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TOXICITY OF BROMINATED FLAME RETARDANTS WITH FOCUS ON RETINOID SYSTEM DISTURBANCES

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Toxicity of brominated flame retardants with focus on retinoid system disturbances

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Transcendental Meditation is that one simple procedure which can raise the life of every individual and every society to its full dignity, in which problems are absent and perfect health, happiness, and a rapid pace of progress are the natural features of life.

- Maharishi Mahesh Yogi

In today's time we neither accept nor speak about the 'dark night of the soul' because the soul is all bliss, is all light, is all infinite, and no darkness is there.

- Maharishi Mahesh Yogi

A new humanity will be born, fuller in conception and richer in experience and accomplishments in all fields. Joy of life will belong to every man, love will dominate human society, truth and virtue will reign in the world, peace on earth will be permanent and all will live in fulfillment in fullness of life in God Consciousness.

- Maharishi Mahesh Yogi

Life will give you whatever experience is most helpful for the evolution of your consciousness. How do you know this is the experience you need? Because this is the experience you are having at this moment.

-Eckhart Tolle - A New Earth: Awakening to Your Life's Purpose

You are the sky. The clouds are what happens, what comes and goes.

(Findhorn Retreat: Stillness Amidst The World)

When you say "yes" to the "isness" of life, when you accept this moment as it is, you can feel a sense of spaciousness within you that is deeply peaceful.

-Echart Tolle - Stillness Speaks

Om du låter omvärlden tro att du är galen är du fri att göra vad du vill.

-Janesh Vaidya

En integrerad individ vet utan att resa, ser utan att titta, och åstadkommer utan att göra.

-Lao Tzu

När medvetenheten riktas utåt uppstår sinnet och världen. När den riktas inåt inser den sin egen Källa och återvänder hem till det Omanifesterade.

-Eckhart Tolle

När du kapitulerar inför det som är och blir fullständigt närvarande upphör det förflutnas makt över dig. Då öppnas Varandets rike, som tidigare har skymts av intellektet. Plötsligt uppstår en gränslös stillhet inom dig, en outgrundlig känsla av frid. Och i den friden finns det en stor glädje. Och i den glädjen finns det kärlek. Och längst in i den innersta kärnan finns det heliga, det omätbara, Det som inte har något namn.

-Eckhart Tolle

Till minne av min far.

ABSTRACT

Background: Brominated flame retardants (BFR) are detected in the environment and biota all over the world. They contribute to the human body burden of industrial chemicals and exposure is mainly via food. Indoor dust contributes substantially in some exposure situations, which involve small children. Regulatory restrictions and bans have been introduced as some BFRs can impact proper development, potentially via the endocrine system.

Objectives: The study aim was to clarify the role of retinoid system disturbances in BFR toxicity. Specific aims were to identify retinoid forms, which are sensitive to BFR exposure, and to identify genes, which can explain observed changes in specific retinoid forms. An additional aim was to evaluate the relevance of using retinoid system endpoints for risk characterization of human BFR exposure.

Methods: Technical decabromodiphenyl ether (decaBDE), hexabromocyclododecane (HBCD), tetrabromobisphenol-A (TBBPA), or pentabromodiphenyl ether (pentaBDE) mixtures, were given orally to male and female Wistar rats at 3-7 different dose-levels. The OECD 28-day repeat dose toxicity study (TG407) and/or the 1-generation toxicity study (TG415) protocols, enhanced to detect endocrine effects were used. Retinoid concentrations and gene expression profiles were determined by HPLC and RT-PCR, respectively. Benchmark doses were established for all endpoints except gene expressions of the decaBDE study. Mode of action and margin of exposure evaluations were performed for decaBDE and HBCD and partly for pentaBDE.

Results and discussion: Reduction of hepatic all-*trans* RA levels was the most sensitive physiological endpoint after decaBDE exposure, and was connected to increased expressions of *Cyp1b1, Cyp26a1, Crabp1* and CYP2B. HBCD exposure resulted in reductions of hepatic 9-*cis*-4-oxo-13,14-dihydro-retinoic acid, retinyl palmitate, and all-*trans* RA levels, which were connected to increased expressions of *Adh1, Aox1, Aldh1, Cyp26a1, Lrat, Ugt1a1, Ugt1a6,* and *Ugt1a9*. The retinoid system characterization of pentaBDE and TBBPA was explorative, and the obtained results confirm that a full characterization is needed; in particular so for pentaBDE. Several lines of evidence, including gender and compound differences in response, as well as previously published data, provide support for key roles of CYP2B and CYP3A in the observed modulations of the retinoid system suggesting that CAR and/or PXR mediated mechanisms are involved. MOE calculations suggest that the observed retinoid system changes are of relevance to human exposure scenarios.

Conclusion: Retinoid forms analyzed in this thesis were sensitive to BFR-exposure and showed gender and compound specificities in response patterns. Most of the 25 selected target genes were needed to explain the observed changes of the analyzed retinoid forms. Additional gene data from the literature was required to provide a good fit for the mechanistic interpretation. Further research is needed to clarify the exact role of CAR, PXR, AhR and/or AhRR, in BFR-induced modulation of the retinoid system.

SAMMANFATTNING

Bakgrund: Bromerade flamskyddsmedel (BFRs), som har använts i stor utsträckning sedan 1970-talet för att öka brandsäkerheten i många konsumentvaror, återfinns nu i miljö, djur och människor över hela världen. BFRs frisätts lätt från varor och produkter, och trots att det nu finns förbud och andra regleringsåtgärder, samt även frivilliga insatser för vissa BFRs, så är bedömningen att BFR-exponeringen kommer att fortsätta långt in i 2000-talet, i och med att dessa kemikalier förekommer i varor och produkter med lång livslängd. Människor exponeras framförallt via animalisk föda, samt genom inandning och kontakt med damm inomhus. Inomhusexponeringen kan vara av särskilt stor betydelse för små barn, som vistas nära golvytor där damm ansamlas. Barn anses även vara en särskilt utsatt grupp i och med att vissa BFRs kan påverka hormonella system och därigenom misstänks störa den normala utvecklingen redan under fostertiden.

Syfte: Syftet med detta avhandlingsarbete var att bidra med kunskap om hur BFRs påverkar retinoidsystemet, som är ett hittills mindre väl karaktäriserat område vad gäller kemikalie-inducerade förändringar i hormonsystemet. Mer specifikt var målet att identifiera vilka retinoidformer, som påverkas vid BFR exponering, och att urskilja vilka gener, som kan ligga bakom de observerade förändringarna. Ytterligare ett syfte var att utvärdera känsligheten av effekter på retinoidsystemet och relevansen av att använda dessa effekter vid human hälsoriskbedömning.

Metoder: Utvalda BFRs i denna avhandling var polybromerade difenyletrar (PBDEs), hexabromcyklododekan (HBCD) och tetrabrombisfenol A (TBBPA) som alla tillhör de vanligaste använda. Tekniska blandningar av dessa gavs oralt till Wistar råttor i följande human-relevanta dosområden: dekaBDE; 0-60 mg/kg kv/dag, HBCD; 0-100 mg/kg kv/dag, TBBPA; 0-3000 mg/kg kv/dag, eller pentaBDE; 0-200 mg/kg kv/dag i 3-7 dosnivåer (5/10 djur/ dos) i antingen 28-dagars toxicitets studier på vuxna djur (OECD 407) och/eller i engenerations toxicitets studier (OECD 415). Studierna var utvidgade jämfört med standardesign enligt regelverken för att även bättre kunna detektera hormonstörande effekter av dessa kemikalier. Retinoidkoncentrationerna mättes med HPLC och genuttryck med Rt-PCR. Benchmark modellering etablerades för alla effekter förutom för genuttryck i dekaBDE studien. Dessutom studerades effektsamband (MOA) och relevansen av effekter för människor (MOE) för dekaBDE, pentaBDE och HBCD.

Resultat och diskussion: Minskade nivåer av all-*trans* retinylsyra i levern utgjorde den mest känsliga fysiologiska effekten av dekaBDE exponering. Denna effekt kunde kopplas till ökat uttryck av *Cyp26a1, Crabp1, Cyp1b1* och CYP2B. HBCD exponering hos vuxna djur resulterade i en helt annorlunda bild, där ändrade nivåer av 9-cis-4-oxo-13,14-retinylsyra, retinyl palmitat, och all-*trans* retinylsyra kunde kopplas till förändrat uttryck av Cyp2b, Cyp3a, *Adh1, Aox1, Aldh1, Cyp26a1, Crabp1, Lrat, Ugt1a1, Ugt1a6* och *Ugt1a9.* Den inledande karaktäriseringen av pentaBDE och TBBPA med avseende på retinoidsystem effekter, som har gjorts inom ramen för detta avhandlingsarbete, visar att det finns behov av

att göra en fördjupad och mer komplett karaktärisering även av dessa BFRs. Detta gäller framförallt för pentaBDE. Taget tillsammans visar de effekt-skillnader, som kan kopplas till substans respektive kön enligt detta avhandlingsarbete, samt tidigare publicerade data, att även generna CYP2B samt CYP3A har en avgörande betydelse för de observerade retinoidsystem förändringarna. I sin tur tyder dessa data på att de nukleära receptorerna CAR och/eller PXR är involverade i bakomliggande mekanismer för BFR-medierad påverkan på retinoid-systemet. Framräknade riskkaraktäriseringsfaktorer för dekaBDE, HBCD och pentaBDE visar att effekter av dessa substanser på retinoidsystemet kan komma att få relevans i samband med human hälsoriskbedömning.

