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CUMULATIVE ASSESSMENT OF PERSISTENT ORGANIC POLLUTANT TOXICITY IN VIVO

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ABSTRACT

Humans are continuously exposed to a multitude of compounds present in the environment and in food. A major challenge in risk assessment is to determine the degree of exposure to multiple chemicals and the hazards associated with such combined exposure. The simultaneous exposure to persistent organic pollutants (POPs), such as dioxins and dioxin-like (DL) compounds, polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs), is one example of a complex group of chemicals which is of concern from a human health perspective.

To assess the cumulative risk related to DL compounds eliciting aryl hydrocarbon receptor (AhR)-mediated biochemical and toxic responses, the WHO TEF/TEQ concept has been developed. Congeners which are assigned a TEF value are thereby covered by the risk assessment for dioxins. The TEF values have been derived using scientific judgments of multiple relative potency values from different studies and for various endpoints including increased liver weight, considered an early and sensitive marker of exposure to organohalogen compounds, decreased liver vitamin A levels, which can be considered a marker of retinoid system modulation, and hepatic EROD induction, which is not a toxic effect per se but is considered an early and sensitive marker of AhR activation. These effects have also been observed after exposure to PCBs, PBDEs and commercial mixtures, but in contrast to the DL compounds several receptors have been suggested to be involved. The similarity in effects, i.e. modulation of a common system or tissue, observed after exposure to several types of POPs indicates that the combined exposure to these chemicals could contribute to cumulative toxicity and that a cumulative assessment based on the biological system or target tissue affected rather than on the mechanism of toxicity might be warranted as a complement to the established TEF concept for DL substances.

The aim of this thesis was to study the feasibility of developing an endpoint-specific cumulative assessment based on effects considered as markers of DL toxicity observed for different POPs in vivo. The studies focused on PCB 180 (Paper I), which is not included in the TEF concept, and the commercial penta-BDE mixture Bromkal 70-5DE (Paper II).

Effects on liver weight, hepatic vitamin A levels and hepatic EROD activity were observed after exposure to PCB 180 as well as observations indicating that the effects were not mediated via the AhR. In a comparison to a series of studies including both congeners assigned a TEF (PCBs 77, 105 and 118) and congeners not assigned a TEF (PCBs 28, 128 and 153) in the WHO concept, relative potency values has been estimated for all included congeners as compared to PCB 126 based on one or more of the endpoints increased liver weight, decreased hepatic vitamin A and hepatic EROD induction, indicating that the observed effects of these congeners were similar to the effects of PCB 126, regardless if they are assumed to act mainly via the AhR or not. Based on a whole mixture approach, Bromkal 70-5DE was found to contain DL contaminants to an extent that could explain the observed effects on liver weight, hepatic vitamin A levels and hepatic EROD induction.

In conclusion, the findings in this thesis support the suggestion to develop endpoint-specific systems for cumulative assessment of POPs based on the criteria to include chemicals with similar effects, i.e. modulating a common system or target tissue via multiple pathways and/or mechanisms of toxicity.

LIST OF PUBLICATIONS

I. Roos R, Andersson PL, Halldin K, Håkansson H, Westerholm E, Hamers T, Hamscher G, Heikkinen P, Korkalainen M, Leslie H, Niittynen M, Sankari S, Schmitz H-J, van der Ven LTM, Viluksela M Schrenk D. 2011. Hepatic effects of a highly purified 2,2´,3,4,4´,5,5´-heptachlorbiphenyl (PCB 180) in male and female rats. Toxicology, doi: 10.1016/j.tox.2011.03.013.

II. Öberg M, Westerholm E, Fattore E, Stern N, Hanberg A, Haglund P, Wiberg K, Bergendorff A, Håkansson H. 2010. Toxicity of Bromkal 70-5DE, a technical mixture of polybrominated diphenyl ethers, following 28 d of oral exposure in rats and impact of analysed impurities. Chemosphere 80(2): 137-143

CONTENTS

LIST OF ABBREVIATIONS

AhR	Aryl hydrocarbon receptor
BMD	Benchmark dose
BMR	Benchmark response
CAR	Constitutive active (androstane) receptor
CYP	Cytochrome P450
DL	Dioxin-like
EFSA	European Food Safety Authority
EROD	Ethoxy resorufin-*O*-deethylase
LO(A)EL	Lowest observed (adverse) effect level
NO(A)EL	No observed (adverse) effect level
PB	Phenobarbital
PBDD	Polybrominated dibenzo-*p*-dioxin
PBDE	Polybrominated diphenyl ether
PBDF	Polybrominated dibenzofurans
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzo-*p*-dioxin
PCDF	Polychlorinated dibenzofuran
PROD	Pentoxy resorufin-*O*-dealkylase
PXR	Pregnane X receptor
REP	Relative potency
RXR	Retinoid X receptor
TCDD	2,3,7,8-tetrachloro dibenzo-*p*-dioxin
TDI	Tolerable daily intake
TEF	Toxic equivalency factor
TEQ	Toxic (TCDD) equivalent
WHO	World Health Organisation

1 GENERAL BACKGROUND

1.1 INTRODUCTION

Humans are continuously exposed to a multitude of compounds present in the environment and in food. A major challenge in health risk assessment is to determine the degree of exposure to multiple chemicals and the hazards associated with such combined exposure (Rider et al. 2010). The simultaneous exposure to several persistent organic pollutants (POPs) is one example of a complex group of chemicals which is of concern from a human health perspective. Within the scope of this thesis, the assessment of effects observed after combined exposure to multiple chemicals is defined as cumulative assessment.

1.2 PERSISTENT ORGANIC POLLUTANTS

POPs are bioaccumulative toxic compounds that are regulated both nationally and internationally in order to protect human health and the environment, but despite regulatory actions, these compounds are still found at considerable amounts in food and in human matrices (Törnkvist et al. 2011; Fürst 2006; Fängström et al. 2005). POPs include pesticides, industrial chemicals and unintentional by-products. The unintentional by-products include dioxins and dioxin-like (DL) compounds and the industrial chemicals include polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs).

1.2.1 Dioxins and dioxin-like compounds

Dioxins and DL compounds are unintentional by-products formed e.g. during the synthesis of organohalogen compounds or during the combustion of chloro-organic material. The group of dioxins and DL compounds includes polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs) (Figure 1). Differing degree and pattern of chlorination generates 75 possible PCDD and 135 possible PCDF congeners, of which 17 are chlorinated in 2,3,7,8- position and are considered to be of toxicological concern. The industrial chemical PCB has 209 possible congeners, twelve of which are considered to be DL. The most toxic and most studied dioxin is 2,3,7,8-tetrachlo dibenzo-*p*-dioxin (TCDD) and it is often used as a model or reference substance for DL compounds.

