Abstracts
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Part 3

Effects and Metabolism
<table>
<thead>
<tr>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effects and Metabolism</td>
<td>123</td>
</tr>
<tr>
<td>Comparison of exposure-effect relationships for phenolic chlorinated and brominated organohalogens A. Brouwer, A. Bergman, I. Brandt, L. Hagmar, P.J.J. Sauer, H. Galligher and H. Lillienthal</td>
<td>125</td>
</tr>
<tr>
<td>Polybrominated diphenyloethers (PBDEs): A novel class of environ-mental neurotoxicants in our environment P. Eriksson, H. Viberg, E. Ankarberg, E. Jakobsson, U Örn and A. Fredriksson</td>
<td>128</td>
</tr>
<tr>
<td>A preliminary characterisation of behavioural alterations following perinatal exposure to a polybrominated diphenylether (PBDE 99) I. Branchi, E. Alleva and L.G. Costa</td>
<td>130</td>
</tr>
<tr>
<td>Capacity of PBDEs to induce CYPIA by the Ah receptor mediated pathway N.J. Bunce, G. Chen, E.M. Joyce and N.C. Bosl</td>
<td>131</td>
</tr>
<tr>
<td>Health effects of polybrominated dioxins and furans L.S. Birnbaum</td>
<td>135</td>
</tr>
<tr>
<td>Cellular disturbance caused by various brominated flame retardants R.H. Boecker, B. Schwind, V. Kraus, S. Pullen and G. Tiegs</td>
<td>138</td>
</tr>
<tr>
<td>Impacts of brominated flame retardants on development and reproduction of two Copepod species, Nitocra spinipes and Acartia tonsa M. Breitholtz, L. Wollenberger, B-E. Bengtsson and K.O. Kusk</td>
<td>140</td>
</tr>
<tr>
<td>Metabolism of polybrominated diphenyl ethers in the rat E. Klasson-Wehler, A. Mörck and H. Hakk</td>
<td>145</td>
</tr>
<tr>
<td>Tissue disposition, excretion and metabolism of 1,2-bis(2,4,6-tribromo- phenoxy)ethane (BTBPE) in male Sprague-Dawley rats H. Hakk, G. Larsen and J. Bowers</td>
<td>150</td>
</tr>
<tr>
<td>Increased susceptibility to adult flame retardant exposure (PBDE 99) in mice neonatally exposed to nicotine E. Ankarberg, A. Fredriksson, E. Jakobsson and P. Eriksson</td>
<td>P17 154</td>
</tr>
<tr>
<td>Congener specific determination of polyhalogenated aromatic hydrocarbons (X=Br, Cl): Relative equivalent potencies (REPs) to 2,3,7,8-TCDD measured by micro-EROD- and DR-CALUX®-biossay P.A. Behnish, K. Hosoe, K. Shiozaki, A. Brouwer and S. Sakai</td>
<td>P18 157</td>
</tr>
<tr>
<td>Thyroidogenic, estrogenic and dioxin-like activity of polybrominated diphenyl ethers (PBDEs) in vitro A. Brouwer, I.A.T.M. Meerts, Å. Bergman and H.T. Besselink</td>
<td>P19 161</td>
</tr>
<tr>
<td>Toxicity of a technical mixture of polybrominated diphenyl ethers following 28 days of oral exposure in the rat E. Fattore, A. Falk-Filipsson, A. Hanberg, A. Bergendorff and H. Håkansson</td>
<td>P20 165</td>
</tr>
<tr>
<td>The effect of polybrominated diphenyl ethers on growth and development in grey seal (Halichoerus grypos) pups A.J. Hall, O.I. Kalantzi, G.O. Thomas and K.C. Jones</td>
<td>P21 167</td>
</tr>
<tr>
<td>Results of a prenatal development toxicity study of decabromodiphenyl oxide in rats M. Hardy, J. Biesemeier and O. Manor</td>
<td>P22 170</td>
</tr>
<tr>
<td>Frog embryo teratogenic assay: Xenopus (FETAX) analysis of the biological activity of tetrabromobisphenol A (TBBPA) E.A.E. Garber, G.L. Larsen, H. Hakk and Å. Bergman</td>
<td>P23 175</td>
</tr>
<tr>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Comparative study of PBDE 99 and Aroclor 1254 neurotoxicity</td>
<td>P24</td>
</tr>
<tr>
<td>M. Llansola, S. Erceg, C. Montoliu and V. Felipo</td>
<td></td>
</tr>
<tr>
<td>Inquiries on the action mechanism of brominated flame retardants: Ah-receptor mediated activity of selected flame retardants</td>
<td>P25</td>
</tr>
<tr>
<td>I. van Overmeire, D. Brown, M. Chu, G. Clark and L. Goeyens</td>
<td></td>
</tr>
<tr>
<td>EROD-induction by polybrominated diphenyl ethers in cultured chick embryo livers</td>
<td>P26</td>
</tr>
<tr>
<td>A. Pettersson, M. Engwall and H. Olsman</td>
<td></td>
</tr>
<tr>
<td>Sublethal effects of the flame retardants hexabromocyclododecane (HBCDD) and tetrabromobisphenol A (TBBPA) in juvenile rainbow trout (Oncorhynchus mykiss)</td>
<td>P27</td>
</tr>
<tr>
<td>D. Ronisz, E. Farmen Finne, H. Karlsson and L. Förlin</td>
<td></td>
</tr>
<tr>
<td>Perturbation by PBDE99 of Calcium homeostasis after in vitro treatment</td>
<td>P28</td>
</tr>
<tr>
<td>K. Smolnikar, M. Dehnhardt and H. Wiegand</td>
<td></td>
</tr>
<tr>
<td>Neonatal exposure to hexabromodiphenyl ether (PBDE 153) affects behaviour and cholinergic nicotinic receptors in brain of adult mouse</td>
<td>P29</td>
</tr>
<tr>
<td>H. Viberg, A. Fredriksson, E. Jakobsson, U. Örn and P. Eriksson</td>
<td></td>
</tr>
<tr>
<td>Brominated flame retardants: Uptake, retention and developmental neurotoxic effects of decabromo-diphenyl ether (PBDE 209) in neonatal mouse</td>
<td>P30</td>
</tr>
<tr>
<td>H. Viberg, A. Fredriksson, E. Jakobsson, U. Örn and P. Eriksson</td>
<td></td>
</tr>
<tr>
<td>Preliminary results of a mass balance study of a commercial pentabromodiphenyl ether mixture in male Sprague-Dawley rats</td>
<td>P31</td>
</tr>
<tr>
<td>H. Hakk, J. Huwe and G. Larsen</td>
<td></td>
</tr>
<tr>
<td>Wildlife and human models to assess the metabolic fate of poly-brominated diphenyl ethers (PBDEs) and metabolite formation and depletion</td>
<td>P32</td>
</tr>
<tr>
<td>R.J. Letcher and E. Bennett</td>
<td></td>
</tr>
<tr>
<td>Metabolism of decabromodiphenyl ether (BDE-209) in the rat</td>
<td>P33</td>
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<td>A. Mörck and E. Klasson Wehler</td>
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Comparison of Exposure-Effect Relationships for Phenolic Chlorinated and Brominated Organohalogenes

A. Brouwer¹, A. Bergman², I. Brandt³, L. Hagmar⁴, P.J.J. Sauer⁵, H, Gallagher⁶ and H. Lillienthal⁷

Summary
This paper will present an overview of effects of phenolic organohalogenes in animal studies, and will give information on the design of a new EU-sponsored comparative study abbreviated as the COMPARE study, including exposure-effect relationships for chlorinated and brominated analogues of organohalogenes in vitro, in long-term animal studies and in human individuals.

Overview on Phenolic Organochlorines
From the mid nineties of last century on the identification and appearance of phenolic metabolites of organohalogenes, in particular OH-PCBs in e.g. human blood plasma and in biota became clear[1]. Moreover, several studies, including the recently finished EU-RENO study reported on their potent interaction with several different endocrine systems and signal transduction pathways, including the thyroid and estrogen system and the Ah-receptor pathway[2]. In an early exposure, long-term effects animal study design it was found that a representative OH-PCB metabolite elicited long-lasting effects at low dose levels on several neurobehavioral paradigms and on oestrous cyclicity in rat offspring exposed to the model OH-PCB during gestation day 10-16. Finally, in both animal and in human infant exposure studies it was observed that OH-PCBs can rapidly transfer from dam to foetus (in animals

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studies, using radiolabelled analogues) and are found in higher concentrations in cord blood plasma as compared to maternal plasma, suggesting that the placenta does not function as a barrier for foetal exposure to OH-PCBs[3,4]. These data, and the realisation that many more organohalogen compounds (both chlorinated and brominated) with similar molecular characteristics are present in human blood plasma prompted us to develop and design the COMPARE study, presented below.

**COMPARE study**
The main objective of the COMPARE study is to improve the understanding of comparative pathways for early life-stage exposure and long-term effects of several classes of organohalogen compounds. A selection will be made of compounds (parent and hydroxy metabolites) representative for e.g., PCBs, chlorobenzenes, brominated bisphenol’s and-diphenylethers. The ultimate goal is to provide a mechanism-based approach for the assessment of human health risks from exposure to complex mixtures of organohalogen substances. Overall the workplan includes an a) environmental chemistry part; b) a toxicology part; and c) a clinical/epidemiological part.

**Environmental chemistry part**
Specific aims of this part are: a) to identify and quantify both neutral, but particularly phenolic organohalogens present in human serum and in some selected food items. Several phenolic PCB metabolites have already been identified in human serum[1] but many more peaks of both chloro and bromo analogues of benzenes, phenols, diphenyl ethers and bisphenol’s are present in the chromatograms awaiting further identification. b) for a selected part of the presently identified organohalogen substances (OHS) and their phenolic metabolites a validation of the methodology will be performed for human serum; c) several representative OHS and their hydroxy metabolites will be synthesized in unlabelled and 14C-labelled versions for toxicology studies. The criteria for selecting the (to be) synthesized compounds include: presence in human serum, relative potency in in vitro tests, availability/synthesis options, urgency from regulatory angle, past information. A list of suggested compounds for synthesis will be presented.

**Toxicology part**
Earlier studies have found several levels of interference of, in particular phenolic organohalogens in the thyroid system (competitive inhibition thyroxine-binding to TTR; UGT
induction, sulfotransferase inhibition) in the estrogen system (estrogen receptor agonists and and/or antagonistic activities, inhibition of estradiol sulphation) and in the Ah receptor pathway (both agonistic and/or antagonistic activities). For details see Poster by Brouwer et al., at this symposium. In the COMPARE project more congeners, identified in the environmental chemistry part will be studied for their in vitro potency profile. In addition, some selected representative OHS, synthesized within the framework of the COMPARE project will be used in early exposure (during gestation) long-term effects study designs. The focus will be on several different neurobehavioral paradigms, estrous cyclicity and testis development. Furthermore, the possible role of TTR in the transplacental and blood-brain barrier transport of phenolic OHS will be studied in detail, using and comparing transfer kinetics in TTR knockout mice and wt mice. Furthermore the exact localization of OHS metabolites in e.g. brain areas and in the fetoplacental unit will be studied in detail using 14C-labelled analogs.

Clinical/epidemiological part
In this part two different clinical/epidemiological study cohorts are included. A) The Swedish East and West Coast Fishermen’s wives cohort has been studied in follow up for many years already. In this project the study will focus on possible associations between exposure to OHS (in particular their hydroxy metabolites) and increased risks for osteoporosis, manifested as increased incidence of skeletal fractures and reduced mineral bone mass, and endometriosis. B) The Dutch-Groningen Infant cohort, which has also been studied already for prolonged times. However, for this particular study, a new cohort of mother-child pairs will be included. The main focus of the study will be on possible associations between early exposure to OHS (particularly hydroxy metabolites) and outcomes in neurobehavioral development, endocrine and immunological indices in the developing child. Further details on study design will be presented.

At the moment possible additional collaborations with other researchers, particularly from the United States is under consideration. Overall the COMPARE project, which runs from March 2001 until January 2004 should provide a scientific basis for a sound human risk assessment of exposure to complex mixtures of organohalogens and their hydroxy metabolites.

References:
4 EU-RENCO project (ENV4-CT96-0170) Final report, 2000
Here we show that developmental (neonatal) exposure to some PBDEs can induce behavioural derangement in mice, a condition that gets worse with age. These are developmental defects that have previously been detected in connection with PCBs. Furthermore, neonatal exposure to low doses of nicotine can potentiate and/or modify reactions to adult exposure to polybrominated diphenylethers. Brominated flame-retardants are a novel group of global environmental contaminants\(^1,2\). Within this group the polybrominated diphenyl ethers (PBDEs) constitute a class that are used at relatively high concentrations in electrical appliances, including television and computer casing, building materials, and textiles\(^3\). PBDEs are persistent compounds that appear to have an environmental dispersion similar to that of polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDT)\(^2\). PBDEs have been found in various wildlife species and in human tissues\(^1,4\), and are now seen increasing in mother's milk\(^5\).

In several studies we have shown that low-dose exposure of environmental toxic agents such as PCBs, DDT, as well as well-known neurotoxic agents such as nicotine, organophosphorous compounds and 1-methyl-4-phenyl-1,2,3,6-tetrahydpyridine (MPTP), during the period of rapid brain growth, known as the "brain growth spurt" ("BGS")\(^6\), in neonatal mice can lead to disruption of the adult brain function\(^7\), and to an increased susceptibility to toxic agents as adults\(^8\). The studies have also shown that there is a critical phase in the neonatal development, when the maturational processes of the developing CNS are at a stage of critical vulnerability, during which these persistent effects are induced\(^7,9\). In humans, this period begins during the third trimester of pregnancy and continues throughout the first 2 years of life; in mice and rats this period is neonatal, spanning the first 3-4 weeks of life.

In our present studies we have found that neonatal exposure to different single PBDE congeners can induce neurotoxic effects that become functionally evident in the adult animal. Induction of permanent aberration in spontaneous behaviour has been observed following neonatal exposure to 2,2',4,4'-tetrabromodiphenyl ether (PBDE 47), 2,2',4,4',5-pentabromodiphenylether (PBDE 99)\(^10\), 2,2',4,4',5,5'-hexabromodiphenyl ether (PBDE 153) and 2,2', 3,3',4,4',5,5',6,6'-decabromodiphenyl ether (PBDE 209). Moreover, this effect
seems to worsen with age, as evident after neonatal exposure to PBDE 47, PBDE 99, PBDE 153 and PBDE 209. Furthermore, neonatal exposure to PBDE 99 and PBDE 153 also affected learning and memory functions in the adult animal. In animals with deficits in learning and memory function following neonatal exposure to PBDE 153, the cholinergic nicotinic receptors in the hippocampus were affected. In the developmental neurotoxic effects of PBDE 99 there appears to be no gender difference.

An environmental mischance commonly occurring in nature is the combination of early exposure (perinatal/neonatal) and later adult exposure to various toxic substances. We have observed that exposure to low doses of nicotine during the BGS in mice can potentiate susceptibility to PBDE 99 in adult life. This combined neonatal and adult exposure caused spontaneous behavioural aberrations, an effect that also was seen to worsen with age. This indicate that neonatal exposure to nicotine - even in low doses - can potentiate and/or modify the reaction to adult exposure to brominated flame-retardants, and thereby accelerate dysfunctional processes.

It is particularly worth noting that these developmental effects of the studied PBDEs, on spontaneous behaviour and habituation processes, are similar to those we have previously observed for certain PCBs and also induced at doses comparable to those used in our earlier PCB studies11. This indicates that PBDEs can be as potent inducers of developmental behavioural aberrations as PCBs. Furthermore, the developmental neurotoxic effects of PBDEs together with its effects on adult animals neonatally exposed to nicotine is of special concern, not only for PDBEs as a single agent, but of possible interactive effects between these new environmental agents and other toxicants affecting the cholinergic system, such as nicotine and PCBs.

References
A preliminary characterisation of behavioural alterations following perinatal exposure to a polybrominated diphenylether (PBDE 99)

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Levels of polybrominated diphenyl ethers (PBDEs), a class of widely used flame retardants, appear to be rising rapidly in the environment. Evidence comes from studies on several sentinel species such as marine mammals and birds, as well as humans, in particular from breast milk monitoring programmes. Use of these compounds has become common practice, despite the paucity of information about the neurotoxic/behavioural teratogenic effects of such compounds on the developing organism.

The purpose of this study was to evaluate the potential effects on neurobehavioural development of perinatal exposure to PBDE in mice. PBDE 99 (2,2',4,4',5-penta BDE; 0.6, 6, and 30 mg/kg/day in corn oil) was administered daily to female CD-1 Swiss mice by gavage from gestational day 6 to weaning at post-natal day (PND) 21. Aroclor 1254 (6 mg/kg/day), a PCB mixture, was administered following the same schedule and served as a positive control. Higher doses of PBDE had an effect on litter viability. No changes in both sensori-motor development (PND 2-20) or ultrasonic vocalisations emission profile (PNDs 4,8,12) were found. At PND 11, the homing test revealed a trend for treated animals, especially the Aroclor 1254 group, to be more active than controls. This spontaneous motor behaviour alteration was confirmed and strongly increased at PND 34 in a open-field test. All PBDE and Aroclor 1254 groups were found to be hyperactive, but the latter showed a greater alteration. Rearing behaviour followed the same profile.

In agreement with earlier studies, these findings show that behavioural alterations due to perinatal PBDE exposure seem to worsen with increasing age, in particular they start to be clearly evident around one month of age. A characterisation of learning and memory performances and social behaviour in mice perinatally exposed to this compound is still warranted.

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Capacity of PBDEs to induce CYP1A by the Ah receptor mediated pathway

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Abstract—PBDE congeners and commercial mixtures bind to the Ah receptor and are weak to moderate inducers of ethoxyresorufin-O-deethylase (EROD) activity. Relative EROD potencies are similar across the vertebrate phylum (human, rat, chicken, and trout cells).

Very little is yet known about the toxicology of PBDEs, but their structural similarities with other classes of halogenated aromatic compounds (HACs), notably the PCBs, suggested that PBDEs might activate the aryl hydrocarbon receptor (AhR) signal transduction pathway, which is a critical toxicological mechanism for many HACs. This response causes the induction of the cytochrome P-450 isozyme CYP 1A1, which can be assayed as 7-ethoxyresorufin-O-deethylase (EROD) activity. These events are relevant to environmental concerns because of the strong rank-order correlation for many HACs between strength of Ah receptor binding, CYP 1A induction, and toxicity.

Ah receptor binding assays: The EC_{50} values for Ah receptor binding of individual PBDE congeners in competition with 1 nM of the reference toxicant 1 nM TCDD were in the μM range. Relative binding affinities (RBAs) ranged from 2 x 10^{-2} (congener 85) to 2 x 10^{-5} (congeners 153 and 154). There was some tendency for the congeners expected to be more “coplanar” (PBDEs 77 and 126) to have stronger binding than those with two or more o-Br atoms, such as 71, 100, 153, and 154.

The most abundant congeners in the commercial mixtures (PBDEs 47, 99, 153, 154, and 183) all had RBAs < 6 x 10^{-4}. Commercial PBDE mixtures also bound weakly to rat hepatic Ah receptor, and their RBAs corresponded closely to those of their major components (PBDE 47 in the case of the “penta” mixture, and PBDE 183 in the case of the “octa”). We could not determine the RBA of the commercial decaBDE because of its very low solubility. The RBAs of the commercial mixtures were unaffected by prior passage through a carbon chromatography column, a treatment intended to remove possible traces of highly active minor components such as brominated dibenzodioxins or dibenzofurans, which would exaggerate the binding strength of the PBDEs (compare PCBs).
For previously examined HAC families, the strength of binding to the AhR is determined by the planarity and lipophilicity of the ligand (5), indicating that the AhR has a non-polar, planar binding site to which ligands must accommodate themselves. To investigate this question for PBDEs we followed the approach of Kodavanti et al. (6), who used molecular mechanics calculations to show, for the PCB family, that the “coplanar” congeners, which bind most strongly to the AhR, exhibited the smallest energy differences between their equilibrium and coplanar geometries. The calculated energies needed to force coplanarity of the PBDEs were larger than for PCBs, and did not parallel the strength of AhR binding. They depended only on the number of o-Br substituents: $\Delta E \sim 13-14$ kcal/mol for zero and one ortho Br; $\Delta E \sim 26-27$ kcal/mol for two or three ortho Br. Our interpretation is that whereas PCBs (for example) must accommodate themselves to the geometry of the AhR binding site, for PBDEs the binding site becomes distorted by the large atomic volume of bromine and renders the issue of ligand planarity inconsequential.