Slutsats: Retinoidformer som analyserades i detta avhandlingsarbete påverkades av BFR-exponering vid human-relevanta exponeringsnivåer. De observerade förändringarna i retinoidsystemet var substans- och könsspecifika på sätt som är i linje med andra toxikologiska respons, som har uppmärksammats vid riskbedömningen av dessa kemikalier. Flertalet av de 25 utvalda generna var väl anpassade för att förklara den köns- och substans specifika påverkan på de olika retinoidformerna. Ytterligare gen-data från litteraturen behövde inkorporeras i den mekanistiska analysen. Mer kunskap behövs för att klarlägga på vilka sätt CAR, PXR, AhR, och/eller AhRR, bidrar till BFR-inducerade förändringar i retinoidsystemet.

LIST OF SCIENTIFIC PAPERS

I. van der Ven L, van de Kuil T, Leonards P, Slob W, Cantón R , Germer S, Visser T, Litens S, Håkansson H, Schrenk D, van den Berg M, Piersma A, Vos J (2008). A 28-day oral dose toxicity study enhanced to detect endocrine effects of decabromodiphenylether (decaBDE). Toxicol letters; 179: 6–14.

II. Litens Karlsson S, Sanchez I, Heinrich P, Roos R, Barber X, van der Ven LT, Esteban J, Håkansson H. High-purity technical decabromodiphenyl ether (decaBDE) modulates the retinoid system in a gender specific manner in Wistar rats at doses of relevance for human exposure.

III. van der Ven L, van de Kuil T, Leonards P, Slob W, Lilienthal H, Litens S, Herlin M, Håkansson H, Cantón RF, van den Berg M, Visser TJ, van Loveren H, Vos JG, Piersma AH (2009). Endocrine effects of hexabromocyclododecane (HBCD) in a one-generation reproduction study in Wistar rats. Toxicol letters. 185: 51-62.

IV. Van der Ven LT, Van de Kuil T, Verhoef A, Verwer CM, Lilienthal H, Leonards PE, Schauer UM, Cantón RF, Litens S, De Jong FH, Visser TJ, Dekant W, Stern N, Håkansson H, Slob W, Van den Berg M, Vos JG, Piersma AH (2008). Endocrine effects of tetrabromobisphenol-A (TBBPA) in Wistar rats as tested in a one-generation reproduction study and a subacute toxicity study. Toxicology, 245: 76-89.

V. Litens Karlsson S, Sanchez I, Martinez Rodriguez S, Barber X, van der Ven L, Esteban J, Håkansson H. Hexabromocyclododecane (HBCD) modulates the retinoid system in a gender specific manner.

VI. van der Ven L, van de Kuil T, Verhoef A, Leonards P, Slob W, Cantón R , Germer S, Hamers T, Visser T, Litens S, Håkansson H, Fery Y, Schrenk D, van den Berg M, Piersma A, Vos J (2008). A 28-day oral dose toxicity study enhanced to detect endocrine effects of a purified technical pentabromodiphenyl ether (pentaBDE) mixture in Wistar rats. Toxicology; 245:109-22.

CONTENTS

LIST OF ABBREVIATIONS

ADH	Cytosolic medium-chain alcohol dehydrogenase
AhR	Aryl hydrocarbon receptor
AhRR	Aryl hydrocarbon receptor repressor
ANOVA	Analysis of variance
ALDH	Retinal dehydrogenase
AOP	Adverse outcome pathway
ARNT	Aryl hydrocarbon receptor nuclear translocator
AOX	Aldehyde oxidase
All-*trans* RA	All-*trans* retinoic acid
BDE	Brominated diphenylether
BFR	Brominated flame retardants
CAR	Constitutive androstane receptor
CED	Critical effect dose
CEDL	Critical effect dose lower confidence limit; 95%-confidence lower bound
CES	Critical effect size
CORA	9-cis-4-oxo-13,14-dihydro retinoic acid
CRABP	Cellular retinoic acid binding protein
CRBP	Cellular retinol binding protein
CYP-450	Cytochrome- P450 superfamily
DE-71	Commercial pentaBDE technical mixture
DecaBDE	Decabromodiphenyl ether
EDC	Endocrine disrupting chemical
ECHA	European Chemicals Agency
EFSA	The European Food Safety Authority
EROD	Ethoxyresorufin
HBCD	Hexabromocyclododecane
HPLC	High Performance Liquid Chromatography
IARC	International Agency on Research on Cancer
LRAT	Lecithin:retinol acyltransferase

MOE	Margin of Exposure
OECD	Organization for Economic Co-operation and Development
PBDE	Polybrominated diphenyl ethers
PCB	Polychlorinated biphenyls
PentaBDE	Pentabromodiphenyl ether
PPAR	Peroxisome Proliferator-Activated Receptor
pQCT	Peripheral Quantitative Computed Tomography
PXR	Pregnane X receptor
QSAR	Quantitative structure–activity relationship
RAR	Retinoic acid receptor
RBP	Retinol binding protein
RDH	Retinol dehydrogenase
ReOH	Retinol
RePA	Retinyl palmitate
RXR	Retinoic X receptor
SVHC	Substance of Very High Concern
T4	Thyroxine
T3	Triiodothyronine
TBBPA	Tetrabromobisphenol-A
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TG	Test Guideline
THR	Thyroid hormone receptor
TTR	Transthyretin
UGT	UDP-glucuronosyltransferase

1 INTRODUCTION

The chemical- and plastic industries have grown tremendously since the 1950´s. In 2007 the chemical industry had more than ten million employees (Forsberg, 2014), introducing new chemicals in a fast pace. Additionally, many chemicals convey endocrine disrupting mode of actions, which can interrupt development during early life, a sensitive time-span which is dependent on exact levels of hormones at correct time points (WHO, 2012).

Brominated flame retardants (BFRs) are persistent, bioaccumulative and used in high quantities. They are found in all biota, including human tissues. The molecular mechanisms underlying their toxicity have yet not been clarified, although growing evidence points towards an endocrine disrupting mode of actions (EFSA, 2011a, b, c).

The retinoid system is a hormonal system with many important roles in many aspects of life such as during development (Mark et al. 2009) and reproduction (Clagett-Dame and Knutson 2011). Various chemicals e.g. BFRs modulate the retinoid system (van der Ven et al.,2006; Novák et al. 2008; Nilsson and Håkansson, 2002). Furthermore, disturbances in the retinoid system have been associated with various severe outcomes such as neurodevelopmental disorders (Elsea and Williams, 2011; Hou et al.,2015), cancer (Shiota and Kanki, 2013), fertility problems (Clagett-Dame and Knutson 2011) and metabolic disorders (Takahashi and Takasu, 2011; Trasino et al.,2015).

2 BACKGROUND

2.1 BROMINATED FLAME RETARDANTS

BFRs are used to prevent fire onset in different consumer products, such as furniture, textile, electronic equipment and building materials. Today three groups of flame retardants are in use; inorganic, halogenated organic and organophosphorus. BFRs belong to the group of halogenated organic flame retardants which since the late 1970´s are the most frequently used. In this thesis, pentabromodiphenyl ether (pentaBDE) technical mixture (DE-71), decabromodiphenyl ether (decaBDE), hexabromocyclododecane (HBCD) and tetrabromobisphenol A (TBBPA) were studied which are the most used substances in the group of BFRs (Figure 1). PentaBDE technical mixtures predominantly consist of BDE congeners with 4-6 bromines with high gastrointestinal absorption (EFSA, 2011a). DecaBDE mainly consists of BDE-209, which consists of 10 bromines. Experimental studies in rodents indicate that decaBDE has a low uptake after oral administration (EFSA, 2011a). HBCD is a technical mixture of primarily γ–stereoisomer with some α– and β– HBCD. Toxicokinetic data suggest that HBCD is absorbed in high extent when orally administered and rapidly distributed in different tissues. TBBPA and its derivatives are easily absorbed by the gastro-intestinal tract (Hakk and Letcher, 2003).

PBDEs and HBCD tend to leach out of the products that they are blended with (reviewed in Alaee et al., 2003; de Wit, 2002) in proportions of 15-30% of the product weight of plastic and polyurethane foam respectively (WHO, 1994). Indeed, as a result of their leakage into the environment and characteristics of being persistent and bio-accumulative, PBDEs (mainly tetra- and hexa BDE) and HBCD have become widespread contaminants observed in all matrices of the environment (EFSA, 2011a, b; Law et al., 2014). In contrast, TBBPA is mainly chemically bound to its product and therefore more unlikely to leach, still TBBPA is found in the environment (reviewed in Alaee et al., 2003) and in human tissues (Cariou et al., 2008).

BFRs are associated with many adverse outcomes, such as liver toxicity, effects on neurodevelopment and reproductive toxicity and growing evidence points towards an endocrine disrupting mode of action. Based on these hazard characteristics, the overall benefit of BFRs has been questioned by scientists (DiGangi et al., 2010), claiming that the hazard of using BFRs overweight the benefits of preventing fire onset (Shaw et al., 2010).

Even though PBDE and HBCD production and use are banned or being phased out worldwide based on their risk profiles (UNEP, 2009, 2013), products are still in use and long-lived consumer products such as domestic furniture, building materials and cars will continue to release BFRs to the environment for many decades to come (Danon-Schaffer et al., 2013). Higher brominated PBDEs such as decaBDE are generally considered less toxic and persistent, however decaBDE can debrominate both in vitro and in vivo to lower- brominated metabolites which are more toxic and persistent (Zeng et al., 2008; Wei et al., 2013; Mörck et al., 2003; Sandholm et al., 2003; Huwe and Smith, 2007; Cai et al., 2011; Noyes et al., 2011).

