Persistency, bioaccumulation and toxicity assessment of selected brominated flame retardants

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Title

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Abstract

Brominated flame retardants (BFRs) are chemicals used in e.g. electronic equipment, textiles and plastics for the prevention of fire. Over recent decades, concern has been raised regarding some heavily used BFRs, since the levels in the environment have been increasing. In the present thesis, persistent, bioaccumulative, and toxic (PBT) properties were studied for a structurally varied set of BFRs selected to represent more than 60 organic BFRs. The studied BFRs include: 2,4,4'-tribromodiphenyl ether (BDE 28), 2,2',3,4,4',5',6-heptabromodiphenyl ether (BDE 183), decabromodiphenyl ether (BDE 209), hexabromocyclododecane (HBCD), tetrabromobisphenol A (TBBPA), tetrabromobisphenol A 2-hydroxyethyl ether (TBBPA OHEE), 2,4,6-tribromophenol (246BrPh), 1,2-dibromo-4-(1,2-dibromoethyl)cyclohexane (TBECH), and hexabromobenzene (HxBrBz).

It is likely that soil will act as a sink for BFRs. Therefore, studies of BFRs were performed on persistence in soil, and on bioaccumulation from soil in the earthworm *Eisenia fetida*. Large variation in the biodegradability in soil among the tested BFRs was observed. The studied brominated diphenyl ethers (BDE 28 and BDE 209) were very persistent under both aerobic and anaerobic conditions, while 246BrPh and TBECH degraded quickly in both aerobic and anaerobic soil. The bioaccumulation in earthworm from soil was high for HxBrBz, TBECH and for tested brominated diphenyl ethers with 4-6 bromine atoms (BDE 47, BDE 99, and BDE 153). Bioaccumulation was also studied in zebrafish after dietary exposure to a mixture of BFRs. It was shown that several metabolites were formed and retained in zebrafish, which highlights the importance of also searching for and identifying persistent degradation products. Maternal transfer was shown for all BFRs present in the female zebrafish. This shows that zebrafish young (fry) are exposed to these BFRs at approximately the same concentrations as female zebrafish during the early-life stages, when fish are usually most sensitive to organic contaminants. Toxicity of individual BFRs and a BFR mixture was studied in *Nitocra spinipes* using a silica gel-based system. Highest toxicities were observed for BDE 28, TBBPA, and TBBPA OHEE. In the mixture toxicity study, simultaneous exposure to low concentrations (individually causing no significant effect) of six BFRs significantly affected the survival of *Nitocra spinipes.* The results from the PBT studies presented in the thesis and literature data were compared with the criteria for PBT classification, as set in the European REACH legislation. Further, some BFRs with physico-chemical properties similar to those of identified PBTs were suggested to be prioritized for future PBT testing.

Keywords

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List of papers

This thesis includes the following papers, which are referred to in the text by their respective Roman numerals (Paper I-V). The thesis also includes some additional unpublished results.

I. **Nyholm JR**, Lundberg C, Andersson PL. Biodegradation of selected brominated flame retardants in aerobic and anaerobic soil. Manuscript submitted for publication, 2009.

II. **Nyholm JR**, Asamoah RK, van der Wal, L, Danielsson C, Andersson PL. Accumulation of polybrominated diphenyl ethers, hexabromobenzene and 1,2-dibromo-4-(1,2-dibromoethyl)cyclohexane (TBECH) in earthworm (*Eisenia fetida*) – effects of soil type and ageing. Manuscript, 2009.

III. **Nyholm JR**, Norman A, Norrgren L, Haglund P, Andersson PL. Maternal transfer of brominated flame retardants in zebrafish (*Danio rerio*). Chemosphere 2008, 73: 203-208.

IV. **Nyholm JR**, Norman A, Norrgren L, Haglund P, Andersson PL. Uptake and biotransformation of structurally diverse brominated flame retardants in zebrafish (*Danio rerio*) after dietary exposure. Environmental Toxicology and Chemistry 2009, 28: 1035-1042.

V. Breitholtz M, **Nyholm JR**, Karlsson J, Andersson PL. Are individual NOEC levels safe for mixtures? A study on mixture toxicity of brominated flame-retardants in the copepod *Nitocra spinipes*. Chemosphere 2008, 72: 1242-1249.

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Abbreviations

246BrPh	2,4,6-tribromophenol
246ClPh	2,4,6-trichlorophenol
2BrSty	2-bromostyrene
B	bioaccumulative
BDE	bromodiphenyl ether
BDE 28	2,4,4'-tribromodiphenyl ether
BDE 47	2,2',4,4'-tetrabromodiphenyl ether
BDE 99	2,2',4,4',5-pentabromodiphenyl ether
BDE 153	2,2',4,4',5,5'-hexabromodiphenyl ether
BDE 183	2,2',3,4,4',5',6-heptabromodiphenyl ether
BDE 209	decabromodiphenyl ether
BFR	brominated flame retardant
BCF	bioconcentration factor
BSAF	biota-soil accumulation factor
BMF	biomagnification factor
C	concentration
CAS	chemical abstract service
CDE 28	2,4,4'-trichlorodiphenyl ether
CMR	carcinogenic, mutagenic or toxic to reproduction
DE	diphenyl ether
DecaBDE	technical product containing mainly BDE 209
dw	dry weight
ECNI	electron capture negative ionization
EI	electron ionization
EINECS	European inventory of existing commercial chemical substances
ELINCS	European list of notified chemical substances
GC	gas chromatography
GPC	gel permeation chromatography
HBCD	hexabromocyclododecane
HPVC	high production volume chemical
HxBrBz	hexabromobenzene
HxClBz	hexachlorobenzene
LC50	the concentration that is lethal to 50% of the test organisms
LDR	larvae development ratio
Log K_{ow}	logarithm of the octanol-water partitioning coefficient
LPVC	low production volume chemical
MS	mass spectrometry
MW	molecular weight

m/z	mass to charge ratio
NOEC	no observed effect concentration
OECD	Organisation of economic co-operation and development
octaBDE-mix	technical product containing hexa- to decabrominated diphenyl ethers
P	persistent
PCB	polychlorinated biphenyl
PCB 153	2,2',4,4',5,5'-hexachlorobiphenyl
PBDE	polybrominated diphenyl ether
PBB	polybrominated biphenyl
PBT	persistent, bioaccumulative, and toxic
PCA	principal component analysis
PCDD	polychlorinated dibenzo-p-dioxins
pentaBDE-mix	technical product containing tri- to hexabrominated diphenyl ethers
PLE	pressurized liquid extraction
POP	persistent organic pollutant
QSAR	quantitative structure-activity relationship
QSPR	quantitative structure-property relationship
RoHS	restriction of the use of certain hazardous substances
REACH	registration, evaluation, authorization, and restriction of chemicals
SAR	structure-activity relationship
SIM	single ion monitoring
S_w	water solubility
T	toxic
TBBPA	tetrabromobisphenol A
TBBPA OHEE	tetrabromobisphenol A 2-hydroxyethyl ether
TBBPA DBPE	tetrabromobisphenol A 2,3-dibromopropyl ether
TBECH	1,2-dibromo-4-(1,2-dibromoethyl)cyclohexane
TMF	trophic magnification factor
UNEP	United Nations environmental programme
V_p	vapor pressure
vPvB	very persistent and very bioaccumulative
w	weight
WHO	World Health Organisation

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1. Introduction

One of Sweden's sixteen environment quality objectives is a non-toxic environment. Today, there are, however, elevated concentrations of many industrial chemicals, metals, pesticides, and pharmaceuticals in the environment. The long-term goal is that, in one generation from now, the environment should be free from man-made compounds posing a threat to the environment or humans, and that the levels of toxic compounds or metals should be close to background levels. A number of actions have been and will be undertaken to approach the goal, e.g. remediation of polluted sites, reduction of emissions from incineration, improvements in the techniques for waste water and sewage treatment, and restriction of production of hazardous chemicals. However, in 2008, the Swedish Environmental Objective Council came to the conclusion that the non-toxic environment objective is difficult or even impossible to achieve within the one generation time-frame (Swedish Environmental Objective Council 2008). Chemicals are transported in the oceans and in the atmosphere, and products containing chemicals are transported all over the world. Therefore international actions are needed to reach a less contaminated environment.

In order to identify hazardous chemicals for regulatory purposes, knowledge of their hazardous properties is obviously needed. Currently, there are major gaps in knowledge on toxicity and environmental fate for a substantial number of the chemicals on the market. All industrial chemicals that were on the European market between 1971 and 1981 are included in the European Inventory of Existing Commercial Chemical Substances (EINECS) list, and chemicals introduced to the market after 1981 are listed in the European List of Notified Chemical Substances (ELINCS). There are more than 100 000 chemicals listed in EINECS. The implementation of the new European chemical legislation Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) will lead to the generation of new data on the industrial chemicals in use. According to REACH regulations, chemicals that are produced or imported in quantities higher than one tonne per year per distributor, need to be registered. Data on chemical and toxicological properties have to be provided by the chemical industry. With increasing production volumes, more comprehensive data on toxicological effects and environmental fate will be required. REACH will be fully implemented by 2018.

Chemicals that are carcinogenic, mutagenic, or toxic to reproduction (CMR) are examples of compounds that will be restricted under the REACH legislation, because of their potential to cause cancer and induce endocrine effects in wildlife and humans. Persistent organic pollutants (POPs) are compounds that

will be restricted under REACH, because of their persistent, bioaccumulative, and toxic (PBT) properties. POPs can easily be transported long distances from their source, accumulate in the food chains and cause long-term effects on humans and the environment. Persistence is the longevity of a compound in the environment, i.e. high persistence means that the compound degrades very slowly. Bioaccumulation refers to the uptake and retention of a compound in living tissue, usually at levels higher than in the surrounding environment, and can lead to exposure of organisms to high concentrations of potentially toxic compounds. For environmental risk assessment of chemicals, PBT data is needed. Generating these is very expensive, and further, the tests required by the legislation for chemicals produced in low volumes will not be sufficient for classification as a PBT chemical. Thus, in the future, data from predictive models will probably become of more importance for predicting chemical properties and behavior and for prioritization of the most important compounds to test.

1.1. POPs and PBT criteria

Many highly chlorinated organic compounds are known to have PBT properties. The United Nations Environment Program (UNEP) on POPs has identified 12 groups of chlorinated organic compounds for a global ban and also set up criteria for identifying new POPs (UNEP 2001). The names and chemical structures of the 12 POPs can be seen in Figure 1. For classification as a POP, four criteria must be fulfilled: P (persistence), B (bioaccumulation), T (toxicity), and potential for long-range transport. Currently, there are only chlorinated compounds included in the UNEP POPs. However, a few brominated organic compounds have recently been suggested for inclusion (http://chm.pops.int/). In the REACH legislation, a substance can be classified as a PBT or very persistent and very bioaccumulative (vPvB), and the criteria are similar to the criteria in the UNEP POP program (European Union 2006). A substance is identified as a PBT chemical if all three criteria for P, B, and T are fulfilled. The criteria for PBT and vPvB classifications can be seen in Table 1.
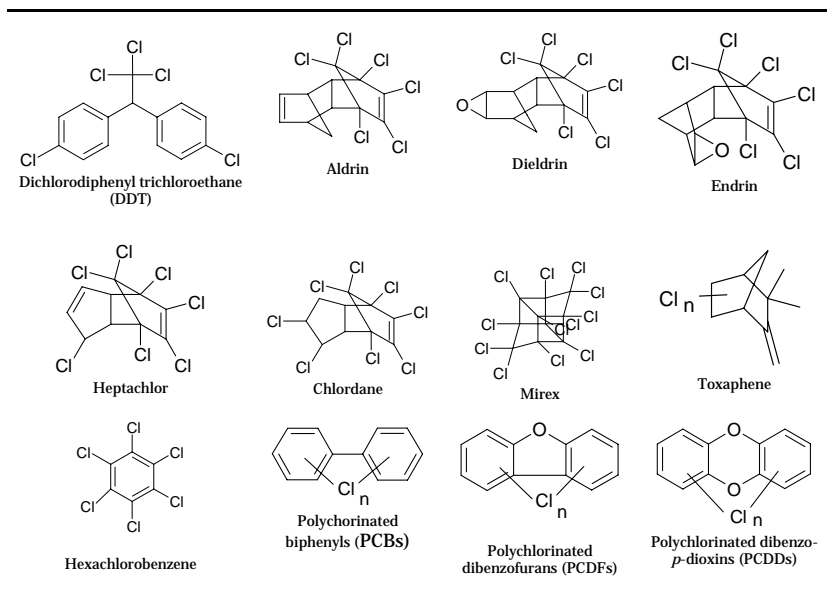
Figure 1. Names and chemical structures of the 12 UNEP POPs.

Table 1. Persistence (P), bioaccumulation (B), and toxicity (T) criteria for classification as a UNEP POP, a PBT and vPvB in the REACH legislation (UNEP 2001, European Union 2006). BCF = bioconcentration factor.

	POP classification according to UNEP	PBT classification according to the REACH legislation	vPvB classification according to the REACH legislation
P criteria	>180 days in soil or sediment or >60 days in water	>120 days in soil or sediment or >60 days in marine water or >40 days in fresh water	>180 days in soil or sediment or >60 days in water
B citeria	BCF >5000 or High accumulation in another species or In absence of measured data, Log Kow >5	BCF >2000	BCF >5000
T criteria	potential for adverse effects on human health or to the environment	NOEC <0.01 mg/l in an aquatic organism or CMR or evidence for chronic toxicity	Not applicable

1.2. Organobromine compounds

Bromine is a halogen element with the atomic number 35. There are two stable isotopes of bromine that exist in almost equal amounts, ^{79}Br and ^{81}Br. In the environment, bromine is mostly found in the form of inorganic salts. The average concentration of bromide in sea water is 65 mg/l. For comparison, the concentration of chloride in sea water is 19000 mg/l (Gribble 1999).