**EROD induction**: The ability of PBDEs to induce EROD activity was studied in chick and rat hepatocytes, in liver cell lines from rainbow trout (RTL-W1), rat (H4IIE) and human (HepG2), and in a human intestinal cell line (Caco-2). PBDEs 77, 100, 119 and 126 induced the greatest EROD activity in all cell types. Compared with TCDD, their maximal EROD activities were less and their EC$_{50}$s were much larger. PBDEs 153 and 183 were weak inducers in all cells. PBDEs 66 and 85 were very weak inducers in rat hepatocytes, but were inactive in the other cells. The environmentally prominent congeners 47 and 99 were not inducers in any cell line; neither were PBDEs 28, 47, 99 and 154 and the commercial PBDE mixtures.

The relative potencies (REPs) of individual PBDEs for EROD induction were expressed as the EC$_{50}$ for TCDD/EC$_{50}$ for PBDE congener, where the EC$_{50}$ is the concentration needed to induce 50% of the maximal EROD response (Table 1). The REP values are empirical only, because of the variation among congeners and cell lines of the maximal EROD induction, which we took as 100% in order to carry out the probit analysis for calculating EC$_{50}$s. Most REPs were similar in cells from different species. Exceptions were PBDEs 66 and 85, which were weakly active in rat liver cells but not in the other cells, and PBDEs 77 and 126, which were approximately 10-fold more potent in chick hepatocytes, which are also particularly sensitive to mono ortho PCBs.

**Parallel between AhR binding and EROD induction**: Ah receptor binding is only the first stage of signal transduction leading to CYP 1A1 formation. Many substances bind the AhR without inducing EROD activity, often because the liganded Ah receptor fails to become
activated towards DNA binding. We explored this issue for PBDEs using the gel retardation assay, using a synthetic oligonucleotide that contained the consensus sequence for the dioxin response enhancer. Congeners that failed to induce EROD activity also failed to produce a shifted band in the gel retardation assay. This was particularly striking for PBDE 85, which had the largest RBA for Ah receptor binding, yet failed completely to activate the AhR towards binding to the synthetic oligonucleotide.

PBDEs are thus another class of HACs that potentially contribute to the total “dioxin-like” activity of environmental samples. Fortunately, they are much less active than potent HACs such as PCDDs, PCDFs, and coplanar PCBs, and the most prominent congeners environmentally appear to be the least active. Nevertheless, PBDEs are persistent, bioaccumulative, and increasing in abundance in environmental biota (7), implying that exposed organisms experience their biochemical or toxic effects for protracted periods. In the context of risk assessment, the similarity of EROD response across the whole vertebrate phylum means that it is not necessary to evaluate the REP of every PBDE congener or mixture for every species of interest.

**Acknowledgement:** We thank the Natural Sciences and Engineering Research Council of Canada and the Canadian Toxic Substances Research Initiative for financial support.

**References**

Table 1. Relative binding affinities (RBAs) for rat Ah receptor and relative induction potencies (REPs) for PBDE congeners in different cell cultures

RBA=EC$_{50}$ of TCDD/ EC$_{50}$ of PBDE for Ah receptor binding  
REP=EC$_{50}$ of TCDD/ EC$_{50}$ of PBDE for EROD induction

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<td>47</td>
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<td>28</td>
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</table>

$^1$RTL-W1= rainbow trout liver cell line; $^2$CEH = primary chick hepatocytes; $^3$PRH= primary rat hepatocytes; $^4$H4IE = rat hepatoma cell line; $^5$Caco-2= human intestinal cell line; $^6$Hep G2 = human hepatoma cell line
Health Effects of Polybrominated Dioxins and Furans

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While there is only limited information, the polybrominated dibenzo-p-dioxins and dibenzofurans appear to have a similar spectrum of biological effects as their chlorinated congeners. The potency of the most toxic brominated congeners is similar to that of TCDD. Although the polychlorinated dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs) have been studied extensively for many years, there is a paucity of data on both the environmental and toxicological significance of the related polybrominated compounds (PBDDs/PBDFs). Interest in these compounds, however, has grown over the past decade with their detection in environmental samples, the realization that they occur as unwanted byproducts of combustion reactions and certain industrial processes, and the limited amount of data demonstrating a common spectrum of effects to that observed with TCDD, the prototypical polyhalogenated aromatic hydrocarbon. Production and combustion of brominated flame retardants (BFRs) appears to be a significant source for the release of PBDDs/PBDFs into the environment. In many cases, mixed brominated-chlorinated dioxins and furans can also be formed. There are several recent reviews dealing with the effects of PBDDs and PBDFs. Relatively few papers have appeared in the peer-reviewed literature since these reports were completed. Therefore, the focus of this report will be on an overview of the earlier reports plus new data not captured earlier.

PBDDs, PBDFs, and mixed chloro-bromo congeners (potentially nearly 5000 compounds) have similar characteristics, both physical-chemical and biological, to those of the chlorinated compounds. However, the large size of the bromine atom, relative to chlorine, and the difference in the strength of bromine-carbon bond, as compared to the chlorine-carbon bond, alter the biochemical properties of the bromine-containing congeners, resulting in altered susceptibility to enzymatic attack.

There is little data available on the pharmacokinetic properties of PBDDs/PBDFs other than that of 2,3,7,8-TBDD. TBDD is well absorbed, similar to TCDD, in rats after oral and pulmonary exposure. Dermal absorption is more limited than that of TCDD. Once absorbed, TBDD is distributed throughout the body with liver and adipose tissue being the major depots.

TBDD
is sequestered in a dose-dependent manner in hepatic tissue, as is TCDD. Feces is the major route of elimination, for both metabolites and parent compound. The half-life of TBDD in rats and humans appears to be similar to that of TCDD. TBDD is slowly metabolized by hydroxylation and debromination.

Even less data is available on the PBDFs. Studies on 1,2,7,8-TBDF demonstrated that it was rapidly metabolized and eliminated, with a short half-life, which is not a surprise given its similarity to 1,2,7,8-TCDF. In contrast, 2,3,7,8-TBDF appears to be more resistant to metabolism and has a much longer half-life than TCDF, likely due to steric hindrance of the bromine atom blocking metabolism. 2,3,4,7,8-PBDF appears to be extremely persistent.

The biological effects of PBDDs/PBDFs are similar to those for the chlorinated compounds. This is due to a common mechanism of action, involving binding to the Ah receptor. For congeners with similar position and degree of bromination, the binding affinity is similar to that seen for the chlorinated congeners. However, likely due to the large size of bromine, tribromo congeners have higher binding affinity than tri-chloro compounds; in contrast, the binding affinity of congeners with 5 or more bromines is less than that of the chlorinated compounds. The mixed bromo-chloro congeners had comparable binding to the fully chlorinated congeners. In vitro studies have mainly focused on the ability of PBDDs/PBDFs to induce CYP1A1 mediated enzyme activity in various cell systems. Behnisch and coworkers have recently examined the relative potency of multiple PBDD/PBDF congeners to that of their chlorinated analogs using the CALUX bioassay, and compared their results to those obtained using other in vitro models reflecting CYP1A1 activation. TBDD and 2,3-diBr,7,8-diCl-DD was essentially equipotent to TCDD; PeBDD was slightly less potent than TCDD; 2,3,7,8-TBDF was nearly equipotent to TCDD, while 2,3,4,7,8-PeBDF was less potent. In contrast, in vivo measures of CYP1A1 activity show greater discrepancies between the brominated and chlorinated congeners. While TBDD and the mixed dibromo-dichloro congener are more active in vivo in the rat than TCDD, PeBDD is considerably less active. This difference is even greater when subchronic studies were conducted in mice. TBDD was only 20% as active as TCDD in the liver, based on administered dose. However, if compared on a liver dose, TBDD was more potent than TCDD. TBDD, like TCDD, has also been shown to be anti-estrogenic in human breast cancer cells and able to transform murine peritoneal macrophages into tumorigenic cells.

In addition to the classical enzyme induction effects, TBDD causes other typical effects of TCDD, such as liver toxicity, thymic atrophy, and the wasting syndrome. Similar doses result in lethality. TBDD has been shown to alter reproductive parameters, decrease circulating thyroid hormone and vitamin A levels, and increase hepatic porphyrins. TBDD was also immunotoxic in monkeys, altering lymphocyte subsets similarly to TCDD. TBDD was also developmentally toxic in mice, inducing cleft palate and hydronephrosis with a similar potency to TCDD. However, TBDD was much less chloracnegic in rabbits than was TCDD.
In contrast, the PBDFs, while causing similar spectrum of effects to TCDD, are more divergent in their potencies. 2,3,7,8-TBDF causes lethality at similar doses to TCDF in the guinea pig, likely because of the lack of metabolism of TCDF in this species. However, it is more toxic in rats than is TCDF. This was also seen in the induction of terata in mouse, where TBDF was more potent than TCDF, and slightly less potent than TBDD or TCDD. 2,3,4,7,8- and 1,2,3,7,8-PeBDF were approximately equipotent, which is in contrast to their chlorinated partners in which the 2,3,4,7,8-PeCDF was more potent than 1,2,3,7,8-PeCDF, largely due to a differential rate of metabolism and elimination. However, both PeBDFs were less teratogenic than the PeCDFs.

Studies in fish embryos demonstrated that TBDD was as toxic, or more toxic than TCDD. TBDF had about 1/4 the toxicity of TCDD, and 2,3,4,7,8-PeBDF was approximately 1/10 as toxic as TCDD.

Effects of PBDDs/PBDFs in human have received little study, in part because there is no evidence of general population exposure. There have been two cases reported of health problems related to acute exposure in people, with demonstration of chloracne and persistence of TBDD. An occupational cohort has also been followed. These workers were involved with the use of BFRs and were tested for immunological function, as well as other general clinical chemistry parameters. There were minor changes in immune parameters, but no major impact on health status could be detected due to the TBDD/TBDF exposure.

Thus, the limited data base on the effects of PBDDs/PBDFs supports the hypothesis that they have similar biological properties to their chlorinated relatives. With the increasing environmental presence of BFRs, it is likely that human, and wildlife, exposure to the PBDDs/PBDFs will increase. Given the common mechanism of action and effects, it is reasonable to predict that their presence will incrementally add to the dioxin body burden of this class of highly persistent, bioaccumulative toxicants.

(This abstract does not reflect EPA policy.)

References
2Mennear, J.H.; Lee, C-C., Environ. Health Perspect., 1994, 102 (S1), 265-74.
7Behnisch, P.A.; Hosoe, K. Shiozaki, K. et al., This meeting, 2001, in press.
Among the various brominated flame retardants Tetrabromobisphenol A is one of the most frequently used compound. TBBPA causes at higher concentrations (100 µM) cytotoxicity and at lower concentrations (5-10µM) inhibition of specific Cytochrome P450 mediated reactions and at concentrations around 1 µM the suppression of T-cell specific surface proteins (IL-2).

Polybrominated hydrocarbons are frequently used as flame retardants in various materials which are present in our daily life, like electronic or electrical devices (computer or TV-housings) plastic materials and also in clothing. They are used to prevent burning. But in case of a fire corrosive gases or dangerous compounds can be formed (1). Therefore, flame retardants seem to be essential. Polybrominated flame retardants are lipophilic and some of them very stable compounds which can accumulate in fatty tissue of higher organisms including men (2,3). During production, during the time of use and during recycling processes several yet not always known compounds with lower molecular weight and lower bromine-content in comparison to the parent compounds are formed. Only little is known about the toxicological behaviour of the parent compounds and of the break-down products. Therefore we investigated the influence of several polybrominated flame retardants, some of their degradation products and also some of their with other halogene atoms substituted congeners on several functions of the cell (cytotoxicity, interaction with cytochrome P450 enzymes and the immune system). As flame retardants were investigated: Tetrabromobisphenol A (TBBP A), its bisallyl-ether and ist propyl-ether, as well as the decabromo-biphenyl, the decabromo-biphenylether and 2,4,6-tribromophenol.

Among the compounds investigated TBBPA causes cytotoxic effects on isolated rat hepatocytes in the 100 µM range what can also be seen by electron microscopy and what is routinely measured by a dose dependent leakage of lactate-dehydrogenase from the cells into the medium. A possible mechanism could be detected (inhibition of oxygen consumption of the cell).
At lower concentrations (5-10 µM) TBBPA as well as 2,4,6-tribromophenol inhibit predominantly cytochrome P450 2C9 and the orthologic enzyme of the rat with an apparent Ki of 3 µM. The halogenated congeners of the phenols are less potent (the compounds substituted with fluorine are activators for the enzymes).

At concentrations around 1 µM TBBPA as well as its bisall-ether and bis-propyl-ether congeners affect the immune system in spleen cells. While all other compounds exhibited no effect on several immunotoxicological relevant markers the above mentioned compounds inhibited the expression of CD 25 (the α-chain of IL-2) what could be demonstrated by immunohistochemical quantification in a laser scanning cytometer with a confocal microscope. At a concentration of 3 µM the expression of this protein which is one of the proteins responsible for the immunomediated defense of an organism against bacteria or viruses and it is also possibly involved in the defense of tumor cells is nearly completely inhibited.

References:
1 Troitsch, J., Flammschutzmittel, Kunststoffe 86, 1996, 629-635;
3 De Wit, C. A., Report 5065 SEP, 2000

This work was financially supported by grant F137 (The Bavarian Minister for the environment)
Impacts of Brominated Flame Retardants on Development and Reproduction of two Copepod Species, *Nitocra spinipes* and *Acartia tonsa*

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Summary

We are investigating impacts of Brominated Flame Retardants (BFRs) on the development of two copepods, the brackish water copepod *Nitocra spinipes* and the marine copepod *Acartia tonsa*. Our preliminary observations show that the development in both copepods is relatively insensitive to sublethal concentrations of tetrabromobisphenol-A (TBBPA) and its metabolite tribromophenol (TBP). Development rate was, however, affected in juvenile *A. tonsa* exposed to low levels of 2,2’,4,4’-tetrabromodiphenyl ether (BDE-47). We are also investigating impacts on the reproduction of *N. spinipes*, but we have not yet seen any adverse effects on copepod reproduction in experiments with TBBPA and TBP. Nevertheless, our conclusion is that BFRs tested so far are relatively toxic to both species.

Introduction

BFRs are used in a wide variety of commercial products, such as electrical equipment, textiles and building materials. In Sweden, levels of BFRs have increased drastically in human breast milk during the last ten-year period and BFRs have shown to elicit neurotoxic activity in mice at low levels. In the aquatic environment levels of BFRs are relatively high in e.g. fish and mammals, as well as in sediment. The trend is that levels of some BFRs are still rising.¹

The aim of the studies is to evaluate the impact on copepod development and reproduction of BFRs, which are present in the biota and that share structural features (polycyclic, halogenated etc.) with known pollutants. *Nitocra spinipes* and *Acartia tonsa* were considered suitable test species since they have been applied in acute and sublethal ecotoxicological bioassays for many years and represent an ecologically important category of aquatic organisms. The bioassays used in the present studies are rapid, cost-effective and powerful tools for screening of sublethal effects on crustaceans and have a great potential to play an important role in future risk assessment concerning BFRs as well as other substances.
Materials and Methods

Chemical substances have either been kindly provided by prof. Å. Bergman, Stockholm University, or were obtained by Promochem S. S. AB, Kungsbacka, Sweden.

DEVELOPMENT-REPRODUCTION TEST WITH NITOCRA SPINIPES: The development-reproduction test with N. spinipes was carried out, with minor modifications, as described earlier. Briefly, nauplii <36 hours old were randomly allocated to vials containing 5 ml of brackish water. Each vial, with 10-15 nauplii, was then randomly allocated to 8 replicates per group, added with 5 ml of two times the desired final test concentration. Copepods were fed with 30 µl of a feed suspension (prepared from commercial salmon feed). Test solutions were renewed (70 %) and copepods were fed (15 µl) every second day. On day 7-8, the larval development rate (LDR) was calculated as the per cent of copepodites among all offspring divided by the total number of juveniles at the beginning of the experiment. (At this time about 50 % of the offspring has normally reached the copepodite stage and offers a useful breakpoint to record alterations in LDR.) Day 15-18 (22 for TBBPA) gravid females became visible and were individually transferred to vials containing test solution. Exposure was terminated on day 18 (22) but individually isolated gravid females were left to hatch until day 22 (26). Nauplii were counted using light microscopy (LM). Remaining copepods were fixed in formaldehyde (4 %), sexed and examined for morphological malformations using LM. Adult acute toxicity tests (4-day-LC50) were performed according to Swedish Standard procedures.

LARVAL DEVELOPMENT TEST WITH ACARTIA TONSA: The larval development test with Acartia tonsa was carried out as described earlier. Briefly, the experiment started by adding 30-40 eggs to 100-ml glass beakers containing 80 ml of test solution. Test solutions were renewed (50%) on day 3. On day 0 and 3 copepods were fed microalgae (5·10^4 cells·ml^{-1}). The exposure was run for 5 days at 20 ± 0.5 °C and at a low light intensity under a 12:12 light: dark photoperiod. On day 5 (about 50 % copepodites in the control group at this time), 0.8 ml Lugol’s solution was added to each beaker. This kills, stains and preserves unhatched eggs, nauplii and copepodites, which were collected on a filter and counted by LM. LDR was expressed as the ratio of copepodites to the sum of nauplii and copepodites. The test was performed in a fully defined saltwater medium (18 ‰ salinity). Adult acute toxicity tests (2day-LC50) with A. tonsa were performed according to International Standards.

STATISTICAL TREATMENT OF DATA: For the acute toxicity tests LC50-values were calculated by probit analysis, using PROBIT. For calculation of EC50-values for the
inhibition of larval development, a statistical PC software program assuming continuos response data and a logarithmic normal distribution was used.

Results
In the acute toxicity test with adults, A. tonsa was most sensitive to TBBPA, followed by TBP and BDE-47. N. spinipes was also more sensitive to TBBPA than to TBP and BDE-47 (Table 1).

### Table 1

<table>
<thead>
<tr>
<th>Substance</th>
<th>Nitocra spinipes (4-day-LC50)</th>
<th>Acartia tonsa (2-day-LC50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBBPA</td>
<td>0.35 (0.30-0.41) mg/l</td>
<td>0.40 (0.37-0.43) mg/l</td>
</tr>
<tr>
<td>TBP</td>
<td>4.42 (3.62-5.55) mg/l</td>
<td>1.50 (1.06-1.83) mg/l</td>
</tr>
<tr>
<td>BDE-47</td>
<td>4.40 (3.70-5.40) mg/l</td>
<td>2.37 (1.44-21.7) mg/l</td>
</tr>
</tbody>
</table>

95% confidence intervals are shown in brackets

The 5-day-EC50-values on LDR in A. tonsa as well as Acute-to-Chronic-Ratios (ACR=LC50/EC50) for each substance are presented in Table 2. The sublethal effects of TBBPA and TBP were very close to the levels of general toxicity seen in the LC50-tests. For BDE-47, however, the ACR was as high as 182.