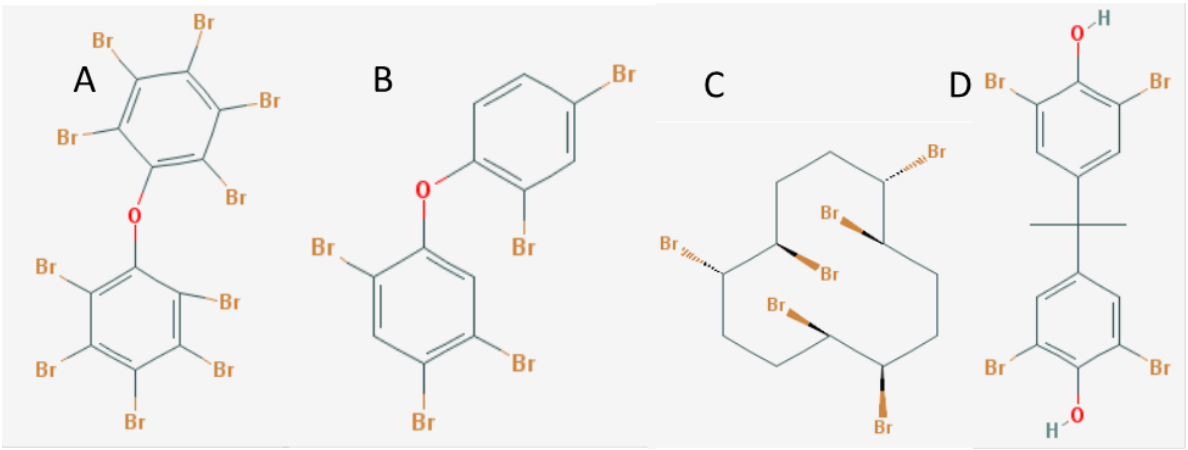

Figure 1. A) Decabromodiphenyl ether (decaBDE) B) Pentabromodiphenyl ether (pentaBDE) C) Hexabromocyclododecane (HBCD) D) Tetrabromobisphenol-A (TBBPA)

2.2 HUMAN EXPOSURE AND TISSUE LEVELS

Humans are mainly exposed to BFRs via food from animal sources (EFSA, 2011a, b). Lately it has also been shown that ingestion from household dust can be a significant source of daily PBDEs and HBCD intake, especially in toddlers who have a high hand-to-mouth action (Stapleton 2012; Johnston-Restrepo and Kannan, 2009; de Wit et al., 2012; EPA, 2010). Johnston-Restrepo and Kannan also showed that dermal absorption from household dust is a significant source in toddlers (Johnston-Restrepo and Kannan, 2009). Since the BFRs are airborne, inhalation can also be a significant source especially in aircrafts (Strid et al., 2014) and in new cars (Lagalante et al., 2011). Occupational exposure to BFRs is higher than the exposure in the general population for electronic recycling workers (Jin et al., 2009) and workers in carpet and furniture production (Stapleton et al., 2008; Chen et al., 2014).

Due to the bioaccumulative characteristics of most BFRs, the estimated half-life in human tissues is up to several years for lower BDE congeners (Geyer et al., 2004). BFRs (mainly tetra- hexa BDEs and HBCD) are found in human adipose tissues, liver, serum, breast milk, indoor dust, indoor and outdoor air, and food all over the world (de Wit 2002; Guvenius et al., 2002, 2003; Law et al., 2003, 2006; Norstrom et al., 2002; EPA, 2010; EFSA, 2011a). PBDE concentrations in human samples have decreased in Europe since the ban of penta- and octaBDE mixtures in 2004, but the trend for HBCD is less clear, and the trends in North America and Asia need more time trend studies (Law et al., 2014). However, it has been known that in general the US population has 10-100 times higher body burdens of PBDEs than the European population has (Ni et al., 2013) (Figure 2), and exposure levels among e-waste workers in Asia and Africa are above estimated risk levels (Law et al., 2014).

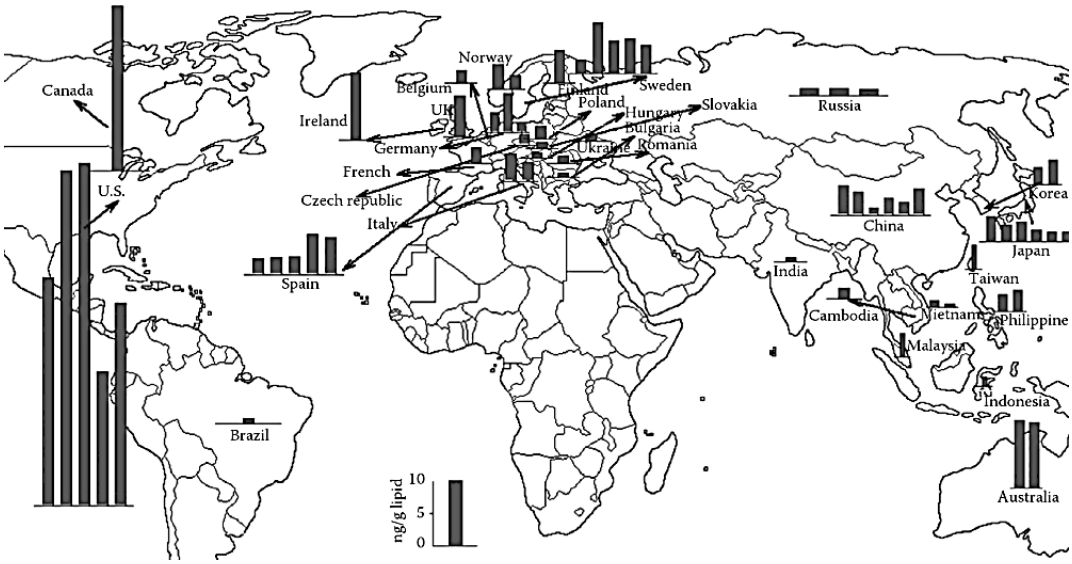

Figure 2. Global distribution of PBDE levels (after year 2000) in human milk. Re-printed from Global Contamination Trends of Persistent Organic Chemicals; 2012. With permission from Taylor and Francis Group LLC Books.

2.3 EXPERIMENTAL STUDIES AND OBSERVATIONAL STUDIES

2.3.1 Animal studies

Liver appears to be a key target organ for pentaBDE and HBCD and the number of studies on neurodevelopmental disorders is growing (Table 1). Other effects observed are reproductive changes, modifications of the immune system as well as disruptions of thyroid- and retinoid hormonal systems. In addition, decaBDE has been classified by IARC as group 3, based on studies in rats and mice where large doses resulted in liver adenoma and carcinoma (EFSA, 2011a).

2.3.2 Epidemiological data

BFRs are not well studied in epidemiological observations (Table 1). Of the few studies performed, PBDEs are the most well studied, especially the lower brominated PBDEs (EFSA, 2011a) (Table 1), where pre- and postnatal PBDE exposure in children are associated with impaired neurobehavioral development (EFSA, 2011a; Eskenazi et al., 2013). However, other halogenated compounds may have interfered with these epidemiological results (EFSA, 2011a).

Table 1. Summary of sub-chronic and chronic toxicity and observational studies in rats from Scientific opinions by EFSA in 2011 (EFSA, 2011a,b,c)

Effects	**PentaBDE**		**DecaBDE**		**HBCD**		**TBBPA**	
	Animal	Human	Animal	Human	Animal	Human	Animal	Human
Liver toxicity								
Weight	↑				↑			
Drug metabolism	↑		↑		↑			
Histopathology	↑				↑		↑	
Neurodevelopment								
Learning and memory	↓	↓	↓		↓		NE	
Attention deficits	**X**	X	X		X[a]		NE	
Reproduction								
Reproductive organ weights	↓		↑		↓		NE	
Spermatogenesis								
Immune system	X[a]				X[a]			
Thyroid hormones								
T4	↓	↓↑↔	↓↔		↓		↓↔	
T3	↓↔	↑↔	↓↑		↓[a]			
TSH	↑↔	↓↔	↓↑	↓[a]	↑		↑↔	
Retinoid system[b]								
Polar retinoids								
Apolar retinoids	↓[c]				↓↔[a]			

[a] One or a few studies performed; [b] before this thesis; [c] Fernie et al., 2005; Ellis-Hutchings et al., 2006. **Bold**: stronger evidence.

Other epidemiological findings related to PBDE exposure are diabetes and metabolic syndrome and reproductive effects, however in all cases EFSA conclude that the evidence is weak due to confounders, such as other contaminants or that the studies were too small (EFSA, 2011a).

Furthermore, there appears to be a causal link between PBDE or its metabolites and disrupted thyroid hormone levels and functions in humans, however, more studies are needed (EFSA, 2011a).

Bone is affected by endocrine disrupters such as organic pollutants e.g. dioxins (Jämsä et al., 2001). It is also well known that retinoids have an important role in bone formation, where retinoid signaling play a role in skeletogenesis by regulating the emergence of chondroblasts from skeletal progenitors (Weston et al., 2000). Effects on bone by BFR exposure have not been studied before, except for one previous sub-acute study in rats treated with HBCD (van der Ven et al., 2006), which showed sensitive effects both on bone and hepatic retinoids.

2.3.3 Receptor mediated toxicity

In spite of the significant number of experimental studies, the molecular toxicological evidence underlying BFR toxicity and the most susceptible endpoints have not yet been completely clarified. However, AhR- , CAR and PXR receptor mediated mechanisms have frequently been discussed in literature to be involved in BFR mediated toxicity outcomes. Additionally, their associated CYP inductions are associated with toxicity outcomes related to disrupted retinoid and thyroid hormone systems.