Bromine gas is produced from bromide ions from bromide rich brines or from the Dead Sea. To avoid transportation of the toxic bromine gas, the bromine industry is usually located near the brines. In the 1960s and 70s, the main use of bromine was for gasoline additives. Nowadays, brominated flame retardants (BFRs) are one of the major applications of bromine. Organobromine compounds can also be used as pharmaceuticals, pesticides, and drilling fluids (Alaee et al. 2003). In the EINECS list, there are more than 2000 organobromine compounds registered (Stenberg et al. submitted manuscript). Over past decades, concern has been raised regarding the heavily used BFRs, the polybrominated diphenyl ethers (PBDEs). This is because of their structural resemblance to chlorinated POPs and since PBDE levels have been increasing in the environment recently (de Wit 2002). However, BFRs comprise a structurally diverse group of chemicals, including more than 75 aliphatic, cycloaliphatic, and aromatic compounds (Alaee et al. 2003).

Organobromine compounds are also naturally produced from inorganic bromine by marine organisms, such as sponges, algae, and worms. In 1999, there were more than 1600 known organobromines naturally occurring in marine organisms (Gribble 1999). These naturally occurring organobromine compounds are very diverse in chemical structure, and some of them are very similar or the same as anthropogenic BFRs. Examples of naturally occurring organobromines are bromophenols, methoxylated polybrominated diphenyl ethers, and brominated dioxins (Vetter et al. 2002; Teuten et al. 2005; Vetter and Janussen 2005; Haglund et al. 2007). Some naturally produced organobromines bioaccumulate in the food web and are present in fish, birds and marine mammals. In the marine environment, the levels of the naturally occurring organobromines are often higher than the levels of their anthropogenic counterparts. For example, in liver from two species of deep-sea fish, the amount of the naturally produced polybrominated hexahydroxanthene derivatives accounted for more than 90% of the total amount of measured organobromines (Covaci et al. 2008). The fact that organobromines are naturally produced does not imply that they are harmless; in fact, one of the suggested functions of the naturally produced organobromines is chemical protection against predators. For example, Konig et al. (1999) showed that the naturally produced mixed halogenated monoterpene (MHC-1) was cytotoxic.

Thus, naturally occurring compounds with POP resemblances increase the chemical load in marine biota, which further motivates the restriction of anthropogenic compounds with PBT properties reaching the environment.

1.3. Aim of the thesis

The overall aim of the work underlying this thesis was to determine PBT properties of BFRs and to identify PBTs within the group of BFRs. A second aim was to suggest non-tested compounds for further PBT testing. The underlying assumptions were that BFRs with similar structure and physico-chemical properties have similar PBT properties, and that relationships between chemical structures and PBT properties could be found if selecting and testing BFRs with highly diverse chemical structures.

The aims of the papers included in the thesis were:
- To study the persistence of selected BFRs by determination of half-lives in aerobic and anaerobic soil, and also to study the effect of temperature and application of different kinds of sewage sludge on the biodegradation kinetics in soil (paper I).
- To study the bioaccumulation potential of selected BFRs by determination of Biota-Soil Accumulation factors (BSAFs) of BFRs in earthworms (*Eisenia fetida*), and to study the effect of ageing the soil, and the use of soils with different properties on the accumulation in earthworms (paper II).
- To study the transfer of BFRs from female zebrafish (*Danio rerio*) to their offspring (paper III).
- To study the uptake kinetics of BFRs in zebrafish (*Danio rerio*) (paper IV).
- To study the toxicity of ten selected individual BFRs to the copepod *Nitocra spinipes*, and also the mixture toxicity of selected BFRs (paper V).

2. Brominated Flame Retardants

In ancient Egypt, alum was used to reduce the flammability of wood (about 450 BC), and ever since that time flame retardants have been used in various materials. Today, inorganic compounds, phosphorous containing compounds, nitrogen containing compounds, and halogen containing compounds are used as flame retardants in electronic equipment, textiles and plastics (WHO 1997). Retardants prevent fires and thus save costs and human lives. In the United States, there were over one million reported fires in 2007, resulting in over three thousand civilian deaths (Karter 2008). Even though the number is high, the fatalities in the United States have decreased by about 50% during the last 30 years (Karter 2008). The same trend is seen in Sweden, where the death rate in fires has fallen by about 35% since 1945 to an average 100 fatal fires per year (Swedish chemical inspectorate 2006). The decrease in fatalities could be related to the increased use of flame retardants. However, it could also be explained by e.g. decreased smoking rate, increased use of smoke detectors and increased awareness.

The halogen-based flame retardants include brominated and chlorinated compounds. In BFRs, the bromine atoms are the active components, preventing fire eruptions by capturing free radicals. While bromines are most important for the function of a BFR, the role of the carbon skeleton is to ensure the stability of the compound and the compatibility with the material where it is to be used (Alaee et al. 2003; Birnbaum and Staskal 2004). BFRs are mainly used in electronic equipment, but also in textiles, carpets, and paint. For example, in a computer, BFRs could be used in the printed circuit boards, in the plastic cover, in cables, and in connectors (D'Silva et al. 2004). However, several reports state that the use of halogenated flame retardants could be substantially reduced without decreasing fire safety (e.g. Swedish Rescue Service Agency 2002; Swedish chemical inspectorate 2005; Swedish chemical inspectorate 2004).

Firemaster, a commercial BFR containing mainly hexabrominated biphenyls, was produced in large quantities in the early 70s. However, the production was stopped in 1974 in the United States after an incident in Michigan, when Firemaster was accidentally mixed into animal feed. The mistake was discovered after severe effects (such as reduction in feed consumption, abnormal hoof growth, lameness and death) were observed among the exposed animals (WHO 1994). The production of octa- and decabrominated biphenyls was stopped in 1979 in the United States (WHO 1994). Another group of heavily used BFRs are the PBDEs that have been produced in three technical mixtures: the pentaBDE-mix, the octaBDE-mix and decaBDE. Concern about

the PBDEs has grown over the last ten years, as studies showed that levels of PBDEs in the environment increased rapidly between the 1970s and the 1990s. Some of the lower brominated PBDEs were also found to biomagnify in the food web (de Wit 2002). As a result, the manufacture of penta- and octa-brominated diphenyl ethers was voluntarily ended in 2004 in the United States (Cleet 2004). In the European Union, the use of PBDEs and polybrominated biphenyls (PBBs) in electronic equipment has been restricted since 2006 under the RoHS ('Restriction of the use of certain Hazardous Substances') directive (Directive 2002/95/EC). DecaBDE was at first exempted from the restriction, but since 2008 it also under restriction (www.rohs.gov.uk).

The BFRs can be monomers, additives or reactive. Brominated styrenes and butadienes are examples of monomers, which are incorporated into polymers. PBDEs and hexabromocyclododecane (HBCD) are examples of additive BFRs and tetrabromobisphenol A (TBBPA) is a reactive BFR. HBCD and TBBPA are also among the most widely produced BFRs. The total market demand in 2003 for decaBDE, TBBPA, and HBCD according to Bromine Science and Environmental Forum (BSEF) was 56418, 145113, and 21951 tonnes, respectively (Andersson et al. 2006). Current production volumes are not publicly available.

2.1. Selection of a training set

BFRs comprise structurally diverse compounds with broad range of physico-chemical properties. Therefore, the environmental fate and toxicity between compounds included in the group of BFRs can be very different. Toxicological and fate testing is very time-consuming and costly, and therefore quantitative structure-activity relationships (QSARs), quantitative structure-property relationships (QSPRs) or structure-activity relationships (SARs) can be used to predict these properties or to prioritize which compounds to test. A QSA/PR model is a mathematical correlation between molecular descriptors and the biological activities or physico-chemical properties of the compounds.

Andersson et al. (2006) identified 65 different brominated organic compounds that are or have been used as brominated flame retardants. Their aim was to get an overview of the commercially used BFRs, chemically characterize them, and to select compounds that are representatives for the different groups of BFRs. A set of ten BFRs was selected that could be used in persistence, bioaccumulation and toxicity screening, or as a training set for building QSAR or SAR models for prediction of untested BFRs. The set was designed to cover the range of physico-chemical properties and molecular variation spanned by the group of 65 BFRs, but also commercial availability, production volumes

and environmental relevance were considered. The ten selected compounds were: 2,4,4'-tribromodiphenyl ether (BDE 28), 2,2',3,4,4',5',6-heptabromodiphenyl ether (BDE 183), decabromodiphenyl ether (BDE 209), HBCD, TBBPA, tetrabromobisphenol A, 2-hydroxyethyl ether (TBBPA OHEE), 2-bromostyrene (2BrSty), 2,4,6-tribromophenol (246BrPh), 1,2-dibromo-4-(1,2-dibromoethyl)cyclohexane (TBECH), and hexabromobenzene (HxBrBz). The molecular structures and the chemical abstract service (CAS) numbers of the ten selected BFRs are shown in Figure 2.

The selections of BFRs used in the studies included in the present thesis were all based on the set presented by Andersson et al. (2006). However, some of the ten BFRs were omitted in papers I and II, and other compounds were included instead. In paper I, the biodegradability of BFRs was tested and 2BrSty, BDE 183, and TBBPA OHEE were not included in the study. 2BrSty was omitted because this compound has a high vapor pressure and it was assumed that this compound would evaporate during the experiment. BDE 183, and TBBPA OHEE were omitted because it was assumed that BDE 209 could degrade into BDE 183, and that TBBPA OHEE could degrade into TBBPA. The inclusion of these compounds would thus complicate the interpretation of the results. In paper II, bioaccumulation in earthworms was studied. Large amounts of chemicals were needed to spike the soil and therefore the three technical mixtures of PBDEs and also TBECH and HxBrBz were tested. In papers III, IV, and V, the ten BFRs suggested by Andersson et al. were tested. In papers III and IV, an additional compound was included, namely, tetrabromobisphenol A 2,3-dibromopropyl ether (TBBPA DBPE).
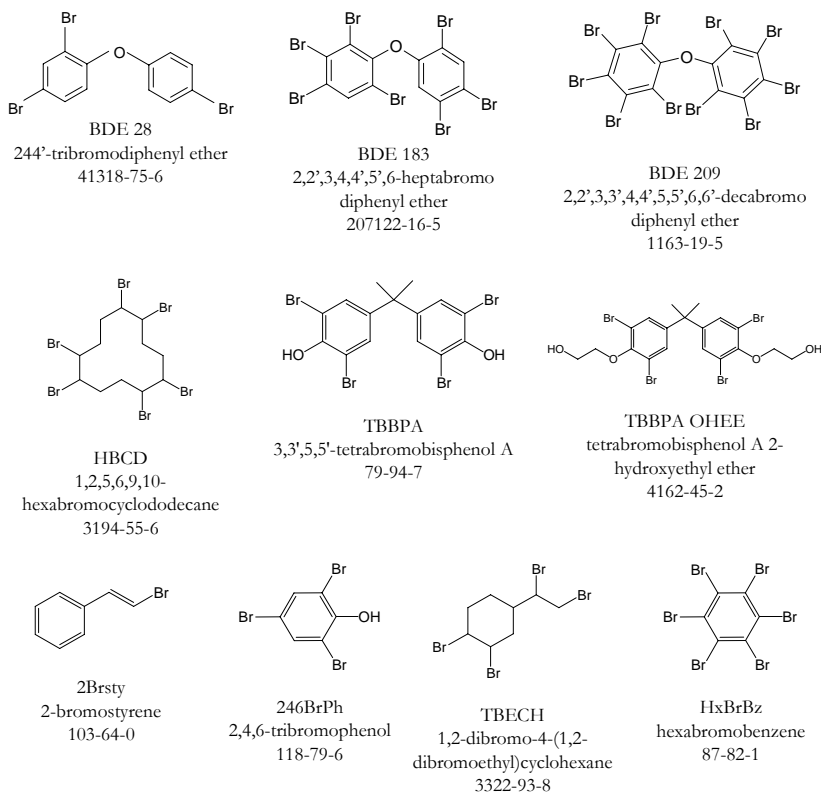
Figure 2. Molecular structures, chemical names, and CAS-numbers of representatives for the group of BFRs (suggested by Anderson et al. 2006).

2.2. Properties, production, and environmental occurrence of the selected BFRs

BDE 28 is a tribrominated diphenyl ether, which is present in the commercial pentaBDE-mix (Bromkal DE-70), although in very low concentrations (Sjödin et al. 1998; Korytar et al. 2005). The BDEs in highest concentrations in a batch of Bromkal DE-70 (Great Lakes Chemical Corp., Indianapolis, IN, USA) were BDE 47 and BDE 99, which accounted for 35 and 37% of the mass, respectively; the corresponding mass of BDE 28 was only 0.11% (Sjödin et al. 1998). Despite the low concentration in the commercial product, BDE 28 is ubiquitously found in the environment and has been detected in sewage sludge, Zebra mussels, fish, and in humans (Knoth et al. 2007; Binelli et al. 2008; Covaci et al. 2008; Sjödin et al. 2008; Jiao et al. 2009). The mean concentration

of BDE 28 in human serum from 2062 humans aged 12 and older, sampled in 2003-2004 in the United States, was 1.2 ng/g lipid (Sjödin et al. 2008). CAS number, molecular weight, Log K_{ow}, water solubility, and vapor pressure of BDE 28 and the other selected BFRs can be seen in Table 2. The calculated Log K_{ow}, water solubility, and vapor pressure were derived using EPI Suite 3.12 software (U.S. Environmental Protection Agency, Washington, DC, USA).

BDE 183 is an octabrominated diphenyl ether which is one of the major BDEs in the commercial product Bromkal 79-8DE from Great Lakes Chemical Corp (Korytar et al. 2005). Like BDE 28, BDE 183 is widespread in the environment and has been detected in various matrices, in biota and in humans, e.g. in sewage sludge, fish, birds, and human milk (Chen et al. 2007; Knoth et al. 2007; Antignac et al. 2009).