### Table 2

<table>
<thead>
<tr>
<th>Substance</th>
<th>5-day-EC50</th>
<th>ACR (LC50/EC50)</th>
</tr>
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<tr>
<td>TBBPA</td>
<td>0.125 (0.065-0.238) mg/l</td>
<td>3.2</td>
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<tr>
<td>TBP</td>
<td>0.811 (0.673-0.978) mg/l</td>
<td>1.9</td>
</tr>
<tr>
<td>BDE-47</td>
<td>0.013 (0.011-0.014) mg/l</td>
<td>182</td>
</tr>
</tbody>
</table>

95% confidence intervals are shown in brackets

In N. spinipes exposed to sublethal concentrations (≤4-day-LC50/10) of TBBPA and TBP, larval development rate, fecundity and sex ratio was not affected compared with the controls (data are not shown here).
Discussion

Our results indicate that BDE-47 seems to have a mode of action that manifests a biological response at a much lower concentration (ACR=182) in juvenile A. tonsa compared with adults. This may indicate a specific toxic effect of BDE-47 on larval development in this species. Juveniles shed their exoskeletons in order to grow (i.e. moulting) which is a sensitive and energy-consuming event during development. The fifth moult is connected to a metamorphosis from the nauplia into copepodite. Moulting as well as metamorphosis is regulated by ecdysteroids and the latter process is presumably also controlled by compounds similar to insect juvenile hormones (Andersen et al. 2001). The observed effect of BDE-47 on juvenile A. tonsa may be a result of disruption of processes controlled by the ecdysteroids or juvenile hormones. Interaction with receptors for signal molecules or modification of activity of key enzymes could also be possible.

It may also be of importance that in the acute toxicity test no food was given to the copepods, whereas in the subchronic test juveniles were fed algae. Presence or absence of organic matter may have dramatic impact on the bioavailability of toxic compounds. Since BDE-47 is very lipophilic it has a high potential for bioaccumulation, which will increase the uptake of the substance via the feeding organism.

TBBPA and TBP did not have any effects on LDR, reproduction or sex ratio in N. spinipes at concentrations below the acutely toxic levels. This, together with the low ACRs from the tests with A. tonsa, indicates that these substances do not have any specific mode of action in copepods. However, since the observed LC50 and EC50-values are ranging between 0.12 and 4.4 mg/l TBBPA as well as TBP must be classified as toxic to very toxic to copepods.

BDE-47 has not yet been tested on N. spinipes but will be during 2001 together with BDE-99 and -100. The latter two compounds will also be investigated in the larval development test with A. tonsa.

References

Metabolism of polybrominated diphenyl ethers in the rat

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Summary
A review on tissue distribution, metabolism and excretion of 2,2′,4,4′-tetra-, 2,2′,4,4′,5-penta- and decabromodiphenyl ether in the rat is presented.

Introduction
Polyhalogenated organic compounds are often persistent in the environment due to their low elimination rates from organisms, which results in their bioaccumulation. The potential for metabolism is generally considered to depend on the degree of halogenation and on the positions of the halogen atoms in the molecule. Polychlorinated biphenyls (PCBs) are among the most well studied pollutants in this respect. The persistence of PCB congeners increases with the number of chlorine atoms and if the para-positions are substituted whereas free meta-/para-positions facilitate metabolism.

Polybrominated diphenyl ethers (PBDEs) are less well studied but from the behaviour of, at least, tetra- to hexabrominated congeners in the environment and in the exposure data for humans, as well as from the tissue half-lives of tetra- to hexaBDEs in rats, similar trends as for PCBs have been indicated. Decabromodiphenyl ether (decaBDE), however, has been reported to be rapidly cleared from rats after oral administration, even though also a very low absorption of this compound was indicated. However, also after intravenous administration, a radiolabelled dose was rapidly excreted via faeces.

We have studied the metabolism, tissue distribution and excretion of 2,2′,4,4′-tetraBDE, 2,2′,4,4′,5-pentaBDE and decaBDE in rats after a single oral dose. The experimental conditions were principally similar, except for the inclusion of bile duct cannulated rats in the two latter studies.

Materials and Methods

Compounds; 2,2’,4,4’-Tetrabromo-[14C]diphenyl ether (BDE-47), specific activity 1 Ci/mol, purity >98%, and 2,2,4,4’,5-pentabromo-[14C]diphenyl ether (BDE-99), specific activity 0.25
Ci/mol, purity 98%, were synthesised\(^6\). Decabromo-[\(^{14}\)C]diphenyl ether (BDE-209), specific activity 15 Ci/mol, purity >98%, was synthesised by bromination with Br\(_2\) and AlBr\(_3\).

**Animal experiments:** In all the experiments, male Sprague-Dawley rats (3-4 rats/group) were kept individually in metabolism cages where urine and faeces were collected in 24h intervals ( bile as shown in Table 1). Tissues collected and for which data are presented are listed in Table 2. Food and water *ad libitum* and a 12h/12h dark/light cycle was standard.

Table 1. Experimental details of dose, formulation and experiment length.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose µmol/ kg bw</th>
<th>Formulation</th>
<th>Survival time</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDE-47</td>
<td>30</td>
<td>Corn oil</td>
<td>5 days</td>
</tr>
<tr>
<td>BDE-99</td>
<td>15</td>
<td>Corn oil</td>
<td>3, 6,12 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bile for 3 days</td>
</tr>
<tr>
<td>DecaDBE</td>
<td>3</td>
<td>Lutrol F127, soya phospholipid, water</td>
<td>3, 7 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bile: 0-4, 4-12, 12-24, 24-48 and 48-72h</td>
</tr>
</tbody>
</table>

**Extraction, clean-up and analysis:** Generally, tissue and faecal samples were homogenised and extracted with either chloroform/methanol or hexane:acetone. The extracts were washed with acidic water and subjected to size exclusion chromatography\(^7\). The essentially lipid free fraction containing parent PBDE and metabolites was further purified using chromatographic methods (silica gel column or TLC) and the samples analysed by GC/MS as such and after methylation. GC/MS analyses were performed on ITS40 for tetraBDE; GCQ and TSQ 700 for decaBDE and Micromass Autospec. Excretion and tissue distribution were determined by the radioactivity content in the samples (liquid scintillation counting (tetraBDE and decaBDE, and fluids from the pentaBDE study) and a tissue oxidiser (tissues and faeces from the pentaBDE study). Urine samples (tetra- and pentaBDE, decaBDE urine samples were not analysed) were partitioned with hexane and subjected to solid phase extraction (C\(_{18}\) cartridge), respectively.

**Result and Discussion**

**Excretion:** For the rat, urinary excretion is almost non-existing, with <0.5, <0.9 and <0.05% of the tetra-, penta and decaBDE dose, respectively, recovered in urine\(^8\). Urine excretion is low in the rat also for many other persistant pollutants, e.g. PCB\(^1\) and chlorinated diphenyl ethers\(^9,10\). However, in the mouse, tetraBDE is excreted mainly via urine (33% of an oral dose) which shows that it is important to be aware of potential species differences\(^8\).
For tetraBDE, a total of 14% of the radioactivity was excreted via faeces within 5 days (5.7% during the first 24h), of which a maximum of 9% corresponded to parent compound, and the remaining 5% represented different types of metabolites.

PentaBDE, a mean of 65% of the oral dose was excreted within 12 days but 44% was excreted within 3 days and 24% during the first 24h. Analysis of faeces day 1-3, showed that 26% of the dose (18% day 1) was excreted as parent compound and thus may represent unabsorbed dose. The bile duct cannulated rats excreted a total of 3.9% in the bile and 86% in faeces (52.5, 30.4 and 3.6% day 1, 2 and 3, respectively). This material was not analysed and it is thus not known whether it represents parent compound, and therefore possibly unabsorbed material, or metabolites excreted over the gut wall. However, as no bile salts were added to the rat to substitute the sampled bile, absorption may be expected to be lower than in a conventional rat.

DecaBDE was excreted to a larger extent than the tetra- and pentaBDEs, with 84% of the dose recovered in faeces within 7 days (72% the first 24 h and another 17% during the 24-48h interval). The excreted radioactivity consisted to 22% of parent compound (20% in the first 24h interval) and, thus, approximately 65% of the oral dose represented different types of metabolites. The bile duct cannulated rats excreted 9.5% of the dose via the bile and 87% via faeces (not further analysed).

The obtained results for decaBDE can not easily be compared to those reported by ElDareer et al as they used repeated oral dosing or single intravenous dosing where we used single oral doses. The intravenously administered decaBDE was reported to be rapidly excreted via faeces with 70% of the dose recovered within 3 days. Also after intravenous dosing, 18% of the dose was excreted as parent compound, 29% (20% of dose) was not extractable and thus a total of 57% of the dose excreted as different types of metabolites. Thus, the results are quite comparable to those obtained from a single oral dose (see above). Bile was collected for 4h from intravenously dosed rats and 7.2% were recovered, representing one metabolite. Less than 1% was parent compound.

**Tissue distribution:** The tissue distribution of lipophilic compounds is governed by the lipid content in the tissues, unless other factors contribute, e.g. protein binding. The data in Table 2 show that BDE-47 is mainly stored in the adipose tissues whereas for BDE-99 and decaBDE, the liver has a higher concentration. Noticeable is also the high concentration in the adrenals. For decaBDE, the observed results are similar to those previously reported, when literature data is recalculated to lipid weight and a similar lipid content is assumed.
Table 2. Tissue distribution determined as radioactivity (expressed as nmol/g lw).

<table>
<thead>
<tr>
<th></th>
<th>BDE-47 Nmol/g lw</th>
<th>BDE-99 nmol/g lw</th>
<th>DecaBDE nmol/g lw</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (µmol/kg bw)</td>
<td>30</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>Adipose tissue</td>
<td>693</td>
<td>116</td>
<td>0.17</td>
</tr>
<tr>
<td>Liver</td>
<td>128</td>
<td>224</td>
<td>13.9</td>
</tr>
<tr>
<td>Kidney</td>
<td>203</td>
<td>19</td>
<td>1.9</td>
</tr>
<tr>
<td>Lungs</td>
<td>134</td>
<td>80.5</td>
<td>2</td>
</tr>
<tr>
<td>Adrenals</td>
<td>n.d.</td>
<td>157</td>
<td>4.4</td>
</tr>
</tbody>
</table>

n.d. = not determined

Metabolism: For tetraBDE, five mono-hydroxylated metabolites were found in faeces and in tissues. The primary site of oxidation was indicated to be in the ortho-positions according to MS fragmentation data⁸. This is consistent with the results reported for chlorinated diphenyl ethers⁹,¹¹.

PentaBDE was metabolised to both mono-hydroxy tetra- and pentaBDEs, and to 3 dihydroxylated metabolites. In addition, two isomers of pentaBDE-thiols were indicated observed.

DecaBDE was metabolised to several hydroxylated metabolites, with mainly dihydroxylated debrominated metabolites observed in faeces⁷. No thiols were observed. However, indications of reactive intermediates were obtained as non-extractable radioactivity in primarily liver and gut wall tissues but also in faecal residues. The results obtained are consistent with those observed by ElDareer et al who reported low recoveries in the extraction of some tissues, e.g. liver, and faeces⁵. However, no structure information was obtained in that study due to the limitations in analytical techniques. In spite of this, at least three metabolites were indicated by HPLC and, by their retention times, to be more polar than the parent compound.

Non-extractable radioactivity was observed in tissues and faeces for all three PBDE congeners, being indicative of metabolism via reactive intermediates. In addition, unstable metabolites, reverting back to parent compound, was observed for all three compounds.

Acknowledgement

The results presented are partly based on separate studies on BDE-99 and decaBDE for which data is being compiled to be published. Special thanks to Dr. Gerald Larsen, Ulrika Örn and professor Åke Bergman for their valuable contributions.
References
Summary

A single oral dose of 1,2-bis(tribromophenoxy)ethane (BTBPE) was almost entirely excreted in the feces of rats at 72h, and tissue retention was minimal. Metabolites were excreted in the urine, bile and feces, but at an exceedingly low rate. It was concluded that BTBPE would not be appreciably absorbed in mammals following acute exposure via ingestion.

Introduction

BTBPE has the chemical structure shown in Figure 1, and is a brominated flame retardant (BFR) used in the production of plastic materials that require high manufacturing temperatures and light stability. Production figures are difficult to obtain, but US production in 1977 was between 100,000-1,000,000 pounds.¹ BTBPE is very hydrophobic, and, like many BFRs, would be expected to be persistent in the environment. However, it is possible that ether cleavage of BTBPE may yield 2,4,6-tribromophenol, or that it may serve as a good alkylating agent through displacement of a bromine atom. Environmental levels of BTBPE have not been reported to our knowledge.

The purpose of the present study was to administer a single oral dose of BTBPE to male rats, and measure the adsorption, tissue distribution, and excretion behavior of BTBPE. Metabolites in the urine and feces will be characterized by chromatographic and spectral means.

Figure 1. Chemical structure of the brominated flame retardant, 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE).
Materials and Methods

$[^{12}\text{C}]$ and $[^{14}\text{C}]$1,2-Bis(2,4,6-tribromophenoxy) ethane (BTBPE) were synthesized in-house by adding bromine water to phenol which yielded 2,4,6-tribromophenol quantitatively. Tribromophenol was refluxed with ethylene dibromide in acetone-DMF using K$_2$CO$_3$ as catalyst. The radiolabel was administered orally (8.0 mg/rat in peanut oil; 1.23 µCi) to seven conventional male rats and six bile-duct cannulated male rats (283-328 g; Sprague-Dawley, Taconic Labs, Germantown, NY, USA). The rats were housed in stainless steel metabolism cages. Urine, feces, and bile were collected at 24h intervals for 72h. The rats were anesthetized with halothane and killed by exsanguination. Adrenals, epididymal fat, G.I. tract, heart, kidneys, liver, lungs, spleen, testes, and thymus were removed. Urine, bile, and blood were assayed for radioactivity by counting aliquots in a liquid scintillation counter (LSC). Lyophilized feces and tissues were combusted in a tissue oxidizer, and the $^{14}$C counted by LSC.

The pooled, lyophilized feces were extracted with anisole, acetone, methanol, and water. Reversed phase C-18 HPLC analyses (C-18 DeltaPak, Waters Associates, Milford, MA, USA) were performed using a linear gradient of 20% water/methanol to 100% methanol. HPLC fractions were evaporated to dryness on a rotary evaporator, derivatized with Regisil® (Regis Technologies, Inc., Morton Grove, IL, USA) and submitted for GC/MS analysis. Protein in urine and bile was removed by methanol precipitation. The supernatants were acidified then applied separately to solid phase extraction columns (ENVI-18, Supelco, Bellafonte, PA, USA), eluted with water and methanol, and the eluants were applied to a Silica gel TLC plate (Analtech, Inc., Newark, DE, USA) with various mobile phases.

Results and Discussion

In the urine of conventional rats in 72h, only 1.6% of the BTBPE dose was excreted, and less than 0.03% was excreted in the urine of bile-duct cannulated rats (Table 1). Fecal excretion was high at 24h for conventional and bile-duct cannulated rats, i.e. 93% and 58%, respectively, and declined rapidly with time. Cumulative fecal excretion was greater than 100% and 94%, respectively. It was concluded that intestinal absorption of BTBPE was very poor, and that BTBPE largely passed out of the rat unchanged.

As a consequence of the low absorption, low tissue levels of BTBPE were observed. Only the carcass and GI tract contained greater than 0.1% of the dose at 72h in conventional rats (Table 1). Skin from the carcass only contained 0.005% of the dose/g, and therefore, the
bulk of the carcass $^{14}$C was presumably associated with muscle, abdominal fat, or bone. Less than 0.03% of the BTBPE was found in the liver and epididymal fat.

Approximately 39% of the $^{14}$C from 0-24h feces in conventional rats was non-extractable. Three unconjugated metabolites in 0-24h feces were observed which accounted for 1.3% of the extractable $^{14}$C. GC/MS analysis of fecal metabolites did not detect any brominated clusters, therefore, spectral characterization remains in progress. The remainder of the extractable fecal $^{14}$C was parent BTBPE [$\text{GC/MS: } M^+ 682 \text{ (6 Br), M-327 (355; 3 Br), M-355 (327; 3 Br), M-383 (299; 3 Br); } ^1\text{H-NMR: } 7.67 \text{ (s) and 4.43 (s)}$]. Analysis of urine and bile by TLC showed that no parent compound was present. In the bile, both conjugated and unconjugated metabolites were suggested by utilizing different solvent systems.

Previous work demonstrated that BTBPE was very poorly absorbed from the gut when administered for one day at 0.05%, 0.5%, and 5% of the diet. Less than 1% was eliminated in the urine while greater than 99% was excreted in the feces as parent. Adipose tissue, skin and thymus were the only tissues with measurable radioactivity following any single feeding of BTBPE. However, following 10 consecutive days of dosed feeding, all tissues contained $^{14}$C, and stomach, intestines, adipose tissue, kidney, and skin containing the highest concentrations. Another hexabrominated BFR, hexabromobiphenyl, was readily absorbed (>90%) from the intestines following a single oral dose. The half-life of hexabromobiphenyl exceeded the life of the rat, and it was not subject to appreciable metabolism.

BTBPE was soluble to only a limited extent in all the common vehicles used in oral dose preparations. Peanut oil was used for dosing, but was warmed to keep BTBPE in solution. Toluene and anisole were the best solvents for BTBPE, but could not be diluted with peanut oil without precipitation. Lutrol-Phospholipon 80 (Astra-Zeneca, Stockholm, Sweden) also was unsuccessful as a vehicle because preparation in water caused an immediate precipitation from solution. As a result of these observations, it was concluded that mammalian absorption and tissue disposition of BTBPE via ingestion would be minimal.

**Acknowledgments**

The authors would like to gratefully acknowledge the technical assistance of Barbara Magelky and Colleen Pfaff.

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Table 1. Recoveries of $^{14}$C from male rats dosed orally with 1,2-bis (2,4,6-tribromophenoxy) ethane (BTBPE) in a conventional and bile-duct cannulated study.

<table>
<thead>
<tr>
<th>Tissue/Excreta</th>
<th>Conventional (n=7)</th>
<th>Cannulated (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-24h</td>
<td>1.1 ± 0.22</td>
<td>0.01 ± 0.009</td>
</tr>
<tr>
<td>24-48h</td>
<td>0.4 ± 0.11</td>
<td>0.005 ± 0.011</td>
</tr>
<tr>
<td>48-72h</td>
<td>0.09 ± 0.05</td>
<td>0.02 ± 0.031</td>
</tr>
<tr>
<td>Bile</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-24h</td>
<td>----</td>
<td>0.2 ± 0.11</td>
</tr>
<tr>
<td>24-48h</td>
<td>----</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>48-72h</td>
<td>----</td>
<td>0</td>
</tr>
<tr>
<td>Feces</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-24h</td>
<td>92.8 ± 16.2</td>
<td>58.1 ± 33.6</td>
</tr>
<tr>
<td>24-48h</td>
<td>20.5 ± 6.7</td>
<td>30.8 ± 26.8</td>
</tr>
<tr>
<td>48-72h</td>
<td>0.7 ± 0.2</td>
<td>5.8 ± 6.8</td>
</tr>
<tr>
<td>Adrenals</td>
<td>0.002 ± 0.0005</td>
<td>0.0002 ± 0.003</td>
</tr>
<tr>
<td>Adipose (epididymal)</td>
<td>0.01 ± 0.004</td>
<td>0.0001 ± 0.0009</td>
</tr>
<tr>
<td>Blood plasma</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Carcass</td>
<td>1.5 ± 0.2</td>
<td>0.6 ± 0.6</td>
</tr>
<tr>
<td>G.I. tract</td>
<td>0.4 ± 0.07</td>
<td>1.5 ± 2.3</td>
</tr>
<tr>
<td>Heart</td>
<td>0.03 ± 0.05</td>
<td>0.0001 ± 0.0002</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.008 ± 0.002</td>
<td>0.0005 ± 0.0007</td>
</tr>
<tr>
<td>Liver</td>
<td>0.03 ± 0.003</td>
<td>0.06 ± 0.1</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.009 ± 0.005</td>
<td>0.0006 ± 0.0007</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.003 ± 0.003</td>
<td>0.0003 ± 0.0007</td>
</tr>
<tr>
<td>Testes</td>
<td>0.003 ± 0.002</td>
<td>0.0001 ± 0.0002</td>
</tr>
<tr>
<td>Thymus</td>
<td>0.02 ± 0.02</td>
<td>0.00001 ± 0.00003</td>
</tr>
<tr>
<td><strong>Total Recovery</strong></td>
<td><strong>117.6</strong></td>
<td><strong>97.1</strong></td>
</tr>
</tbody>
</table>

**References**

Increased susceptibility to adult flame retardant exposure (PBDE 99) in mice neonatally exposed to nicotine

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Summary
Low dose exposure of neonatal mice to nicotine has been shown to induce an altered behavioural response to nicotine in adult mice. Also the widely used flame retardant PBDE 99 (2,2’,4,4’,5-pentabromodiphenylether) has been shown to cause behavioural aberrations on spontaneous behaviour in adult mice neonatally exposed to PBDE 99. This study shows a behavioural disturbance in adult animals exposed neonatally to nicotine and adult to PBDE 99, a disturbance that also worsens with age.