2.3.3.1 AhR

AhR is a transcription factor, where the prototype ligand is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), with a planar molecule structure. Planar or co-planar aromatic hydrocarbons have the highest affinity to bind to the AhR-pocket. Due to the bulky non-planar molecule structures of PBDEs have been considered not to bind and activate the AhR with high affinity (Luthe et al., 2008) in the same way as TCDD. When AhR is activated, it translocates together with ARNT into the nucleus and bind to the XRE where it can alter expression of notably CYP1A and CYP1B. Several experimental studies have shown AhR-dependent activity e.g. CYP1A1 induction by PBDEs but those effects have been discussed to be caused by contaminants (Sanders et al., 2005; Wahl et al., 2008; Luthe et al., 2008). Still, several PBDE congeners have shown AhR agonistic effects both in vivo (Chen et al., 2001; 2003) and in vitro (Hamers et al., 2006; Su et al., 2012) and these results have been supported lately by QSAR modelling, where it was shown that para- and meta bromination, results in the higher AhR affinity for PBDEs (Gu et al., 2012). For HBCD and TBBPA, there are no AhR mediated activity observed (EFSA, 2011; Hamers et al., 2006).

2.3.3.2 CAR and PXR

CAR and PXR are nuclear receptors which are activated by e.g xenobiotic substances as ligands. When activated CAR and PXR form heterodimers with the retinoid X receptor

(RXR) and further bind to the hormone response elements on the DNA, they alter expression of phase I (CYP2B and CYP3A) (di Masi et al., 2009) to III enzymes involved in detoxification and elimination of xenobiotics. Both in vitro (Sueyoshi et al., 2014; Fery et al., 2009; Fery et al., 2010; Wahl et al., 2008; Pacyniak et al., 2007) and in vivo (Sueyoshi et al., 2014; Szabo et al., 2009; Fery et al., 2009; Sanders et al., 2005) studies have suggested that PBDEs are inducers of CAR and PXR mediated gene expressions since they result in CYP2B and CYP3A inductions. In addition, Sueyoshi and co-workers showed that BDE-47 induced translocation of human CAR from cytoplasm into the nucleus (in vitro). CYP2B and CYP3A inductions have also been observed with HBCD which probably are associated with CAR and PXR mediated gene expressions (Germer et al., 2006). Activation of PXR by HBCD is also supported in vitro, where PXR activity was induced (Fery et al., 2010). So far, no studies have reported any CAR och PXR mediated activities by TBBPA.

2.4 RETINOID SYSTEM

The most well studied hormone receptor pathways for BFR toxicity are the thyroid-, estrogen- and androgen receptors (Ren and Guo, 2013; Hamers et al., 2006) but also the retinoid system (Ellis-Hutchings et al., 2006; 2009; Fernie et al., 2005; van der Ven et al., 2006; 2008; Öberg et al., 2010) has been addressed.

2.4.1 Dietary retinoids and transport

Retinoids (vitamin A and its analogues) are non-steroid hormones and derivatives of retinol (ReOH) (Figure 3). Retinoids are derived from vitamin A which is an essential vitamin.

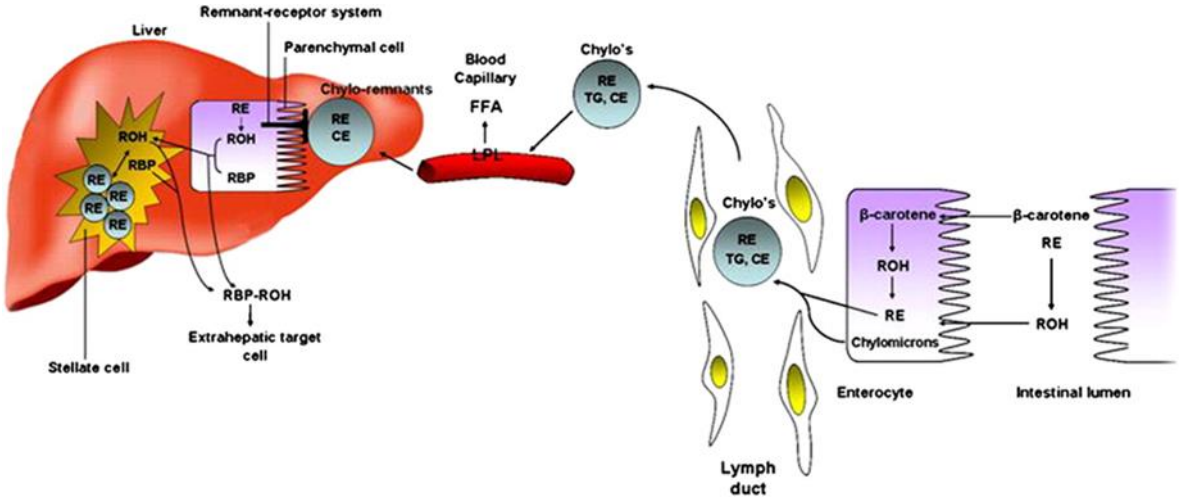

Figure 3. Figure 3. Retinoid transport in the body. RE: retinol ester; ROH: retinol; TG: triglycerides; CE:cholesterol ester; LPL: lipoprotein lipase; RBP: retinol binding protein. For description see text below. Reprinted from van Berkel TJ (2009) Bringing retinoid metabolism into the 21st century. J Lipid Res.50 (12): 2337-9. With permission from ASBMB (American Society for Biochemistry and Molecular Biology).

There are two types of vitamin A sources in food; 1) retinyl esters (REs) from animal sources and 2) carotenoids from vegetables. Both REs and carotenoids are in several steps transformed to either retinol or REs which are transported via the intestines, lymph and circulation to the liver where 50-80% is stored in the stellate cells, if concentrations in circulation are enough (van Berkel, 2009). Two proteins are mainly responsible for storage: lecithin:retinol acyl transferase (LRAT) and cellular retinol binding protein-I (CRBP-I). Mice lacking either of them have impaired storage of retinyl esters, possibly leading to impaired retinoid homeostasis. When plasma concentrations of retinol, which are strictly regulated and maintained at about 2 μM, are lowered, stored RE will be released as retinol to the blood. In the blood retinol binds to retinol binding protein (RBP) in a complex with transthyrethin (TTR) to prevent glomerular filtration of the small retinol-RBP molecule (Blomhoff and Blomhoff, 2006; van Berkel, 2009).

2.4.2 Retinoid metabolism

The retinoic acid molecules, which are the active forms of retinoids required for signaling, are gained via conversion of retinol in target cells (Figure 4), and the major source is retinol from plasma. The synthesis of the major active cellular retinoid compound, all-*trans* retinoic acid (all-*trans* RA), occurs from retinol in a two-step reaction. First, retinol is oxidized to retinal, by Cytosolic Medium-Chain Alcohol Dehydrogenases (ADHs). Retinal can then be oxidized to all-*trans*RA, normally by Retinal dehydrogenases (RALDH1 -4), which is tissue and cell type specific and essential in the regulation of RA signaling, but also COX1, CYP1B1 and CYP1A1 appears to catalyze the oxidation of retinal to all-*trans* RA. Furthermore, the catabolism of all-*trans* RA via enzymes of the cytochrome P450 (CYP) family, mainly CYP26, is an important mechanism for controlling retinoic acid levels in cells and tissues (Blomhoff and Blomhoff, 2006). Finally, UGTs are involved in further degradation and excretion of the formed all-*trans* RA metabolites.

2.4.3 Retinoid signaling

Hundreds of genes are thought to be regulated by all-*trans* RA directly or indirectly via transcriptional activation of retinoic acid receptors (RARs) and retinoid X receptors (RXRs) in the nucleus (Figure 4), belong to the nuclear hormone receptor superfamily and are ligand dependent transcription factors (Amann et al., 2011). Among others, this superfamily also includes steroid and thyroid hormone and vitamin D receptors (Chambon 1996).

Both the RAR and the RXR family consist of three isotypes (α, β, γ) (Bastien and Rochette-Egly 2004). In vitro studies have shown that all-*trans* RA and 9-cis retinoic acid (9-cis-RA) are high affinity ligands of RAR whereas only 9-cis-RA binds with high affinity to RXR. It is generally accepted that all-*trans* RA is the physiological ligand of RAR, while it is still controversial whether 9-cis-RA is the physiological ligand of RXR since it has so far not been reliably detected in vivo. An explanation for this could be rapid degradation of the molecule after production for signaling (Blomhoff and Blomhoff, 2006). Recently, another possible endogenous RAR ligand has emerged, 9-cis-4-oxo-13,14-dihydro retinoic acid (9 cis-4-oxo-

13,14-dh-RA). This compound was shown to induce transcription of several all-*trans* RA related genes, however with less potency than all-*trans* RA itself. Experimental results also indicate that it binds to RARs in vitro. In wild type mice and rats, 9 cis-4-oxo-13,14-dh-RA was even found to occur in higher concentrations than all-*trans* RA (Schuchardt et al., 2009).

In order for RAR to act as a transcription factor it has to dimerize with RXR, the RAR:RXR heterodimer then binds to RAREs in the DNA.