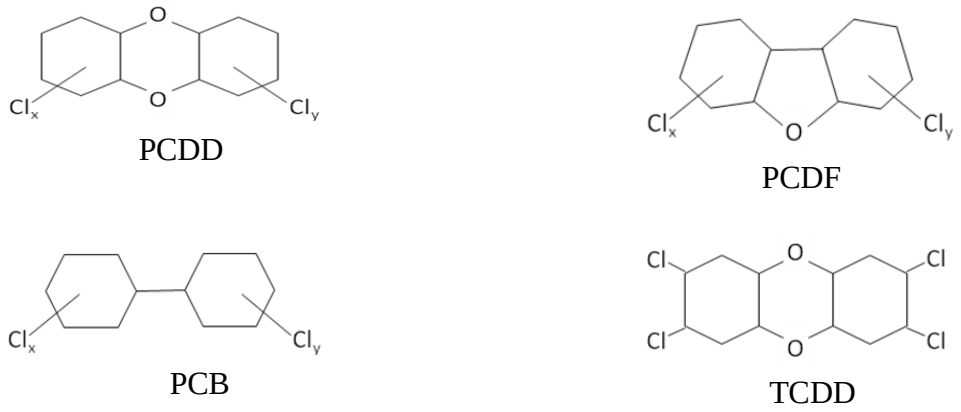


Figure 1. Chemical structure of polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs). The most toxic dioxin is 2,3,7,8 tetrachloro dibenzo-p-dioxin (TCDD).

The dioxins and DL compounds bind to and activate the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor mediating the biological responses to DL compounds. In the absence of a ligand, AhR is present in the cytosol associated with the molecular chaperone heat shock protein (HSP) 90 and co-chaperones p23 and immunophilin-like protein XAP2. Upon ligand binding, the chaperones dissociate and the ligand-bound AhR is translocated to the nucleus where it forms a dimer with the aryl hydrocarbon nuclear translocator (Arnt) protein. The formed complex recognises specific xenobiotic or dioxin responsive elements (XRE, DRE) on DNA and the binding to these elements activates the transcription of a battery of dioxin-responsive genes (Figure 2). One of the most studied and highly inducible events is the induction of drug-metabolising enzymes such as cytochrome P450 (CYP) 1A1 (Flaveny et al. 2009; Mimura and Fujii-Kuriyama 2003).

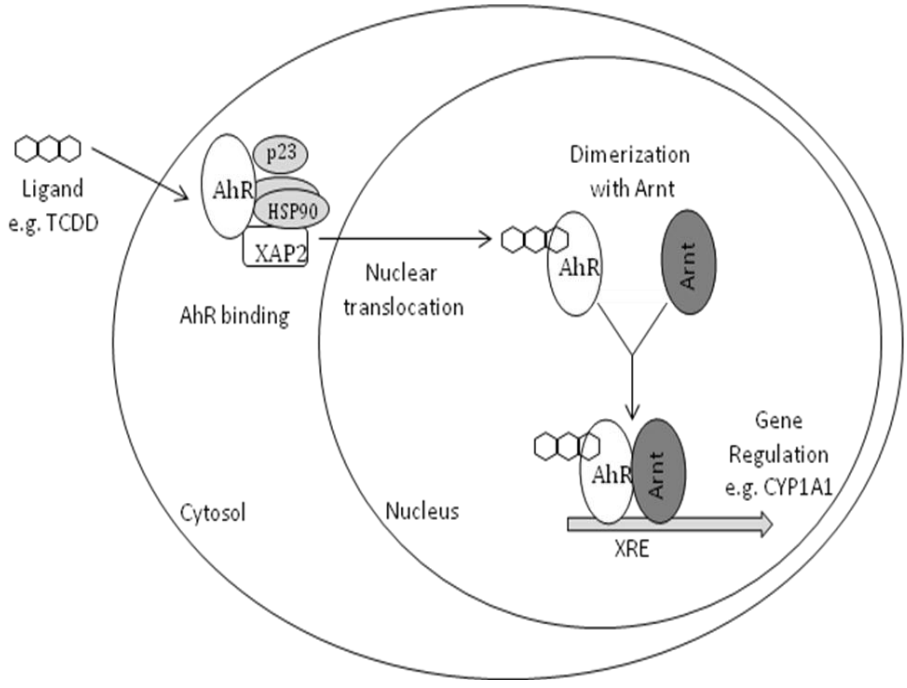


Figure 2. Mechanism of transcriptional aryl hydrocarbon receptor (AhR) activation.

The common mechanism of action, initiated by activation of the AhR, is used as a criterion for including compounds in the established World Health Organisation (WHO) toxic equivalency factor (TEF) concept for cumulative risk assessment of dioxins and DL compounds. The TEF values for DL compounds (Table 1) only apply to AhR mediated response and in order to be included in the TEF system a compound must 1) show a structural relationship to the PCDD/Fs, 2) bind the AhR, 3) elicit AhR-mediated biochemical and toxic responses, and 4) be persistent and accumulate in the food chain (Van den Berg et al. 2006). The potency of individual DL compounds fulfilling the criteria is compared to the most toxic dioxin TCDD or the potent DL PCB 126 and a TEF is assigned to each congener. Based on an assumption of additivity, the individual TEF values of DL compounds can be used to summarize the dose of a complex mixture of individual DL compounds as one single number, the toxic equivalent (TEQ) dose. Congeners which are assigned a TEF value are thereby incorporated in the risk assessment for dioxins (EU 2001).

Table 1. WHO 2005 TEF values

Congener	**WHO-TEF[a]**	**Congener**	**WHO-TEF[a]**
Chlorinated dibenzo-p-dioxins		**Non-*ortho*-substituted PCBs**	
2,3,7,8-TCDD	1	PCB 77	0.0001
1,2,3,7,8-PeCDD	1	PCB 81	0.0003
1,2,3,4,7,8-HxCDD	0.1	PCB 126	0.1
1,2,3,6,7,8-HxCDD	0.1	PCB 169	0.3
1,2,3,7,8,9-HxCDD	0.1	**Mono-*ortho*-substituted PCBs**	
1,2,3,4,6,7,8-HpCDD	0.01	PCB 105	0.00003
OCDD	0.0003	PCB 114	0.00003
Chlorinated dibenzofurans		PCB 118	0.00003
2,3,7,8-TCDF	0.1	PCB 123	0.00003
1,2,3,7,8-PeCDF	0.03	PCB 156	0.00003
2,3,4,7,8-PeCDF	0.3	PCB 157	0.00003
1,2,3,4,7,8-HxCDF	0.1	PCB 167	0.00003
1,2,3,6,7,8-HxCDF	0.1	PCB 189	0.00003
1,2,3,7,8,9-HxCDF	0.1		
2,3,4,6,7,8-HxCDF	0.1		
1,2,3,4,6,7,8-HpCDF	0.01		
1,2,3,4,7,8,9-HpCDF	0.01		
OCDF	0.0003		

[a]Van den Berg et al. 2006

Both polybrominated dibenzo-*p*-dioxins (PBDDs) and dibenzofurans (PBDFs) have been shown to have AhR agonist properties and to cause DL effects (Behnisch et al. 2003; Birnbaum et al. 2003; Kuiper at al. 2006). Human exposure levels of PBDDs and PBDFs are lower than the levels of PCDDs, PCDFs and PCBs but could still contribute significantly to the total amount of TEQ and PBDDs and PBDFs should be given high priority for evaluation of a possible inclusion in the TEF concept (Van den Berg et al. 2006). Mixed haologenated dibenzo-*p*-dioxins and dibenzofurans have also been discussed in this context, and even though these

congeners seem to follow the same structure-activity rules as the PCDD/Fs the relevance for human exposure are not known (Van den Berg et al. 2006).