Introduction
Present days environment is increasingly afflicted by vast numbers of hazardous contaminants and exposure to different environmental toxic agents can occur throughout life. This can include exposure to both persistent and non-persistent xenobiotics, which can induce brain disruption when administered during a critical phase of neonatal brain development⁴. One of the critical periods for normal maturation of the central nervous system (CNS) is known as "the brain growth spurt"⁴. This period does not take place at the same time in all mammalian species, in man, this period begins during the third trimester of pregnancy and continues throughout the first 2 years of life. However, in rodents, this period is neonatal, spanning the first 3-4 weeks of life. The brain undergoes several developmental phases during this period of rapid growth, such as major axonal and dendritic outgrowth, synaptogenesis, multiplication of glia cells, myelinization and numerous biochemical changes such as the development of the cholinergic transmitter system⁵. Earlier studies have shown that low-dose exposure to different environmental agents during the rapid development of the neonatal mouse brain can lead to irreversible changes in adult brain function⁴. The induction of these disturbances occurs at doses that apparently have no permanent effects when administered to the adult animal. These studies also indicate that
there is a defined critical period during neonatal development of the mouse brain when these persistent effects are induced. In a series of studies, Eriksson and co-workers have shown that different PBDE congeners have developmental neurotoxic effects. For example, PBDE 99, have been shown to cause altered spontaneous behaviour and changed learning and memory functions. Also nicotine, a well known addictive drug and former insecticide, has been shown to cause behavioural changes as well as changes in nicotinic receptors.

PBDE constitute a group of polybrominated flame-retardants that appears to have an environmental dispersion similar to that of PCBs and DDT. PBDE 99 is a congener that has been found in human plasma samples. With regard to some of the similarities in effects caused by PBDE 99 and nicotine when given during neonatal life the present study was undertaken to determine the interaction effects between neonatal nicotine exposure and adult exposure to PBDE 99.

Methods

Ten-day-old male NMRI mice received s.c. injections of (-)nicotine-base. (33 µg in 0.9% NaCl (10 ml/kg b.w.) twice daily for 5 consecutive days. Control animals received 0.9% NaCl (10 ml/kg b.w.) in the same manner. At the age of 5 months the animals received one single oral dose of PBDE 99 (8 mg/kg b.w. in 20% fat emulsion vehicle (10 ml/kg b.w.)) or 20% fat emulsion vehicle (10 ml/kg b.w.). 24 hours and 2 months after the PBDE 99 exposure the animals were tested in a spontaneous behaviour test.

Spontaneous behaviour was measured for 3 x 20 min in an automated device consisting of cages placed within two series of infrared beams (Rat-O-Matic, ADEA Elektronik AB, Uppsala, Sweden). The test measures locomotion (horizontal movement), rearing (vertical movement), and total activity (all types of vibration within the test cage, i.e. those caused by mouse movements, shaking and grooming).

The (-)nicotine-bi-(+)-tartrate where obtained from Sigma, St. Louis USA and the PBDE 99 was synthesized at Wallenberg Laboratory, Stockholm University, Sweden.

Results and Discussion

The present study showed that neonatal exposure to nicotine and adult exposure to PBDE 99 affected the behaviour at adult age. This aberration in behaviour also worsened with age. This behaviour change is not seen in animals treated with saline neonatally and exposed to PBDE 99 as adults nor in animals only exposed to nicotine in neonatal age and emulsion as adults. It
seems to be a combination of these two treatments that induces the behavioural change, indicating that the neonatal nicotine exposure made the animals more susceptible to the adult PBDE 99 exposure. In earlier studies we have reported behavioural changes in adult animals after neonatal exposure to PBDE 99. These animals showed altered spontaneous behaviour and memory and learning aberrations (11). In spontaneous behaviour tests, information about the animal’s ability to habituate to a novel environment can be obtained. Normal habituation is defined as a decrease in locomotion, rearing and total activity variables in response to the diminished novelty of the test chamber over the 60 min test period. In the present study normal habituation was demonstrated in all animals except the animals neonatally exposed to nicotine and exposed to PBDE 99 as adults. In these animals a hypoactive behaviour was displayed in the beginning of the test period, while toward the end, they became hyperactive. This behaviour is even more pronounced in the 7 months old animals, indicating a disturbance that worsens with age. The increased susceptibility to PBDE 99 at adult age indicates that neonatal exposure to nicotine, affecting the cholinergic system, can potentiate and/or modify reactions to adult exposure to brominated flame-retardants, and thereby accelerate neurodegenerative and/or aging processes.

References
Congener specific determination of polyhalogenated aromatic hydrocarbons (X=Br, Cl):
Relative equivalent potencies (REPs) to 2,3,7,8-TCDD measured by Micro-EROD- and
DR-CALUX®-bioassay

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b Kyoto University, Environment Preservation Center, Kyoto, Japan
c Kaneka Techno Research, Kobe, Japan
d Free University of Amsterdam, Inst. for Environmental Studies, The Netherlands
e BioDetection Systems, The Netherlands
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E-mail address: behnisch@takaken.kaneka.co.jp

Summary
The aim was to compare the activity of PBDD/F-congeners and their chlorinated homologues in in vitro CYP1A1- (Micro-EROD-bioassay) and luciferase induction (DR-CALUX®-bioassay). In case of PXDDs similar relative equivalent potencies (REP) could be analyzed, whilst for the PXDFs significant differences could be measured: 2,3,7,8-TBDF (EROD: 0.62/DR-CALUX®: 0.49 compared to WHO-TEF PCDD/F 0.1), 1,2,3,7,8-PBDF (DR-CALUX®: 0.41; compared to WHO-TEFPCDD/F 0.05) and 2,3,4,7,8-PBDF (EROD: 0.047/ DR-CALUX®: 0.088; compared to WHO-TEFPCDD/F 0.5). The decrease of the dioxin-like-potency with increase of halogenation was confirmed (see WHO, 1998)1.

Introduction
Incineration and wastewater treatment are a common method of waste recycling. Public concern regarding production and release of halogenated aromatic compounds such as dibenzo-p-dioxins and –furans (PXDD/Fs; X=Cl, Br, F) has increased. PBDD/DFs in waste recycling processes have been already determined in several studies by chemical analysis1-6. The comparison of dioxin-like potency measured by bioassays/biomarkers and chemical analysis of incinerator related emissions and residues have already been examined2,3,4. Therefore, the additional constitution of these brominated dioxin-like compounds to the sum of dioxin-like potency in waste related samples such as fly ashes/emissions or sewage sludge needs further investigations.
In a first step, the DR-CALUX®-bioassay was established and validated in comparison to the Micro-EROD bioassay by analyzing relative equivalent potencies (REPs) for several PXDD/PXDFs/PCBs-congeners and mixtures (see also literature 5). In addition, the present study reports on REP values from several other Ah receptor agonists and mixtures such as polyaromatic hydrocarbons (PAHs), polybrominated diphenylethers (PBDE) and brominated flame retardants (Bromkal BP6/Dow Firemaster 250).

**Validation study:** At first, the DR-CALUX® technology was validated with several dioxin-like compounds and mixtures in a cross-validation study with BioDetection Systems (BDS). The DR-CALUX® technology was established according to the guidelines from BDS (www.biodetectionsystems.com) and recently published studies8,9. The EC₅₀ value [in pM and the coefficient of variation CV], correlation coefficient [R²] value of the EC₅₀ curve, minimal quantification limit [MQL] and minimal detection limit [MDL] of 2,3,7,8-TCDD analyzed by DR-CALUX® in the cross-validation study with BDS [EC₅₀: 10 pM/ R²: 0.99/ MQL: 1.0 pM/ MDL: 0.29 pM; n=10]; analyzed by Bovee et al (1998)⁶ [EC₅₀: 7 pM, limit of detection: 0.27 pM]; Murk et al. (1996)⁸ [EC₅₀: 10 pM] and in the present study analyzed at KC [EC₅₀: 14.9 pM, n=88, CV:14% / R²: 0.99; MQL: 1.4 pM; MDL: 0.38 pM; n= 20 independent test] were comparable. REP values analyzed at BDS and KC showed similar values for 2,3,4,7,8-PCDF [BDS: 0.84; n=3; CV=23%; KC: 0.75, n=4, CV=8%], 2,3,4,7,8-PBDF [BDS: 0.099; n=2; KC: 0.088, n=5, CV=30%] and PCB-126 [BDS: 0.073; n=3; CV=22%; KC: 0.072, n=6, CV=19%]. Several reference mixtures of PCDD/Fs (BDS: 294, n=2; KC: 263, n=16; CV=15%; chemical origin: 242); of a mixture of non-, mono- and di-ortho- PCBs (BDS: 137, n=5; KC: 98, n=3; CV=21%; chemical origin: 161), a mixture of coplanar PCBs (BDS: 334, n=2; KC: 266, n=11; CV=22%; chemical origin: 242) and a mixture of PCBs/PCDDs/PCDFs (BDS: 145, n=1; KC: 164, n=7; CV=20%; chemical origin: 146) were also similar between these two laboratories [all data in pg TEQ/ml].

**Results and discussion:** In the second step several dioxin-like compounds and other Ah receptor agonists were analyzed by DR-CALUX® and Micro-EROD bioassay. The resulted REP values are listed in Table 1 and were compared to already published data (for reference see literature 8). Additionally, the responding dose-response curves are shown in Graph 1. The following list of compounds did not show activity in the DR-CALUX® in the applied concentration ranges and the resulting REP values are listed in brackets: 22′45′6-PBB [only maximum concentration active ≤ 3.8E-5]; p-bromo-phenol [only maximum concentration
active: ≤ 3.3E-4]; 2,4-Bromo-phenol [< 3.8E-3]; TBBPA [< 0.02]; 2,2,4,4’-T4BDE [< 3.6E-4]; 2,2,4,4’,5-P5BDE [< 4.2E-4]; 2,2,4,4’,5,5’-H6BDE [< 4.8E-4]; 2,3,3’,4,4’5,6-H7BDE [< 5.3E-4]. In case of the PBDE-congeners REP values analyzed by DR-CALUX® have been already reported, indicating that the in the present study used concentrations were not high enough: 1) 2,2’,4,4’-TBDE (7.1 x 10⁻⁷); 2) 2,2’,4,4’,5-PBDE (5.9 x 10⁻⁶); 3) 2,2’,4,4’,5,5’-HBDE (4.3 x 10⁻⁶); and 4) Bromkal 70-5-DE (4.8 x 10⁻⁶)¹⁰.

Future research is need to understand the impact of these brominated dioxin-like compounds to the biopotency in samples of waste incineration²-⁶, sewage sludge¹⁰ and in environmental samples¹.

References

Graph 1: Dose-response curves obtained in the DR-CALUX®-bioassay with a number of different PXDD/Fs (X=Br, Cl), PCBs and PAHs. Curves were fitted using a one-ligand curve-fit. Data points are means of three independent measurements.
Table 1: Relative equivalents potencies (REP) of several AhR agonists compared to 2,3,7,8-TCDD analysed by DR-CALUX® [present study (24 h kinetic) and Sanderson et al., 1996], in vitro AHH/EROD [Present study (72 h kinetic) with rat hepatoma H4IE cells; Safe et al., 1991, Schramm et al. 2000], in vivo by AHH induction in the rat (Safe et al., 1991) and in a rainbow trout early life stage mortality bioassay (ELS; Hornung et al., 1996). [the here cited references see literature 9].

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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>12378-PCDD</td>
<td>0.44 [3; 26]</td>
<td>0.51 [6; 32]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.79</td>
</tr>
<tr>
<td>2378-TCDF</td>
<td></td>
<td>0.079 [5; 29]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23478-PCDF</td>
<td>0.75 [4; 8]</td>
<td>0.43 [12; 32]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.69</td>
</tr>
<tr>
<td>2,3,7,8-TBDD</td>
<td>0.54 [3;26]</td>
<td>0.65 [3; 9]</td>
<td>2.3</td>
<td>5.3</td>
<td>0.65</td>
<td>0.35</td>
<td>1.1-2.5</td>
<td></td>
</tr>
<tr>
<td>2,3-diBr-7,8-diCDD</td>
<td>0.72 [6; 12]</td>
<td>0.69 [3; 38]</td>
<td>3.4</td>
<td>8.2</td>
<td>1.4</td>
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<tr>
<td>3,7-diBr-2,8-diCDD</td>
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<td>8-Br-2,3,7-triCDD</td>
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<td></td>
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<tr>
<td>2-Bromo-3,7,8-triCDD</td>
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<td>0.94 [3; 38]</td>
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<td>0.30 [3; 43]</td>
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<td>0.16</td>
<td>0.11</td>
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<td>0.60 [3; 52]</td>
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<td>2,3,7,8-TBDF</td>
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<td>0.62 [3; 44]</td>
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<td>0.41 [3; 13]</td>
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<td>0.047 [3; 49]</td>
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<td>Dow FR 250</td>
<td>1.3E-5 [3; 30]</td>
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<tr>
<td>Benzo[a]pyrene</td>
<td>4.4E-4 [3; 23]</td>
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<td>Phenanthrene</td>
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<td>PCB-126</td>
<td>0.072 [6; 19]</td>
<td>0.049 [6; 36]</td>
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<td>8.4E-6 [3; 7]</td>
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<td>PBB-77</td>
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<td>9E-4 to 1.4E-3</td>
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<tr>
<td>PBB-169</td>
<td>0.011 [2]</td>
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<td>1.2E-4</td>
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Thyroidogenic, estrogenic, and dioxin-like activity of Polybrominated Diphenyl Ethers (PBDEs) in vitro

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Summary
In the present study, the in vitro endocrine disrupting and dioxin-like activity of pure PBDEs and three synthetic PBDE metabolites was investigated by means of the DR-CALUX® and ER-CALUX® bioassays. In addition, binding of PBDEs to the thyroid hormone transport protein transthyretin (TTR) was studied using a T4-TTR competition binding assay.

Introduction
Polybrominated diphenyl ethers (PBDEs) are widely used as additive flame retardants in many different polymers, resins and substrates. Because of the widespread production and use of PBDEs, and their lipophilic characteristics, several PBDE congeners bioconcentrate and bioaccumulate in the environment¹. PBDEs have been detected in various biotic samples such as birds, seals, whales, and even in human blood, adipose tissue and breast milk²,³. One of the most sensitive end points of PBDE toxicity in vivo are effects on thyroid function, observed as induction of thyroid hyperplasia and alteration of thyroid hormone production in rats and mice⁴. Furthermore, PBDEs are shown to induce cytochrome P450 1A1 and 1A2 in vitro and in vivo⁵,⁶. Recently, studies have shown that many industrial chemicals such as bisphenol A and various phthalates possess (anti)estrogenic activity⁷. To date there have been few reports investigating the (anti)estrogenic activities of PBDEs and HO-PBDEs.

In the present study, we report on the possible endocrine disrupting and dioxin-like activity of pure PBDEs and (synthetic) PBDE metabolites by means of different in vitro assays
Material and Methods

Pure PBDE-congeners were synthesised as described before\(^{(8,9)}\). Hydroxylated metabolites of PBDEs were obtained by 1) \textit{in vitro} metabolic activation and 2) synthesised as described by Marsh \textit{et al}\(^{(10)}\). The T\(_4\)-TTR competition binding studies were performed as described before\(^{(11)}\). PBDEs, PBDE-OH, and PBDE-metabolites obtained from \textit{in vitro} metabolic incubations were finally dissolved in methanol. Competition binding curves were made by plotting relative \(^{125}\text{I}\)-T\(_4\)-protein binding (% of control, with control incubations of microsomes set to 100%) against the dilution factor. For estrogenic and dioxin-like activity studies, PBDEs were dissolved in dimethylsulfoxide (DMSO 99.9%; Janssen Chimica, Geel, Belgium). The Ah-receptor agonist and antagonist activities of the PBDEs were determined in the DR-CALUX\textsuperscript{®} bioassay, a recombinant H4IIE rat hepatoma cell line showing Ah receptor mediated expression of a luciferase reporter gene\(^{(12)}\). The estrogenic activities of the PBDEs were determined in the ER-CALUX\textsuperscript{®} bioassay, a human T47D breast cancer cell line stably transfected with an estrogen responsive luciferase reporter gene construct\(^{(13)}\).

Results and Discussion

![Figure 1 Structures of synthesized HO-PBDEs](image)

\textit{T\(_4\)-TTR competition binding studies.}

In table I the T\(_4\)-TTR binding inhibition potency of the tested PBDEs incubated with the three different microsome types are presented. The potency of a PBDE to compete with T\(_4\)-TTR binding appears to be both congener and cytochrome P450 specific. No competition was observed for any of the parent compounds (not shown). The 3 tested synthetic HO-PBDEs show competition with T\(_4\) for binding to TTR with the highest affinity observed with the T\(_4\)-like HO-PBDE. In table I it can also be seen that no T\(_4\)-TTR inhibition occurs with the higher brominated diphenyl ethers (e.g. BDE-166 and 190) after microsomal incubations. It is hypothesized that these PBDEs are not metabolized during the microsomal incubations. Further studies will be necessary to confirm this.
Table 1 Inhibition of T₄-TTR binding by PBDE-metabolites obtained after incubation with PB-, NF- or CLOF-induced microsomes

<table>
<thead>
<tr>
<th>BDE</th>
<th>PB-micr.</th>
<th>NF-micr.</th>
<th>CLOF-micr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4,6</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>2,2',4,6'</td>
<td>++</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2,3',4',6</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2,4,4',6</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2,2',4,4',6</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2,3',4,4',6</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2,3,4,4',5,6</td>
<td>+</td>
<td>-</td>
<td>-</td>
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</table>

Synthetic HO-PBDEs

<table>
<thead>
<tr>
<th>Synthetic HO-PBDEs</th>
<th>Rel. potency (T₄ set at 1.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₂-like</td>
<td>0.41</td>
</tr>
<tr>
<td>T₃-like</td>
<td>1.22</td>
</tr>
<tr>
<td>T₄-like</td>
<td>1.42</td>
</tr>
</tbody>
</table>

Inhibition potencies are given from the undiluted extract. ++ = 60% -100% inhibition, + = 20% -60% inhibition, - = 0-20% inhibition.

Estrogenic activity studies.

The most potent PBDE-congener tested [BDE-100 > BDE-75 > BDE-51 > BDE-30 > BDE-119] showed EC₅₀ values within a small concentration range of 2.5 to 3.9 µM (Table 1). These PBDE agonists were 250,000 to 390,000 times less potent than the natural ligand, E₂. The T₄-like HO-BDE compound demonstrated no estrogenic effect up to 10 µM. In contrast, the T₃-like and T₂-like HO-BDE showed the highest estrogenic potencies (EC₅₀ 0.5 and 0.1 µM, respectively) among all compounds tested in this study (Table 2). Antiestrogenic potency of PBDEs was determined in the ER-CALUX® bioassay by treating T47D.Luc cells with 0.01 to 10 µM concentrations of PBDEs in the presence of 10 pM of E₂ (not shown). Only BDE-166 and BDE-190, which did not induce luciferase activity alone (up to 10 µM), reduced E₂-induced luciferase activity.