BDE 209 is the primary compound present in the technical product decaBDE. DecaBDE is listed as a high production volume chemical (HPVC) in the 2004 Organisation for Economic Co-operation and Development (OECD) list for chemicals whose production exceeds 1 000 tonnes per year in one or more member countries (http://www.oecd.org). DecaBDE is also listed as an HPVC in the EU, which means that it is placed on the market in volumes above 1 000 tonnes per year per distributor (http://ecb.jrc.ec.europa.eu/esis). BDE 209 has been detected in various matrices and in biota, e.g. sewage sludge, fish, birds, and human milk (Öberg et al. 2002; Chen et al. 2007; Knoth et al. 2007; Antignac et al. 2009; Shaw et al. 2009).

HBCD was listed as an HPVC both by the EU and the OECD. The technical product of HBCD contains three diastereomers, α, β, and γ. HBCD is widespread in the environment and has been detected in various matrices and in biota, e.g. sewage sludge, fish, and human serum (Remberger et al. 2004; Meijer et al. 2008; Shaw et al. 2009).

TBBPA is a heavily used reactive BFR, listed in the EU and OECD as a HPVC. TBBPA has been detected in sewage sludge (Sellström et al. 1999) and in human milk (Cariou et al. 2008). However, the levels of TBBPA in biota are generally lower than those of HBCD and the most abundant PBDEs.

TBBPA OHEE is a TBBPA derivative that is used as an additive in engineering polymers and coatings (WHO 1995), and is currently a low production volume chemical (LPVC) in the EU, which means that it is placed on the market in volumes between 10 and 1000 tonnes per year per distributor. The production volume in the US was between 4 and 226 tonnes in 1998. In 2002, there was no reported use in the US.

2BrSty has been used as a BFR monomer (Andersson et al. 2006) and also as an additive in soaps and washing powder (IVL Swedish Environmental Research Institute 2006). 2BrSty is currently not listed as a LPVC or an HPVC in EU. In a screening study, 2BrSty was not detected in any of the analyzed environmental samples (IVL Swedish Environmental Research Institute 2006). 2BrSty is a liquid at room temperature; other physicochemical properties are presented in Table 2.

246BrPh is used as a reactive flame retardant and its trade names are Great Lakes PH-73 or FR-613. The world-wide production was approximately 2500 tonnes in 2001 (Screening Information Data Sheets for High Production Volume Chemicals, available at: http://www.inchem.org/pages/sids.html), and it is listed as a HPVC in the EU and was on the 2004 OECD list for HPVCs. 246BrPh has been detected in sewage sludge in Sweden at levels up to 0.9 ng/g wet weight (Öberg et al. 2002). Bromophenols are also naturally produced by marine organisms, and in brown algae levels up to 5780 ng/g dry weight (dw) have been measured (Chung et al. 2003).

TBECH is a cycloalifatic tetrabrominated compound, that is commercially produced as an additive flame retardant (Saytex BCL 462). The commercial mixture contains mainly two isomers, α and β, in about equal amounts (Arsenault et al. 2008). TBECH is currently not listed as a LPVC or a HPVC in the EU. The production volume in the United States was between 4 and 226 tonnes in 2002 (http://www.epa.gov/oppt/iur/tools/data/2002-vol.htm). TBECH was recently identified in whales from the Canadian Arctic (Tomy et al. 2008), and in eggs of herring gull (Gauthier et al. 2009).

HxBrBz was one of the most commonly used flame retardants in the 70s and early 80s (Yamagushi 1988), but no information on current production volumes was found (it is not listed on the EU LPVC or HPVC lists). The production volume in the United States was between 4 and 226 tonnes in 1998, but in 2002 there was no reported use (http://www.epa.gov/oppt/iur/tools/data/2002-vol.htm). Despite this relatively low production, HxBrBz was recently found in relatively high levels (median concentration 55 ng/g dry matter) in sewage sludge (Kuch et al. 2005), and in eggs of herring gull (Gauthier et al. 2007; Gauthier et al. 2009). Other possible sources of the HxBrBz present in the environment are emissions from polymeric BFRs (Gouteux et al. 2008) or formation during thermolysis of decabromodiphenyl ether (Buser 1986).

BDE 209, HBCD, TBBPA, TBBPA OHEE, 2BrSty, 246BrPh, TBECH, and HxBrBz are all preregistered in REACH regulations

(http://apps.echa.europa.eu/preregistered), and HBCD is also on the REACH candidate list of substances of very high concern for authorization (http://echa.europa.eu/chem_data/candidate_list_table_en.asp).

Table 2. Molecular weights (MW), calculated and experimental Log K_{ow}, calculated and experimental water solubilities (Sw), and calculated vapor pressures (Vp) for the selected BFRs. The calculated data were derived from EPI Suite 3.12 software (U.S. Environmental Protection Agency, Washington, DC, USA).

	MW *(g/mol)*	*Log K_{ow}* *(calc)*	*Log K_{ow}* *(exp)*	*S_w (mg/L)* *(calc)*	*S_w (mg/L)* *(exp)*	*Vp (mm Hg)* *(calc)*
BDE 28	406.9	5.9	5.9 [a]	0.026	0.07 [f]	2.3×10^{-6}
BDE 183	722.5	9.4	8.3 [a]	2.2×10^{-7}	0.0015 [f]	3.3×10^{-10}
BDE 209	959.1	12.1	9.9 [b]	2.8×10^{-11}		4.7×10^{-12}
HBCD	641.7	7.7	5.3/4.9/5.0 [c]	2.1×10^{-5}		1.7×10^{-8}
TBBPA	543.9	7.2	6.5/4.8 [d]	0.0010	0.17/4.2 [d]	3.5×10^{-11}
TBBPA OHEE	632.0	6.8		0.00016		3.9×10^{-14}
2BrSty	183.1	3.2		108		0.12
246BrPh	330.8	4.2	4.2/3.14 [e]	9.1	61/600 [e]	0.00030
TBECH	427.8	5.2		0.069		0.00011
HxBrBz	551.5	7.3	7.1 [b]	0.0022	0.0030 [e]	1.7×10^{-8}

a) Braekevelt et al. 2003

b) Watanabe and Tatsukawa 1989

c) Log K_{ow} for α-, β-, and γ-HBCD from Hayward et al. 2006

d) Log K_{ow} and water solubility at pH 3/7.5 from Kuramochi et al. 2008a

e) Log K_{ow} and water solubility at pH 3/7 from Kuramochi et al. 2008b

f) Tittlemier et al. 2002

3. Chemical analysis of BFRs

Determination of BFRs in environmental samples requires several steps and techniques. The ten BFRs under consideration were chosen to represent wide variation in chemical properties. The project then aimed to find one streamlined analytical method for these 10 BFRs, although this proved difficult, as described below.

The analytical steps used in the projects for determination of BFRs in the soil, worm, fish, fish egg, feed, and frog samples were pretreatment, extraction, fractionation of polar and non-polar (non-hydroxylated) compounds, derivatization of the hydroxylated compounds, and chemical analysis with gas chromatography – mass spectrometry (GC-MS). Worm, fish, and frog samples, which contain substantial amounts of lipids, were cleaned up with gel permeation chromatography (GPC) before fractionation. The individual steps are further described below.

Internal standards were added to the samples (and to a quantification standard) before extraction, and recovery standard was added before injection on the GC-MS. These compounds should behave similarly to the target analytes and the results will, thereby, allow correction for losses during clean-up and for instrument variations.

3.1. Extraction

In papers I and II, Soxhlet apparatus were used for extraction of soil samples. The soil samples were first dried and acidified with sodium sulfate impregnated with 5 % sulfuric acid (w/w). The sodium sulfate was acidified to ensure that the hydroxylated compounds were protonated during the extraction. Acetone:hexane (5:2) was used for the extraction and the Soxhlet was run for 20 hours. In papers III and IV, the biota samples were first homogenized with sodium sulfate impregnated with 1% sulfuric acid (five times the weight of the fish), according to a method described by Berger et al. (2004). Then the homogenate was packed in columns which were eluted with acetone:hexane (5:2) and hexane:diethyl ether (9:1).

Pressurized Liquid Extraction (PLE) was used for extraction of frog and frog feed samples (unpublished data). The samples were first homogenized by grinding the samples with sodium sulfate in the following ratios: whole body of frogs:sodium sulfate (1:9 w:w) and feed:sodium sulfate (1:30 w:w). Heptane:acetone (3:2) was used for the extraction and the temperature was first

set to 75ºC and the pressure was set to 11 MPa. The samples were extracted in three static cycles. Then the temperature was raised to 120 ºC and the samples were extracted again.

3.2. Clean-up

GPC was used to reduce the amount of lipids in earthworm, zebrafish, zebrafish egg, and frog samples. In a GPC system, molecules are separated mainly based on size, and the large sized lipid molecules elute before the smaller BFRs (Rozemeijer et al. 1996). The GPC system used in the studies for the present thesis was wet-packed in-house, with 25 g of Bio-beads. The Bio-beads were allowed to pre-swell in cyclohexane:ethyl acetate (3:1) for 30 minutes before packing the column. The column inner diameter was 1.5 cm, the column height was 40 cm and it was fed cyclohexane:ethyl acetate (3:1). The first fraction contained the bulk of lipids and was discarded. The second fraction, which contained the BFRs, was collected.

3.3. Fractionation

In papers I and V, non-polar and polar (hydroxylated) compounds were fractionated using liquid-liquid extraction with equal volumes of hexane and an alcoholic 0.5M KOH solution. After agitation, the polar analytes partition to the aqueous phase and the non-polar to the organic phase, as described in Hovander et al. (2002). The water phases were combined, acidified with 2M hydrochloric acid and back-extracted to hexane:methyl-tert-butylether.

In papers III and IV, non-polar and polar compounds were fractionated on Florisil, an upscaled version of a method described in Berger et al. (2004). The Florisil columns were wet-packed with Florisil that had been deactivated with de-ionized water (1.2% w/w), and were overlain with a layer of activated sodium sulfate. A first fraction containing non-polar compounds was eluted with hexane:dichloromethane (3:1), and a second fraction containing hydroxylated compounds was eluted with hexane:acetone (85:15) and dichloromethane:methanol (88:12).

3.4. Derivatisation of hydroxylated compounds

Hydroxylated compounds are retained in a GC-column because of interactions between the stationary phase and the hydroxyl group, which leads to poor chromatography. Derivatisation of the hydroxyl groups with methylation,

acetylation, or silylation agents before GC-MS analysis improves the chromatography. In papers I and III-V, 246BrPh, TBBPA, and TBBPA OHEE were silylated using *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 1% of trimethylchlorosilane (TMCS) before analysis. The silylation method was similar to the method described in Zafra et al. (2003). In the analysis of frog samples, the samples were heated at 60°C for 30 minutes to improve the efficiency of the silylation reaction (Zhang et al. 2006; Xie et al. 2007).

3.5. GC-MS analysis

The most common analytical technique for separation of PBDEs is gas chromatography (GC) and the most widely used detectors are mass spectrometers (Covaci et al. 2003). In the projects for this thesis, the samples were injected using a programmable temperature vaporizing (PTV) injector operated in pulsed splitless mode, which has been shown to give higher yield of BDE 209 as compared with the constant temperature mode (Björklund 2003). A short column (15 m DB5-MS column, 0.25 mm i.d. × 0.10 μm film thickness; J&W Scientific, Folsom, CA, USA) was used for separating the analytes, with helium as the mobile phase carrier. The electron ionization (EI) mode was used for analysis of 2BrSty, TBECH, BDE 28, HxBrBz, BDE 183, 246BrPh, TBBPA, and TBBPA OHEE and molecular or fragment ions were recorded in the single ion monitoring (SIM) mode. The advantage with EI is that the specificity is higher than for the alternative electron capture negative ionization (ECNI), and that isotopic dilution quantification can be used. The drawback with this technique is that the sensitivity for the higher brominated PBDEs is relatively low. Consequently, ECNI was used for analysis of BDE 209, TBBPA DBPE and HBCD. Methane was employed as the reagent gas, and the ion source temperature was set to 180°C to promote dissociative electron captures that produce bromide ions with high yields.

HBCD has been reported to start to degrade at 240-270°C (Barontini et al. 2001), so the residence time in the injector and on the column should be as short as possible. This is one of the reasons that pulsed splitless injection was used. The three isomers of HBCD cannot be separated by gas chromatography, but the total amounts of HBCD can be determined. However, the HBCD peak is broader than near eluting peaks for PBDEs and the standard deviation is higher. The fragments monitored for the selected BFRs in EI or ECNI mode are given in Table 3.

Table 3. Monitored m/z (mass-to-charge ratio) of brominated flame retardants and ^{13}C-labeled internal standards.