The risk assessment of dioxins and DL PCBs in food is based on developmental effects in rat male offspring, which is the most sensitive effect of TCDD in experimental animals (EU 2001), but the database used as a basis for the establishment of TEF values contains a wide array of effects (Haws et al. 2006). The toxic responses observed after exposure to dioxins and DL compounds include effects on the immune system, carcinogenicity, disturbed reproduction and development, behavioural changes and disturbances of endocrine systems including thyroid hormones and retinoids. In laboratory animals, early and sensitive effects include increased relative liver weight, considered an early and sensitive marker of exposure to organohalogen compounds (Van Birgelen et al. 1995; Håkansson et al. 1991) resulting from hypertrophia and hyperplasia of hepatocytes (Bock and Köhle 2009) and AhR-mediated alterations in hepatic retinoid stores and enzyme induction (Murphy et al. 2007). The hepatic ethoxyresorufin-*O*-deethylase EROD activity catalyzed by CYP1A1, is not a toxic effect per se but is often used as an early and sensitive marker of AhR activation (Van Birgelen et al. 1995; Pohjanvirta and Tuomisto 1994; Brunström et al. 1991). In addition, alterations of the retinoid homeostasis have been suggested to be involved in the underlying mechanism of action of DL compounds and decreased liver vitamin A levels can be considered a marker of such alterations (Fattore et al. 2000; Nilsson and Håkansson 2002; Novák et al., 2008). REP values calculated based on sensitive subchronic effects including liver lesions have also been found to be similar to REP values derived based on hepatic vitamin A reduction for several DL PCDDs and PCDFs (Fattore et al. 2000).

1.2.2 PCB

Polychlorinated biphenyls (PCBs, Figure 1) are abundant environmental contaminants also present in food (Törnkvist et al. 2011) and human tissues and milk (Fürst 2006; Fängström et al. 2005; Norén and Meironyté 2000). PCBs have previously been extensively used for industrial application, but the production and use is now prohibited (Giesy and Kannan 1998). The toxicity and mechanism of action of individual congeners vary depending on the number and pattern of chlorination. Many of the effects observed after exposure to DL compounds as well as PCBs are similar, but in contrast to the DL compounds, several mechanism of actions have been suggested for subclasses of PCBs including activation of the AhR (Van den Berg et al. 2006), the constitutive active (androstane) receptor (CAR) and/or the pregnane X receptor (PXR) (Kretschmer and Baldwin 2005).

Cytosolic CAR is activated upon ligand binding, resulting in its dissociation from HSP90 and the co-chaperone cytoplasmic CAR retention protein (CCRP). Ligand-bound CAR is translocated to the nucleus, presumably dependant on the activity of the protein phosphatase PP2A, followed by association with the retinoid X receptor (RXR) and binding to phenobarbital (PB) responsive enhancer modules (PBREM), resulting in transcription of responsive genes including the induction of CYP2B (Figure 3; Timsit and Negishi 2007; Saito et al. 2010). Similar to CAR, PXR is retained in the cytosol by CCRP and upon ligand binding the receptor dissociates and translocates to the nucleus where it forms a heterodimer with RXR and binds the xenobiotic responsive enhancer module (XREM), leading to the induction of

e.g. CYP3A (Timsit and Negishi 2007). Some overlap has been observed for CAR and PXR-mediated gene expression (Sueyoshi and Negishi 2001).

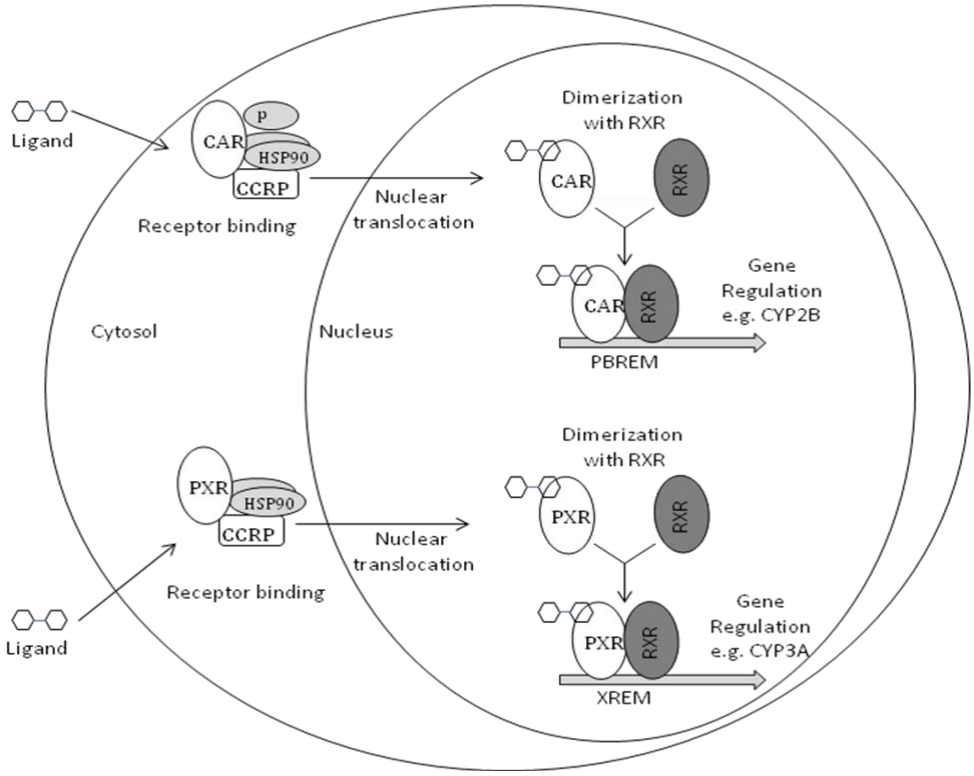


Figure 3. Mechanism of transcriptional constitutive active (androstane) receptor (CAR) and pregnane X receptor (PXR) activation.