Dioxin-like activity studies.

The results in table 2 summarize the EC₅₀-values and maximum luciferase induction of the PBDEs alone and the IC₅₀-values for the inhibitory response observed in cells co-treated with 15 pM TCDD plus different concentrations of PBDEs. At PBDE-concentrations from 0.01 to 25 µM, induction of luciferase activity varied from 0 to 65.8% of the response observed for 100 pM TCDD. Seven of the 9 tested PBDEs induced luciferase expression, indicating that they are able to activate the Ah-receptor. EC₅₀-values could only be determined for BDE-166.
and BDE-190. In cells co-treated with 15 pM TCDD plus different PBDE-concentrations, there was a concentration dependent decrease in TCDD-induced luciferase expression by 3 of the tested PBDEs.

Table 2

<table>
<thead>
<tr>
<th>BDE</th>
<th>LOEC (µM)</th>
<th>EC50 (µM)</th>
<th>Relative potency</th>
<th>EC50 (µM)</th>
<th>Induction (at 25 µM)</th>
<th>IC50 (µM)</th>
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<tr>
<td>2,4,6</td>
<td>1.0</td>
<td>3.4</td>
<td>2.9E-6</td>
<td>-</td>
<td>9.6 ± 0.5</td>
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<td>2,2',4,4'</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>3.3 ± 0.2</td>
<td>3.6</td>
</tr>
<tr>
<td>2,2',4,6'</td>
<td>1.0</td>
<td>3.1</td>
<td>3.2E-6</td>
<td>-</td>
<td>10.1 ± 0.4</td>
<td>-</td>
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<td>2,3',4,6'</td>
<td>2.5</td>
<td>7.3</td>
<td>1.4E-6</td>
<td>-</td>
<td>6.3 ± 0.7</td>
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<tr>
<td>2,4,4',6'</td>
<td>2.5</td>
<td>2.9</td>
<td>3.5E-6</td>
<td>-</td>
<td>13.2 ± 0.2</td>
<td>-</td>
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<td>1.0</td>
<td>2.5</td>
<td>4.1E-6</td>
<td>-</td>
<td>8.0 ± 0.9</td>
<td>n.r.</td>
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<td>3.9</td>
<td>2.6E-6</td>
<td>n.r.</td>
<td>26.9 ± 0.6</td>
<td>n.r.</td>
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<td></td>
<td>65.8 ± 5.0</td>
<td>Induction</td>
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<td>2,3,3',4,4',5,6</td>
<td>0.8</td>
<td></td>
<td>50.3 ± 4.0</td>
<td>-</td>
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T2-like 0.05 0.1 1.0E-4
T3-like 0.5 0.5 2.0E-5
T4-like - - -

LOEC = Lowest Observed Effect Concentration. Relative Potency as compared to estradiol.
Maximal luciferase induction (100%) was obtained with 100 pM TCDD. N.r. = not reached, -- = no induction or inhibition at all. N.d. = not determined.

Acknowledgements

This research was supported by the European Commission grant no: ENV4-CT96-0170.

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12. Aarts JMMJG.; Denison MS.; Cox MA.; Schalk MAC.; Garrison PM.; Tullis K.; de Haan LH.; Brouwer A., European Journal of Pharmacology, Environmental Toxicology and Pharmacology Section, 1995, 293, 463-474.
Toxicity of a technical mixture of polybrominated diphenyl ethers following 28 days of oral exposure in the rat

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Sprague-Dawley rats were exposed by gavage for 28 days to a technical mixture of polybrominated diphenyl ethers (PBDEs). Effects observed were dose-related enzymatic induction, hepatic vitamin A reduction, increased relative liver and kidney weights and hepatic histological changes.

PBDEs are widely used as flame retardants in textiles, computers, television sets and other electrical household appliances. The world production of these compounds in the early 1990’s was estimated to be 40,000 tonnes/year consisting of decaBDE (75%), octaBDE (15%) and pentaBDE (10%)². The low brominated PBDEs (tetra- and penta-) are persistent and lipophilic compounds, which bioaccumulate in the food chain. OctaBDEs are also persistent and lipophilic. However, it is unknown whether the high brominated PBDEs (octa- and deca-) bioaccumulate in the food chain. The PBDEs are ubiquitous pollutants today, found in sediments, wildlife and human tissues, and the PBDE level in human milk (summary of tri- tetra-, penta- and hexaBDEs) has increased exponentially in Sweden during the last 25 years³.

In this study the toxicity of Bromkal 70-5 DE, a technical mixture of PBDEs, was investigated in a 28-days exposure study in the rat. Bromkal 70-5 DE mainly consists of the congeners 2,2’,4,4’-tetaBDE (BDE-47), 2,2’,4,4’,5-pentaBDE (BDE-99) and 2,2’,4,4’,6-pentaBDE (BDE-100) accounting for 37, 35 and 6.8% of the mixture, respectively⁴.

Groups of five male and five female Sprague-Dawley rats, 5-6 weeks old, were exposed by gavage once a day for 28 days to 0, 2.5, 25 and 250 mg Bromkal 70-5 DE /kg bw/day. The animals were acclimatised for one week prior to treatment and they had free access to food and water. Lighting was maintained on 12-h light-dark cycle and room temperature and relative humidity were kept at 22±3°C and 40-60%, respectively.

The behaviour and the health-state of the animals, as well as the growth rate and food consumption were not affected by the treatment. Dose-related increases in relative liver and
kidney weights were observed in both male and female rats. The increases were significant at the highest dose-level. Histopathological findings consisted in dose-dependent liver changes (increase of fat cells, enlargement of hepatocytes). No significant differences were observed in the relative weight of other organs (thymus, spleen, lungs, heart, brain, ovaries, uterus, testis, pancreas, and adrenal glands). Dose-dependent increases in hepatic 7-ethoxyresorufin-\(O\)-deethylase (EROD) and 7-pentoxyresorufin-\(O\)-deethylase (PROD) activities, and reduction of the hepatic vitamin A content were observed in both male and female rats. The increase in EROD activity and the decrease of vitamin A were significant for males and females at the medium and high dose-levels. The increase in PROD activity was significant for males and females at the highest dose-level.

Blood chemical analysis showed an increase of total serum protein and serum cholesterol levels in both male and female rats at the highest dose-level. In addition, increases of alanine aminotransferase activity and calcium levels in male rats, and decreases in plasma levels of creatinine and the ratio albumin/globulin in female rats were observed.

Detailed knowledge about the toxicity and mechanism of action of PBDEs is limited. The present study indicates similarities between the studied PBDEs and dioxinlike compounds with regard to hepatic EROD induction and vitamin A reduction.

References
2 Arias, P. Brominated diphenyloxides as flame retardants, bromine-based chemicals; Consultants report to the OECD, 1992.
The effect of polybrominated diphenyl ethers on growth and development in grey seal (Halichoerus grypus) pups

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The aim of this study was to investigate the accumulation of polybrominated diphenyl ethers (PBDEs) in the blubber of grey seal pups and relate these concentrations to the health, condition, growth rate, circulating growth and thyroid hormone levels of animals during their first year of life.

Little is known about the effect of PBDEs, a widely used class of brominated flame retardant compounds, on the marine ecosystem. Concentrations are increasing in the environment¹ and many congeners have been found in marine top predators such as cetaceans and seals²,³. They bioaccumulate through the food chain⁴ and high levels were recorded in the livers of some fish from the Tees estuary, on the east coast of the UK⁵. Recent laboratory studies have indicated certain PBDE congeners have immunologic and endocrine effects, particularly affecting thyroid hormone transport and metabolism, similar to those reported for specific PCB congeners⁶,⁷. In this study we investigated the accumulation and potential effect of some of these compounds on a marine top predator, the grey seal, found on the east coast of the UK.

Animals were studied at weaning and during their first year of life, when growth rates are high and many developmental and physiological changes are occurring.

Grey seal pups are born largely on remote, uninhabited islands. Females give birth to a single white coated pup which it abandons at weaning. Pups then remain on the breeding beach and undergo a period of post-weaning fast, until they go to sea to forage for the first time. During this period of aphagia, pups may lose on average 0.5 kg per day body weight, most of which is blubber loss, used for energy requirements⁸. The physiological reason for this fast is not well understood and the factors which determine when animals go to sea are unknown.

During the 1998 and 1999 breeding seasons at the Farne Islands on the east coast of the UK, 170 and 45 pups respectively, were tagged after weaning with brightly coloured individually
numbered passive head tags\textsuperscript{9} as part of an ongoing mark-recapture study. This allowed individual animals to be identified both during the remainder of the fast and when animals were at sea or hauled out on land during their first year of life. This allowed them to be identified and recaptured when hauled out on land. Blood samples for clinical chemistry and hormone levels and blubber biopsies for PBDE congener analysis were obtained from all individuals at marking during the post-weaning fast and on recapture. A sub-sample of animals were re-bleed and re-weighed during the post-weaning fast until departure from the colony. 14 tagged animals were recaptured between 3 and 12 months after leaving the breeding colony. In addition 33 pups were sampled repeatedly during the breeding season and 41 untagged animals from the same birth cohort were sampled sometime during their first year of life. This gave us two groups of samples, longitudinally sampled pups and cross-sectional, cohort samples.

Serum, plasma and blubber samples collected were stored at below \(-20^\circ\text{C}\) until assayed for various blood parameters and hormones important in growth and metabolism and for PBDE congeners. Animals were weighed and measured and condition indices (mass/length as a surrogate for body fat) were calculated. Pups lost mass during the post weaning period (up to 20 days pre-departure) at a mean rate of 0.65 kg/day and gained mass over the next 12 months at a mean rate of 0.039 kg/day.

Clinical chemistry blood parameters investigated included calcium, cholesterol, creatinine, glucose, albumin, total protein, blood urea nitrogen and alkaline phosphatase. Changes in these parameters were largely associated with the periods of growth, fasting and feeding, such as glucose, creatinine, cholesterol and albumin which decreased during the post-weaning period and increased during the first year of life. Three classes of thyroid hormones, total thyroxine, total triiodothyronine and free triiodothyronine were also measured in blood samples. Total thyroxine and triiodothyronine were positively correlated with concentrations of albumin, a carrier protein for these hormones and plasma concentrations of the two hormones were significantly correlated to each other. They also decreased with age, both showing in general two-fold decreases in circulating levels during the first 3-4 months of life. Four individuals showed anomalies in these general patterns. Growth hormone levels were also measured although no distinct patterns of change were seen.

The relationship between the circulating levels of these hormones and the other clinical chemistry parameters and the concentrations of 15 PBDE congeners in the blubber will be investigated.
References

Results of a Prenatal Developmental Toxicity Study of Decabromodiphenyl Oxide in Rats

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Albemarle Corporation, Baton Rouge, LA, USA; Great Lakes Chemical Corporation, West Lafayette, IN, USA; Dead Sea Bromine Group, BeerSheva, Israel

Summary. DBDPO has undergone a wide range of toxicology tests in mammalian species with the results indicating a no-adverse-effect-level in repeated dose studies of ~1000 mg/kg/day. An oral prenatal developmental toxicity study performed under current guidelines and good laboratory practices was recently completed. Female rats (25 mated females/group) received 0, 100, 300 or 1000 mg DBPDO/kg/day via gavage in corn oil from Gestation Day 0-19. The NOEL (No Observable Effect Level) for maternal and developmental toxicity was 1000 mg DBPDO/kg/day, the highest dose level tested, and is consistent with 2 year chronic oral dose studies that showed no effect of DBDPO on the reproductive system of rats and mice at doses up to 2,550 and 7,780 mg/kg/d, respectively.

Introduction. Decabromodiphenyl oxide (DBDPO, CAS# 1163-19-5), also known as decabromodiphenyl ether (DBDPE), is a highly effective flame retardant that is primarily used in electrical and electronic equipment with a very important secondary application in upholstery textiles. Global market demand in 1999 for DBDPO was estimated at 54,800 tons, making DBPDO the second largest volume brominated flame retardant in use today and ~82% of all polybrominated diphenyl oxide (ether) flame retardants sold. DBDPO has undergone a wide range of toxicology tests in mammalian species with the results indicating a no-adverse-effect-level in repeated dose studies of ~1000 mg/kg/day. An oral prenatal developmental toxicity study performed under current guidelines and good laboratory practices was recently completed and is described.

Materials and Methods. The test article was a composite of 3 lots of commercial DBDPO produced by Albemarle Corporation, Great Lakes Chemical Corporation, and Ameribrom (Dead Sea Bromine Group). Male and female Sprague Dawley rats (CD[CrI:CD(SD)GS BR], 1

1Study sponsored by the American Chemistry Council’s Brominated Flame Retardant Industry Panel (BFRIP).
Charles River Laboratories, Portage, MI) were acclimated for approximately 1 week after arrival and prior to mating. Male rats were used only for mating. The animal room was maintained between 67-70°F and 52-70% humidity, and monitored daily. Fluorescent lighting provided illumination for approximately 12 hours per day. Diet (Certified Rodent Chow, #5002, PMI Nutrition International, Inc., St. Louis, MO) and tap water were available ad libitum.

The study consisted of 3 treatment groups and 1 vehicle (corn oil) control group of 25 mated female rats/group. Female rats were mated in-house and were treated daily on gestation days 0-19 with DBDPO via gavage at dose levels of 0 (vehicle control), 100, 300 or 1000 mg/kg/day at a constant volume of 5 ml/kg. Individual doses were based on the most recent body weight. The day on which evidence of mating was observed was considered day 0 of gestation. Dams were observed daily and maternal body weight and food consumption measured at appropriate intervals. Females were euthanized on day 20 of gestation and necropsied. Gravid uterine and liver weights were recorded. Litters were delivered by cesarean section. The total number of corpora lutea, uterine implantations, early and late resorptions, viable and nonviable fetuses, and the sex and individual weights of fetuses were recorded. All fetuses were examined grossly. Approximately one-half of the fetuses in each litter were stained with Alizarin Red S and Alcian Blue and evaluated for skeletal/cartilaginous malformations and ossification variations. The remaining fetuses of each litter were processed for cross sectional soft tissue evaluations. The maternal day 20 gestation examinations and cesarean sections, and subsequent fetal evaluations were performed blind to treatment. Appropriate statistical methods for each end point were used.

The study was designed to meet or exceed the United States EPA OPPTS Guideline No. 870.3700 of August 1998, and the OECD Guideline Study No. 414, August 1996, and was conducted in accordance with the United States EPA TSCA Good Laboratory Practice Standards, 40 CFR Part 792 and OECD Principles of Good Laboratory Practice [C(97)186 (Final)].

**Results (Table 1).** Chemical analysis of the neat test article demonstrated a purity of 97.3% DBDPO. Chemical analyses of the DBDPO suspensions demonstrated homogeneity, stability, and correct concentrations. No clinical signs related to treatment were observed in any group. All animals survived until scheduled sacrifice. Mean treatment group maternal body weights

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171
during gestation and mean body weight gain between each weighting interval and over the entire day 0-20 gestation period for the treated groups was statistically comparable to controls. DBDPO-treatment did not induce an adverse effect on food consumption. Mean treatment group maternal liver weights, absolute and relative to the adjusted day 20 gestation body weights, were statistically comparable to controls. No adverse effect was evident at gross necropsy at any DBDPO dose level.

Pregnancy rates in the control, 100, 300 and 1000 mg/kg/day dosage groups were 96%, 100%, 96% and 96%, respectively, and were statistically comparable to the control mean. The number of litters with viable fetuses in the vehicle control, 100, 300 and 1000 mg/kg/day groups was 24, 25, 23, and 24, respectively. No abortions or early deliveries occurred. No effect of treatment was detected on the number of dams pregnant, pregnancy index, number of dams with viable fetuses, corpora lutea/dam, implantation sites/dam, % preimplantation loss/dam, or % viable fetuses/dam or implant, fetal sex ratio, % postimplantation loss/dam, nonviable fetuses/dam, % early resorptions/implant or late resorptions/dam. The mean number of early resorptions in the 1000 mg/kg/day group was statistically higher than controls (1.4 vs. 0.6 in controls), but was within the historical range for the laboratory.

No effect of treatment was found on gravid uterine weights, adjusted Day 20 gestation body weights, adjusted body weight gain for days 0-20 of gestation, the male, female, and combined male and female fetal body weights, and fetal sex distribution. No external malformations or variations were seen in fetuses in the treated groups, and no effect of treatment was evident from the fetal visceral or skeletal examinations.

**Discussion and Conclusion.** Results from an earlier developmental study in rats using the 1970s commercial DBPDO product (77.4% DBPDO, 21.8% NonaBDPO, and 0.8% OctaBDPO) at doses up to and including 1,000 mg/kg administered on days 6-19 of gestation were consistent with the present study and negative for maternal toxicity and developmental effects. In the 1970s study, a statistical increase in the number of resorptions/implant site and the number of litters with resorptions at the 1,000 mg/kg dose was detected, but was comparable to the historical control, did not show a dose-response relationship and was considered due to the unusually low control value. Also, some fetal variations consisting of subcutaneous edema and delayed ossification of the interparietal bones of the skull were observed. A one-generation reproductive study, using a top dose of 100 mg/kg/d of the 77% DBDPO product, was also negative for reproductive effects. Likewise, 2 year chronic
studies using a DBDPO test article of ≥96% purity provided no evidence of toxicity on the reproductive system in rats and mice at doses up to 2,550 and 7,780 mg/kg/d, respectively. In the current study, female rats (25 mated females/group) received 0, 100, 300 or 1000 mg DBPDO/kg/day via gavage in corn oil from Gestation Day 0-19. No morality or evidence of toxicity was noted. Pregnancy rates in the control and treated groups ranged from 96-100% and provided 23 or more litters in each group for evaluation on gestation day 20. No effect of treatment was seen in maternal clinical findings, gestational parameters (body weight, body weight gain and food consumption), uterine implantation data, liver weights or necropsy findings. Likewise, no effect of treatment was seen in fetal body weights, fetal sex distribution, or on the fetal external, visceral, or skeletal examinations. The NOEL (No Observable Effect Level) for maternal and developmental toxicity was 1,000 mg DBPDO/kg/day, the highest dose level tested and administered from day 0-19 of gestation. Again, the results of this study are consistent with the lack of effect of DBPDO on the reproductive system in other repeated dose studies, and DBDPO’s minimal absorption and rapid elimination by the rat.

<table>
<thead>
<tr>
<th>Table 1. Abbreviated results from the DBDPO developmental toxicity study.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DBDPO Dose (mg/kg/day) Day 0-19 Gestation</strong></td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td><strong>Maternal Endpoints</strong></td>
</tr>
<tr>
<td>Body Wt, D 20 (g)</td>
</tr>
<tr>
<td>Liver/Body Weight (%)</td>
</tr>
<tr>
<td>Pregnancy Index (%)</td>
</tr>
<tr>
<td>No. Dams w/Viable Fetuses D 20</td>
</tr>
<tr>
<td><strong>Fetal Endpoints</strong></td>
</tr>
<tr>
<td>Total No. Malformations</td>
</tr>
<tr>
<td>Total No. Variations</td>
</tr>
<tr>
<td>No. Examined: Visceral</td>
</tr>
<tr>
<td>Total No. Malformations</td>
</tr>
<tr>
<td>Total No. Variations</td>
</tr>
<tr>
<td>No. Examined: Skeletal</td>
</tr>
<tr>
<td>Total No. Malformations</td>
</tr>
<tr>
<td>Total No. Variations</td>
</tr>
<tr>
<td>No. Examined: External, Visceral &amp; Skeletal Malformations</td>
</tr>
<tr>
<td>Total No. Malformations</td>
</tr>
</tbody>
</table>

L/F= number of litters examined/number of fetuses examined
No statistically significant differences between treatment and control groups (p<0.05)
Reference List

1 M. Hardy, *Organohalogen Compounds*, 2000, 47, 41-44.
Frog Embryo Teratogenic Assay: *Xenopus* (FETAX) analysis of the biological activity of tetrabromobisphenol A (TBBPA)

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The biological activity of tetrabromobisphenol A and bisphenol A were examined using the Frog Embryo Teratogenesis Assay: *Xenopus* (FETAX) bioassay. These analyses were conducted employing both the standard FETAX media and a mineral media which contains concentrations of sodium and potassium that barely prevent developmental retardation (S5511). Under both growth conditions 0.1, 1.0, 10, 100, and 500 ppb tetrabromobisphenol A and bisphenol A had no effect on *Xenopus* development.