Compound	EI (quantification, qualification ion)	ECNI (quantification, qualification ion)
BDE 28	405.8, 407.8	
BDE 183	721.4, 723.4	
2BrSty	182.0, 184.0	
246BrPh	386.8, 388.8	
TBECH	266.9, 264.9	
HxBrBz	551.5, 549.5 or 549.5, 547.5	
TBBPA	672.8, 670.8	
TBBPA OHEE	672.8, 670.8	
BDE 209		485, 487
HBCD		79, 81
TBBPA DBPE		79, 81
[13]C BDE 77	497.8, 495.8	
[13]C BDE 209		495, 497
[13]C HxBrBz	559.5, 561.5	
[13]C TBBPA	699.9, 697.9	
[13]C 246BrPh	392.9, 394.9	

Identification of unknowns. In the studies for paper IV, several unknown compounds were observed in the bromide ion traces of exposed fish samples, and some of these peaks were tentatively identified using full scan EI-MS as a pair of hexabromodiphenyl ethers, a tetrabromobenzene, and a tribromoanisole. The mass spectra are shown in Figure 3. For identification of these unknowns, analytical standards were purchased and run on two columns coated with stationary phases of complementary selectivity to verify the identity through retention time matching.
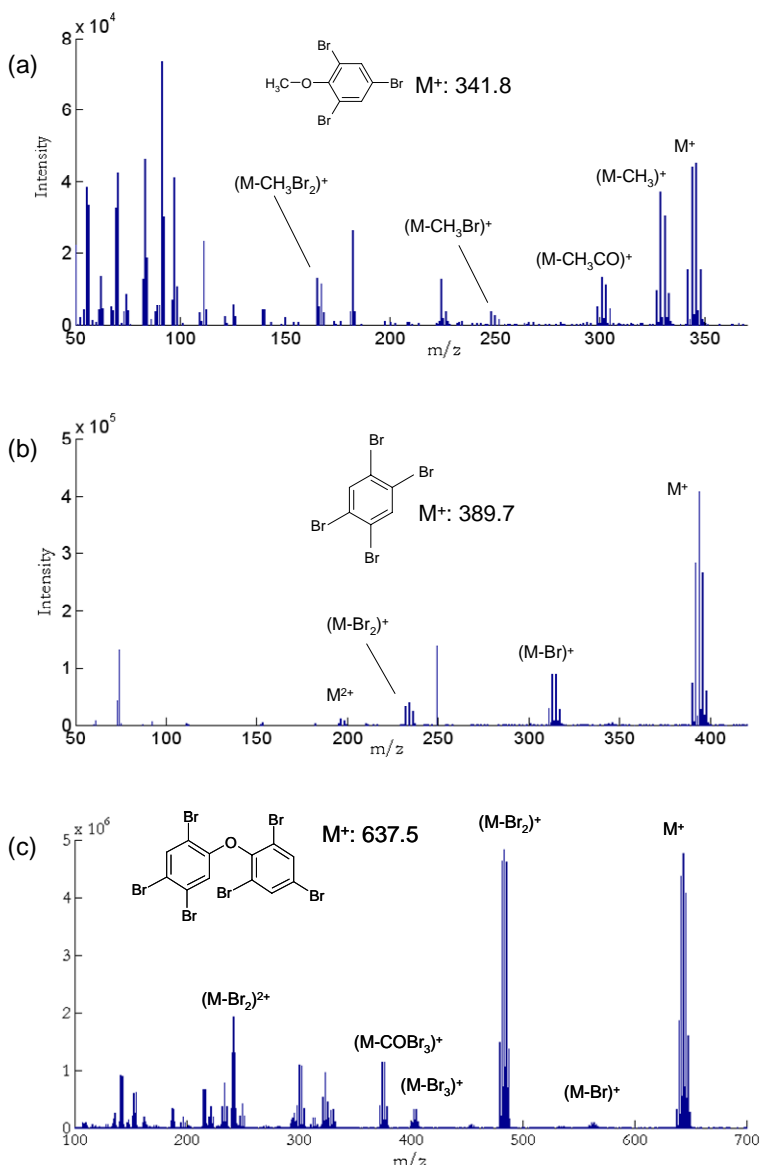
Figure 3. Electron ionization (EI) mass spectra of a (a) tribromoanisole, (b) tetrabromobenzene, and (c) hexabromodiphenyl ether found in zebrafish exposed to a mixture of eleven BFRs (the ten BFRs presented in Figure 2 and tetrabromobisphenol A 2,3-dibromopropyl ether).

3.6. Performance evaluation of the methods used

The compounds covered in these analyses have very different chemical properties. The aim was to include as many compounds as possible in one streamlined analysis with as few as possible parallel steps.

In paper IV, the recoveries following extraction, clean-up and fractionation of the ten selected BFRs in spiked fish fat samples were tested. The tests indicated that the analytical method applied (including e.g. column extraction, GPC, Florisil fractionation) gave unacceptable losses of 2BrSty, TBBPA DBPE, TBBPA OHEE, and TBBPA. The recoveries of HBCD were relatively low and showed high variation (36 $\pm$ 27 %). Although the reasons for the low recoveries were not fully evaluated, it appeared to be high volatilization losses of 2BrSty, and the other analytes may have been lost through adsorption or degradation processes during extraction or Florisil fractionation. High average recoveries (46-111%) were observed for 246BrPh, TBECH, HxBrBz, BDEs 28, 183, and 209.

Thus, the data indicated that 2BrSty, TBBPA DBPE, TBBPA OHEE, and TBBPA could not be accurately determined using the method. Therefore, 2BrSty and TBBPA DBPE were not included in the studies underlying papers I, II, or V. Further work was carried out for simultaneous analysis of TBBPA and PBDEs. A method using Soxhlet extraction and direct isolation of hydroxylated compounds through hexane:KOH partitioning and back-extraction was used in paper I and showed high average recoveries (>65%) of the internal standards ^{13}C-labeled TBBPA, 246BrPh, BDE 77, BDE 209, HxBrBz, HxClBz, 246ClPh, and PCB 194. In the frog study, a method using PLE extraction and hexane:KOH partitioning resulted in average recoveries above 45% for the internal standards ^{13}C-labeled TBBPA, 246BrPh, BDE 77, BDE 209, and HxBrBz.

4. Persistence of BFRs

A persistent organic compound degrades very slowly in the environment, i.e. it has high stability. Stability, as a property, is inversely related to the reactivity of a compound, i.e. the tendency to degrade via oxidation, reduction, photolysis, reaction with free radicals and hydrolysis, elimination and also substitution reactions (Green and Bergman 2005). A compound is not equally stable in different compartments (soil, sediment, water or air), because of the different probability of the reactivity mechanisms occurring there. Thus, the partitioning of a chemical between compartments is important for the overall persistency in the environment.

There are several definitions of persistency (P) found in the literature: persistence can refer to the stability in one compartment, but can also refer to the stability in the whole environment. The term overall persistency is used to clarify that all compartments are considered. For example, van Leeuwen and Hermens (1995) describe persistence as an "attribute of a substance which describes the length of time that the substance remains in a particular environment before it is physically removed or chemically or biologically transformed". UNEP has explained persistence as "lasting for years or even decades before degrading into less dangerous forms" (UNEP 2005). Green and Bergman (2005) have suggested the definition "The persistence of a chemical is its longevity in the integrated background environment as estimated from its chemical and physicochemical properties within a defined model of the environment".

The overall persistency may be predicted by multimedia models, as for example "The OECD software tool for screening chemicals for overall persistence and long-range transport potential" available at: http://www.oecd.org (Wegmann et al. 2009). Input data for prediction of overall persistence are log K_{ow}, Henry's law constant, and half-lives in soil, ocean water and air. However, according to the current legislation, an estimation of the overall persistence is not needed for P classification, only determination of half-life in soil, sediment or water (e.g. in REACH).

4.1. Abiotic degradation

BFRs are generally hydrophobic and fugacity models show that a large part of the BFRs released to the environment will end up in soil or sediment (Palm et al. 2002; Gouin and Harner 2003). In soil, abiotic oxidation could be of importance in the top layer of soil, while reduction is probably of more

importance in soil covered with water, and in sediment. In studies by Bastos et al. (2008a, b) PBDEs were shown to be resistant to abiotic oxidative transformations (by using $KMnO_4$ as an oxidizing agent in water), while investigated hydroxylated PBDEs, and TBBPA were susceptible to these reactions. Abiotic reductive debromination of PBDEs has been investigated (using zerovalent iron as a reducing agent), resulting in a positive correlation between the degree of bromination and the debromination rate (Keum and Li 2005). Hydrolysis, elimination and substitution reactions could also be of importance in soil, sediment and water. However, aromatics are generally resistant to hydrolysis, while alkyl halides are susceptible to hydrolysis. The half-lives of the non-aromatic TBECH and HBCD in water predicted by the HYDROWIN program, EPI Suite 3.12 software (U.S. Environmental Protection Agency, Washington, DC, USA) were very long (presented in Table 4). However, highly brominated DEs have been shown to be susceptible to substitution (of one or more bromines for a methoxyl group using the reagent sodium methoxide in methanol:dimethylformamide), while lower brominated DEs were resistant to this reaction (Rahm et al. 2005).

In the atmosphere, the most important degradation process PCBs, and chlorinated dibenzo-*p*-dioxins (PCDDs) are gas phase reactions with hydroxyl radicals (Andersson and Hites 1996; Brubaker and Hites 1997). The hydroxyl radicals are formed during ozone photolysis reactions in the troposphere and they then react with organic molecules. The atmospheric degradation rates of PCBs and PCDDs have been shown to decrease with increasing degree of chlorination (Andersson and Hites 1996; Brubaker and Hites 1997; Taylor et al. 2005). The same relationship is probably also valid for their brominated counterparts. Raff and Hites (2006) studied the gas phase reactions with hydroxyl radicals of mono- and dibromodiphenyl ethers and also predicted the atmospheric half-lives of tri- and hexabromodiphenyl ethers to be 3 and 46 days, respectively. These half-lives are in the same range as the half-lives predicted by the AOPWIN program, EPI Suite 3.12 software (U.S. Environmental Protection Agency, Washington, DC, USA). The half-lives in air of the ten selected BFRs under consideration, calculated by the AOPWIN program, are presented in Table 4. If the half-life is longer than 2 days, the compound is considered to have potential for long-range transport according to UNEP (2001). Thus, these data indicate that for most BFRs the degradability in air is low enough to give the potential for long-range transport. Further, BFRs have been found in remote areas (e.g. Jiao et al. 2009), which also suggests that atmospheric transport occurs. However, highly brominated BFRs have very low vapor pressures (see Table 2) and therefore it may be more likely that they are particle-bound in the atmosphere, rather than present in the gas phase (Wania and Dugani 2003).

Direct photolysis could be significant for brominated aromatics at the surface of airborne particles, as well as in the gas phase. Several studies have investigated the photodegradation of brominated aromatics in different matrices (Eriksson et al. 2004a; Eriksson et al. 2004b; Söderström et al. 2004; Ahn et al. 2006; von der Recke and Vetter 2007; Fang et al. 2008) and shown that the degradation rates generally increase with increasing degree of bromination. In a study by Söderström et al. (2004), the half-life of BDE 209 adsorbed to sediment and exposed to natural sunlight was 80 hours (3.3 days). However, the study by Ahn et al. (2006) showed longer half-lives of BDE 209 absorbed to clay minerals, metal oxides and sediment. For example, the half-life of BDE 209 absorbed to sediment was 990 days in natural sunlight (Ahn et al. 2006). In both these studies, less brominated PBDEs were formed.

Table 4. Half-lives of ten BFRs in air (hydroxyl radical reactions) and in water (hydrolysis) and ready biodegradability predictions from EPI Suite 3.12 software (U.S. Environmental Protection Agency, Washington, DC, USA).

BFR	Half-lives in water (years) Hydrolysis	Half-lives in air (days) Hydroxyl radical reactions	Ready degradable? (time frame for ultimate biodegradation)
2BrSty	-	3.9	No (weeks)
BDE 28	-	7.6	No (months)
BDE 183	-	64	No (recalcitrant)
246BrPh	-	23	No (months)
TBECH	86.4 years (pH 7) 8.64 years (pH 8)	2.2	No (weeks-months)
HxBrBz	-	934	No(recalcitrant)
TBBPA	-	3.6	No (recalcitrant)
TBBPA OHEE	-	0.42	No(recalcitrant)
BDE 209	-	318	No(recalcitrant)
HBCD	$1.2*10^{11}$ (pH 7) $1.2*10^{10}$ (pH 8)	1.7	No (months)

4.2. Biodegradation in soil

Biodegradation is the transformation of a compound by a living organism. Usually, biodegradation of organic compounds is studied using Ready Biodegradability tests. These include e.g. the closed bottle test and the MITI-I test, which are simple and relatively fast methods to assess if a compound will be easily degraded by microorganisms in the environment. In the MITI-I test, the test bottle, including the test compound and an aqueous solution with sludge, is incubated for 28 days while the biological oxygen demand is measured. If the biological oxygen demand is >60% of the theoretical oxygen demand, the test compound is considered ready degradable (Sedykh and Klopman 2007). None of the BFRs tested, BDE 209, HBCD, TBBPA, HxBrBz, and 246BrPh, passed the MITI-I test. The biological oxygen demands were 0, 1, 0, 0 and 49% for BDE 209, HBCD, TBBPA, HxBrBz, and 246BrPh, respectively ("Biodegradation and bioconcentration of the existing chemical substances under the chemical substances control law" database of National Institute of Technology and Evaluation, Japan, available at: http://www.safe.nite.go.jp). Predictions on ready biodegradability for the ten BFRs from Biowin, EPI Suite 3.12 software (U.S. Environmental Protection Agency, Washington, DC, USA) can be seen in Table 4. These tests were, however, not designed for very hydrophobic chemicals. For most BFRs, the test concentrations exceed their water solubility. Therefore, biodegradation data derived from more environmentally relevant tests are needed.

