not be quantified in biota. Also BDE 209 could only be measured in eight samples, while this congener was often the most abundant one in the sediments. This is most likely related to congener specific uptake and metabilisation differences.

In the second part of this thesis, the presence of PBDEs was investigated in terrestrial environments. Biological samples of representative wildlife avian and mammalian top-predators, such as birds of prey and foxes, were investigated for PBDEs. Concentrations and tissue distribution of PBDEs were examined in various tissues of buzzard and sparrowhawk.

PBDE levels were significantly higher than those seen in the aquatic environment. Further, the tissue levels in sparrowhawks were in general one order of magnitude higher than in the tissues of common buzzards. This was expected and could be attributed to the birds' feeding habits. The PBDE distribution between various organs was not only lipid-content dependant, suggesting that active transport or blockage mechanisms are present in certain organs, i.e. PBDE levels in the brain, a fatty tissue, were unexpectedly low. BDE 209 was detected in nearly all serum and in some liver samples, but not in any other bird tissues. This observation suggests that exposure to BDE 209 is low or that this congener is poorly accumulated, as was seen in the aquatic samples. However, these data are convincing evidence that BDE 209 is bioavailable and accumulates in animals.

Also terrestrial mammals were investigated. Various tissues of the terrestrial top predator red fox were analysed for PBDEs. The concentrations of the lower brominated PBDEs (up to BDE 183) were low, but BDE 209 could be determined at relatively high concentrations and was even the most abundant congener whenever it was present above the LOQ. In general, this congener was detected in around 40 % of all samples analysed. Such congener profile, with BDE 209 as the dominant congener, had only once been reported earlier, namely in Grizzly bears. The distinct PBDE pollution profile and low levels of lower brominated PBDEs as seen in the fox was ascribed to highly developed metabolic capacities in this species. The breakdown products that are formed are yet unknown, but they are undoubtedly of interest seeing their suspected hormonal homeostatic disruption capacity. This data on BDE 209 again confirms unambiguously that this congener does bioaccumulate in terrestrial top predators, such as the red fox.

Samples of avian and mammalian prey species were also investigated. Concentrations in all tissues of small rodents could be considered low and in the same range as the levels measured in the foxes. These low levels were explained by the granivorous feeding pattern of these small mammals. Additionally, passerines were also investigated and they were higher contaminated than the small rodents (approximately one order of magnitude). Small rodents are an important part of the diet of buzzard and fox, while passerines are considered the major prey of sparrowhawks. Due to the nature of PBDEs (lipophilic and persistent), they are suspected to biomagnify to a great extent from prey to predator. Our results however showed that this is not always true. Biomagnification could be undoubtedly demonstrated from small rodents to buzzards and from passerines to sparrowhawks, respectively, with BMFs ranging from 2 to 34. However, biomagnification could not be observed for PBDEs in the fox samples (BMF < 1). This was unexpected but in accordance with the high xenobiotic turnover capacity of the fox. These results indicate that foxes, in contrast to what was already proposed for other pollutants, are not suited as biomonitor for PBDE pollution assessment.

It is noteworthy that the PBDE pollution profiles in both aquatic and terrestrial ecosystems differ significantly. In aquatic samples, BDE 47 generally dominates the congener profile (30-

40 % of the total PBDEs), with only little contribution from higher brominated congeners, such as BDE 153. The hepta-brominated congener BDE 183 was never found in the aquatic biota, while it was present in the sediments. In terrestrial samples, higher brominated congeners are more commonly detected; the contribution of BDE 47, 99 and 153 are more or less comparable and BDE 183 is present in the majority of the samples. Whether these differences are related to exposure, uptake, metabolism or excretion is currently not clear.

In the third part of this thesis, PBDEs were investigated in relation to Man. A method for the determination of PBDEs, including BDE 209, in (human) serum was developed. The method was applied to a small sample set. The results were low and within the range of PBDE levels in other non-exposed population from Europe. These low levels in human serum raised interest in possible exposure pathways. A principal route of uptake is assumed to occur through food. Therefore a small study was set up to investigate and assess the human PBDE intake based on the analysis of selected food items for sale at Belgian supermarkets. Representative samples were taken from food most likely to contain the highest levels (fatty foods). Various fish, meat and dairy products were analysed. Concentrations were very much dependent on the nature and origin of the food, with fish products containing the highest levels. In general however, levels in food are very low and comparable to other food products throughout Europe, but concentrations were significantly lower than food from the United States. Preliminary intake assessment based on these data yielded an average daily PBDE intake between 23 and 48 ng, wherefrom it could be concluded that the dietary intake is low. However, additional exposure from (dust) inhalation can be less controlled and therefore this route of exposure can contribute substantially to the total human exposure.

In the fourth and final part of this thesis, the emphasis was placed upon another BFR, namely HBCD. This flame-retarding compound is gaining market share due to the ban of certain BFRs in the EU As a consequence, this isomeric compound is increasingly reported in the environment. Unfortunately, this compound is inherently coupled to analytical difficulties that are sometimes difficult to overcome. Therefore, data on environmental distribution of HBCD was rather scarce until recently. We were able to report for the first time on the enantiomeric composition of HBCD in muscle and liver of several fish species from the SE. The total content of HBCD (sum of α-, β- and γ-diastereoisomers), as well as the distribution of diastereoisomers and enantiomers, varied between the species. The levels of HBCD found in fish tissues were higher than those measured in fish from European rivers with no known point sources of HBCD. The α-HBCD diastereoisomer was most abundant in all fish samples with a higher contribution to the total HBCD levels in liver compared to muscle for bib and whiting. The γ-HBCD diastereoisomer accumulated less in liver than in muscle of sole, bib and whiting. This can have implications for toxicity, but the current toxicity data is inadequate to make any assessments. For the first time, enantiomer fractions were determined for diastereoisomers of HBCD in liver of three fish species and in muscle of two fish species. A

significant enrichment of the (+)α-HBCD enantiomer was found in whiting and bib liver samples. A high enantioselectivity has also been seen for the γ-HBCD diastereoisomer in whiting liver. However, the implications of this diastereoselective behaviour still remain unclear.

Exposure of BFRs to animals and humans will continue as long as these chemicals are a part of our daily life and as long as they are present as contaminants in the surrounding environment. The present scientific knowledge of the potential health risks of these BFRs still needs to be further developed. This aspect was beyond the scope of this thesis, but is fortunately investigated elsewhere. The author hopes that this thesis and the related scientific publications add to the global knowledge on environmental BFR distribution and behaviour, and that these data will be useful for future risk assessments concerning BFRs.

8

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당신을 감사하십시오

Thank you

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9

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