Introduction:

Bisphenol A (BPA) is a ubiquitous compound employed in the production of epoxy resins used to line food and beverage cans and as a building block for polycarbonate plastics. BPA has been demonstrated to exhibit estrogenic activity though it is not definite whether it poses an environmental concern (1). In contrast little is known about the biological activity of tetrabromobisphenol A (TBBPA) which is employed as a flame retardant.

A commonly employed bioassay for the study of hormonally active agents is the Frog Embryo Teratogenesis Assay: *Xenopus* (FETAX). The FETAX bioassay examines the effects of aqueous agents on *Xenopus* embryo development during the first 96 hours of development. The endpoints examined including mortality, malformation rate, and growth inhibition / acceleration as indicated by a change in embryo length and the presence of features indicative of earlier / later stages.

Research has indicated that sodium and potassium content influence *Xenopus* embryo development and that the mineral content of the media may influence the susceptibility of frogs to biologically active compounds (2,3). This has raised questions regarding the
conditions under which bioassays are performed to screen environmental samples for the presence of hormonally active agents. As a result, the ability of BPA and TBBPA to effect *Xenopus* development was examined using the standard mineral media and a media (S5511) in which the sodium and potassium concentrations are barely sufficient to prevent developmental retardation. Under both conditions BPA and TBBPA displayed no activity in the FETAX bioassay at concentrations of 0.1, 1, 10, 100 and 500 ppb. Additional studies are necessary to determine whether these results are reflective of the narrow sensitivity of the FETAX bioassay or the compounds' biological activity.

**Materials and Methods:**
The FETAX bioassay was performed according to ASTM guidelines (4) as previously modified (5). In addition, the embryos were reared using both the commonly employed FETAX mineral media (10.7mM NaCl / 1.14mM NaHCO3 / 0.403mM KCl / 0.135mM CaCl2 / 0.349mM CaSO4·2H2O / 0.623mM MgSO4) and a mineral media (S5511) whose composition is barely sufficient to prevent developmental retardation and is similar to pond water encountered in Minnesota, USA. The composition of S5511 is 0.22mM NaCl/0.13mM KCl/0.41mM MgSO4/0.25mM CaCO3. No differences in the results obtained were observed upon using plastic petri plates in place of glass.

Inasmuch as the stock solutions of BPA and TBBPA were prepared in ethanol, the concentration of ethanol in the growth media was maintained at 0.015 %, which in control experiments had no effect on *Xenopus* embryo development during the time course of the FETAX bioassay.

**Results and Discussion:**
*Xenopus laevis* eggs harvested in the mid-blastula stage were exposed to 0.1, 1.0, 10, 100 and 500 ppb BPA and TBBPA in FETAX mineral media and S5511 mineral media. Table 1 summarize the results in mineral media and S5511 media for BPA and TBBPA. The slight, statistically insignificant decrease in the embryo lengths obtained in S5511 media versus the standard FETAX mineral media is characteristic of S5511 and reflected in the presence of a slight residual cement gland in the embryos. As indicated in Table 1, the presence of BPA and TBBPA had no effect on the survivability of the embryos, the malformation rate, or the length of the embryos.

A limitation of the FETAX bioassay is that it screens for biological activity during only the first 96 hours of embryo development when organ development is occurring. As such,
hormonally active compounds that only effect later stages of development appear to lack biological activity. In order to fully ascertain the biological activity of a compound it is therefore necessary to determine its activity on several organisms and during various stages of development.

Acknowledgments:
The authors greatly appreciate the technical assistance of Amy McGarvey, Paula Fernholz, Mark Davis, Andrew Jackson, and Jennifer Levi without whose assistance none of the research could have been accomplished. Financial support was obtained from MISTRA for synthesis of the TBBPA.

Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others than may also be suitable.

References:
**Biological Activity of BPA and TBBPA In The FETAX Bioassay**

<table>
<thead>
<tr>
<th>conc. (ppb)</th>
<th>Lethality (%)</th>
<th>Malformation (%)</th>
<th>length* (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineral Media</td>
<td>9</td>
<td>3</td>
<td>$9.5 \pm 0.5$ (100/2)</td>
</tr>
<tr>
<td>+ 0.015% EtOH</td>
<td>8</td>
<td>7</td>
<td>$9.7 \pm 0.5$ (50/2)</td>
</tr>
<tr>
<td>BPA</td>
<td>0.1</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>3</td>
<td>$9.6 \pm 0.4$ (50/2)</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>8</td>
<td>$9.4 \pm 0.5$ (50/2)</td>
</tr>
<tr>
<td>100</td>
<td>4</td>
<td>4</td>
<td>$9.6 \pm 0.3$ (50/2)</td>
</tr>
<tr>
<td>500</td>
<td>0</td>
<td>7</td>
<td>$9.6 \pm 0.5$ (75/3)</td>
</tr>
<tr>
<td>TBBPA</td>
<td>0.1</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>4</td>
<td>$9.7 \pm 0.4$ (50/2)</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>6</td>
<td>$9.7 \pm 0.4$ (150/4)</td>
</tr>
<tr>
<td>100</td>
<td>3</td>
<td>4</td>
<td>$9.7 \pm 0.4$ (50/4)</td>
</tr>
<tr>
<td>500</td>
<td>4</td>
<td>6</td>
<td>$9.6 \pm 0.4$ (150/4)</td>
</tr>
<tr>
<td>S5511</td>
<td>3</td>
<td>1</td>
<td>$9.4 \pm 0.4$ (50/2)</td>
</tr>
<tr>
<td>0.015% EtOH</td>
<td>1</td>
<td>3</td>
<td>$9.4 \pm 0.4$ (50/2)</td>
</tr>
<tr>
<td>BPA in S5511</td>
<td>0.1</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>4</td>
<td>$9.5 \pm 0.2$ (50/2)</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>5</td>
<td>$9.5 \pm 0.4$ (50/2)</td>
</tr>
<tr>
<td>100</td>
<td>2</td>
<td>1</td>
<td>$9.4 \pm 0.3$ (50/2)</td>
</tr>
<tr>
<td>500</td>
<td>2</td>
<td>4</td>
<td>$9.5 \pm 0.4$ (150/4)</td>
</tr>
<tr>
<td>TBBPA in S5511</td>
<td>0.1</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>3</td>
<td>$9.4 \pm 0.5$ (50/2)</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>4</td>
<td>$9.4 \pm 0.4$ (150/4)</td>
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<tr>
<td>100</td>
<td>7</td>
<td>4</td>
<td>$9.2 \pm 0.5$ (50/2)</td>
</tr>
<tr>
<td>500</td>
<td>6</td>
<td>6</td>
<td>$9.3 \pm 0.4$ (125/4)</td>
</tr>
</tbody>
</table>

* length is expressed as mm ± standard deviation (# embryos screened / # different clutches)
COMPARATIVE STUDY OF PBDE 99 AND AROCLOR 1254 NEUROTOXICITY

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We compared neurotoxicity of Polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) in primary culture of cerebellar neurons and we carried out a learning test to study possible alterations in learning of offspring of rats injected with PCB or PBDE during pregnancy.

Polychlorinated biphenyls (PCBs) have been used in the past in several technical and industrial applications, e.g., as flame retardants, in lubricants or in plastics. Because of their physical and chemical properties, PCBs are resistant to degradation, and their use has resulted in contamination of environment. It has been shown that PCBs are toxic compounds which induce hepatotoxicity, immunotoxicity, and cancer\textsuperscript{1}. There are also neurotoxicological effects that induce neurological dysfunction, and impair neuronal plasticity in experimental animals\textsuperscript{2}. To avoid the toxic effects of PCBs they have been substituted by polybrominated diphenyl ethers (PBDEs). These compounds are now used as flame retardants in plastic materials, as computer and cable insulators\textsuperscript{3} or in polymers and textiles, and they also accumulate in the environment\textsuperscript{4}. However, the toxicity of these compounds has not been studied in detail.

We are studying the possible neurotoxicity of one of these compounds, PBDE 99 and comparing its effects with those of a well known and studied PCB, Aroclor 1254.

We have carried out a comparative study of the neurotoxicity of PBDE 99 and Aroclor 1254 in cerebellar neurons in culture. Different concentrations of Aroclor 1254 or PBDE 99 were added to the culture medium during maturation in vitro. We have studied viability of neurons at 12-16 days in vitro. None of these compounds induced significant neuronal death at concentrations lower than 10 $\mu$M. At 10 $\mu$M PBDE 99 induced the death of 23% of the neurons, while Aroclor 1254 did not induce significant death. At higher concentrations both compounds induced significant neuronal death. 30 $\mu$M Aroclor 1254 induced death of 36% of neurons, and 30 $\mu$M PBDE induced death of 24% of neurons. PBDE 99 or Aroclor 1254 at 50 $\mu$M induced death of 75% of neurons. These results indicate that long-term exposure to Aroclor 1254 or PBDE 99 induce similar neuronal death in the same range of concentrations.
We have also studied neurotoxicity of PBDE 99 and Aroclor 1254 when we added the compound during 4 hours in Locke’s solution to cultured neurons at 11-14 days in vitro. We have observed that neither PBDE 99 or Aroclor 1254 induce significant neurotoxicity at concentrations lower of 10 \(\mu\)M. Higher concentrations of PBDE 99 or Aroclor 1254 induced significant neurotoxicity which increased with concentration. Aroclor 1254 induced death of 75\% of neurons at 50 \(\mu\)M while PBDE 99 50 \(\mu\)M induced death of 25\% of neurons, and PBDE 99 at 100 \(\mu\)M induced death of 38 \% of neurons. Thus, PBDE 99 is slightly less neurotoxic that Aroclor 1254 under these conditions (short-term exposure).

We are also studying the effects of PBDE 99 and Aroclor 1254 in rats in vivo. We have injected pregnant rats with PBDE 99 or Aroclor dissolved in olive oil. Control rats were injected only with olive oil. We injected the pregnant rats with a daily dose of 30 mg./kg. of PBDE or Aroclor 1254 during pregnancy: one group of rats in the E11-E19 period and another group in the period E2-E9.

We have observed that injection with Aroclor 1254 in the period E11-E19 induces a decrease of the body weight of offspring of either sex, while PBDE 99 injected in this period does not induce change of body weight. However, if we injected the PBDE 99 in the period E2-E9 of pregnancy we observed a significant decrease of the body weight of offspring.

When these rats reached adulthood, we carried out a learning test to study if there were differences in learning ability between offspring of rats injected with PBDE 99 or Aroclor 1254 and their controls. We used the learning test of conditional discrimination. To carry out this test a wooden Y-maze has been used. The whole area of the arms was covered by black or white inserts. Rats were rewarded for choosing the left arm when the inserts were black and the right arm when the inserts were white. The reward for the correct response was three food pellets placed in the food cup at the end of the correct arm.

References
Inquiries on the action mechanism of brominated flame retardants: Ah-receptor mediated activity of selected flame retardants

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2 Xenobiotic Detection Systems, Inc., 1601 East Geer Street, Suite S, Durham, NC 27704, US

Summary
A number of frequently used flame retardants were tested for AhR agonist activity with the CALUX bio-assay. Some compounds showed low but significant responses, indicating dioxin-like activity. Brominated dibenzo-p-dioxins and biphenyls were also tested and were readily detected with activities similar to their chlorinated analogues.

Introduction
Brominated flame retardants (BFRs) such as polybrominated diphenylethers (PBDEs) and polybrominated biphenyls (PBBs) are known to bioaccumulate in nature similar to polychlorinated dibenzo-p-dioxins, dibenzofurans and biphenyls and, consequently, pose a threat to environmental quality and human health. Levels of PBDEs in environmental matrices and in human milk, blood and adipose tissue are well documented.1,2 Although effects on thyroxine hormone levels, behaviour and interference with the Ah-receptor and estrogen receptor have been reported for PBDEs,1,3 the current knowledge of the toxicological actions of flame retardants is rather limited.

Objectives
The aim of this study was to investigate whether commonly used flame retardants elicit dioxin-like toxicity in the CALUX bio-assay. By using this assay all compounds acting through the Ah-receptor are detected. It is a suitable assay for estimating dioxin-like activity present in any sample, whether the sample is a pure compound or contains a mixture of compounds. We investigated some products for their potential dioxin-like activity:
1. pure compounds used as flame retardant or present at high concentrations in the biosphere (e.g. brominated diphenylethers, pentabromotoluene, tetrabromo-o-chlorotoluene, tetrachlorobisphenol A),
2. commercial technical mixtures containing brominated biphenyls, and
3. pure compounds such as brominated dioxins and biphenyls, which are known contaminants in synthetic flame retardant blends.

Discussion
Pure flame retardant compounds showed activities several orders of magnitude (10^5-10^6) less than the activity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (molar concentrations). Only for the highest concentrations measured in environmental samples would a significant contribution to the overall TEQ value be expected. However, it is known that polybrominated dibenzo-p-dioxins and dibenzofurans occur as trace contaminants in BFRs and are produced during combustion of these chemicals. In the CALUX assay the brominated analogue of TCDD and non-ortho substituted brominated biphenyls showed equal or greater activity as compared to their chlorinated analogues. It cannot be ruled out that the dioxin-like activity observed in some of the BFR-formulations is due to the presence of polybrominated dioxins and/or furans. Therefore the health risks associated with the use of BFRs and their waste incineration could be substantially caused by the presence of contaminating dioxin-like compounds. The CALUX assay provides a relatively simple way for estimating dioxin-like activity in a sample. Application of this assay will contribute to the collection of data on occurrence and contribution of polybrominated dioxins, furans and biphenyls to the TEQ value in environmental, biological or human samples. However, other mechanisms than the AhR pathway, which are as yet insufficiently known, could also be involved in the toxicity of BFRs.

References
EROD-induction by polybrominated diphenyl ethers in cultured chick embryo livers

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Summary

The PBDE congeners PBDE #47, PBDE #99, PBDE #153 and the PBDE mixture Bromkal 70 were tested for their EROD induction potency in a sensitive avian bioassay. We found that PBDE #99 and PBDE #153 had Ah-receptor agonist properties. However, their EROD induction potencies in relation to TCDD were low. Thus, the dioxin-like toxicity of these PBDE congeners does not seem to be an immediate threat to avian fauna.

Introduction

It is known that some of the polybrominated diphenyl ethers (PBDEs) are weak agonists for the Ah-receptor, having cytochrome P4501A inducing potency\(^1\). From the extensive research on PCBs and PCDDs/F, it is known that the absolute potencies and potency rank orders of the congeners differ greatly between species. This may also be the case for PBDEs. In addition, the increasing concentrations of some PBDEs in parts of the external environment, stresses the need to assess their potential as avian toxicants. Hence, it is of interest to study the dioxin-like toxicity of PBDE congeners in birds. To this date, very few data have been produced concerning this specific topic.

The chicken (\textit{Gallus domesticus}) is one of the most sensitive avian species to TCDD and related aromatic hydrocarbons\(^2\). We have developed a very sensitive avian \textit{in vitro} bioassay, based on the induction of the P4501A-related enzyme activity 7-ethoxyresorufin-O-deethylase (EROD), using whole embryonic chick liver\(^3\). Chicken embryos are extremely sensitive to dioxin-like compounds, and there is a good correlation between the toxicity of these compounds and their potencies as EROD inducers in chicken embryos \textit{in ovo}\(^3\). Using this bioassay, we have tested a few individual PBDE congeners and the PBDE mixture Bromkal 70.
Material and Methods

The PBDE congeners 2,2’,4,4’-tetrabromodiphenylether (#47), 2,2’,4,4’,5-pentabromodiphenylether (#99), 2,2’,4,4’,5,5’-hexabromodiphenylether (#153) and the industrial PBDE mixture Bromkal 70 were dissolved in dimethylsulfoxide (DMSO) for addition to whole organ cultures of livers, from chicken embryos. The bioassay was carried out as previously described\(^3\)\(^-\)\(^4\). Essentially, livers of 8-day-old chicken embryos were exposed to the compounds in organ cultures for 48 h. Seven concentrations of each chemical were used and four chicken livers were exposed to each concentration. In each assay, one group of four livers was also exposed to the positive reference TCDD in a concentration that causes maximal EROD induction (\(10^{-9}\) M). EROD activities in the livers were determined by measuring the deethylation of 7-ethoxyresorufin to the fluorescent product resorufin using a fluorescence multiwell plate reader\(^5\). The maximal induction rates of the concentration-response curves for the PBDEs were lower than the positive control (\(10^{-9}\) M TCDD). In addition, one of the congeners (#153) did not reach a maximum EROD induction plateau at its highest concentration. Therefore, the effective concentration values were calculated in relation to the maximum induction caused by \(10^{-9}\) M TCDD rather than the maximum induction of each compound. The concentration that caused 10% of the TCDD-induced maximum EROD activity was defined as EC\(_{10\text{TCDD}}\) for the PBDE congeners. The EC\(_{10\text{TCDD}}\)-values were used for the calculations of the TEF-values for the compounds. The TCDD EC\(_{10}\) value used in the TEF calculation was 0.001-nM, a mean value derived from many concentration-response curves.

Results and Discussion

All PBDE congeners and Bromkal 70 induced EROD to varying degrees in the chicken livers (figure 1). The EC\(_{10\text{TCDD}}\) values, TEF values and induction efficacies for the compounds are summarised in table 1. From the concentration-response curves it can be seen that PBDE #99 and PBDE #153 were able to induce EROD to a significant degree, while PBDE #47 and Bromkal 70 only produced small increases in EROD activity (figure 1). The most potent of the tested congeners was PBDE #99, which had an EC\(_{10\text{TCDD}}\) value of 250 nM, which is quite high in comparison with the EC\(_{10}\) for TCDD (0.001 nM). The TEF value for PBDE #99 was calculated to be 0.000004. This makes it as potent an EROD inducer as benzo(k)fluoranthene, which is the most potent PAH we have tested\(^6\). Thus, this congener seems to be a rather efficient Ah-receptor agonist, but its potency is still low compared to other dioxin-like compounds. Regarding Bromkal 70, its potency in relation to TCDD was estimated using weight normalisation instead of the correct molar basis, since Bromkal 70 is a mixture with an
undefined molar weight. In this estimation, we found that the Bromkal 70 mixture had a low potency as an EROD inducer (figure 1).

Figure 1. Concentration-response curves for EROD induction in the *in vitro* chicken embryo liver bioassay by the PBDE congeners 2,2',4,4'-tetrabromodiphenylether, 2,2',4,4',5-pentabromodiphenylether, 2,2',4,4',5,5'-hexabromodiphenylether and the industrial mixture Bromkal 70. Each square represents the mean of four livers ± SEM.

Table 1. EROD induction of three PBDE congeners and the commercial mixture Bromkal 70, in cultured chick embryonic liver. EC$_{10\text{TCDD}}$ values are shown in nM for the congeners and in ng/ml for Bromkal 70.

<table>
<thead>
<tr>
<th>Congener</th>
<th>EC$_{10\text{TCDD}}$ (nM)</th>
<th>TEF</th>
<th>Efficacy (% of maximum EROD induction)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,2',4,4'-tetraBDE (nM)</td>
<td>790</td>
<td>0.000001</td>
<td>17</td>
</tr>
<tr>
<td>2,2',4,4',5-pentaBDE (nM)</td>
<td>250</td>
<td>0.000004</td>
<td>63</td>
</tr>
<tr>
<td>2,2',4,4',5,5'-hexaBDE (nM)</td>
<td>2000</td>
<td>0.0000005</td>
<td>≥49</td>
</tr>
<tr>
<td>Bromkal 70 (ng/ml)</td>
<td>0.22</td>
<td>0.0000014$^a$</td>
<td>16</td>
</tr>
</tbody>
</table>

$^a$ Maximum EROD induction was obtained by dosing the chicken livers with 10$^{-9}$ M 2,3,7,8-TCDD.