As mentioned before, a large part of the BFRs released to the environment will probably end up in soil. Thus, this matrix is relevant for persistency studies on BFRs. Paper I investigated the microbial degradation of BFRs and some chlorinated analogues in aerobic and anaerobic soil. The BFRs included in the study were: BDE 28, BDE 209, HBCD, TBECH, HxBrBz TBBPA, and 246BrPh. The chlorinated compounds added for comparison were: 2,4,4'-trichlorodiphenyl ether (CDE 28), hexachlorobenzene (HxClBz), 2,4,6-trichlorophenol (246ClPh), and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153). To mimic land application of sludge, the BFRs were first added to sewage sludge and thereafter blended with soil, to final concentrations at about 50 ng/g dw for each test compound and sewage sludge concentrations at 0.5% dw/dw. Three types of sludge were studied: activated, digested, and hygienized sludge. Microcosms with spiked soil (about 50 g dw) were incubated at room temperature for up to 160 days, and microcosms with autoclaved soil served as controls for the study of abiotic degradation.
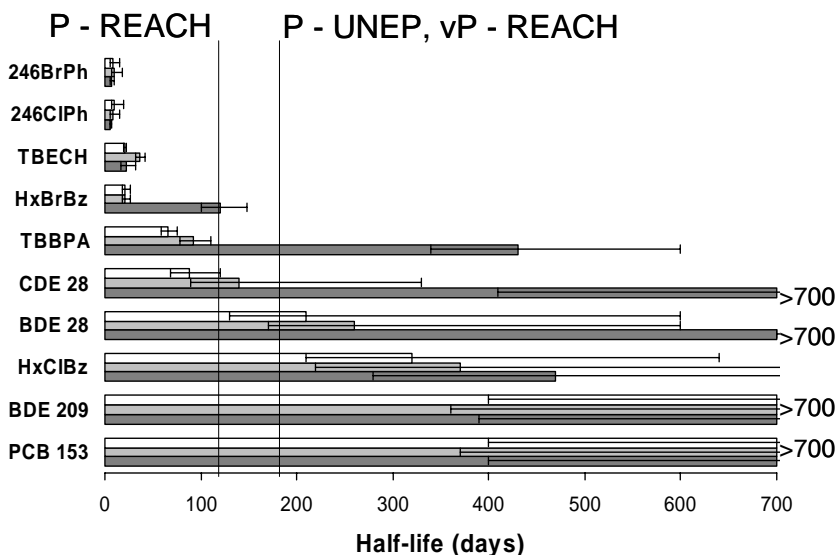
Figure 4. Half-lives of the tested BFRs and chlorinated analogues: 2,4,6-trichlorophenol (246ClPh), 2,4,6-tribromophenol (246BrPh), 1,2-dibromo-4-(1,2-dibromoethyl)cyclohexane (TBECH), hexabromobenzene (HxBrBz), tetrabromobisphenol A (TBBPA), chlorodiphenyl (CDE) 28, bromodiphenyl ether (BDE) 28, hexachlorobenzene (HxClBz), BDE 209, and hexachlorobiphenyl (PCB) 153 in anaerobic soil with application of 0.5% activated sludge (dark grey bars), in aerobic soil with application of 0.5% digested sludge (light grey bars), and aerobic soil with application of 0.5% activated sludge (white bars). Error bars denote 95% confidence intervals. The persistence (P) criteria as set in UNEP, and P and very persistent (vP) criteria as set in REACH are marked in the Figure.

Large variation in the biodegradability between the tested BFRs in soil was found, as depicted in Figure 4. BDE 209 showed no significant degradation during the entire incubation period of 160 days (95% confidence intervals give half-life >360 days in aerobic soil and >390 days in anaerobic soil). BDE 28 also degraded very slowly (half-life 260 days in aerobic soil and >3400 in anaerobic soil). The result for BDE 209 is in agreement with Sellström et al. (2005) who noted that BDE 209 could last for more than 20 years in soil amended with contaminated sewage sludge. Gerecke et al. (2006) also reported a BDE 209 half-life of 700 days in digested anaerobic sewage sludge. The half-life of TBBPA was 93 days in aerobic soil, and HxBrBz, 246BrPh, and the cycloaliphatic TBECH also degraded quickly in aerobic soil (half-lives less than 40 days). TBBPA and HxBrBz degraded much slower in anaerobic soil, with half-lives fulfilling the REACH P criteria (half-lives 430 and 120 days, respectively), while the degradation of TBECH and 246BrPh was in the same

range as under aerobic conditions. Compounds with higher water solubility are generally more bioavailable to microorganisms, which could explain why the dicyclic aromatic compounds degraded slower than HxBrBz, TBECH, and 246BrPh.

Preliminary results for HBCD showed that only a minor part of the nominal concentrations were recovered in day 0 samples. Further work is needed, including new extractions of samples spiked with labeled internal standards of the diastereoisomers, and better clean-up and analysis (using HPLC-MS-MS), before evaluating this data. Davis et al. (2005, 2006) have previously studied the half-lives of HBCD in anaerobic soil and sediment. In the first study, the half-lives of γ-HBCD in aerobic and anaerobic soil were determined as 63 and 7 days, respectively. In sediment, half-lives ranged from 11 to 32 days under aerobic conditions and from 1.1 to 1.5 days under anaerobic conditions. In the second experiment using higher concentrations, the half-lives of total HBCD in aerobic and anaerobic sediment were determined as 101 and 66 days, respectively. The differences between their results indicate that the concentration of the test compound is an important factor in the degradation kinetics, or indicates the challenges involved in the analysis of these compounds.

In relation to chlorine or bromine substitution, no differences were observed for tribromophenol and trichlorophenol; both degraded quickly. CDE 28 seemed to degrade faster than BDE 28 in aerobic soil, which may be related to higher water solubility and bioavailability of CDE 28. The largest differences in degradation rates were observed between HxBrBz and HxClBz. A possible explanation could be that the carbon-bromide bond is weaker than the carbon-chlorine bond.

Paper I also showed that for most compounds there was no differences in degradation between aerobic microcosms amended with the three different sludges (activated, digested, and hygienized sludge). HxBrBz alone showed significantly higher concentrations in soil amended with hygienized sludge after 30 days incubation. In another sub-study, aerobic microcosms were incubated at 8°C for 30 days and compared to microcosms incubated at room temperature. The degradation of HxBrBz, TBECH, 246BrPh, and 246ClPh in microcosms incubated at 8°C were significantly lower than in microcosms incubated at room temperature (paired t-test, $p<0.05$). For HxBrBz and TBECH, degradation was much slower at the colder temperature, which highlights the importance of considering temperature to a greater extent in biodegradation tests and in regulatory work.

In autoclaved controls, there was no or relatively low degradation of BFRs, compared with the viable soil, even though measured density of microorganisms showed that the soil in autoclaved controls was viable again after one to two weeks. Among the autoclaved controls, highest degradation was seen for TBECH, 246BrPh, and 246ClPh in anaerobic soil. Even though the autoclaved controls were not sterile, the low degradation in the controls showed that the abiotic degradation in soil was relatively minor in comparison with the microbial degradation.

In conclusion, large variation in the biodegradability in soil among the tested BFRs was observed. Generally, lower degradation was seen in the anaerobic soil compared with the aerobic soil. 246BrPh and TBECH degraded quickly in both aerobic and anaerobic soil, while the brominated diphenyl ethers (BDE 28 and BDE 209) were very persistent under both aerobic and anaerobic conditions. TBBPA and HxBrBz, that were predicted to be recalcitrant to biodegradation in the EPI Suite program, were both shown to degrade in aerobic soil, and did not fulfill the P criterion (in aerobic soil). Although several studies have shown that BFRs are susceptible to abiotic transformations, biodegradation is probably of most importance in soil for the studied BFRs (as no or only low degradation was seen in controls). Among abiotic processes, photochemical reactions are probably of most importance for the degradation of highly brominated diphenyl ethers in the environment.

5. Bioaccumulation of BFRs

Bioaccumulation is the net uptake and retention of a compound in living tissue from all routes of exposure (van den Berg et al. 1995) resulting in higher concentration within the organism compared with ambient concentration. Living organisms can be exposed to organic contaminants via air, water, food, and soil and uptake of contaminants could occur through, for example, the respiratory system, the skin, and the digestive system. Bioaccumulation can lead to exposure of organisms to high concentrations of potentially toxic compounds.

There are several accumulation factors found in the scientific literature, e.g. bioaccumulation factor (BAF), bioconcentration factor (BCF), biota-soil accumulation factor (BSAF), biomagnification factor (BMF), and trophic magnification factor (TMF). The bioaccumulation factor is the ratio between the concentration in an organism and the concentration in the surrounding environment at steady state after exposure from any source. Bioconcentration is defined as the accumulation through respiratory system or dermal contact after exposure in water, and is measured after laboratory tests. However, both BAF and BCF are usually calculated as the concentration (c) of a compound (in lipid weight) in an organism divided by the concentration in the surrounding water.

$$BAF, BCF = \frac{c_{organism}}{c_{water}}$$

BSAF is calculated as the concentration in an organism (usually on lipid basis) divided by the concentration in soil (usually on organic carbon basis).

$$BSAF = \frac{c_{organism}}{c_{soil}}$$

BMF is calculated as the concentration in an organism divided by the concentration in its feed.

$$BMF = \frac{c_{organism}}{c_{feed}}$$

TMF is the average biomagnification in several trophic levels, and it can be derived from the slope of the line in a plot of $c_{organism}$ vs trophic level.

Several studies have investigated the bioaccumulation potentials of PBDEs. For example, Gustafsson et al (1999) have shown that BDE 47 and BDE 99 are very bioaccumulative in blue mussels (*Mytilus edulis*) with BAFs of 1.3 and 1.4×10^6 (mg dw/l), respectively. Booij et al. (2002) have also shown that BDE 28, BDE 100, and BDE 153 bioaccumulate in blue mussels to a significant extent. Two investigations of the TMFs of PBDEs in the Canadian arctic marine food web showed that only BDE 47 had a TMF significantly above one, indicating that it is a biomagnifying compound (Kelly et al. 2008; Tomy et al. 2008). The TMF of BDE 209 was 0.3 and the values were significantly decreased at higher trophic levels. In a similar study of a freshwater food web in south China, three PBDEs showed a TMF significantly above one, namely BDE 47, 100, and 153 (Wu et al. 2009). The TMFs were generally lower than those of investigated PCBs, and their magnification seemed to be influenced more by metabolism.

According to REACH guidelines, a measured BCF in an aquatic species is needed to fulfill the B (bioaccumulation) classification (European Chemical Agency 2008a). In a typical test system for determining BCF, the test chemical is dissolved directly into water or in a solubilizing agent. This system may introduce large uncertainties, when very hydrophobic compounds are assessed. Compounds, such as highly brominated BFRs, might stick to glass walls or particles in the test system, or partition into feed and the test organism (Breitholtz et al. 2006). In a semi-static system where the water is changed, this could lead to a higher load of chemicals in the system with time. Therefore, studying the BMF or the BSAF could be a better alternative for the determination of bioaccumulation potentials of compounds that are super hydrophobic. In the present thesis, Paper II addressed the bioaccumulation of BFRs from soil in earthworms and paper III and IV addressed the accumulation and maternal transfer of BFRs after dietary exposure to BFRs.

5.1. Bioaccumulation in earthworms

In paper II, the accumulation potentials of selected PBDEs, hexabromobenzene, and TBECH in earthworms (*Eisenia fetida*) were investigated. The tested BFRs were bioavailable and accumulated in the earthworms and thus could reach higher trophic levels in the environment. BSAFs for earthworms exposed for 28 days at three exposure levels (~10 000, 100, or 10 ng/g soil) can be seen in Figure 5. BSAFs were calculated on the basis of lipid weight in earthworms and organic matter in soil.
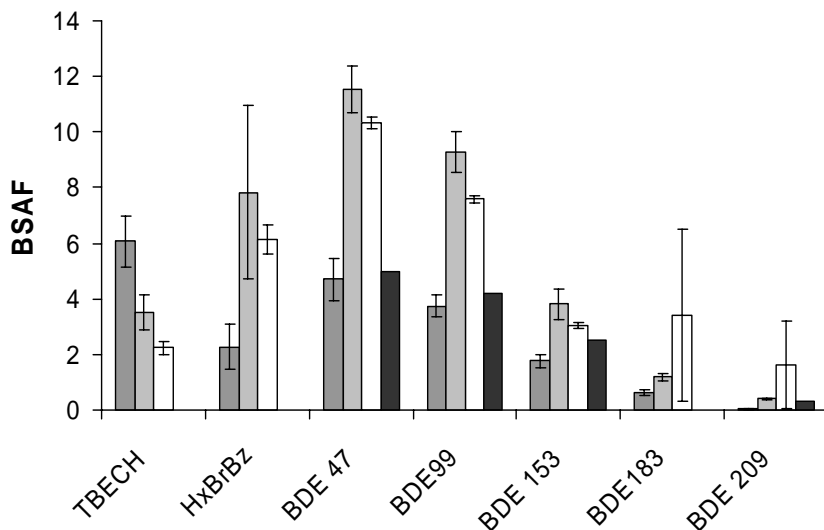
Figure 5. Biota-Soil Accumulation factors (BSAFs) for 1,2-dibromo-4-(1,2-dibromoethyl)cyclohexane (TBECH), hexabromobenzene (HxBrBz), and bromodiphenyl ethers (BDE) 47, 99, 153, 183, and 209 in earthworms exposed for 28 days in OECD soil spiked with BFRs in three different concentrations (dark grey bars: 10 000 nmol/g soil, grey bars: 100 nmol/g soil, light grey bars: 10 nmol/g soil of each BFR). BSAFs determined in a study by Sellström et al. (2005) are shown with black bars. Error bars denote one standard deviation.

The BSAF in the present study were higher or in the same range as mean BSAFs reported for these compounds in earthworms in contaminated soil from the field (5.0, 4.2, 2.5, and 0.3 for BDE 47, 99, 153 and 209, respectively) (Sellström et al. 2005). The accumulation factors at the two lower concentrations are more relevant, since the concentrations are closer to environmentally realistic levels. The highest concentration was very high compared with levels in the environment and it is possible that the high concentrations affected the worms. The pattern for accumulation factors for PBDEs was the same in the three exposure groups; BSAFs decreased with increasing degree of bromination, which is also in line with the study by Sellström et al. (2005).

In paper II, the effect of ageing the soil for approximately two years was studied, indicating decreased accumulation of BDEs with six or less bromine atoms, HxBrBz and TBECH. Ageing can be described as a process where the bioavailability of organic contaminants in soil decreases, the longer time that

has passed since the contamination (Alexander 2000). No effect of ageing was seen for BDE 183 or BDE 209, which could be related to low mobility in soil for these compounds. Paper II also showed that the soil type used in the bioaccumulation test affects the bioaccumulation in earthworms. Three soils were tested: the standard artificial OECD soil, a natural heavy clay soil from Lanna, Sweden, and one forest soil from Kloten, Sweden with a high content of organic matter. These natural soils are among the 13 Nordic reference soils that represent the most common soil types in the Nordic countries (Tiberg 1998). The results showed significantly higher accumulation in earthworms from the OECD soil than from the Kloten soil. This could be explained by higher bioavailability of the organic contaminants in the OECD soil and possibly also by starving (weight loss) of the earthworms in OECD soil, because of lack of feed in the medium.