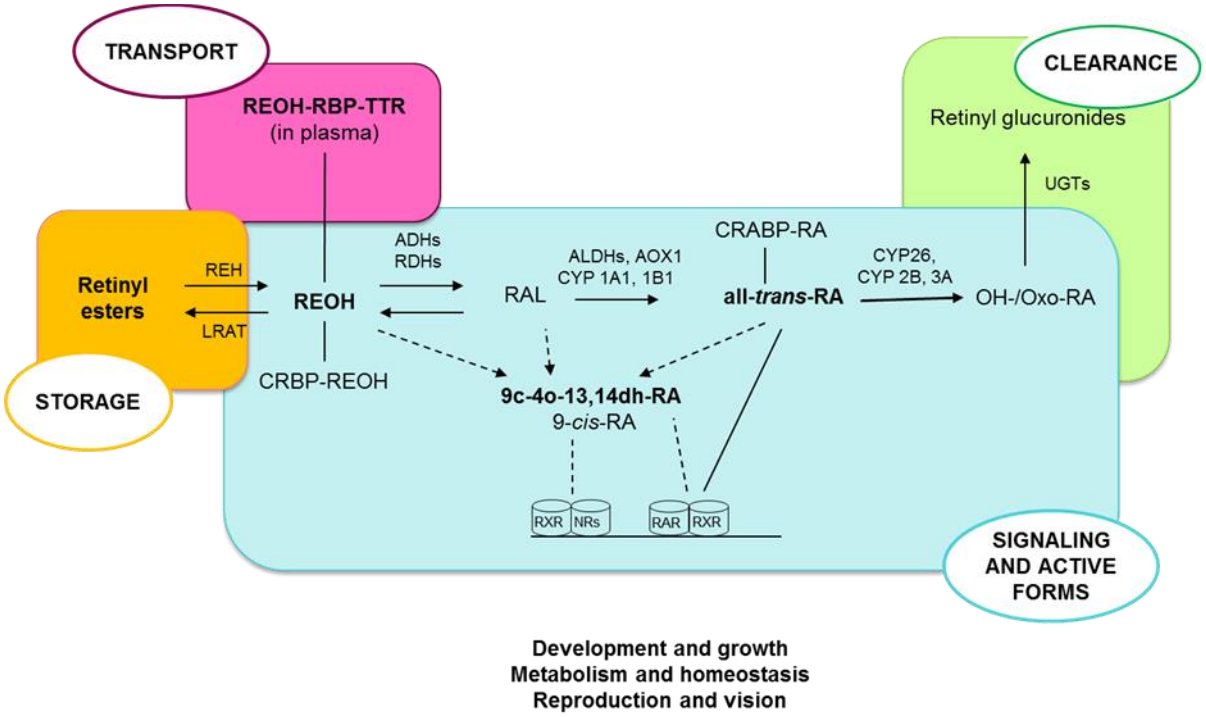

Figure 4. Retinoid metabolism. ADH: Cytosolic medium-chain alcohol dehydrogenase; CRABP: Cellular retinoic acid binding protein; CRBP: Cellular retinol binding protein; CYP: Cytochrome P450 superfamily; LRAT: Lecithin:retinol acyltransferase; NRs: Nuclear receptors (e.g. PPAR: peroxisome proliferator-activated receptor, or THR: Thyroid hormone receptor); RA: Retinoic acid; RAL: Retinal; ALDH: Retinal dehydrogenase; RAR: Retinoic acid receptor; RBP-TTR: Retinol binding protein-transthyretin-complex; RDH: Retinol dehydrogenase; REH: Retinyl ester hydrolase; REOH: Retinol; RXR: Retinoid X receptor; UGT: UDP-glucuronosyltransferase; Biotransformation; Binding equilibrium. Dashed lines indicate uncertainties in current knowledge.

2.4.4 Retinoid function

It is well known that retinoids are essential for many vital processes e.g. embryonic development of the heart and brain and for many aspects of adult life, including normal growth and metabolism, growth and differentiation of many cell types, hematopoiesis, vision, reproduction and bone formation (Brtko and Dvorak, 2011; Novák et al., 2008; Theodosiou et al., 2010; Simandi et al., 2015). Beyond the well-known consequences of vitamin A deficiency (Nilsson and Håkansson, 2002) or excess (Jacobs et al 2011) accumulating data also point to severe outcomes in genetic disorders of the retinoid system in many aspects of life;

- Neurodevelopment and nervous system disorders (hindbrain development, learning and memory, neurobehavioral development, Alzheimer´s) (Wendling and Ghyselinck et al. 2001; Hou et al., 2014; Elsea and Williams, 2011; Sodhi and Singh, 2014; Sucov and Evans, 1995)
- Congenital heart disease (Unolt et al., 2013)
- Congenital diphragmatic hernias (Mey et al., 2013)
- Cranial developmental defects (Jacobs et al., 2011)
- Eye defects (Mark et al., 2009)
- Fertility, sex differentiation and determination (Clagett-Dame and Knutson 2011)
- Immune system alterations (Ross, 2012)
- Cancer (Shiota and Kanki, 2013)

2.4.5 Chemicals affecting the retinoid system

Several studies have shown that the retinoid system responds to different chemicals (Novák et al., 2008), at very low doses as compared to several other biochemical and adverse effects (Nilsson and Håkansson 2002). TCDD is the most well studied environmental contaminant in relation to disturbances on the retinoid system. Moreover, it has been well established that dioxins induce health effects that correlate to altered tissue levels of signaling retinoid forms. For BFRs the retinoid system has been less well studied with only a few studies for the PBDEs and one for HBCD, which demonstrated effects on the retinoid storage system (Öberg et al., 2010; Fernie et al., 2005; Ellis-Hutchings et al., 2006, 2009; van der Ven et al., 2006), at low doses compared to several other biochemical and adverse effects, however, dioxinlike impurities might have played a role in the studies on the pentaBDE technical mixtures. To date, no study has investigated altered retinoid homeostasis at the level of retinoic acid or its retinoic acid metabolite 9 cis-4-oxo-13,14-dh-RA following exposure to purified technical mixtures of pentaBDE, decaBDE and HBCD or TBBPA in liver or kidneys and further the associations of those to other sensitive endpoints. Moreover mode of actions behind the retinoid disturbances after exposure to BFRs by looking at gene expressions of retinoid related genes needs clarification. Gender differences and the relevance of using altered hepatic retinoid levels as critical effects have not been studied either.

3 PRESENT STUDY

3.1 HYPOTHESIS AND AIM

Hypothesis: Disruption of retinoid system homeostasis contributes to the toxicity of brominated flame retardants.

The overall aim of this thesis was to identify compound- and gender specific disturbances in the retinoid system.

To meet the overall aim of the thesis project, a series of test guidelines studies were analyzed with *the specific aims*:

- To identify retinoid forms which are sensitive to BFR exposure
- To identify genes which can explain observed changes in retinoid homeostasis
- To evaluate the relevance of retinoid system endpoints for human risk characterization

3.2 RELEVANCE

This project is important for the development of methods of use for regulatory agencies in Sweden and internationally when assessing human health risks due to chemical exposure. The articles published in this thesis were used in the risk assessment by EFSA (The European Food Safety Authority) of PBDEs, HBCD and TBBPA in food (EFSA, 2011 a,b,c) and ECHA (The European Chemicals Agency) has considered the published HBCD paper in their proposed identification of HBCD as a so called substance of very high concern. Furthermore, using Benchmark dose modelling (BMD) in study design and data evaluation in combination with Partial Least Square analyses (PLS), additional new knowledge has been developed in this thesis project and will be published to reach a broader audience. In addition to quantitative data this thesis also provides new mechanistic information about BFR interferences with retinoid system related gene expression, which have been used in the started discussions within OECD whether to implement retinoid testing for suspected EDCs.

3.3 METHODOLOGICAL CONSIDERATIONS

3.3.1 Animals and Study design

All studies were performed in Wistar rats in accordance with OECD test guidelines for toxicity studies enhanced to detect endocrine effects (Andrews et al., 2001) and to accommodate benchmark-dose analysis (Kavlock et al., 1996; Slob et al., 2002). Paper I, II, IV, V, VI were performed according to OECD TG407 and paper III and IV according to OECD TG415 (Figure 3). Study designs are summarized in table 1 and 2.

Table 1. Study design comparison of the 28 day studies (OECD TG 407)

	DecaBDE (paper I, II)	**HBCD** (van der Ven et al., 2006, paper V)	**PentaBDE** (paper VI)	**TBBPA** (paper IV, V)
Age at necropsy	14 w	14 w	11 w	11 w
Dosing procedure	Gavage	Gavage	Gavage	Mixed in food
Vehicle	Enhanced emulsion for optimal uptake	Corn oil	Corn oil	-
Dose range (mg/kg bw/day)	0, 1.87, 3.75, 7.5, 15, 30[c]	0, 0.3, 1, 3, 10, 30, 100, 200	0, 0.27, 0.82, 2.47, 7.4, 22.2, 66.7, 200	0, 0, 30, 100, 300
Animals/ dose / gender	5	5	5	10
Hepatic apolar[a] retinoid analyses	X	X	X	Paper V
Hepatic polar[b] retinoids analyses	Paper II	Paper V	-	Paper V[d]
Bone measurements	Paper I	Van der Ven et al., 2006	X	Paper IV

[a] retinyl palmitate and retinol; [b] all-*trans*-retinoic acid and 9-cis-4-oxo-13,14-dihydroretinoic acid; [c] 30 mg/kg bw was maximum soluble concentration. 60 mg/kg bw was also used however split into two doses of 30 mg/kg bw/day. One extra control was added for comparison to the twice dosage maximum dose; [d] only ctrl + high dose in males.

3.3.2 Chemicals

Purified technical mixtures were used for the decaBDE and pentaBDE 28 day studies. Briefly, the pentaBDE and HBCD mixtures were dissolved in corn oil. DecaBDE is less soluble and was mixed with an optimized formula for higher bioavailability. In contrast, TBBPA in both studies (paper IV) as well as the HBCD in the 1 generation study (paper III) were mixed with the food (Table 1, 2).

Dose ranges were selected to cover known toxic doses at the high end, and to include human exposure ranges at the low end as much as possible. Solubility was a limiting factor in the case of HBCD and decaBDE.

Table 2. Study design comparison 1 generation studies (OECD TG 415)

	HBCD (paper III)	**TBBPA** (paper IV)
Age at necropsy	11 w	14 w
Dosing procedure	Mixed in food	Mixed in food
Vehicle	-	-
Dose range (mg/kg bw/day)	0, 0.1, 0.3, 1, 3, 10, 30, 100	0, 3, 10, 30, 100, 300, 1000, 3000
Animals/ dose / gender	5	5
Hepatic apolar[a] retinoid analyses	X	[c]
Hepatic polar[b] retinoids analyses	[c]	[c]
Bone measurements	X	X

[a] retinyl palmitate and retinol; [b] all-*trans*-retinoic acid and 9-cis-4-oxo-13,14-dihydroretinoic acid; [c] In progress, not included in the papers.