Both CAR and PXR are functionally versatile and respond to distinct but overlapping groups of chemicals (Sueyoshi and Negishi 2001) but lack known physiological ligands (Kretschmer and Baldwin 2005). The activated receptors act as regulators of detoxification and elimination of both endogenous substances and xenobiotics, affecting the induction of several phase I enzymes such as CYPs, phase II enzymes such as uridine diphospho-glucuronosyltransferases (UGTs) and phase III transporters (Kretschmer and Baldwin 2005; Timsit and Negishi 2007; Saito et al. 2010).

In addition to the classic CYP2B inducer PB (Waxman and Azaroff 1992), compounds such as retinoic acid and highly chlorinated PCBs have been found to activate CAR (Timsit and Negishi 2007), highly chlorinated PCBs are also PXR agonists in rodents (Kretschmer and Baldwin 2005). Observations in vitro also indicate that highly chlorinated PCBs such as PCB 153 activate the CAR and PXR (Kretschmer and Baldwin 2005; Tabb et al. 2004; Sueyoshi et al. 1999; Schuetz et al. 1998). It has been suggested that in general, PCBs with 5-10 chlorines activate PXR and the potency to do so increase with the number of ortho chlorines (Tabb et al. 2004).

Endpoints such as carcinogenicity, alterations in circulating thyroid hormone concentration and neurotoxicity could arise by both AhR and non-AhR mediated

mechanisms. Non-planar ortho-substituted PCBs have been shown to elicit a diverse spectrum of toxic responses believed to be non-AhR-mediated in experimental animals, including neurobehavioural (Boix et al. 2010), neurotoxic (Honma et al. 2009; Piedrafita et al. 2008), carcinogenic (Jeong et al. 2008; Knerr and Schrenk 2006) and endocrine changes. The endocrine changes include alterations in thyroid hormones (Hedge et al. 2009; Kobayashi 2009) and the retinoid system. Endpoints such as altered body and organ weights, altered tissue vitamin A levels and EROD induction included in the database used as a basis for the establishment of WHO TEFs for DL compounds (Van den Berg et al. 2006; Van den Berg et al. 1998) have also been observed after exposure to PCB congeners not included in the TEF concept (Chu et al. 1996a, b; Lecavalier et al. 1997). The most sensitive effects seen in studies with individual PCB congeners not included in the TEF concept were liver and thyroid toxicity (Chu et al. 1996a; Chu et al. 1996b; EFSA 2005; Lecavalier et al. 1997). However, since the simultaneous exposure to PCBs and DL compounds hampers the interpretation of toxicological findings and the database on effects of individual PCB congeners not included in the TEF concept is rather limited, no health-based guidance value has been established for PCBs so far (EFSA 2005).

The toxic responses observed after exposure to commercial PCB mixtures are dependent on several factors including the chlorine content and purity of the commercial mixture (Burgin et al. 2001; Kodavanti et al. 2001). These complex mixtures have been shown to induce both PB-type and DL-type enzyme activity, which could be due to the different action of individual components (Burgin et al. 2001). Data on toxicity after exposure to a variety of commercial PCB mixtures indicate that the liver is a common target organ and various symptoms of hepatotoxicity have been observed, but effects also include acute lethality, body weight loss, dermal toxicity, thymic atrophy, immunosuppressive effects, reproductive and developmental toxicity, carcinogenesis, other genotoxic responses, modulation of diverse endocrine-derived pathways, including effects on several levels of thyroid hormone regulation, and neurotoxicity (Burgin et al. 2001; Kodavanti et al. 2001; Ma and Sassoon 2006; Royland and Kodavanti 2008; Safe 1994; Silkworth et al. 2008; Steinberg et al. 2007).

Analysis of environmental samples has shown that the PCB composition is highly variable and does not resemble the composition of the commercial mixtures (Ishikawa et al. 2007). However, PCB congeners which are not included in the WHO TEF concept for DL compounds represent by far the largest portion of constituents both in commercial PCB mixtures (Mayes et al. 1998; Schmitz et al., 1996) and in human (Fürst 2006) and food samples (EFSA 2005; Törnkvist et al. 2011), illustrating the need for a reliable risk assessment of these compounds. The high levels in food are also reflected on the human dietary intake levels (Table 2, Törnkvist et al. 2011), where the intake of PCBs (∑PCB) is much larger on a bodyweight basis than the intake of dioxins and DL compounds. The intake has decreased concerning both total TEQ and total PCB between 1999 and 2005 (Törnkvist et al. 2011). Based on effects observed after exposure to PCB congeners not included in the TEF concept, the European food safety authority (EFSA) estimated a factor 10 margin of body burden for humans which can be considered rather small. The margin is based on average intake and more highly exposed groups such as breast-fed infants and subpopulations with a dietary intake

exceeding the average could have an even smaller margin of body burden (EFSA 2005).

Table 2. *Total intake of dioxin-like compounds, PCBs and PBDEs from food in Sweden in 1999 and 2005.*

	Intake[a], pg TEQ/day	
	1999	**2005**
PCDD/F TEQ[b]	54.4	24.7
PCB TEQ[b]	41.4	26.2
Total TEQ[b]	95.8	50.9
	Intake[a], ng/day	
	1999	**2005**
∑PCB	615	362
PCB 153	139	85.3
∑PBDE	50.9	50.6
BDE 47	26.5	20.6

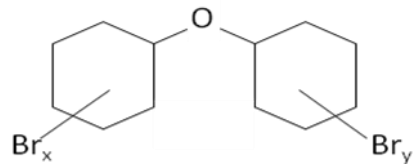
[a]Törnkvist et al. 2011
[b]TEQ calculation based on 1998 WHO TEFs, Van den Berg et al. 1998

1.2.2.1 PCB 180

PCB 180 is a di-ortho heptachlorinated congener classified as a PB-type inducer of CYPs (McFarland and Clarke 1989). PCB 180 has previously been included in the TEF concept and was assigned a TEF in the 1994 establishment of values based on one acute in vivo study as compared to TCDD (Ahlborg et al. 1994). The TEF value for PCB 180 was however withdrawn in the 1998 WHO re-evaluation (Van den Berg et al. 1998). PCB 180 is commonly found in environment and food and is also included as one of the six analytical indicator PCB congeners (EFSA 2005). In a comparison between the congener composition of the commercial PCB mixture Aroclor 1260 and the congeners found in breast milk samples the congener composition was found to differ. However, PCB 180 was abundant in both the commercial mixture and in the human milk samples (Safe 1994).

1.2.3 PBDE

Commercial mixtures of polybrominated diphenyl ethers (PBDEs, Figure 4), including Penta-BDE products such as Bromkal 70-5DE, have been widely used as flame retarding additives. The concern for human and environmental health due to increased use of PBDEs combined with their tendency to bioaccumulate in humans has prompted the ban of penta-PBDE flame retardants both in the EU and in various states in the USA (EU 2003a; EU 2003b; BSEF 2009). However, some major applications relate to long-lived consumer products and continued release to environment and humans may be anticipated for many years to come. Similar to the PCBs, several receptors, including the AhR, the CAR and/or the PXR, have been suggested to be modulated by PBDEs (Figure 3; Fery et al. 2009; Pacyniak et al. 2007).