In conclusion, high bioaccumulation potential in earthworms was observed for BDE 47, BDE 99, BDE 153, HxBrBz, and TBECH, and no apparent debromination of these compounds were observed in earthworms. To our knowledge, the current study is the first on bioaccumulation potential of TBECH in terrestrial biota.

5.2. Uptake, biotransformation and maternal transfer in zebrafish

Papers III and IV investigated the accumulation parameters and the transfer of BFRs from female zebrafish to their offspring, after dietary exposure to a mixture of the following BFRs: BDE 28, BDE 183, BDE 209, HBCD, TBBPA, TBBPA OHEE, 2BrSty, 246BrPh, TBECH, HxBrBz, and TBBPA DBPE.

In addition to the dosed chemicals, several metabolites were observed in zebrafish (Figure 6) and in zebrafish eggs. These were most likely metabolites of BDE 183, 246BrPh, and HxBrBz and they were identified as 2,2',3,4',5',6-hexabromodiphenyl ether (BDE 149), 2,2',4,4',5,6'-hexabromodiphenyl ether (BDE 154), 2,4,6-tribromoanisole and 1,2,4,5-hexabromobenzene. Debromination of PBDEs in fish has been observed in a number of studies investigating bioaccumulation after dietary exposure (Andersson et al. 1999; Kierkegaard et al. 1999; Stapleton et al. 2004; Stapleton et al. 2004; Stapleton et al. 2004; Tomy et al. 2004; Isosaari et al. 2005).
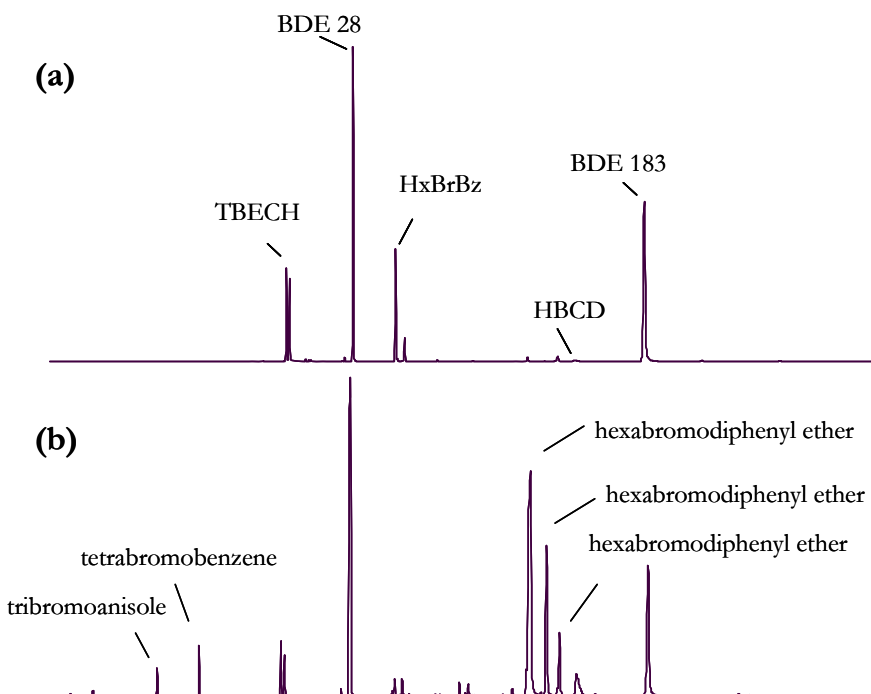
Figure 6. ECNI-chromatograms for samples of (a) spiked feed and (b) male zebrafish exposed to spiked feed for 42 days.

Uptake and elimination rates in zebrafish were calculated for five of the studied BFRs (Paper IV): BDE 28, BDE 183, BDE 209, TBECH, and 246BrPh. The highest uptake was observed for BDE 28 and lowest for BDE 209 (Table 5). Half-lives shorter than 2 days were observed for TBECH and 246BrPh, and calculated BMFs ranged from 0.88 for BDE 28 to 0.0012 for BDE 209. Low accumulation potentials were observed for TBBPA, TBBPA OHEE, and HxBrBz, which is in line with previously published studies. No accumulation of HxBrBz was observed in juvenile Atlantic salmon by Zitko and Hutzinger (1976) and minimal accumulation of TBBPA was observed in zebrafish by Kuiper et al. (2007). The BAF of TBBPA OHEE in carp after exposure to water concentrations of 0.25 and 0.025 mg/L was 10-36 and 15-53, respectively (WHO 1995). The accumulation of HBCD seemed to be high in zebrafish comparing the HBCD peak in zebrafish and feed. However, levels in zebrafish were not calculated in paper IV due to analytical problems. In a bioaccumulation study on HBCD in rainbow trout (*Oncorhynchus mykiss*), high levels of dietary accumulation were observed (Law et al. 2006). The BMF of the three diastereomers were 9.2, 4.3, and 7.2 for α-, β-, and γ-HBCD, respectively.

Table 5. Uptake, elimination, and BMFs in male zebrafish (*Danio rerio*) for five of the tested BFRs at low dose (LD) and high dose (HD).

	Uptake constant (day^{-1})		Elimination constant (day^{-1})		Half-life (days)		BMF	
	LD	HD	LD	HD	LD	HD	LD	HD
BDE 28	0.019	0.023	0.030	0.027	23	25	0.66	0.86
BDE 183	0.0018	0.0019	0.050	0.070	15	9.8	0.040	0.027
TBECH	0.012	0.024	0.76	0.52	0.9	1.3	0.016	0.047
246BrPh		0.0090	-	0.54	-	1.3		0.017
BDE 209		0.00012	-	0.11	-	6.5		0.0012

Maternal transfer was observed for all BFRs detected in female zebrafish and for the metabolites. The egg/fish concentration ratios were significantly above 1 for several compounds. Generally, high egg/fish concentrations were observed for BFRs with high Log K_{ow} values. These compounds seemed to be more efficiently transferred to the eggs.

In conclusion, highest accumulations were observed for BDE 28. BDE 183 showed lower accumulation, but substantial amounts of hexabromodiphenyl ethers that probably were debromination products of BDE 183 accumulated in zebrafish. Maternal transfer was shown for all BFRs present in the female zebrafish. This shows that zebrafish fry are exposed to these BFRs at approximately the same concentrations as female zebrafish during the early-life stages, when fish are usually most sensitive to organic contaminants.

5.3. Bioaccumulation in frogs

The accumulations of BFRs in claw frog (*Xenopus tropicalis*) tadpoles were studied following exposure to feed spiked with the BFRs: BDE 28, BDE 183, BDE 209, HBCD, TBBPA, TBBPA OHEE, 2BrSty, 246BrPh, TBECH, and HxBrBz. The frogs were obtained from Gunnar Carlsson at the Swedish University of Agriculture, Uppsala, where a four-week exposure had been performed. The frogs had been fed spiked feed at two concentrations (~1 and 10 nmol/g feed per each compound) at about 4 % of their body weight per day for 28 days. Frog/feed concentration ratios are shown in Figure 7; these should not be regarded as biomagnification factors, since no steady-state conditions can be assumed. The results showed that BDE 183 had the highest frog/feed concentration ratio, followed by BDE 28 and BDE 209. About one third of the total applied dose of BDE 183 was measured in the frogs after 28 days of exposure. The concentrations of HBCD, TBECH, and 2BrSty were not analyzed. In comparison with the fish, frogs seem to accumulate more of BDE 183 and BDE 209, and no apparent debromination was observed.
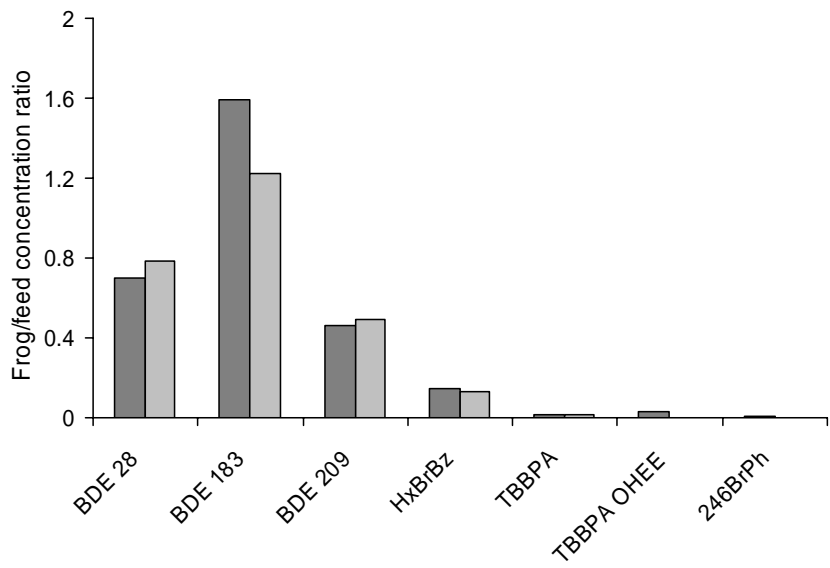
Figure 7. Lipid based frog/feed concentration ratios of bromodiphenyl ethers (BDE) 28, 183, and 209, hexabromobenzene (HxBrBz), tetrabromobisphenol A (TBBPA), tetrabromobisphenol A 2-hydoxyl ether (TBBPA OHEE), and 2,4,6-tribromophenol (246BrPh) in frog tadpoles exposed to BFR spiked feed (dark grey bars: 10 nmol/g feed of each BFR, light grey bars: 1 nmol/g feed of each BFR) for 28 days.

6. Toxicity of BFRs

Neurotoxicity and adverse developmental effects seem to be the most negative effects following exposure to PBDEs (Darnerud 2003). For example, neurotoxic effects in mice have been observed after neonatal single exposures to several PBDEs (Eriksson et al. 2002; Viberg et al. 2003; Viberg et al. 2006; Viberg et al. 2007). Lema et al. (2007) demonstrated that early life exposure of BDE 47 alters growth, morphology and cardiac and neural functions in zebrafish. Further, environmentally relevant concentrations of TBBPA in zebrafish reduced their reproductive success (Kuiper et al. 2007), and brominated indols and phenols affected the development of embryos (Kammann et al. 2006). There are also several studies that have shown that BFRs have the potential to cause endocrine disruption (Darnerud 2008; Legler 2008). A recent study by Larsson et al. (2006) investigated androgen effects of the ten BFRs in focus in the present thesis, and found that TBECH was an androgen agonist that activated the androgen receptor in human hepatocellular liver carcinoma cells.

According to REACH guidelines, the T (toxicity) criterion is fulfilled if the no observed effect concentration (NOEC) is less than 0.01 mg/l in an aquatic species (European Chemical Agency 2008a). The NOEC is the concentration at which no significant effects can be observed as compared with the controls (without any addition of the tested chemical). LC50 is the concentration that is lethal to 50% of the test organisms. A typical dose-response curve can be seen in Figure 8. As discussed in chapter 5, the low water solubility of most BFRs can lead to a low exposure of the test organism. Therefore, in paper V, a silica gel-based system described in Breitholtz et al. (2007) was used for toxicity testing of BFRs in the copepod *Nitocra spinipes*.
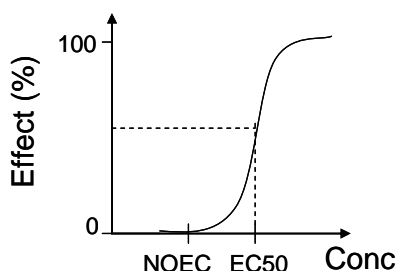

Figure 8. A typical dose-response curve. The no observed effect concentration (NOEC) is the concentration where no significant effects can be observed as compared with the controls. The EC50 is the concentration where 50% of the test organisms show an effect.

6.1. NOECs for *Nitocra spinipes*

Crustaceans are commonly used in ecotoxicological tests, since they comprise a large group of both ecological and economical important organisms. The Crustacean subphylum is the second largest invertebrate subphylum after insects, and includes very small animals such as copepods, as well as larger animals such as crabs and lobsters. Copepods include 12 000 described species and in the food chain they are consumed by invertebrates, fish and whales. *Nitocra spinipes* is a harpacticoid copepod; a benthic species that is commonly found in shallow waters worldwide (Dahl 2008). *Nitocra spinipes* undergoes several larvae (naupliar) and juvenile (copepodite) stages, during their generation time of approximately 16-18 days at 20ºC.

The aim of Paper V was to establish acute LC50s and sub-chronic NOECs for *Nitocra spinipe*s for the 10 BFRs: BDE 28, BDE 209, HBCD (technical product containing α-, β-, and γ-HBCD), TBBPA, TBBPA OHEE, 2BrSty, 246BrPh, TBECH (technical product containing α-, β-TBECH), and HxBrBz, and the technical octa-mix (Octa LM, Bromine Co. Ltd, Israel, technical product that contains BDE 183, and can also contain several hexa- to decabrominated DEs). These tests studied if different concentrations of individual BFRs significantly changed the larvae development ratio (LDR) and mortality of *Nitocra spinipes*. The LDR is the number of animals that has reached the copepodite stage as compared with the total number of animals at the end of the test (6 days).

The established LC50s and NOECs can be seen in Table 6. A factor of three was used between each tested concentration, which means that the NOEC is three times lower than the concentration that caused a significant effect as compared with the control. In these tests, BDE 28 was the most toxic with the LDR NOEC value of 2 µg/l. Other active compounds were TBBPA, TBBPA OHEE, TBECH, HBCD, 246BrPh, and HxBrBz. Results for 2BrSty were inconclusive and no NOEC was established. The octaBDE-mix and BDE 209 was not toxic even at a concentration of 100 mg/l (expressed in relation to the volume of water in the test beaker, even though being well above the water solubility).