3.3.3 Retinoid analyses

Both polar (all-*trans*-retinoic acid and 9-cis-4-oxo-13,14-dihydro retinoic acid) and apolar (retinol and retinyl palmitate) retinoid levels were analyzed with high performance liquid chromatography (HPLC) in liver (female and male rats) (paper I-III, V-VI) and kidney samples (paper II, V) (only males in paper V). These tissues were chosen since they represent an important part of overall retinoid metabolism and regulation.

3.3.4 Gene expression analyses

In papers II and V, a total number of 25 genes were selected for hepatic mRNA expression analysis with RT-PCR, in order to provide further mechanistic and functional information about the impact of BFR exposure on key aspects of the retinoid system. The genes were selected to cover the complexity of the retinoid system in terms of retinoid specific receptors (Rars and Rxrs) (six), binding proteins (two), or enzymes (ten), or indirectly as Ahr-related genes (three), or receptors forming dimers with RXRs (four), which are present in virtually all cells from the earliest stages of development. The following genes were examined; *Adh1, Ahr, Ahrr, Aox1, Arnt, Car, Crabp-1, Crbp-1, Cyp26a1, Lrat, Pxr, Aldh1a1,Rar α, Rar β, Rar γ, Rxr α, Rxr β, Rxr γ, Thr α, Thr β, Ugt1a1, Ugt1a6, Ugt1a 9.*

Different housekeeping genes (HKG) were tested for each study to avoid artefacts due to possible influences on the HKGs by exposures. B-actin was used as a HKG in the decaBDE study (paper II) since it was not affected by dose, GAPDH was also tested with similar results. For HBCD (paper V) the geometrical mean of the following HKGs; B-actin, GAPDH and Eef1a1 were used to affirm that no single HKG affected the results. For TBBPA (paper V), GAPDH was used as HKG. The ratios were calculated using the ΔΔCT method for decaBDE (paper II), whereas for HBCD and TBBPA, the standard curve method was used (paper V). Negative controls were included in each run for both studies.

3.3.5 Bone measurements

In papers I, III, IV and VI peripheral quantitative computed tomography (pQCT) was used to examine the geometrical and densitometrical properties of femur and tibia. The pQCT is an X-ray based technique that distinguishes between cortical and trabecular bone tissues and the bone mineral content and density together with geometry of different bone compartments can be calculated. In addition, total length of the bones was measured using an electronic sliding caliper.

3.3.6 Benchmark dose modeling

Dose-response relationships were established with benchmark modeling for tissue retinoids, gene expressions and for bone parameters (all papers), except for gene expressions in paper II.

Critical effects sizes (CES) for the published papers (I, III, V and VI) were set at 10% as a default, whereas for the papers II and V, a CES of 5% was used for retinoid measurements, based on the recommendation by EFSA (EFSA, 2009). For the gene expression analyses we decided to use an overall CES of 20 % after testing the relevance of using either 10%, 20%, 50% or 100%. The use of CES 20% was also based on literature studies, where higher CES% are often used for mRNAs, combined with the fact that nuclear receptors and AhR were included, which are normally not affected to the same extent as for example enzymes.

3.3.7 Partial Least Square analyses

To evaluate if the hepatic retinoid system modulations were statistically associated with other observed effects i.e. established data from paper I and van der Ven et al., 2006 for endocrine parameters, clinical chemistry, hepatic drug metabolism together with organ-, and body weights (paper II and V) by using a multivariate partial least squares regression (PLS-2), as previously described (Elabbas et al., 2014), using the "enter" method with R software (R Core Team, 2014). Furthermore, PLS was used in the mode of action analyses to explain which genes were altering the hepatic tissue levels.

3.3.8 Relevance to humans – Margin of exposure

Margin of exposure methodology was used to evaluate if the observed effects on the retinoid system could be comparable to other endpoints and of relevance for human risk characterization. This was performed by the use of observed tissue levels of the different BFRs in humans from open literature. Margins of exposure (MOEs) were calculated as internal liver lipid doses of the BFRs for hepatic retinoids and other sensitive effects. As rat and human hepatic BFR concentrations were used for MOE calculations, there was no need for an interspecies toxicokinetic extrapolation factor (EFSA, 2011a,b). However, a toxicodynamic factor of 2.5 was applied to cover interspecies variability and a factor of 10 to cover the inter-individual variability. Thus, in these studies an uncertainty factor (UF) of 25 was considered adequate to extrapolate from animal to human data for the analysed endpoints.

We wanted to cover a wide range of the populations and scenarios included were; general population, occupational exposure and populations living in production areas. Criteria were also to find well conducted studies with enough individuals. Internal critical effect doses (lower bound) CEDLs in the animals were re-calculated to serum levels by a distribution factor (Huwe et al., 2007) in the decaBDE study to compare with exposure levels found in the humans. For HBCD, a distribution factor was missing in literature thus only one human scenario of relevance was found with exposure levels in liver of healthy aborted fetuses.

3.3.9 Ethical approvals

All animal experiments were conducted according to OECD TG407 and OECD TG415, respectively, at RIVM as part of the EU- project FIRE (QLRT-2001-00596), after approval by the institutional Committee on Animal Experimentation according to Dutch legislation under ISO 9001-2000 quality standards (Ethical permits: decapapers I, II: 2004276; HBCD paper III: 2004566; HBCD paper V: 2003082; TBBPA paper IV, V: 2003083, 2002630; pentaBDE paper VI: 2004265).

3.4 RESULTS AND DISCUSSION

Results from this thesis, which was performed to provide information about the toxicity of selected BFR, with focus on the retinoid system, are summarized in Table 3-8 and Figure 5-6. Both hepatic retinoids and associated gene expressions were altered in a compound- and gender specific manner, which could be explained by differences in *Adh1, Aldh1, Aox1, Crabp1, Cyp26a1, Lrat, Ugt1a1, Ugt1a6, Ugt1a9* and CYP enzyme inductions associated with AhR, CAR or PXR mediated mode of actions.

3.4.1 Hepatic retinoid forms

3.4.1.1 28 day studies

Hepatic all-trans RA (paper II and V)

By regulating hundreds of genes via the RAR-RXR receptor mediated pathway, all-*trans* RA has a pivotal role during development but also for many aspects of adult life, and it is normally kept under steady state (Kedishvili 2013). Thus, the observed dynamic type of strong reductions up to 50% from lowest dose levels in male rats after decaBDE exposure were remarkable findings (Table 3). Female rats had a similar dynamic type of response but less pronounced (Table 3). In line with the decaBDE treatment also HBCD treatment resulted in all-*trans* RA level reductions in male rats, although from higher doses and less prominent. All-*trans* RA reductions were also observed in the HBCD exposed females, however only with internal doses with a maximum reduction of 54% (Table 3). A few additional studies have measured all-*trans* RA levels after exposure to organic pollutants in rats. Consistent with our results on HBCD and decaBDE, one sub-chronic toxicity study with TCDD, resulted in reductions of all-*trans* RA concentrations in female Long-Evans rats (Table 3). On the contrary, increased all-*trans* RA has been observed in a short term study on female rats with TCDD (Schmidt et al., 2003) and a 28 day study in adult rats with PCB180, although from considerably higher dose levels and in male rats alone (Table 3).

In summary, all-*trans* RA levels were reduced by decaBDE and HBCD exposure, in line with a sub-chronic TCDD study. Whereas, higher dose-levels of PCB180 and short term TCDD exposure resulted in *increased* levels. Thus it appears that all-*trans* RA levels are altered in a compound specific manner by AhR, CAR and PXR mediated mechanisms which also dependent on the duration of the exposure.

Hepatic 9-cis-4-oxo-13,14-dh RA (Paper II and V)

9-4o-13,14-dh-RA is an all-*trans* RA metabolite which needs to be further elucidated since formation, biotransformation, and functional role has only briefly been evaluated both for biochemical and toxicological relevance (Elabbas et al., 2014; Esteban et al., 2014; Fletcher et al., 2005; Hoegberg et al., 2005; Schuchardt et al., 2009; Viluksela et al., 2014). In the HBCD adult animals (paper V), we observed a strong reduction of 9c-4o-13,14-dh-RA hepatic concentration from low doses in both genders, most pronounced in female rats (Table 3), while

no effect was observed in the decaBDE treated rats (paperII). As for the HBCD rats, reductions have also been observed in sub-chronic (Table 3) and short term studies with TCDD (Schmidt et al., 2003) as well as in a PCB180 study in female rats (Table 3). Thus, it appears that 9-4o-13,14-dh-RA, in line with all-*trans* RA can be reduced by chemicals with different mode of actions (Ahr, CAR and/or PXR).