$^b$ The TEF value for Bromkal 70 was estimated using weight normalisation instead of the more correct molar basis.
Conclusion

We found that some PBDE congeners had Ah-receptor agonist properties, but their EROD induction potencies in relation to TCDD were low. The concentrations of PBDEs found in the environment are still relatively low in comparison to PCB congeners, which are much more potent as Ah-receptor agonists than the PBDEs tested. Thus, the dioxin-like toxicity of these PBDE congeners does not seem to be an immediate threat to avian fauna.

References

Sublethal effects of the flame retardants hexabromocyclododecane (HBCDD) and tetrabromobisphenol A (TBBPA) in juvenile rainbow trout (*Oncorhynchus mykiss*)

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Summary: The effects of HBCDD and TBBPA on several biomarkers were studied in rainbow trout. HBCDD induced catalase activity after 5 days, and increased Liver Somatic Index after 28 days of treatment. This could point to a peroxisome proliferator mode of action of HBCDD in fish. TBBPA induced glutathione reductase activity suggesting a possible role of TBBPA in inducing oxidative stress. Both compounds seemed to inhibit the EROD activity of CYP1A.

Due to extensive computerisation and increased demands for fire-safety, brominated flame retardants leak out in the environment, including the aquatic one, in an accelerating manner. Despite this, physiological and especially sublethal effects of these chemicals are generally poorly investigated. In this study a screening of selected biomarkers in juvenile rainbow trout (*Oncorhynchus mykiss*) was performed after exposure to the brominated flame retardants hexabromocyclododecane (HBCDD) and tetrabromobisphenol A (TBBPA). Biomarkers are biological responses (e.g. changes in liver enzyme activities,) that work as “early warning”, signalling exposure to and/or effects of pollutants. In this study we investigated the activities of selected hepatic detoxification and antioxidant enzymes; we also measured liver somatic index (LSI), levels of the yolk precursor vitellogen in blood plasma and DNA-adducts.

TBBPA is the dominating brominated flame retardant with world production of about 60,000 tons. It is used mainly in epoxy polymers (e.g. circuit boards). HBCDD is a relatively new flame retardant used in polystyrene foams for building industry, textile coatings, electric cable coatings and latex. It has been reported in sediment and fish in the Swedish river Viskan\(^1\) and also in airborne particles a long distance from major sources\(^2\).

HBCDD: Fish were injected intraperitoneally with 50 or 500mg/kg HBCDD solved in peanut oil, and experiments lasted for 5 and 28 days. The investigated biomarkers included CYP1A,
glutathione-S-transferase (GST), glutathione reductase (GR) and catalase; LSI was also measured. The short-term experiment showed a dose-related increase in the activity of catalase, but this effect had disappeared after 28 days. Instead, a 40% increase in LSI could be observed for the 500mg/kg dose of HBCDD after this time of exposure. These changes are all numerously reported in connection with peroxisome proliferators (PPs) and it is therefore suggested that HBCDD might act in a PP-like mode of action in fish. PPs are a structurally dissimilar class of chemicals that are closely related to the development of non-mutagenic cancer. Analysis of DNA-adducts was also performed on selected samples from the control and 500mg/kg group. The results showed no adducts in any of the samples, which suggests that HBCDD probably is not genotoxic. HBCDD did also seem to have an antagonistic effect on the important detoxification enzyme CYP1A (measured as ethoxyresorufin-O-deethylase or EROD-activity), which possibly could have an impact on detoxification in wild fish exposed to multiple pollutants. The antagonistic effect could be observed for the 500mg/kg dose, both in a combination experiment with the model EROD-inducer β-naphthoflavone (BNF) and in the 28 days study.

TBBPA: The fish were injected with different doses of TBBPA solved in peanut oil and experiments lasted for 1, 4, 14 and 28 days. The biomarkers investigated included CYP1A, GST, uridine diphosphate glucuronosyl transferase (UGT), GR, catalase and glutathione peroxidase. LSI was also measured as was the level of vitellogenin in the blood plasma. The EROD-activity was significantly inhibited after 4 days at the doses of 100 and 500 mg/kg. There was also a trend towards inhibition of EROD in a combination experiment where TBBPA was injected together with BNF. The activity of GR decreased after 1 day at 100 mg/kg, but it increased significantly after 4, 14 and 28 days at the same dose suggesting a possible role of TBBPA in inducing oxidative stress. There was no elevation of vitellogenin levels in the fish suggesting no or not detectable oestrogenicity of TBBPA. There is clearly a need for further experiments e.g. investigations of the putative PP-like mode of action of HBCDD, mechanistic studies of the EROD inhibition and studies of more long-term, low-dose effects of the flame retardants in fish.

References
Intracellular calcium \([\text{Ca}^{++}]_i\) is an important second messenger the homeostasis of which is disrupted by in vitro treatment with polychlorinated hydrocarbons (PCBs) as shown recently (Inglefield, J.R.; Shafer, T.J., 2000). We have investigated the effects of the polybrominated diphenyl ether congener PDBE99 in vitro on the \([\text{Ca}^{++}]_i\)-levels in human macrophages, rat astrocytes and rat PC12 cells, respectively, using microspectrofluorometry of Fura-2. Acute exposure of the cells in vitro using micromolar concentrations of PDBE99 dissolved in 0.1% DMSO induced elevations of the basal \([\text{Ca}^{++}]_i\)-level as well as an additional occurrence of recurring peak-like \([\text{Ca}^{++}]_i\)-oscillations, which were nonperiodic and arised from the elevated basal \([\text{Ca}^{++}]_i\)-levels. The induction of these \([\text{Ca}^{++}]_i\)-homeostasis perturbations commenced after starting the PDBE99 treatment with a time delay which seemed to depend on the PDBE concentration applied. Stimulation of human macrophages or rat astrocytes using ATP (10-50 \(\mu\)M) or of rat PC12 cells using high potassium concentration (50mM) resulted in peak-like \([\text{Ca}^{++}]_i\)-elevations which seemed to be unaffected by acute PDBE99 treatment of 60 min duration.

These results show that in vitro treatment using PDBE results in simular changes of the \([\text{Ca}^{++}]_i\)-homeostasis as compared to PCBs.

Reference

Neonatal exposure to hexabromo-diphenyl ether (PBDE 153) affects behaviour and cholinergic nicotinic receptors in brain of adult mouse

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Summary

This study shows that neonatal exposure to PBDE 153 can induce irreversible, dose-response related, behavioural disturbances in adult mice, disturbances that worsen with age. In addition, changes in nicotinic receptors in the adult mouse brain are also induced after neonatal exposure to PBDE 153.

Introduction

Polybrominated diphenyl ethers (PBDEs) are a group of chemical substances, used as additive flame-retardants, with chemical and physical characteristics very similar to those of the polychlorinated biphenyls (PCBs) (1, 2). Products that contain flame-retardants are electrical appliances such as computers and television sets, textiles, building materials and other objects (2). PBDEs are not fixed in the polymer products and can thus leak into the environment (3, 4). Studies have shown that PBDEs are present in the global environment (5) and that levels of PBDEs are increasing in the Swedish environment (6, 7, 8). A recent report has shown the presence of PBDEs in Swedish mother’s milk and also that the PBDEs have increased exponentially since 1972, whereas PCBs are decreasing steadily (9). Samples of human blood have also been shown to contain PBDEs (10), including workers in the electrical dismantling industry who show levels of PBDEs in their blood, including the hexa-brominated congener PBDE 153 (11). This indicates that humans are exposed to PBDEs both as infants and as adults. In recent studies we have shown that neonatal exposure to PCB congener, PCB 153 (12), as well as flame-retardants, during the period of rapid brain growth, known as the “brain growth spurt” (“BGS”), can induce persistent dysfunction in adult mice, manifested as deranged spontaneous behaviour, an effect that also worsens with age (13) and that these effects are inducible during a restricted period of neonatal life (14). In view of the increasing amounts of PBDEs in the environment and in mother’s milk and the fact that other PBDE
Congeners have been shown to induce persistent dysfunctions in mice, this study was undertaken to study possible behavioural effects of PBDE 153, when given during the rapid development of the brain. A further objective was to investigate whether neonatal exposure to PBDE 153 can affect the nicotinic receptors in the adult mouse brain, as earlier seen for certain PCBs.

Materials and methods
The PBDE 153 was synthesized at the Wallenberg Laboratory, University of Stockholm, Sweden, and was orally administered as one single dose to neonatal NMRI mice on postnatal day 10. The three doses used were 0.45, 0.9 and 9.0 mg PBDE 153/kg b.wt. (0.7, 1.4 or 14 µmol PBDE 153/kg b.wt.). Mice serving as controls received 10 ml/kg b.wt. of the 20% fat emulsion vehicle.

Spontaneous motor behaviour test was conducted at 2, 4 and 6 months of age. The test measures locomotion: horizontal movements, rearing: vertical movements, and total activity: all types of vibrations within the test cage.

Swim maze test was performed at 6 months of age. The swim maze was of Morris water maze type and the mice’s ability to locate a submerged platform was studied for 5 days. The test measures spatial learning ability and memory.

Nicotinic receptors were analysed by measuring α-Bungarotoxin binding sites in the P2-fraction of the hippocampus in 6-months-old mice.

Results and discussion
The spontaneous motor behaviour data showed a dose-response related disruption of habituation in adult mice, neonatally exposed to PBDE 153. Habituation, defined here as a decrease in locomotion, rearing and total activity variables in response to the diminishing novelty of the test chamber over the 60 min test period, was demonstrated in the control group at 2, 4 and 6 months of age. Mice exposed to the two highest doses of PBDE 153 displayed a non-habituating behaviour at 2, 4 and 6 months of age, and in addition mice exposed to the highest dose of PBDE 153 showed the most pronounced deviation from normal behaviour. This non-habituating behavioural profile has also been reported for PCB 153 (12) and PBDE 47 and 99 (13, 14). Mice exposed to the lowest dose of PBDE 153 showed normal habituation at 2 and 4 months of age, but at 6 months of age the spontaneous behaviour was significantly altered compared to the control animals. The results from the spontaneous behaviour tests further indicate that the functional disorder worsen with increasing age. This change in
spontaneous behaviour profile, both time dependent and dose related, indicates the advance of a brain dysfunction process induced at the time of rapid brain development in the neonatal mouse.

The ability of adult mice to learn and memorize spatial navigation task was studied using a Morris water maze type of swim maze. The swim maze allowed a 4-day acquisition period followed by reversal learning on the fifth day. All mice improved their ability to locate the platform during the acquisition period, but animals exposed neonatally to 0.9 or 9.0 mg PBDE 153/kg b.wt. displayed significantly longer latencies to locate the platform on day 2 and 3 during the acquisition period. The changes in swim maze performance have earlier been seen for animals treated neonatally with certain PCBs (12) and PBDE 99 (13).

α-Bungarotoxin binding sites were assayed in the P2-fraction of the hippocampus in 6-months-old mice using \(^{3}\text{H}\)-α-bungarotoxin/α-bungarotoxin. Mice exposed to the highest dose of PBDE 153 (9.0 mg/kg b.wt.) showed a significant decrease in the density of specific \(^{3}\text{H}\)-α-Bungarotoxin binding sites in the hippocampus. Neonatal exposure to certain PCBs has also been shown to affect the nicotinic receptors in the adult mouse brain (15).

The present study shows that neonatal exposure to PBDE 153 can induce neurotoxic effects in the adult animal. Permanent aberrations in spontaneous motor behaviour are induced by neonatal exposure to PBDE 153, aberrations that are dose-response related and worsen with age. Furthermore, learning and memory functions are also affected by neonatal exposure to PBDE 153 and these changes are also dose-response related. In addition, nicotinic α-Bungarotoxin binding sites are also affected in the adult mouse, by the neonatal exposure to PBDE 153 on postnatal day 10.

Acknowledgements

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during a critical phase of neonatal brain development.” Submitted.
Brominated Flame Retardant: Uptake, retention and developmental neurotoxic effects of decabromodiphenyl ether (PBDE 209) in the neonatal mouse

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Summary

This study shows that PBDE 209 can be taken up and retained in the neonatal mouse brain. In addition, neonatal exposure to PBDE 209 induces behavioural disturbances in adult mice, disturbances that worsen with age.

Introduction

Polybrominated diphenyl ethers (PBDEs) are group of chemical substances used as additive flame-retardants. Products that contain flame-retardants are electrical appliances such as computers and television sets, textiles, building materials and other objects. PBDEs are not fixed in the polymer products and can thus leak into the environment (1, 2). Studies have shown that PBDEs are present in the global environment (3) and that levels of PBDEs are increasing in the Swedish environment (4, 5, 6). A recent report has shown the presence of PBDEs in Swedish mother’s milk and also that the PBDEs have increased exponentially since 1972 (7). Samples of human blood have also been shown to contain PBDEs (8), including workers in the electrical dismantling industry who show levels of PBDEs in their blood, including the deca-brominated congener PBDE 209 (9). This indicates that humans are exposed to PBDEs both as infants and as adults. In recent studies we have shown that neonatal exposure to flame-retardants, during the period of rapid brain growth, known as the “brain growth spurt” (“BGS”), can induce persistent dysfunction in adult mice, manifested as deranged spontaneous behaviour, an effect that also worsens with age (10) and that these effects are inducible during a restricted period of neonatal life (11). In view of the increasing amounts of PBDEs in the environment and in mother’s milk and the fact that other PBDE congeners have been shown to induce persistent dysfunctions in mice, this study was undertaken to study uptake and retention of PBDE 209 in neonatal mice, as well as possible behavioural effects of PBDE 209 when given during the rapid development of the brain.

Materials and methods

Both [¹⁴C]PBDE 209 and PBDE 209 were synthesized at the Wallenberg Laboratory, University of Stockholm, Sweden, and were administered as one single oral dose to neonatal NMRI mice on postnatal day 3, 10 or 19.
In order to study uptake and retention two litters in each age categories were given 1.5 \([^{14}\text{C}]\text{PBDE MBq/kg body weight (40.5 \mu Ci/kg body weight). Each of the two litters from the three different age categories was sacrificed, by decapitation, 24 h or 7 days, respectively, after administration. The skull was opened and the brain sectioned just behind the cerebellum. The brains were solubilized and the radioactivity was counted in a scintillation analyser. In the behavioural study 3-days-old and 19 days-old mice were given 2.22 or 20.1 mg PBDE 209/kg b.wt. (2.3 or 21 \mu mol/kg b.wt.) and 10 days-old mice were given 1.34, 13.4 or 20.1 mg PBDE 209/kg b.wt. (1.4, 14, or 21 \mu mol/kg b.wt.). Mice serving as controls received 10 ml/kg b.wt. of the 20% fat emulsion vehicle in the same manner. Each group contained 3-5 litters. The spontaneous behaviour test was conducted at 2, 4 and 6 months of age. The test measures *locomotion*: horizontal movements, *rearing*: vertical movements, and *total activity*: all types of vibrations within the test cage.

**Results and discussion**

The data from the uptake and retention study showed that \([^{14}\text{C}]\text{PBDE can be taken up and be distributed in the neonatal mouse, but that there is a difference in the amount of radioactivity found in the mouse brain in the different age categories. Mice exposed to \([^{14}\text{C}]\text{PBDE on postnatal day 3 or 10 displayed around 4\% of the administered amount of radioactivity in the brain 24 h after administration, whereas mice exposed to \([^{14}\text{C}]\text{PBDE on postnatal day 19 had only about 0.6\% of the administered amount of radioactivity in the brain 24 h after administration. 7 days after administration the amount of radioactivity in the brain had increased almost two-fold in mice exposed to \([^{14}\text{C}]\text{PBDE on postnatal day 3 or 10, whereas mice exposed on postnatal day 19 showed the same amount of radioactivity as they did 24 h after administration. This shows that PBDE 209 can be taken up and find its way to the brain during the critical “BGS”, but the retention pattern differs from other similar compounds during this period, for example PBDE 99 (11) and some PCBs (12).**

The spontaneous motor behaviour data states a disruption of habituation in adult mice exposed to PBDE 209 on postnatal day 3, but this disruption in habituation can not be seen in mice exposed to PBDE 209 on postnatal day 10 or 19. Habituation, defined here as a decrease in *locomotion, rearing* and *total activity* variables in response to the diminishing novelty of the test chamber over the 60 min test period, was demonstrated in the control groups of the three age categories as well as in the animals exposed to PBDE 209 on postnatal day 10 or 19. The animas exposed to the highest dose of PBDE 209, on postnatal day 3, showed a non-habituating behaviour profile at 2, 4 and 6 months of age. At 6 months of age mice exposed to the lower dose of PBDE 209, on postnatal day 3, showed this non-habituating behavioural profile. Certain PCBs (12) as well as PBDE 99 (11, 12) have been shown to induce this type of behavioural profile when administered on postnatal day 3, but this response is always accompanied by a response in animals exposed to the toxic compound on postnatal day 10. In this study the explanation can be that the amount of substance reaching the brain is not
enough to induce disturbances but as time goes by the amount is increasing, which is seen in the retention study. Another possible explanation is that PBDE 209 is metabolised to a metabolite that reaches the brain just in time for the critical window of “BGS” and induces the persistent effect. In addition, the neurotoxic effects of PBDE 209 were more pronounced in the older the animals, which indicate the advance of a brain dysfunction process induced at the time of rapid brain development in the neonatal mouse.

The present investigation shows that $[^{14}C]$PBDE can be taken up in the neonatal mouse and that the uptake is more efficient in younger animals. Radioactivity is found in the brain and increases during the first week after administration. This study also shows that neonatal exposure to PBDE 209 can induce neurotoxic effects, manifested as aberrations in spontaneous motor behaviour in the adult animal, effects that also worsens with age.

Acknowledgements

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Preliminary Results of a Mass Balance Study of a Commercial Pentabromodiphenyl Ether Mixture in Male Sprague-Dawley Rats

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Summary

A 21 day feeding study with environmental levels of a commercial penta bromodiphenyl ether mixture in rats was performed. Preliminary tissue retention results indicate that BDE-47, 100, and 153 accumulated in the carcass at a higher rate than other BDE congeners present in the penta formulation. These results may help to explain the prevalence of these congeners in biota. Future fecal results will allow a full mass balance study to be conducted.

Introduction

Polybrominated diphenyl ethers (PBDEs) are common additive flame retardants used in high impact polystyrene, polyurethane foam, and textile coating. The most recent production figures indicate that 40,000 tons are manufactured worldwide.\(^1\) They are structurally similar to other environmentally persistent aromatics, i.e. dioxins, PCBs, and furans, therefore, there is a growing belief that this family may be the next environmental contaminant of concern. Environmental sampling for the past 20 years has shown that PBDEs are persistent in sediment and bioaccumulate in tissues.\(^2,3\) Human milk levels are increasing,\(^4\) as are levels in organisms that inhabit the deep oceans.\(^5\) BDE-47 is the most abundant PBDE in environmental tissues, followed by BDE-99, 100, 153, and 154. Production of commercial penta-BDE formulations, which contain predominantly BDE-47 and 99, is very minor, while production of octa- and deca-BDE mixtures are substantially higher. Commercial octa-BDE mixtures contain tetra- and pentabrominated diphenyl ethers only as minor contaminants. A question to be addressed is whether observed environmental levels are due to preferential bioaccumulation of lower brominated PBDEs, or debromination of higher brominated congeners.

The purpose of this experiment was to perform a mass balance study in male rats that received a low dose of a commercial penta BDE mixture for 21 days. Preliminary results on the retention of PBDE congeners in liver and carcass are presented.