Table 6. Acute LC50s and subchronic NOECs determined for *Nitocra spinipes*, expressed as concentrations in μg/l.

	Acute 96-hr-LC$_{50}$	*6-day Mortality* NOEC	*6-day LDR* NOEC
BDE 28	72	2	2
BDE 183	>100000	>100000[a,b]	>100000[a,b]
BDE 209	>100000	[c]	[c]
HBCD	820	226[d]	226[d]
TBBPA	390	70	7
TBBPA OHEE	620	13	40
2Brsty	[e]	[e]	[e]
246BrPh	920	>1000	300
TBECH	654	360	40
HxBrBz	>100000	33400[b]	33400[b]

a) In this test the commercial product (Octa LM, Bromine Co. Ltd, Israel) was used.

b) In these tests there were one week equilibrium period between water, silica and substance before addition of algae and copepods.

c) This substance did not have any effect over 4 days exposure in an acute test up to 100 mg/l, and was therefore not tested in the sub-acute studies.

d) No significant LOEC could be found. However in the highest tested concentration (226 μg/l), both mortality and LDR was affected as compared with the control (p=0.07). Hence, 226 μg/l was used as a NOEC.

e) Animals disappeared from the test beakers during testing.

6.2. Effects of BFR mixtures

The primarily aim of paper V was to study the toxicity of mixtures of BFRs in *Nitocra spinipes*. Six of the BFRs that were active were mixed, based on their respective LDR NOEC-values: BDE 28, TBBPA, TBBPA OHEE, TBECH, HBCD, and 246BrPh. A factor of five between each tested concentration was used, starting at 0.008×NOEC for each of the six BFRs. The highest tested concentration was 5×NOEC, and at that concentration all test animal were dead after 6 days exposure. In the NOEC×1 proportions, significantly increased mortality was observed in juvenile *Nitocra spinipes* after 6 days exposure. None of the other NOEC proportions caused significant negative responses related to development, reproduction or mortality after 6 days or after 26 days (full-life cycle test) of exposure. Paper V shows that simultaneous exposure to low concentrations of several substances can cause effects, and that toxicity of mixtures should be considered to a greater extent in toxicity testing and regulatory work.

7. Identification of PBTs

In this chapter, the PBT properties of the studied BFRs (except for 2BrSty and TBBPA DBPE) are assessed and compared with the test criteria in the REACH legislation, in order to identify PBT compounds. The results from paper I-V are summarized in Table 7; these data are considered, as well as other data taken from the literature. However, it has to be emphasized that the available knowledge about PBT properties is still very limited for several of the BFRs. In summary, the assessment showed that BDE 28 is the only one of the selected BFRs that meets the PBT criteria, as set in the REACH legislation. If debromination and degradation products of the assessed compound are also considered, then BDE 183, and BDE 209 can be considered as PBT compounds. HBCD is a borderline case, and further studies on persistency are needed for this BFR.

The P criterion is fulfilled for **BDE 28**, as the half-life in soil exceeded 120 days (paper I). In the zebrafish study (paper IV), BDE 28 showed the highest accumulation potential among the tested BFRs, but the BMF was not above 1. However, other studies have shown higher bioaccumulation potential of BDE 28, e.g. bioaccumulation in mussels was very high (BAF≈10^7 ml/g lipid, Booij et al. 2002). Further, biomagnification has been observed in fish by Burreau et al. (2004) and in food webs. The TMF was 1.64 in a freshwater food web in south China (Wu et al. 2009) and 0.96 in a Canadian arctic marine food web (Kelly et al. 2008). The T criterion was fulfilled, as the NOEC for larvae development was below 0.01 mg/l (paper V). BDE 28 also fulfils the vPvB criterion, since the half-life in soil exceeded 180 days (paper I), and the BAF in mussels exceeded 5000.

Table 7. Summary of the results of the PBT studies for this thesis.

	Half-lives in aerobic soil (days)	BSAF in earthworm	BMF in zebrafish	Frog/feed C-ratio in frog	NOECs in Nitocra Spinipes (mg/l)
BDE 28	260	-	0.86	0.70	0.002
BDE 183	-	1.2	0.027	1.6	>100
BDE 209	>360	0.4	0.0012	0.46	-
HBCD	-	-	-	-	0.226
TBECH	36	3.5	0.047	-	0.040
HxBrBz	22	6.0	-	0.15	33.4
TBBPA	93	-	-	0.013	0.007
TBBPA OHEE	-	-	-	0.032	0.040
246BrPh	10	-	0.017	0.007	0.300

-=not tested or not quantified

The half-life in soil for **BDE 183** was not tested in paper I. However, by analogy with other PBDEs, BDE 183 can be expected to degrade slowly. The most probable routes of degradation are anaerobic biodegradation, and photolysis, primary degradation that can form lower brominated diphenyl ethers. In zebrafish, high levels of hexabromodiphenyl ethers (BDE 154 and BDE 149) were retained and were probably debromination products of BDE 183 (paper IV), which suggests high uptake of BDE 183. Debromination of BDE 183 into hexabromodiphenyl ethers has also been shown in carp (Stapleton et al. 2004). However, in a study on pike, perch and roach, biomagnification was observed for BDE 183. In frog, no apparent debromination was observed and BDE 183 showed the highest bioaccumulation potential of the tested BFRs. This further supports the premise that the uptake of BDE 183 from food can be high. The acute toxicity of BDE 183 in aquatic species seems to be low. In paper V, no effects of the OctaBDE-mix were seen in concentrations up to 100 mg/l on larval development or survival of the copepod *Nitocra spinipes*. However, BDE 183 is a potential endocrine disruptor, as Hamers et al. (2006) showed an *in vitro* anti-androgenic effect. Further, neurotoxic effects of BDE 183 have been observed in mice by Viberg et al. (2006). In addition, the lower brominated DEs and dibenzo-p-dioxins and furans that can be formed during degradation of BDE 183 (Buser 1986), show higher toxicities. In summary, available data for BDE 183 does not clearly show that BDE 183 is a PBT chemical. However, considering its degradation products, it could well be classified as a PBT and vPvB.

The half-life of **BDE 209** in soil exceeded 160 days and fulfilled the P and the vP criteria. In paper II, uptake of BDE 209 in earthworms was shown and in the frog study, relatively high accumulation potential was observed. The bioaccumulation potential of BDE 209 in fish seems to be low, and no magnification of BDE 209 was observed in a freshwater food web in south China (Wu et al. 2009). However, a study by Chen et al (2007) showed that PBDE profiles were dominated by higher brominated PBDEs in several species of birds of prey from Northern China. High concentrations of BDE 209 were measured in these birds, which indicate that the bioaccumulation potential in terrestrial food webs may be higher than that in aquatic food webs. Moreover, BDE 209 has been shown to debrominate to lower brominated DEs (Kierkegaard et al. 1999, Söderström et al. 2005) that have higher accumulation and toxic potentials. The acute toxicity of BDE 209 seems to be low. In paper V, no effects of decaBDE on larval development or survival of the copepod *Nitocra spinipes* were found, using concentrations up to 100 mg/l. However, BDE 209 is potentially neurotoxic, since a neonatal single dose altered the spontaneous behavior in the adult rat (Viberg et al. 2006). The degradation of BDE 209 into more toxic compounds (lower brominated DEs

and dibenzo-p-dioxins and furans) should also be considered. Dibenzo-*p*-dioxins and furans can be formed during thermolysis of BDE 209 (Buser 1986). BDE 209 is not a PBT, but if degradation products are considered in the assessment of BDE 209, the conclusion is that BDE 209 should be treated as a PBT.

The technical mixture **HBCD** consists of three diastereomers: α-, β-, γ-HBCD. Only α-HBCD was shown to biomagnify (TMF=2.2) in a Canadian arctic marine food web (Tomy et al. 2008). α-HBCD also showed a longer half-life in soil compared with β- and γ-HBCD in a study by Davis et al. (2006). The half-lives in aerobic sediment were 113, 68 and 104 days for α-, β-, γ-HBCD, respectively (Davis et al. 2006). None of the diastereomers fulfilled the P classification; however, α-HBCD is a borderline case. The test was performed at 20°C, and recalculating the half-lives to 12°C gives the half-lives 214, 129, and 197 for α-, β-, γ-HBCD, respectively (European Commission 2007). In paper V, the LDR NOEC in *Nitocra spinipes* was 0.226 mg/l. However, according to the European Commission (2007), several invertebrate and algae tests have shown NOECs below 0.01 mg/l. Therefore, at least the α-HBCD fulfils the B and T classifications. Further studies on HBCD persistency are needed.

The half-life of **HxBrBz** in aerobic soil (which is probably most relevant) was very fast. However, in anaerobic soil, the half-life was 120 days, which fulfilled the P criterion. In zebrafish (paper IV) and in frog, the accumulation potential of HxBrBz was low. However, in earthworms, the accumulation factor was high (paper II). The higher accumulation in earthworms is probably related to a lower biotransformation rate in earthworm, as compared with that in fish and frog. The acute toxicity of HxBrBz was very low in *Nitocra spinipes* (paper V), and also in mice (Szymenska 1997). Therefore, HxBrBz is considered not to fulfill the PBT classification.

The half-life of **TBBPA** in aerobic soil did not fulfill the P criterion, but in anaerobic soil it did. The T criterion was also fulfilled, as the NOEC for larvae development in *Nitocra spinipes* was below 0.01 mg/l (paper V). However, no studies were found showing that TBBPA bioaccumulates to any extent. Therefore TBBPA is not considered as a PBT.

246BrPh and **TBECH** did not fulfill the P criteria, as their half-lives in soil were small in both aerobic and anaerobic soil, indicating rapid degradation. However, paper I showed that TBECH degraded much slower at 8°C, which leads to the conclusion that TBECH probably can persist in soil for a long time in cold climates. Paper II showed high BSAF for TBECH and Paper IV showed that the uptake of TBECH in zebrafish was high. Even though

TBECH might not fulfill the P criteria, more attention should be paid to the toxicity of TBECH, since TBECH is a potential endocrine disruptor (Larsson et al. 2006) and is also potentially genotoxic (Galloway et al. 1997). **TBBPA OHEE** was not identified as a bioaccumulative compound in a study on carp (WHO 1995), and paper IV indicated low bioaccumulation potential in zebrafish. In summary, more studies on TBECH and TBBPA OHEE are needed.

In this assessment of nine structurally varied BFRs, no additional PBTs were identified among the tested BFRs, besides PBDEs that are already restricted within the EU. HBCD is a borderline case and is already on the REACH candidate list of substances of very high concern for regulation. It is however possible that the outcome of the current assessments will be proven wrong, if/when more PBT studies of these compounds are performed.

8. Candidates for future PBT testing

Models can be used to predict properties of non-tested compounds, but also to prioritize which compounds that are most urgent to test. In this chapter, *read-across* was used as a technique to suggest BFRs for future testing from among 55 (in this thesis) non-tested compounds. The principal component analysis (PCA) model originally used for selection of a BFR training set by Andersson et al. (2006) was used for this purpose. *Read-across*, or the transfer of the hazard profile of one substance to another with a similar structure, is a method that can be used for filling data gaps in chemical safety assessments (European Chemical Agency 2008b).

PCA is used to condense and enable visualization of multivariate data by extracting new latent variables (principal components, PCs) that describe the major variation among the original variables. The projections of the observations (in this case BFRs) on the PCs are called scores, and the projections of the descriptors on the PCs are called loadings. In the PCA used in the present study, 15 descriptors were used to capture size, electronic, and steric effects of the BFRs, and the first three PC axes accounted for 84% of the variation in these descriptors (Andersson et al. 2006). Score plots (PC1 vs PC2, and PC1 vs PC3) can be seen in Figure 9. The distances in the three dimensional space from each non-tested BFR to the tested ones were calculated with Pythagoras' theorem, using score-values derived in the PCA. The nearest tested neighbor to each non-tested BFR was identified. If a non-tested BFR had a PBT (BDE 28, BDE 183, or BDE 209) as a nearest neighbor, it was considered as a potential PBT (or can degrade to a PBT) and is suggested to be prioritized for PBT testing.