Table 3. Hepatic retinoid results 28 day studies

	Male rats		Female rats	
	CED mg/kg bw/day	Max. response %	CED mg/kg bw/day	Max. response %
DecaBDE				
all-*trans* RA	0.02	-50	0.31	-40
9-4o-13,14-dh-RA	NE		NE	
Retinyl palmitate	NE		↑	23
Retinol	(↓)[c]	[c]	NE	
HBCD				
all-*trans* RA	1.7	-30	[d]	-54[d]
9-4o-13,14-dh-RA	17	-46	3.3	-60
Retinyl palmitate	9.5[e]	-19[e]	1.4	-34
Retinol	NE		NE	
TBBPA				
all-*trans* RA	NE			
9-4o-13,14-dh-RA	NE			
Retinyl palmitate	(↑)[f]		NE	
Retinol	(↑)[f]		NE	
PentaBDE				
all-*trans* RA	[g]		[g]	
9-4o-13,14-dh-RA	[g]		[g]	
Retinyl palmitate	2.7	-59	2.0	-50
Retinol	22	-38	19	-43
PCB 180[a]				
all-*trans* RA	450	20	NE	
9-4o-13,14-dh-RA	NE		12.2	-78
Retinyl palmitate	22	-63	122	-51
Retinol	32	-47	156	-43
TCDD[b]				
all-*trans* RA			1[h]	↓
9-4o-13,14-dh-RA			1[i]	↓
Retinyl palmitate			1/10[j]	↓
Retinol			1[i]	↓

CED: Critical effect dose at Critical effect size 5% (EFSA); NE: No effect.
[a] A similar study OECD 407 but with Sprague-Dawley rats (Viluksela et al. 2014); [b] TCDD study adult rats Han Wistar and Long Evans dosing 1/week for 20 w (Fletcher et al., 2005); [c] Only low dose effect at 1.87 mg/kg bw/day; [d] significant decrease with internal dose; [e] conflicting results with ANOVA [f] mid dose effects alone; [g] in progress; [h] Long-Evans rats NOEL: ng/kg bw/day; [i] Long-Evans and Han-Wistar rats LOEL ng/kg bw/day; [j] Long Evans/ Han Wistar.
rats; LOEL ng/kg bw/day

Hepatic retinyl palmitate (Paper I-II, V-VI and van der Ven et al., 2006)

Retinyl palmitate is the storage form of retinoids. In paper I, III and VI a method was used to analyse dietary retinoid forms, which mainly constitutes retinyl palmitate but also retinyl stearate and retinol. In the pentaBDE study, hepatic retinyl palmitate concentrations were

reduced to around 50% of the controls in both genders from low doses in comparison to other toxicological observations (Table 3, Table 8). Similarly to the pentaBDE study, retinyl palmitate was reduced in HBCD exposed animals, however much more pronounced in female rats in terms of CEDs and maximum response (Table 3). In contrast with the pentaBDE and HBCD results, treatment with decaBDE or TBBPA resulted in slightly increased retinyl palmitate levels in female and male rats, respectively. Hepatic retinyl palmiate levels are more well studied than all-*trans* RA and 9-4o-13,14-dh-RA and from our results we can conclude that retinyl palmitate reductions after pentaBDE and HBCD exposure are in line with decreased hepatic retinyl palmitate levels observed with chemicals such as TCDD (Fletcher et al., 2005 (Table 3); Håkansson et al., 1987; Kransler et al., 2007; Nilsson et al., 2000; Viluksela et al., 2000; Schmidt et al., 2003), PCB 180 (Viluksela et al., 2014) (Table 3), pentaBDE (Öberg et al., 2010; Ellis-Hutchings et al., 2006; Fernie et al., 2005) as well as PCBs 77, 118 and 126 (Chu et al., 1995; 1994). The tendency of increased retinyl palmitate following decaBDE and TBBPA have not been observed previously.

In conclusion, retinyl palmitate levels were reduced from low CEDs in high magnitudes for both pentaBDE and HBCD studies, whereas in the TBBPA and decaBDE studies the tendency of transcient increase were observed in the males of TBBPA and females of decaBDE. Reduced levels observed in the pentaBDE and HBCD studies were is consistent with exposure studies with other pentaBDE studies as well as for other substances such as TCDD and both non-dioxinlike and dioxinlike PCBs, which shows that retinyl palmitate levels are decreased by chemicals both with typical AhR- and/or CAR or PXR mediated mechanisms.

Table 4. Hepatic retinoid concentrations in control animals.

	Males				Females			
	pentaBDE	decaBDE	HBCD	TBBPA	pentaBDE	decaBDE	HBCD	TBBPA
Age at start (weeks)	8	11	11	8	8	11	11	8
Exposure	Gavage	Gavage	Gavage	Diet	Gavage	Gavage	Gavage	Diet
Vehicle	Corn oil	Special[a]	Corn oil	-	Corn oil	Special[a]	Corn oil	-
At-RA (pmol/g)	32 ± 4	35 ± 4	26 ± 4	**15 ± 2**	**15 ± 3**	**68 ± 32**	26 ± 6	Nt
CORA (pmol/g)	34 ± 8	**75 ± 15**	44 ± 12	**12 ± 4**	93 ± 19	**246 ± 44**	116 ± 22	Nt
Retinol (nmol/g)	14 ± 2	13 ± 1	19 ± 4	**7.5 ± 1.3**	22 ± 6	18 ± 3	19 ± 3	**12 ± 2**
RePa (umol/g)	1.0 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	**0.6 ± 0.07**	1.7 ± 0.1	1.5 ± 0.2	1.7 ± 0.3	1.4 ± 0.1

At-RA: All-*trans* retinoic acid; CORA: 9c-4o-13,14-dh-RA; RePa: Retinyl Palmitate; Nt: Not tested. **Bold** values if comparably high or low; [a] Emulsion for enhanced gastric uptake (Mörck et al., 2003).

Hepatic retinol (Paper I-II, V-VI and van der Ven et al., 2006)

Hepatic retinol levels decreased by pentaBDE exposure from mid doses and to a high degree (38-43%) in male and female rats (Table 3). Similarly, retinol levels have also been decreased in other pentaBDE studies (Öberg et al., 2010; Ellis-Hutchings et al., 2006), as well as in TCDD

studies (Schmidt et al., 2003; Fletcher et al., 2005; Viluksela et al., 2000). DecaBDE, HBCD and TBBPA had no effect on the hepatic retinol levels. Thus, it appears that hepatic retinol reductions might be linked to AhR mediated mechanisms.

Levels of hepatic retinoids in control animals

Variation in retinoid concentrations in the control animals are observed for the different studies and can be explained by differences in age, exposure and/or vehicle (Table 4). In general, females had 3-4 times higher levels of 9c-4o-13,14-dh-RA in comparison to male rats, while TBBPA animals had around 20-50% of the four hepatic retinoid levels in comparison to the other compounds. Moreover, retinol and retinyl palmitate levels appeared to be most comparable between the studies.

3.4.1.2 1-generation studies with HBCD (paper III)

Table 5 summarizes hitherto published data on hepatic retinoid levels following in utero/ lactational exposure to HBCD, Aroclor and NCM. These data show that HBCD exposure similar to Aroclor and NCM reduced hepatic retinyl palmitate levels at 100 and 10 fold higher dose levels as compared to NCM in male and female rats respectively. In the NCM study, retinyl palmitate levels were reduced at similar CEDs for both genders, whereas for the HBCD study (paper V) female rats had 10 fold lower CED values for retinyl palmitate reductions, compared to male rats.

Table 5. Hepatic retinoid results 1 generation studies in rats

	Male off-springs		Female off-springs	
	CED mg/kg bw/day	Max. response %	CED mg/kg bw/day	Max. response %
HBCD				
all-*trans* RA	NE[a]		NE[b]	
9-4o-13,14-dh-RA	NE[a]		↓[b]	
Retinyl palmitate	41[e]	-21	5.1[e]	-33
Retinol				
Aroclor[c]				
all-*trans* RA	↑	+43	↑	+60
9-4o-13,14-dh-RA	↓	-70	↓	-93
Retinyl palmitate	↓	-53	↓	-67
Retinol	↓	-50	↓	-54
NCM[d]				
all-*trans* RA	1.5	+18		
9-4o-13,14-dh-RA	0.32	-55	0.08	-96
Retinyl palmitate	0.52	-39	0.40	-48
Retinol	0.38	-49	0.47	-42

CED: Critical effect dose at Critical effect size 5% (EFSA); NE: No effect; NCM: Northern Contaminant Mixture of 27 contaminants including polychlorinated biphenyls (PCB), organochlorine (OC) pesticides, and methylmercury (MeHg).

[a] Control and high-dose [b] Control, mid-dose and high-dose ; [c] No CEDs available, Post natal day 35 (Esteban et al., 2014); [d] PND 35, NCM present in maternal blood of the Canadian Arctic Inuit population (Elabbas al 2014); total levels; [e] total levels of retinylpalmitate, stearate and retinol.

Consistent with the gender difference observed in the 1-generation study, females were also the more sensitive gender in the adult study (paper V and van der Ven et al., 2006). In conclusion, decreased hepatic retinyl palmitate levels were more pronounced in the female rats in line with the results from the adult study, which indicate the need of more studies to fully characterize developmental exposure for HBCD.

3.4.2 Renal retinoid forms

In this study renal retinoids were measured in decaBDE treated animals (paper II) and HBCD treated male rats (paper V). In contrast to TCDD exposure, which resulted in major increases of renal retinoids in rats (Fletcher et al., 2005; Nilsson et al., 2000; Håkansson et al., 1987; Kransler et al., 2007), renal retinoids were not affected after HBCD and decaBDE exposure. In line with the TCDD studies also exposure to other substances have resulted in increased levels of renal retinoids e.g. PCB 180 (Viluksela et al., 2014), PCB 77 or 118 (Chu et al., 1995), PCB126 (Chu et al., 1994). Increased renal retinol and retinyl palmitate levels are also observed in vitamin A deficient rats and thus it has been discussed as a retinoid saving role of the kidney in rats (Morita and Nakano, 1982; Werner and DeLuca, 2001), while other rodents seems to lack this response in kidneys at least following TCDD exposure (Håkansson et al., 1991). In summary, it appears that exposure to chemicals associated with AhR mediated toxicity, such as TCDD, PCB 126, 77 and 118 results in increased levels of renal retinoids in rats, whereas exposure to HBCD and decaBDE with predominantly CAR- and or PXR mediated mechanisms do not alter renal retinoids in rats.

3.4.3 Mode of action for retinoid system disturbances

3.4.3.1 Hepatic retinoid alterations explained by gene expressions in adult female and male rats exposed to decaBDE (paper II)

Results from paper II, which was performed to provide more detailed information about the effect of decaBDE exposure on the retinoid system, are summarized in Figure 5.