PBDE

Figure 4. *Chemical structure of polybrominated diphenyl ethers (PBDEs).*

Individual PBDE congeners have a low acute toxicity but long-term toxicity studies have shown that the thyroid and liver are major target organs for BDE toxicity (Talsness 2008; Gill et al. 2004; WHO-IPCS 1994). Individual PBDE congeners do not have AhR agonist properties, although similar effects have been observed for DL compounds and PBDEs. The commercial PBDE mixtures however contain PBDD/Fs and have been shown to have AhR mediated activities (Van den Berg et al. 2006). Photolytical and combustion processes of PBDEs can produce PBDD/Fs, but it is unclear to what extent the use of PBDEs contributes to PBDD/F levels. PBDD/Fs are similar in behaviour to their chlorinated analogues and similar effects are also observed for PBDD/Fs and PCDD/Fs (Wahl et al. 2008; Hanari et al. 2006; Birnbaum et al. 2003). The structural similarities between PBDEs and classes of dioxin-like chemicals (e.g. PCBs), suggests that PBDEs might bind the AhR with low affinity. Unlike the DL AhR ligands, PBDEs are usually not coplanar and the AhR binding affinities of PBDEs is reported to be 10^{-2} to 10^{-5} times that of TCDD (Chen et al. 2001). Though a low AhR affinity has been observed, the subsequent XRE binding and induced gene expression was not (Birnbaum and Staskal 2004; Peters et al. 2006; Talsness 2008).

The most toxicity information is available for commercial PBDE mixtures and less for individual congeners. The target systems and organs appear however to be similar for the mixture and the individual congeners, and the effects observed after exposure to brominated flame retardants include developmental neurotoxicity, reproduction and development effects, hormone-related effects, morphological effects on liver and kidney and hepatotoxicity (Dunnik and Nyska 2009; Van der Ven et al. 2008; Darnerud 2008; Talsness 2008; Birnbaum and Staskal 2004; Zhou et al. 2001). The liver effects included liver weight changes, hepatocyte hypertrophy, retinoid alterations, induction of CYP and UGT enzymes as well as EROD and PROD activity.

The commercial PBDE products are not exclusively one pure congener, but contain several PBDE congeners with different amount of bromination. The exact congener pattern is likely to vary slightly between manufacturers and batches. Even though the penta-BDE use is banned, the dominating congener pattern in human serum samples is still similar to the pattern in commercial penta-BDE products, namely BDE 47, BDE 99, BDE 100 and BDE 153 (Harley et al. 2010; Sjödin et al. 2008; Andersson et al. 2008). PBDEs are also found in human breast milk (Lignell et al. 2009; Fängström et al. 2008; Fürst 2006) and in food (Schecter et al. 2004; Törnkvist et al. 2011). The dietary intake of PBDEs is lower than the intake of PCBs, but still much higher than the intake of DL compounds (Table 2). Also the intake of PBDEs has remained quite

constant between 1999 and 2005, although the calculation for 2005 was based on a larger number of PBDE congeners (Törnkvist et al. 2011).

1.3 CUMULATIVE ASSESSMENT

Although some potential environmental hazards involve significant exposure to only a single compound, most instances of environmental contamination involve exposures to a mixture of compounds (Kortenkamp 2007). Though the problem of assessing the combined exposure to multiple chemicals has been acknowledged for decades, only a few cumulative assessment models have been used for regulatory purposes. To assess the effects of exposure to groups of POPs, potency normalisation approaches have been developed. This method includes the selection of a reference or index compound against which the potency of related compounds are normalised, such as the WHO-TEF system for dioxins and DL compounds (Van den Berg et al. 2006) and the Relative Potency Factor (RPF) method developed by the USEPA for pesticides (Boobis et al. 2008; USEPA 2000). The RPF method is quite generalised and has also been used for PAHs (Pufulete et al. 2004). Studies regarding the feasibility of developing cumulative assessments have also been performed for reproductive toxicants acting via diverse mechanisms (Rider et al. 2010) as well as for estrogenic (Kortenkamp 2006) and anti-androgenic chemicals (Kortenkamp and Faust 2010; Müller et al. 2009). The TEF system was developed to assess the total contribution of DL compounds in a mixture in order to assess the combined effects of structurally related compounds with a common mechanism/mode of action (Van den Berg et al. 2006) while the RPF method can be applied in situations where the mode of action appears to be similar, but the exact mechanism is complex and maybe not known in detail (USEPA 2000).

Several of the POPs, including PCBs and PBDEs, have similar effect spectra as the DL compounds on an end-point basis, but since the criteria of acting via the common mechanism initiated by binding to the AhR is not fulfilled, they are not included in the TEF system (Van den Berg et al. 2006). The similarity in effects, i.e. modulation of a common system or tissue, observed after exposure to several types of POPs indicate that the combined exposure to these chemicals could contribute to cumulative toxicity and that a cumulative assessment based on the biological system or the target tissue affected rather than on the mechanism of toxicity might be warranted as a complement to the established TEF concept for DL substances. By focusing only on mechanism-based (AhR) TEF values and not taking effect-related potencies into consideration, the combined effect of e.g. dioxins, PCBs and PBDEs might be underestimated.

The inclusion of compounds modulating a common system or tissue in assessments has also been raised during the establishment of TEF values for DL compounds. Ahlborg and colleagues suggested already in 1994 to explore the possibility of developing endpoint-specific REP values for endpoints where effects are seen after exposure to both congeners included as well as not included in the TEF system (Ahlborg et al. 1994). Endpoint-specific REP values can be calculated based on single studies while the TEF values are based on REP values derived from many types of studies and endpoints. The REP approach seems to be appropriate in order to perform cumulative risk assessment, with the restriction that the REP values

should only be used for the endpoint for which they were obtained and not for all endpoints (Müller et al. 2009).

The assessment of mixtures can be performed on three levels, i.e. using data on the mixture of concern, using data on a toxicologically similar mixture and using data on the mixture component chemicals (USEPA 2000). The whole mixture approach, where a mixture is studied as if it was a single compound, is useful for studying complex mixtures but leads to difficulties in extrapolating from one mixture to another (Kortenkamp 2007).

Cumulative exposure to a group of substances with the same toxicological mode of action could be of concern even if exposure to either of the substances individually does not pose a risk to human health and the same could also hold true for compounds modulating a common system or tissue though acting via separate mechanisms. A cumulative risk assessment should hence include all chemicals which affect a specified system or tissue (Rider et al. 2010). The biological reality of combination effects from exposure to multiple agents at low doses highlights the potential for underestimating risks when mixture effects are not taken into account (Kortenkamp 2007). Also, the information required to establish membership of a common mechanism group is substantial, leading to a risk of failing to consider compounds that should have been included (Boobis et al. 2008).