20 out of 55 non-tested BFRs had a PBT as a nearest tested neighbor. No non-tested compounds had HBCD (the borderline case) as a nearest neighbor. The BFRs that were close to BDE 28, BDE 183 or BDE 209 in the descriptor space and their CAS numbers are listed in Table 8. The use and environmental abundance of the suggested BFRs are further described below, and the chemical structures of the suggested non-PBDEs can be seen in Figure 10.
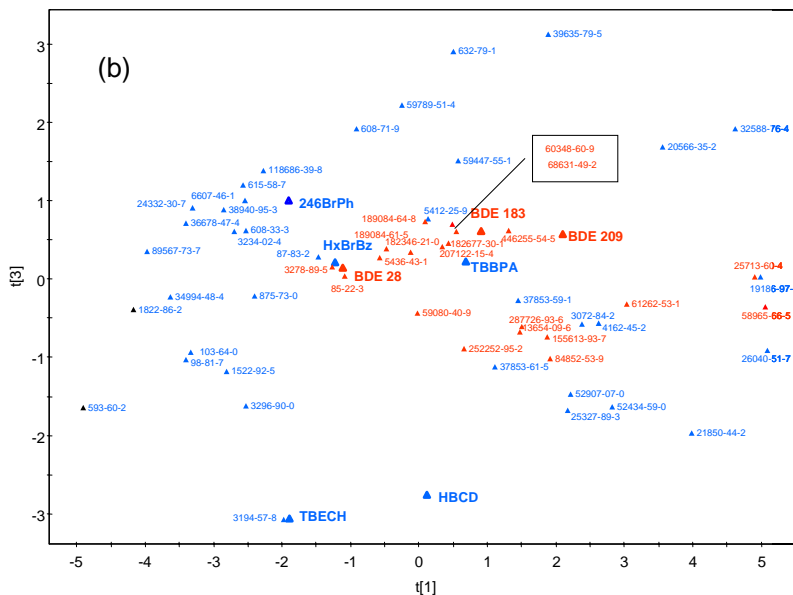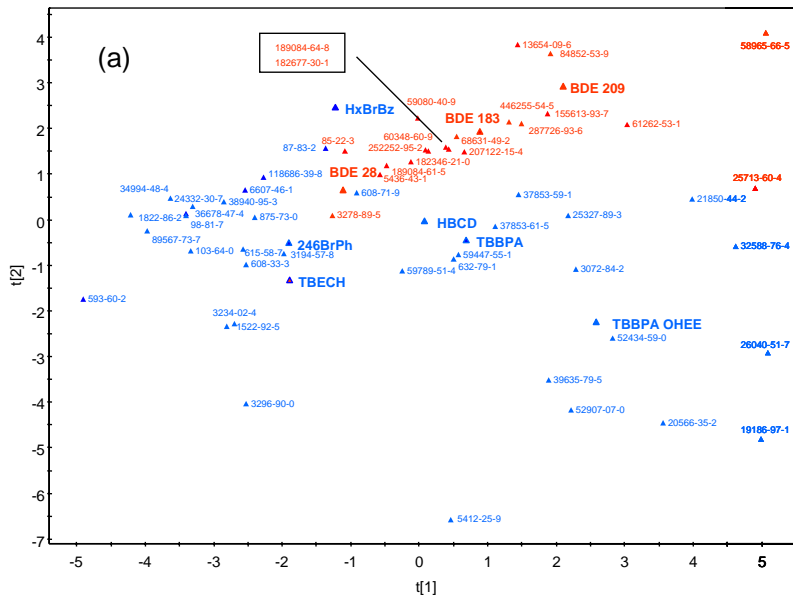
Figure 9. Score plots from the principal component analysis (PCA) of (a) principal component (PC) 1 vs PC2, and (b) PC 1 vs PC 3. The identities of tested BFRs are shown with their abbreviations (see Figure 2), and the identities of the non-tested BFRs are shown with CAS numbers. The BFRs that have BDEs 28, 183, or 209 as a nearest tested neighbor are marked with red.

Table 8. Chemical names, CAS numbers, nearest neighbor, and distance to nearest neighbor of the BFRs with similar physico-chemical properties as BDE 28, BDE 183 or BDE 209.

Chemical name	CAS number	Nearest neighbor	Distance to nearest neighbor
2,2',4,4'-Tetrabromodiphenylether	5436-43-1	BDE 28	0.65
2,3',4,4'-Tetrabromodiphenylether	189084-61-5	BDE 28	0.87
2,2',3,4,4'-Pentabromodiphenylether	182346-21-0	BDE 28	1.18
2,4,6-Tribromophenyl allyl ether	3278-89-5	BDE 28	0.57
Pentabromoethylbenzene	85-22-3	BDE 28	0.88
2,2',4,4',5-Pentabromodiphenylether	60348-60-9	BDE 183	0.88
2,2',4,4',6-Pentabromodiphenylether	189084-64-8	BDE 183	0.89
2,2',3,4,4',5'-Hexabromodiphenylether	182677-30-1	BDE 183	0.61
2,2',4,4',5,5'-Hexabromodiphenylether	68631-49-2	BDE 183	0.36
2,2',4,4',5,6'-Hexabromodiphenylether	207122-15-4	BDE 183	0.60
2,2',3,4,4',5,6,6'-Octabromodiphenylether	446255-54-5	BDE 183	0.49
2,2',4,4',5,5'-Hexabromobiphenyl	59080-40-9	BDE 183	1.39
Pentabromo-2,3-dihydro-1,1,3-trimethyl-3-phenylindane	252252-95-2	BDE 183	1.53
Heptabromo-2,3-dihydro-1,1,3-trimethyl-3-phenylindane	287726-93-6	BDE 183	1.38
2,2',3,3',4,4',5,5',6,6'-Decabromobiphenyl	13654-09-6	BDE 209	1.68
Octabromo-2,3-dihydro-1,1,3-trimethyl-3-phenylindane	155613-93-7	BDE 209	1.44
1,2-Bis(pentabromophenoxy)ethane	61262-53-1	BDE 209	1.54
Decabromodiphenylethane	84852-53-9	BDE 209	1.75
1,4-Bis(pentabromophenoxy)tetrabromobenzene	58965-66-5	BDE 209	3.33
2,4,6-Tris(2,4,6-tribromophenoxy)-1,3,5-triazine	25713-60-4	BDE 209	3.62

2,4,6-tribromophenyl allyl ether is listed as LPVC in EU, which means that it is placed on the market in volumes between 10 and 1000 tonnes per year and distributor. In the US, the production volume was between 226 and 450 tonnes in 1998, and between 4 and 226 tonnes in 2002 (http://www.epa.gov/oppt/iur/tools/data/2002-vol.htm). 2,4,6-tribromophenyl allyl ether has been used as a reactive BFR (Andersson et al. 2006), and was recently identified in seal blubber and brain (von der Recke and Vetter 2007). However, it was shown that the presence of 2,4,6-tribromophenyl allyl ether was possibly due to transformation of the more abundant 2,3-dibromopropyl-2,4,6-tribromophenyl ether (also used as a flame retardant but not considered in the present study).

Pentabromoethylbenzene is listed as a LPVC in EU, and in the US the production volume in 1986 was between 4 and 226 tonnes (there was no reported use in 1990, 1994, 1998, or 2002). Pentabromoethylbenzene has been detected in eggs of herring gull (Gauthier et al. 2007), and in air samples from the US (Hoh and Hites 2005). Further, release of pentabromoethylbenzene has been observed from polymeric flame retardants (Goutex et al. 2008).

2,4,6-Tribromophenyl allyl ether

Pentabromoethylbenzene

Octabromo-2,3-dihydro-1,1,3-trimethyl-3-phenylindane

Decabromodiphenylethane

1,4-Bis(pentabromophenoxy) tetrabromobenzene

2,2',4,4',5,'5,'-hexabromobiphenyl (BB 153)

Decabromobiphenyl (BB 209)

1,2-Bis(pentabromophenoxy)ethane

Pentabromo-2,3-dihydro-1,1,3-trimethyl-3-phenylindane

Heptabromo-2,3-dihydro-1,1,3-trimethyl-3-phenylindane

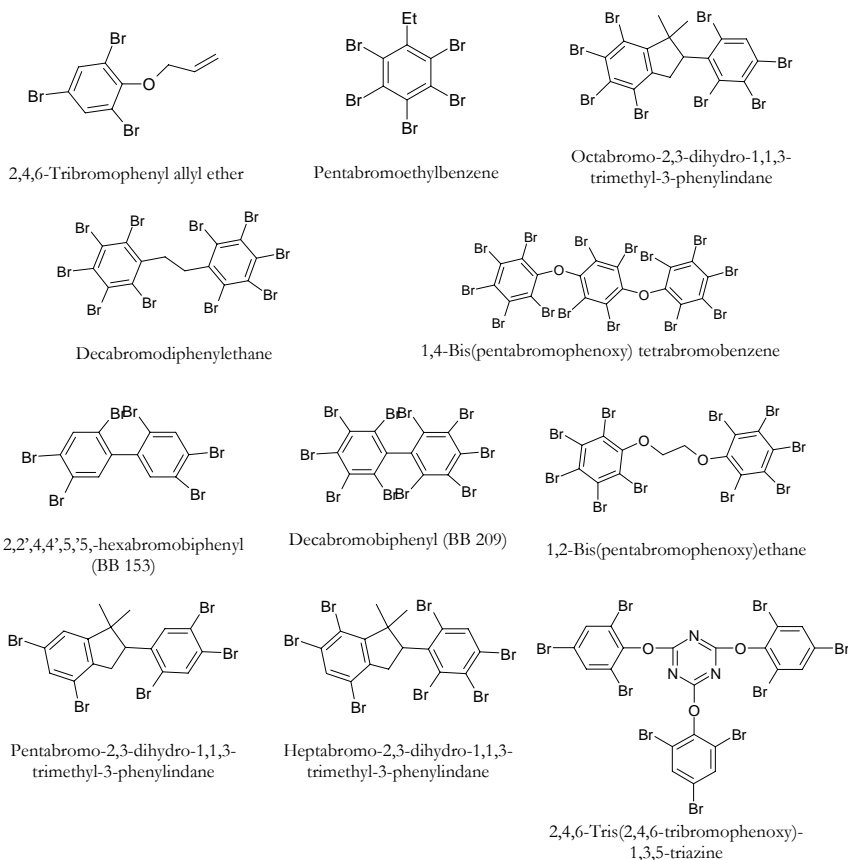2,4,6-Tris(2,4,6-tribromophenoxy)-1,3,5-triazine

Figure 10. . Chemical structures of the BFRs that were found to be similar to BDE 28, BDE 183 or BDE 209 (except for other PBDEs). The two upper rows show BFRs with known production (> 4 tonnes per year in 2002 or later). No information on recent production volumes was found for BFRs on the two lower rows. The indanes are examples of penta-, hepta, and octabrominated congeners.

No information was found on production volumes of **pentabromo-1,1,3-trimethyl-1-phenylindane** and **heptabromo-1,1,3-trimethyl-1-phenylindane**. However, the production of **octabromo-1,1,3-trimethyl-1-phenylindane** was between 450 and 4500 tonnes in 1998, and between 4 and 226 tonnes in 2002 in the US. No information on environmental abundance of octabromo-1,1,3-trimethyl-1-phenylindane was found in the literature. It was searched for but not detected in eggs of herring gull (Gauthier et al. 2009).

Decabromodiphenylethane is a LPVC in the EU, but no information on production in US was found. Decabromodiphenylethane has been detected in sewage sludge (Ricklund et al. 2008), in eggs of herring gull (Gauthier et al. 2009), in fish (Law et al. 2006), and in Chinese captive panda (Hu et al. 2008). Thus, this compound seems to be wide-spread in the environment and it would also be interesting to study its potential debromination products, since no such studies were found in the literature. **1,2-bis(pentabromophenoxy)ethane** is not a LPVC or HPVC in the EU, and in the US the reported use in 1986 was between 226 and 450 tonnes, but there was no reported use in 1990, 1994, 1998 or 2002.

1,4-bis(pentabromophenoxy)tetrabromobenzene is listed as LPVC in EU, and is on the OECD HPVC list. In the US, the reported production volumes were between 450 and 4500 tonnes in 1998 and 2002. However, no information on toxicity or environmental abundance was found in the literature for 1,4-bis(pentabromophenoxy)tetrabromobenzene. For **2,4,6-tris(2,4,6-tribromophenoxy)-1,3,5-triazine**, no information on production volumes were found.

In conclusion, the PCA-nearest neighbor model identified 20 BFRs with similar physico-chemical properties as BDE 28, BDE 183 or BDE 209, of which eleven is already restricted in the EU **(PBDEs, 2,2',4,4',5,5'-hexabromobiphenyl** and **decabromobiphenyl)**. No information on production volumes were found for additionally four BFRs (pentabromo-1,1,3-trimethyl-1-phenylindane, heptabromo-1,1,3-trimethyl-1-phenylindane, 1,2-bis(pentabromophenoxy)ethane, and 2,4,6-tris(2,4,6-tribromophenoxy)-1,3,5-triazine). Thus, the BFRs that are most urgent to PBT test are: 2,4,6-tribromophenyl allyl ether, pentabromoethylbenzene, octabromo-1,1,3-trimethyl-1-phenylindane, decabromodiphenylethane, and 1,4-bis-(pentabromophenoxy)tetrabromobenzene.

9. Conclusions and future aspects

In the work underlying this thesis, PBT properties of structurally diverse BFRs with a large spread in physico-chemical properties were studied. Large variation in biodegradability, bioaccumulation potential and toxicity was observed among the studied BFRs. Comparing the properties of the compounds to the test criteria under the REACH legislation, BDE 28 was the only studied BFR that met all three PBT classification criteria. If also considering degradation products, both BDE 183 and BDE 209 could be classified as PBTs. HBCD is a borderline case and more studies on persistency are needed, in order to classify this BFR. Since only PBDEs were found to fulfill the PBT criteria, BFRs with similar physicochemical properties as PBDEs could be prioritized for future PBT testing. In order to highlight some non-tested BFRs with physico-chemical descriptors similar to the PBDEs, a simple PCA-nearest neighbor model was used. In this analysis, 2,4,6-tribromophenyl allyl ether, pentabromo-ethylbenzene, octabromo-1,1,3-trimethyl-1-phenylindane, decabromodiphenyl-ethane, and 1,4-bis(pentabromophenoxy)tetrabromobenzene were identified as chemicals with similar physicochemical properties to the PBDEs and they are currently or have recently been produced in high volumes.

In environmental risk assessment, classification criteria generally require traditional standardized tests for determination of bioaccumulation and toxicity. However, some of these tests are not relevant for super hydrophobic compounds, like the highly brominated BFRs. Therefore, criteria and guidelines should be defined and developed for tests tailored for hydrophobic compounds, e.g. determination of BSAFs and for tests of aquatic bioaccumulation and toxicity after dietary exposure. Some additional issues that also could be of importance in PBT testing were found in the studies that make up this thesis. Paper I highlighted the effect of temperature on the biodegradation of BFRs. In large parts of the world, the temperature is far below 20ºC for most of the year and therefore degradation tests could be performed at lower or fluctuating temperatures to better simulate environmental conditions. In paper IV, several metabolites were formed and retained in zebrafish, which shows the importance of searching for and identifying persistent degradation products. Differences in bioaccumulation pattern were observed among studied animals (earthworms, zebrafish, claw frog), which shows there is a need to study bioaccumulation and toxicity in more than one organism. In paper V, simultaneous exposure to low concentrations (individually causing no significant effect) of six BFRs significantly affected the survival of *Nitocra spinipes*. The effects of mixtures need to be further studied and also considered to a greater extent in regulatory work.

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