Materials and Methods

A commercial penta-BDE mixture (DE-73; Great Lakes Chemical) was added to peanut oil and administered in the feed for 21 days at the rate of 32 ng/day/rat (672 ng total). The rats were male Sprague-Dawley (n=8; 258-288 g; Taconic Labs), and were trained to consume the dosed feed in 1 hour. Control rats (n=8) consumed peanut oil vehicle in the feed. The rats were housed individually in stainless steel metabolism cages, which allowed for the daily separation of urine and feces, and the room was kept at 25°C with a 12h light:day cycle. The rats were killed; feces, livers and carcasses were frozen at -70°C C until analyzed.
Dosed carcasses were individually homogenized in a Hobart grinder. Livers were diced with a razor blade, and feces were lyophilized. Control carcasses, livers, and feces were pooled, but processed in the same manner. The tissue samples were placed into the stainless steel cell of an Accelerated Solvent Extractor (Dionex, Sunnyvale, CA), a recovery standard was added, and extraction was performed with 50:50 hexane:MeCl₂. The extracts were purified by a modification of EPA Method 1613⁶ including sequential washing of the extracts with 20% aqueous potassium hydroxide, water, concentrated sulfuric acid, and water, followed by chromatography on a triphasic silica column and an alumina column. The PBDEs were eluted from the alumina with 50% hexane/methylene chloride. GC-MS analyses were performed on a VG Autospec instrument operating in the electron impact selective ion monitoring mode at 2500 mass units resolution. Gas chromatography was performed on a 30 meter DB-5MS column (J&W Scientific, Folsom CA) using on-column injection and pressure programming which eluted BDE-209 in under 45 min. Two ions were monitored for each homolog group (mono- to deca-BDEs); ion ratios were found to be within 15% of the theoretical value. PBDEs were quantitated by comparison to the internal standard (¹³C-BDE-139) and the ¹³C-recovery surrogates.

The PBDE congener composition of the DE-73 formulation was also determined by the isotope dilution GC/MS method. PBDEs have been analyzed in 4 dosed rats and the control rats. Rat chow and peanut oil have not been analyzed; background BDE levels from these sources have been accounted for by the control rats.

**Results and Discussion**

The rats received PBDEs in their feed at a low level of 2.9 ppb, a rate designed to mimic environmental levels. Rat liver and carcass retention of the most abundant BDE congeners from a 21 day dietary administration of a commercial penta BDE formulation ranged from 0.15-0.69% in the liver, and from 11-56% in the remaining carcass (Table 1). The BDE congener with the highest bioaccumulation in both the liver and the carcass was BDE-47, i.e. 0.69% and 55.90%, respectively. With the exception of the penta BDE-85, bioaccumulation in the liver generally decreased with increasing bromination. In the carcass, BDE-47, 100, and 153 had similar total accumulations as a percentage of administered dose, while BDE-85, 99, and 154 accumulated at one fifth to one half that rate at 21 days. These chronic feeding study results parallel what has been observed in the limited number of metabolism studies that have been conducted. Rat liver and carcass retention of BDE-99 after a single oral dose of 8.8 mg/kg was 0.9 and 49.1%, respectively, after 3 days.⁷ At 12 days, these levels decreased to 0.4 and 28.9% (unpublished data). Even higher levels of BDE-47, i.e. 86%, remained in the body of rats 5 days after receiving a single 14.5 mg/kg dose.⁸ Most studies, both in vivo and in vitro, have concluded that metabolic activity towards PBDEs is low.⁴ The results in Table 1 at 21 days most likely represent rats approaching steady state, while the short-term, single dose studies represent peak levels of these congeners.

It is assumed that the bulk of the remaining BDEs consumed in the diet were eliminated in the feces. However, fecal analysis for BDEs has not been completed due to a high carryover of impurities, which prevented quantitation according to our present conditions. Re-analysis of fecal extracts is planned, after appropriate cleanup methods are found.

**Acknowledgements**

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Table 1. Average BDE liver and carcass retention data for male rats (n=4) administered a commercial penta BDE formulation for 21 days, i.e. 672 ng total of Great Lakes Chemical DE-73.

<table>
<thead>
<tr>
<th>BDE congener</th>
<th>Total mass consumed(ng)</th>
<th>Congener mass in liver (ng)</th>
<th>% of Dose in liver</th>
<th>Congener mass in carcass (ng)</th>
<th>% of Dose in carcass</th>
</tr>
</thead>
<tbody>
<tr>
<td>47</td>
<td>186.10</td>
<td>1.29</td>
<td><strong>0.69</strong></td>
<td>104.03</td>
<td><strong>55.90</strong></td>
</tr>
<tr>
<td>85</td>
<td>17.04</td>
<td>0.025</td>
<td><strong>0.15</strong></td>
<td>1.81</td>
<td><strong>10.62</strong></td>
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<tr>
<td>99</td>
<td>299.60</td>
<td>1.36</td>
<td><strong>0.46</strong></td>
<td>91.67</td>
<td><strong>30.60</strong></td>
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<tr>
<td>100</td>
<td>49.97</td>
<td>0.20</td>
<td><strong>0.40</strong></td>
<td>28.03</td>
<td><strong>56.09</strong></td>
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<tr>
<td>153</td>
<td>55.57</td>
<td>0.14</td>
<td><strong>0.25</strong></td>
<td>27.66</td>
<td><strong>49.78</strong></td>
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<tr>
<td>154</td>
<td>23.83</td>
<td>0.039</td>
<td><strong>0.16</strong></td>
<td>5.02</td>
<td><strong>21.07</strong></td>
</tr>
</tbody>
</table>

* Identity of BDE-85 was assumed based on ion ratios, despite its slightly shifted retention time.

References

Wildlife and Human Models to Assess the Metabolic Fate of Polybrominated Diphenyl Ethers (PBDEs) and Metabolite Formation and Depletion

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The proposed research will address and investigate the role of biotransformation in the biological fate, toxicokinetics, ecological dynamics and endocrine activation of an environmentally important class of brominated flame retardants (BFRs), the polybrominated diphenyl ethers (PBDEs), in model wildlife species and humans that are known to be naturally exposed to PBDEs.

Due to the high production (about 40,000 tons produced yearly) and use of PBDEs, and their high binding affinity to particles and their lipophilic characteristics, PBDEs have been shown to bioconcentrate and bioaccumulate in the environment in a manner similar to the structurally analogous polychlorinated biphenyls (PCBs).1,2 PBDEs have emerged as an important class BFR environmental contaminants, and have been reported in wildlife species from marine, freshwater and terrestrial environments. PBDEs have been detected in various biological tissue samples from birds, seals, whales, and even in human blood, adipose tissue and breast milk from a limited number of ecosystems. For example, total PBDE levels in the blubber of beluga and ringed seals from the Canadian Arctic were about 1% the concentration of total PCBs.3 The total concentration of PBDEs has increased in the breast milk of Swedish humans from 0.07 ng/g lipid weight in 1972 to 4.02 ng/g lipid weight in 1998.4 In Lake Trout from Lake Ontario PBDE concentrations were recently shown to have increased from < 5 ng/g (lipid weight) in 1983 to abut 700 ng/g (lipid weight) in 1998.5 Mainly tetra-BDE (TeBDE) have been detected in wild animals although TeBDE is but part of the total amount of PBDEs used. The congeners 2,2′,4,4′-tetaBDE (BDE-47), 2,2′,4,4′,5-pentaBDE (BDE-99) and 2,2′,4,4′,6-pentaBDE (BDE-100) are generally the more dominant congeners found in biota and humans.

Cytochrome 450 monooxygenase iso-enzymes and epoxide hydrolases play a major role in the bioaccumulation of persistent contaminants in food chains. Both Phase I enzyme
systems are important determinants of susceptibility to organohalogen exposure, and it is reasonable to hypothesize that differences in metabolic capacity are due to differences in functional enzyme levels and are reflected in bioaccumulation trends of organohalogens. As a consequence, biotransformation processes can modulate organohalogen toxicity. It is now well established that PCBs can be biotransformed in populations of wildlife and humans to persistent and bioaccumulative methyl sulfonyl (MeSO_2-) and retained hydroxyl- (HO-) PCB metabolites. However, very little is known regarding PBDE biotransformation in biota. Unlike analogous PCBs, dehalogenation of highly PBDEs is more facile due the C-Br bond being weaker than the C-Cl bond. The most persistent PBDE congeners are highly brominated and/or contain meta or para, brominated carbons (i.e., BDE-47, BDE-99 and BDE-100). This may indicate that lower brominated PBDEs with non-brominated meta-para carbons are biotransformed. Limited studies have indicated that HO-PBDE metabolites formed via metabolic biotransformation from PBDEs in exposed species. Örn et al. demonstrated that BDE-47 is biotransformed to HO-PBDEs in rats and mice. A number of HO-PCBs and HO-PBDEs were reported in the blood of Atlantic populations of salmon from the Baltic Sea, which are affected by a severe syndrome known as M74A, as well as in human blood.

The formation of endocrine active HO-PBDE metabolites is a possibility in PBDE exposed species. Comparatively, a number of HO-PCBs, several of which have been identified in biota, are known to possess thyroidogenic, estrogenic and androgenic activity. PBDE toxicity in vivo include effects on thyroid function, observed as induction of thyroid hyperplasia and alteration of thyroid hormone production (i.e. lowering of free and total thyroxine (T_4) concentrations) in rats and mice. Consistent with these findings is the recent observation that in phenobarbital-induced liver microsomes of rats, several pure PBDE-congeners were able to competitively displace T_4 from transthyretin (TTR) binding in vitro, after metabolic conversion to hitherto unidentified metabolites. Several HO-PBDE congeners competitively bind to the thyroid hormone receptor. Meerts et al. recently demonstrated for the first time that eleven out of seventeen PBDEs, two HO-PBDEs and three brominated bisphenols are estrogenic in vitro in the ER-CALUX bioassay based on stably transfected human breast T47D.luc carcinoma cells. The eleven estrogenic PBDEs included environmentally relevant BDE-47, -99 and –100. Generally, PBDEs with bromine-unsubstituted, adjacent carbons, and minimal bromination adjacent to these carbon positions were the most estrogenic. Like analogous PCBs, these PBDE congeners would likely be most susceptible to CYP enzyme-mediated, and subsequently epoxide hydrolase-mediated Phase I metabolism. Of three para-HO-PBDEs, the congener (i.e., 4-HO-2’,4’,6’-tribromodiphenyl
ether) with no bromine atoms adjacent to the HO-group was similarly estrogenic as 17β-
estradiol. Therefore, it is important to evaluate PBDE biotransformation and exposure to HO-
PBDEs to assess the endocrine-related health risk to wildlife and humans.

**Hypothesis #1:** The metabolism of selected congeners of PCBs and PBDEs is dependent upon
the congener structure and number and position of chlorine or bromine atoms, and is variable
among species, which affects accumulated PCB and PBDE congener patterns.
Pooled liver microsomes from each of the several model species (i.e., polar bear, ringed seal,
and deepwater sculpin and lake trout for the Great Lakes) and humans (Greenland Inuit and
peoples from southern Canadian populations) will be employed for the *in vitro*
metabolism/inhibition studies. The CYP enzyme-mediated catalytic activities will be
determined using the testosterone hydroxylase and other catalytic assays. Epoxide hydrolase
activities will be determined as well. Commercially available liver microsomes from PB and
3-MC induced rats will initially be used to determine if selected PBDE congeners, PBDE
congener mixtures and PBDE fractions from free-ranging species are metabolized *in vitro.*
PBDE congeners to be considered as substrates include BDE-15 (4,4’), BDE-28 (2,4,4’),
BDE-30 (2,4,6), BDE-32 (2,4’,6), BDE-47, BDE-51 (2,2’,4,6’), BDE-71 (2,3’,4’,6), BDE-75
(2,4,4’,6), BDE-77 (3,3’,4,4’), BDE-85 (2,2’,3,4,4’), BDE-99, BDE-100, BDE-138
(2,2’,3,4,4’,5) and BDE-153 (2,2’,4,4’,5,5’). The selection of the PBDE congeners will be
based on analogous PCB structures known to be suitable substrates for CYP1A and CYP2B-
like iso-enzymes. As a test substance a technical mixture of penta-brominated biphenyl ethers
(PeBDE; Bromkal 70-5DE) will be considered.

**Hypothesis #2:** Different CYP iso-enzymes mediate the biotransformation of PCBs/PBDEs
depending on the structure the patterns of chlorine/bromine substitution. Constituent and
induced metabolic capacity of CYP iso-enzymes, and thus the potential effects and toxicities
from parent and/or HO-containing metabolite exposure, is different among the wildlife
species and humans.
The congener specificity of CYP iso-enzyme(s) involved in the metabolism of PCBs and
PBDEs, and subsequent epoxide hydrolase-mediated metabolism, will be investigated in the
model wildlife species and humans using in vitro metabolism/inhibition assay. Selected
chemical inhibitors and antibodies that are known to be specific to CYP1A, CYP2B, CYP3A
and microsomal epoxide hydrolase will be used in co-incubation studies to elucidate the CYP-
iso-enzyme(s) mediating the metabolism of the organohalogen substrates. Changes in the
congener depletion patterns in the presence of a given inhibitor will show the QSARs of the PCB and PBDE structures as substrates for species-specific CYP iso-enzyme(s). Each of the chosen inhibitors and antibodies will also be co-incubated in the microsomal preparations to determine their CYP iso-enzyme-specific effect on catalytic activities such as testosterone hydroxylase, EROD and other catalytic assays.

PBDE biotransformation and the risk of HO-PBDE metabolite exposure are essential to understand PBDE toxicokinetics and toxicodynamics in wildlife and humans. *In vitro* metabolism as bioanalytical tool to assess the relative importance, metabolic capacity and iso-enzyme specificity of Phase I pathways in a given wildlife species will be developed. These *in vitro* QSARs will be useful in the development of species-specific bioindicators of PCB and PBDE metabolism and *in vivo* in among species and humans.

References

Metabolism of decabromodiphenyl ether (BDE-209) in the rat

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Summary
DecaBDE is excreted mainly, 90% of the dose within 3 days, via faeces. Several types of metabolites, e.g. non-extractable, water soluble, lipid bound and hydroxylated metabolites. GC/MS analysis of conventional hydroxylated metabolites showed that in faeces, all observed metabolites were dihydroxylated. The high relative amount of non-extractable metabolites in liver and jejunum wall is indicative of metabolism via a reactive intermediate.

Introduction
The absorption, metabolism and excretion of pollutants are important factors for the estimation of potential risk. DecaBDE has low solubility in both water and organic solvents (1) and the absorption has been reported to be low (2, 3). The molecular weight of decaBDE is M<sub>w</sub> 959g/mol and according to proposed limits for absorption (M<sub>w</sub><700 g/mol) (4) decaBDE should not be bioavailable. Still, decaBDE is present in human plasma (5) and after an oral dose of radiolabelled decaBDE to rats, radiolabelled materials was found in tissue (2). The excretion and metabolism of decaBDE has previously been studied reporting that the major part of an oral dose was excreted in faeces, 99% of the dose, and only a minor part in urine, <0.5% (2, 3). Although, metabolites have been found in faeces and bile, the information regarding characterisation of the metabolites is limited (2).

The present study was primarily aimed to identify metabolites excreted in faeces, after an oral dose of radiolabelled decaBDE to rats as well as to investigate metabolites retained in tissues.

Material and methods
Animals: Two groups of male Sprague-Dawley rats, conventional (n=4) and bile duct cannulated (n=2), were dosed orally with <sup>14</sup>C-labelled BDE-209 (3 µmol/kg, 15 Ci/mol). DecaBDE was dispersed in a suspending vehicle, consisting of Lutrol F127, soya phospholipid and water. The animals were kept in metabolism cages and excreta were collected in 24h intervals; 0-24h, 24-48 and 48-72h. Bile from the bile duct cannulated rats
was collected at 0-4 h, 4-12 h, 12-24 h, 24-48 h and 48-72 h after dose. The radioactivity content was determined in the faeces, urine and bile. From the conventional rats liver, adipose tissue, lung, kidney, adrenals, skin, muscle, spleen, testis, thymus, heart, plasma, red blood cells (RBC), colon wall and content, jejunum content and jejunum wall were collected and radioactivity content measured.

**Extraction and clean up:** The extraction and clean up steps used have been described previously (6) but a brief outline of the methods is given.

**Faeces:** Freeze-dried and ground faecal samples were extracted with chloroform/methanol (2/1, 200 ml) in a Soxhlet-apparatus (7). The extract was redissolved in hexane washed with H₃PO₄ (0.1M in 0.9% NaCl) (8) and the lipid weight determined. The radioactivity content in extracts, water phases and residues were determined. The extracted compounds were fractionated by gel permeation chromatography (GPC) and one fraction containing lipids (GPC-LF, 0-130 ml) and one essentially lipid-free fraction containing decaBDE and non-conjugated metabolites (GPC-MF 130-260 ml) were collected. The radioactivity content in GPC-LF and GPC-MF were determined. The GPC-MFs was pooled by day for each group and partitioned into neutral and phenolic compound (6). The phenolic fraction was derivatised with diazomethane, and both the neutral and phenolic fractions were analysed by GC/MS (NICI (negative ion chemical ionisation)) (9), in the mass range m/z 50-1000.

**Tissues:** Liver, adipose tissue, lung, kidney and intestinal tissues were pooled for each group and homogenised in hexane/acetone (1/3.5, 45ml) followed by extraction with hexane/MTBE (9/1, 25ml) (10). The solvent was evaporated and the lipid weight determined. The radioactivity content in extracts, water phases and residues were determined. The extracts, pooled for each group, were dissolved in hexane/dichloromethane (1/1) separated by GPC, into GPC-LF, 0-130ml and GPC-MF, 130-260ml.

**Results**

The major route of excretion for decaBDE was faeces, approximately 90% of the dose were excreted within three days and only traces were excreted in urine, <0.05% of the dose. Excretion via the bile from bile duct cannulated rats corresponded to approximately 9.5% of the dose within three days. The tissue distribution of radioactivity, as % of dose, is presented in Figure 1.
The radiolabelled material in faeces and tissues was characterised by the distribution in various fractions in the sample work-up. The relative distribution of non-extracted, water soluble, lipid bound and decaBDE/non-conjugated metabolites are presented in Figure 2. Notable is the high relative proportion of non-extracted radioactivity in the jejunum wall (60%) and in the liver (30%). The faecal samples from different rats and time points were very similar and are therefore presented as a mean value in Figure 2. 22%, 42% and 45% of the radioactivity in faecal GPC-MF corresponded to phenolic metabolites in samples from day 1, day 2 and day 3, respectively. Eight phenolic metabolites were identified as their methyl derivatives by GC/MS (NICI), as di-metoxylated penta- to octabrominated diphenyl ethers. The neutral substances corresponded mainly to decaBDE, but trace of amounts of three nonabrominated diphenyl ethers was also observed. Analysis of tissue samples is on going.
Discussion

In the present study, decaBDE was shown to be absorbed from the GI tract and 9.5% of the oral dose was excreted in bile within three days. Approximately 3% of the dose was retained in the tissues (Figure 1). DecaBDE is metabolised via oxidative debromination, as deduced from the presence of debrominated dihydroxylated diphenyl ethers, the dihydroxylation was always on one phenyl ring, according to mass fragmentation. Oxidation to an epoxide and further to a diol could explain the formed metabolites. However, arene oxide formation is difficult when bromine atoms occupy all carbons. Debrominated diphenyl ethers was not observed except for trace amount of three nonaBDEs. Hexabromobenzene have shown to be metabolised to debrominated-, hydroxylated- and sulphur-containing metabolites (11). Dihydroxylated metabolites of PCB may form reactive quinone intermediates and further react with both sulphur and nitrogen nucleophiles in the cell (12). Transformation of decaBDE to reactive intermediates could explain the non extractable radioactivity and lipid bound metabolites. Lipid bound metabolites have previously been shown for polychlorinated biphenyls (13). The potential reactive intermediate metabolites require further studies.

References