2 PRESENT STUDY

2.1 AIM

The aim of the thesis was to study the feasibility of developing endpoint-specific cumulative assessments based on effects considered as markers of DL toxicity observed for different POPs in vivo.

Paper I: The aim of paper I was to characterise the hepatotoxicity of PCB 180, not included in the TEF concept, with a specific focus on liver weight, hepatic vitamin A levels and hepatic EROD induction which are endpoints used as markers of DL toxicity.

Paper II: The aim of paper II was to characterise the toxicity of the commercial PBDE mixture Bromkal 70-5DE based on a whole mixture approach and to assess the presence of DL compounds and their influence on the observed effects focusing on liver weight, hepatic vitamin A levels and hepatic EROD induction as markers of DL toxicity.

2.2 EXPERIMENTAL DESIGN

General study design
The studies were performed according to the OECD 407 Guideline on Repeated Dose 28-day Oral Toxicity Study in Rodents, which was enhanced for biochemical endpoints. The study design in Paper I was also improved to facilitate the assessment of dose-response relationships and subsequent calculation of benchmark doses (Slob 2002). Groups of 5 male and 5 female Sprague-Dawley rats were exposed by oral gavage to PCB 180 dissolved in corn oil (Paper I) or Bromkal 70-5DE dissolved in peanut oil (Paper II).

Clinical observations and chemistry, body and organ weights
Recording of final body weight as well as complete necropsies, including macroscopic observations and tissue sampling for molecular biology, biochemistry, histopathology, analytical chemistry and organ weights, were performed on each rat. Blood was collected for haematological analyses and serum chemistry. In paper I, total mRNA for real-time PCR analysis was isolated from frozen liver samples.

Biochemistry
In papers I and II, *O*-Dealkylation of 7-ethoxyresorufin (EROD) and 7-pentoxyresorufin (PROD) were determined in liver microsomes, while in paper I the microsomal UGT activities were also determined using thyroxine (T4) as a substrate and beta-naphthoflavone as internal reference.

Apolar retinoids were extracted from liver homogenates and retinol and retinyl esters were separated. Quantification was performed by the use of internal (retinyl acetate) and external (retinol and retinyl palmitate) standards. The summarized levels of apolar retinoids was computed as sum of retinol and retinyl esters. In paper II, retinoid levels were also measured in kidneys and lungs.

Chemical characterization and residues
The dosing solutions were analysed for chemical impurities with GC-MS. The levels of DL-PCBs and PCDD/Fs in PCB 180 (Paper I) were found to be 2.7 ng TEQ/g PCB 180. For the analysis of Bromkal 70-5DE (Paper II), all the seventeen 2,3,7,8-substitued PCDFs and PCDDs and ten of the 2,3,7,8-substituted PBDFs and PBDDs were used as reference compounds. The TEQ dose was calculated based on weight adjusted potency relative to TCDD adapted from Behnisch and colleagues (Behnisch et al. 2003) and was found to be 524 ng TEQ/g Bromkal 70-5DE.

Tissue residue concentrations of PCB 180 (Paper I) were determined in perirenal adipose tissue and in liver tissue.

2.3 RESULTS

Paper I
At the highest dose level a temporary decrease in body weight during loading dosing was observed in both genders, but no other signs of general toxicity were observed after exposure to PCB 180. Absolute liver weights were dose-dependently increased in both males and females and observations were made at a lower dose in males than in females. Liver histopathology showed centrilobular hypertrophy in exposed animals. Significant decreases in liver apolar retinoid concentrations as well as total amounts were observed in both male and female rats after exposure to PCB 180. The decrease was larger in males and also appeared at a lower dose (Figure 5).

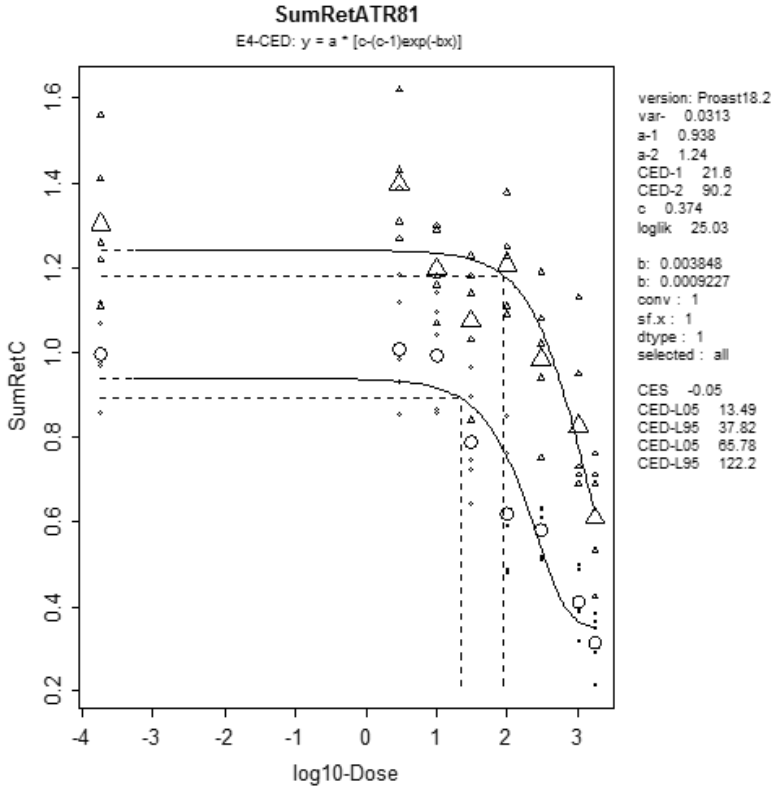


Figure 5. *Dose-response relationship analysis and estimated benchmark doses based on decreased hepatic apolar retinoid concentration observed in male (○) and female (Δ) Sprague-Dawley rats after exposure to PCB 180.*

Hepatic microsomal EROD and PROD activities were significantly induced in both males and females. The induction was seen at a lower dose in males and the PROD induction was more pronounced in males. Hepatic UGT activity towards T4 as substrate was significantly induced in males and females and at a lower dose in males. The observed induction of CYP and UGT activities was also analysed on mRNA and protein level. A slight CYP1A2 protein induction was found in the livers of both male and female rats at the higher doses only but on the level of mRNAs, CYP1A1 was only induced at the highest dose in females and no induction was observed for CYP1A2 or CYP1B1 mRNAs. In contrast, strong induction of CYP2B1/2 and CYP3A1 protein as well as CYP2B1 and CYP3A1 mRNA was observed in males and in females with the extent of induction being more pronounced in males. Also UGTs 1A1 and 1A6 were induced in males and females on both protein and mRNA level with males being more responsive to PCB180. The extent of induction was particularly high for UGT 1A6 protein in male rats.

Paper II

No clinical signs of toxicity were observed after exposure to Bromkal 70-5DE and no animals died during the study. The relative liver weights were increased in a dose-dependent manner in male and female rats. Observed histological hepatic changes consisted of centrilobular hepatocellular hypertrophy of different grade and patchy fatty changes (lipidosis). Altered serum parameters associated with hepatotoxicity were also observed. The hepatic EROD activity was dose-dependently elevated in both male and female rats and the PROD-activity was also markedly elevated in a dose-dependent manner. Hepatic vitamin A content was decreased in a dose-dependent manner in both male and female rats.

3 CONCLUSIONS

Liver is a major target organ for DL compounds as well as for PCBs and PBDEs and endpoints such as liver weight, hepatic vitamin A levels and AhR-mediated enzyme induction are sensitive effects observed at low doses, with reported LOELs in rats of 1 ng TCDD /kg b.w./day (lowest dose tested) for induction of hepatic EROD activity (Viluksela et al. 2000) and depletion of hepatic vitamin A (Fletcher et al. 2005). An increased liver weight was found from 10 ng TCDD /kg b.w./day (Viluksela et al. 2000). These effects have previously been used for deriving REP values included in the database for the establishment of TEF values (Van den Berg et al. 2006; Van den Berg et al. 1998). However, the assessment of these effects after exposure to PCBs and PBDEs has been hampered by the use of a whole mixture approach employed in many studies, making it hard to attribute the effects to specific components, and the uncertainty regarding the level of potent DL compounds in mixtures or as contaminants in single compound preparations. Often an induced EROD activity has been regarded as evidence of the presence of DL compounds.

3.1 PCB

Effects observed after exposure to PCB 180 include liver enlargement, centrilobular hepatocellular hypertrophy, decreased hepatic apolar retinoid levels and liver enzyme induction. The effects observed were in general more prominent in males (Paper I).

An induction of hepatic microsomal AhR-marker EROD and the CAR-marker PROD activity was observed. The CAR-activating potency of PCB 180 was also verified on mRNA and protein level with strong inductions of CYP2B1 and CYP3A1. In contrast, the transcripts of CYP1A2, and 1B1 were not induced, while CYP1A1 mRNA was induced at the highest dose level in females only and a weak inducing effect on CYP1A2 protein was observed (Paper I).

The data suggest that PCB 180 can act as a CAR/PXR agonist, since strong effects were observed for enzymes that have been related to an activation of CAR/PXR. The nuclear transcription factor CAR has been reported to be more prominent in male than in female rats (Yoshinari et al. 2001), and the observed gender differences in decreased hepatic vitamin A levels and induced PROD activity adds further support to the importance of CAR in mediating effects of PCB 180. The analysis of hepatic PCB 180 levels did not reveal differences between male and female rats and the levels were roughly proportional to the applied dose. The CAR-dependent mode of action was also supported by an increased UGT activity towards T4 accompanied by an induction of UGTs 1A1 and 1A6 on protein and mRNA level (Barter and Klaassen, 1992; Shelby and Klaassen 2006; Vansell and Klaassen 2002).

A weak AhR agonistic effect was also observed after PCB 180 exposure, with EROD induction observed at lower doses than those needed to induce the corresponding enzymes on mRNA and protein level. The analysed DL contamination was 2.7 ng TEQ/g PCB180, which would correspond to a total contaminant dose of 0.027 ng TEQ/kg b.w. in the 10 mg PCB 180/kg b.w. dose group where the EROD induction was significantly increased in males (Paper I). This TEQ dose is lower than the reported LOAEL for EROD induction, indicating that the induction might instead be

due to an enzymatic specificity overlap resulting from the strongly induced CYP2B and 3A with possible minor catalytic EROD activity (Burke et al. 1994). It has also been suggested that conditions affecting the formation or metabolism of natural AhR ligands may modify the AhR activity and lead to misinterpretation of experimental data, such as EROD induction (Bergander et al. 2005; Wincent et al. 2009).

The selected endpoints increased liver weight, decreased hepatic vitamin A levels and hepatic EROD induction were also evaluated by calculating REP values using data from a series of single compound experiments including PCBs 77, 105, 118 and 126 (Chu et al. 1998; Chu et al. 1995; Chu et al. 1994) included in the TEF concept, as well as PCBs 28, 128 and 153 (Chu et al. 1996a, b; Lecavalier et al. 1997) not included in the TEF concept. Endpoint-specific REP values were estimated as NOEL ratios compared to the potent DL PCB 126 (TEF=0.1, Table 1).

Significant dose-response relationships, analysed with a non-linear regression model in which dose-related changes in mean responses were described by a Hill function, were observed based on liver weight data after exposure to PCBs 105, 128 and 153, based on hepatic vitamin A data after exposure to PCBs 77, 105, 128, and 153 and based on hepatic EROD activity data after exposure to PCBs 28, 77, 105, 118, 128 and 153 (Kalantari et al. 2010 abstract). In all cases where a significant dose response relationship was observed for an individual PCB congener the effect on the studied endpoint was similar to the effect observed after exposure to the reference compound PCB 126 and only differing in potency. Endpoint-specific REP values could hence be established both for congeners assigned a TEF (PCBs 77, 105 and 118) and congeners not assigned a TEF (PCBs 28, 128 and 153) based on one or more of the endpoints (Table 3).

Table 3. *Relative potency (REP) values estimated as NOEL ratios based on liver weight (%), hepatic vitamin A (µg) and hepatic EROD activity (nmol/mg protein/hr) observed in male and female Sprague-Dawley rats after exposure to individual PCB congeners*[1].

PCB congener	TEF[a]	Liver weight NOEL REP[b]		Hepatic vitamin A NOEL REP[b]		Hepatic EROD activity LOEL[c] REP[b]	
		MALE	FEMALE	MALE	FEMALE	MALE	FEMALE
28						$2.6e^{-07}$	$2.5e^{-07}$
77	$1.0e^{-04}$			$1.1e^{-04}$	$1.1e^{-04}$	$1.3e^{-06}$	$1.1e^{-05}$
105	$3.0e^{-05}$	$2.0e^{-05}$	$1.9e^{-04}$	$2.0e^{-05}$	$2.3e^{-05}$	$2.3e^{-07}$	$2.5e^{-07}$
118	$3.0e^{-05}$					$1.5e^{-06}$	$5.9e^{-06}$
128			$2.0e^{-04}$	$1.9e^{-05}$	$2.3e^{-06}$	$2.4e^{-07}$	$2.3e^{-07}$
153		$2.3e^{-05}$	$2.0e^{-04}$	$2.3e^{-05}$	$2.3e^{-06}$	$2.9e^{-05}$	$2.4e^{-05}$

[1]Chu et al. 1998; Chu et al. 1996a, b; Chu et al. 1995; Chu et al. 1994; Lecavalier et al. 1997
[a]WHO TEF values (Van den Berg et al. 2006).
[b]The REP values have been adjusted to account for the use of PCB 126 as a reference compounds, i.e. by multiplying with the TEF for PCB 126 (TEF=0.1).
[c]For hepatic EROD activity data, a NOEL value could not be established for PCB 126 and the calculations have been based on LOEL values for all congeners.

The potency of each PCB congener differed from endpoint to endpoint, with the highest NOEL REP values mainly corresponding to the endpoint relative liver weight

and NOEL REP values based on decreased hepatic vitamin A levels found to be slightly lower (Table 3). The lowest REP values were in general estimated based on hepatic EROD activity data. Based on liver weight and hepatic vitamin A data, the estimated potency of PCBs 77 and 105 were similar to their corresponding established WHO-TEF value, while the REP values based on EROD induction data for PCBs 77 and 105 as well as PCB 118 were lower than the corresponding TEF values (Table 3). Based on liver weight and hepatic vitamin A data, the estimated potency of PCBs 128 and 153 was similar to the common TEF established for low-potency mono-ortho PCBs in the WHO concept (Table 3). For PCBs 28 and 128 a low potency effect was observed based on hepatic EROD induction data while PCB 153 was the most potent congener in inducing this effect and the REP value was in the same range as the TEF value for mono-ortho PCBs (Table 3).

The low chlorinated PCB 28 did not show a high potency in inducing the effects studied, while PCB 153 on the other hand had a similar or slightly higher potency than congeners included in the TEF concept in causing increased relative liver weight, decreased hepatic vitamin A levels and hepatic EROD induction. Observations in vitro indicate that highly chlorinated PCBs such as PCB 153 activate the CAR and PXR (Kretschmer and Baldwin 2005; Tabb et al. 2004) while an activation of CAR/PXR was not observed for the low chlorinated congener PCB 28. PCB 153 has previously been classified as a PB-type inducer (Mc Farland and Clarke 1989) and after exposure to PCB 153 in ovariectomized mice, different gene expression pattern was observed as compared to TCDD and PCB 126 including CAR/PXR regulated genes but not AhR regulated genes (Kopec et al. 2010). The high EROD induction observed after exposure to PCB 153 could hence be a resulted of a marked induction of CAR/PXR-related enzymes or secondary effects in a similar manner as was described for PCB 180.

3.2 PBDE

Effects observed after exposure to the commercial penta-BDE mixture Bromkal 70-5DE include a marked increase in relative liver weight, hepatic lipidosis, centrilobular hypertrophy of hepatocytes, hepatic vitamin A depletion and hepatic EROD and PROD enzyme activity induction (Paper II). The increased liver weight and the decrease in hepatic vitamin A was observed from 25 mg Bromkal 70-5DE/kg b.w./day, corresponding to an estimated daily PBDD/F-TEQ intake of 13 ng/kg b.w./day, while the hepatic EROD enzyme activity was increased from 2.5 mg Bromkal 70-5DE /kg b.w./day, corresponding to an estimated daily dose of 1.3 ng PBDD/F-TEQ/kg b.w. The estimated contribution of DL compounds found in the mixture may explain the effects observed on liver weight, hepatic vitamin A and EROD activity, at least it cannot be ruled out. The hepatic PROD enzyme activity was increased from and 25 mg/kg b.w./day, which may suggest a CAR-dependant activity of Bromkal 70-5DE.

Van der Ven and colleagues studied a purified preparation of DE-71, another commercial penta-BDE mixture, and observed increased liver weights and centrilobular hepatocellular hypertrophy, decreased hepatic levels of retinoids as well as induction of both EROD and PROD activities and associated enzymes (Van der Ven et al. 2008). Their conclusion was that the enzyme activities, induced to similar

extent and at low doses, indicated activation of both AhR and CAR and that some mixture constituents may act via CAR while others have weak AhR agonistic activity.

The Bromkal 70-5DE and DE-71 studies were performed under similar experimental conditions, following the OECD 407 28-day subacute toxicity guideline. However, in the Bromkal 70-5DE study Sprague-Dawley rats were used (Paper II) while in the DE-71 study Wistar rats were used (Van der Ven et al. 2008). Effects on liver weight and hepatic EROD and PROD induction were observed at similar dose levels in the two studies. Decreased hepatic vitamin A levels were observed at similar dose levels in female rats, while in male rats DE-51 was found to cause an effect at a 50 times lower dose. After exposure to Bromkal 70-5DE, the EROD induction was observed at 10 times lower doses than the PROD induction in both males and females, the maximal induction was however similar and only differed 2.5 fold (Paper II). A similar maximal induction of EROD and PROD was also observed after exposure to DE-71, but the EROD induction was observed at a 10 times higher dose than the PROD induction in males and at a 2 times lower dose in females (Van der Ven et al. 2008).

3.3 CONCLUSION

Effects on liver weight, hepatic vitamin A and hepatic EROD activity were observed after exposure to PCB 180 as well as observations indicating CAR/PXR activation. These findings support the influence of CAR and PXR activation on effects such as increased liver weight and modulations of the retinoid system. Based on a whole mixture approach, Bromkal 70-5DE was found to contain DL contaminants to an extent that could explain most of the effects observed. In a comparison to a series of PCB experiments, REP values could be estimated for all included congeners as compared to PCB 126 based on one or more of the endpoints increased liver weight, decreased hepatic vitamin A and hepatic EROD induction, indicating that the observed effects of these congeners were similar to the effects of PCB 126, regardless if they are assumed to act mainly via the AhR, i.e. have been assigned a TEF value, or not. The hepatic EROD activity does not appear discriminating enough to distinguish between AhR-mediated and non AhR-mediated enzyme induction.

In conclusion, the findings in this thesis support the suggestion to develop endpoint-specific systems for cumulative assessment of POPs based on the criteria to include chemicals with similar effects, i.e. modulating a common system or target tissue via multiple pathways and/or mechanisms of toxicity.

4 FUTURE PERSPECTIVES

A next and important step after identifying endpoints that are affected in a similar way, but most likely involving several different mechanistic pathways, includes developing suitable methodology for assessing the cumulative effects observed after combined exposure to bioaccumulative and toxic substances. It is also important to incorporate uncertainty into the final risk estimates and this can be achieved by using REP values derived as BMD ratios including confidence intervals. The use of BMD methodology also allows for a statistical analysis of e.g. gender differences and detailed compound comparisons.

Several studies characterising the toxic effects of PCBs and PBDEs report neurotoxicity and endocrine alterations, including effects on thyroid hormones. The study on PCB 180 (Paper I) also supports effects on thyroid hormone metabolising UGTs. Many different mechanisms have been suggested to be related to the neurotoxic as well as the endocrine modulating effects and it would be difficult if not impossible to define chemicals affecting these systems in terms of strict molecular similarity. Therefor it would be interesting to investigate such effects from a similar perspective as has been done in this thesis.

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