In this study, marked reduction of hepatic all-*trans* RA levels was the most sensitive physiological endpoint, more prominent in male as compared to female rats; a finding, which is in line with the more pronounced toxicity profile of decaBDE in male rats. Together with data from literature, several lines of evidence suggest that decaBDE itself or its metabolites reduces all-*trans* RA levels via CAR and perhaps PXR mediated inductions of CYP2B and 3A (paper I and II) and *Cyp26a1* and *Crabp*1 inductions for the low doses (paper II) (Figure 5). In response to the increased degradation of all-*trans* RA, *Cyp1b1* was increased (paper II) probably as an attempt to maintain "normal" all-*trans* RA homeostasis. Failure to reach control levels of all-*trans* RA in decaBDE exposed rats despite *Cyp1b1* induction could be due to 1) no need, 2) dynamic response.

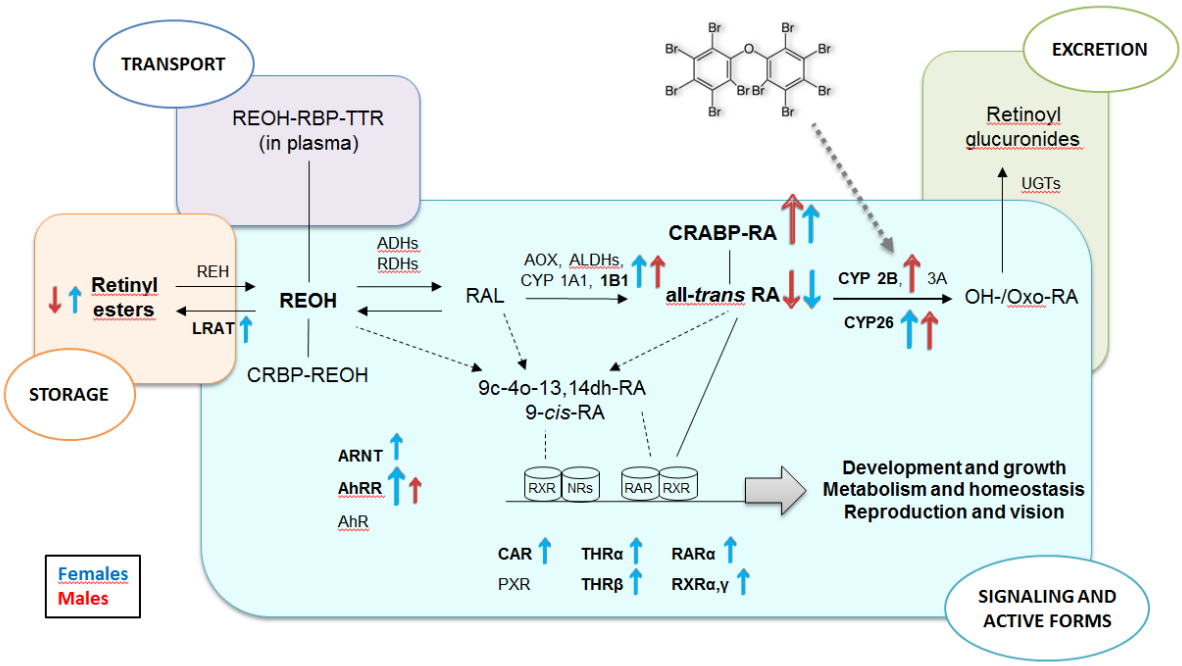

Figure 5. Mode of action for decaBDE retinoid system modulations. ADH: Cytosolic medium-chain alcohol dehydrogenase; AhRR: Aryl hydrocarbon receptor repressor; ARNT: Aryl hydrocarbon receptor nuclear translocator; CAR: Constitutive androstane receptor; CRABP: Cellular retinoic acid binding protein; CRBP: Cellular retinol binding protein; CYP: Cytochrome P450 superfamily; LRAT: Lecithin:retinol acyltransferase; NRs: Nuclear receptors (e.g. PPAR: peroxisome proliferator-activated receptor, or THR: Thyroid hormone receptor); RA: Retinoic acid; RAL: Retinal; RALDH: Retinal dehydrogenase; RAR: Retinoic acid receptor; RBP-TTR: Retinol binding protein-transthyretin-complex; RDH: Retinol dehydrogenase; REH: Retinyl ester hydrolase; REOH: Retinol; RXR: Retinoid X receptor; UGT: UDP-glucuronosyltransferase; Biotransformation; Binding equilibrium. Dashed lines indicate uncertainties in current knowledge.

We speculate that 50% reduction of the control levels of all-*trans* RA was due to a dynamic response down to a minimum level for necessary functions, where the other 50 % is replaced by accumulating all-*trans* RA-metabolites which can contribute to RAR/RXR signaling. The gender differences are partly explained by the observed differences in biotransformation of the decaBDE compound (paper I), resulting in a gender specific profile of decaBDE metabolites/ degradation products which seems to activate CAR and perhaps also PXR, AhR and AhRR mediated mode of action. However, further research is needed to clarify the exact role of hepatic AhR, AhRR, CAR, and PXR, in the decaBDE-induced modulation of hepatic all-*trans* RA levels and/or turnover.

3.4.3.2 HBCD (paper V)

Results from paper V, which was performed to provide more detailed information about the effect of HBCD exposure on the retinoid system, are summarized in Figure 6.

Reduction of hepatic 9-cis-4-oxo-13,14-dh-RA levels was the most pronounced physiological effect following HBCD-exposure, while reduction of hepatic retinyl palmitate and all-*trans* RA levels were the most sensitive physiological effects in terms of CEDs, in female and male rats, respectively. Candidate genes behind the modulated retinoid levels include *Adh1*, *Aldh1*, *Aox1*, *Cyp26a1*, *Lrat*, *Ugt1a1*, *Ugt1a6*, and *Ugt1a9* (Table 6, 7 and Figure 6). *Aldh1a1* and *Ugt1a1* appear to be of higher importance, since both were markedly up-regulated after HBCD treatment and more so in female rats (Table 7). In addition, several lines of evidence provide support for key roles of CYP2B and CYP3A in the observed retinoid system modulations following HBCD-exposure in both male and female rats. Where CYP3A where more highly induced in female rats and CYP2B expression and PROD in male rats, while CYP2B protein was more highly induced in female rats, advocating a CAR mediated mode of action in both genders and a more prominent PXR mediated mode of action in the female rats (Table 7).

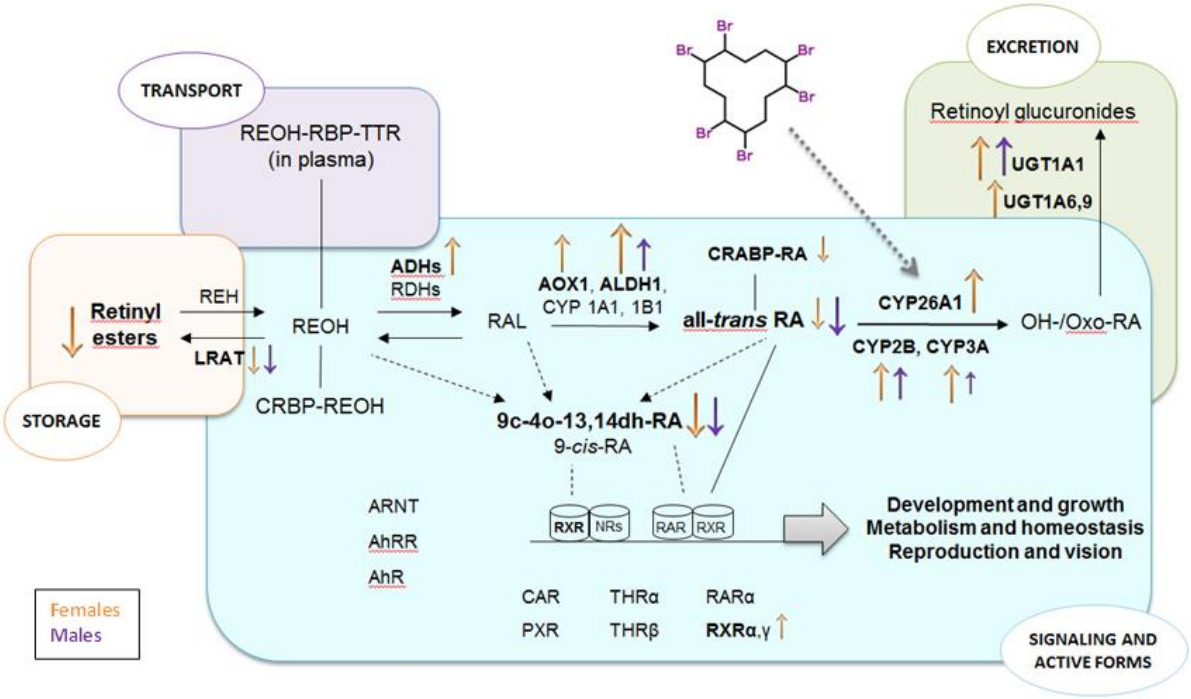

Figure 6. Mode of action for HBCD retinoid system modulations. ADH: Cytosolic medium-chain alcohol dehydrogenase; AhRR: Aryl hydrocarbon receptor repressor; ARNT: Aryl hydrocarbon receptor nuclear translocator; CAR: Constitutive androstane receptor; CRABP: Cellular retinoic acid binding protein; CRBP: Cellular retinol binding protein; CYP: Cytochrome P450 superfamily; LRAT: Lecithin:retinol acyltransferase; NRs: Nuclear receptors (e.g. PPAR: peroxisome proliferator-activated receptor, or THR: Thyroid hormone receptor); RA: Retinoic acid; RAL: Retinal; RALDH: Retinal dehydrogenase; RAR: Retinoic acid receptor; RBP-TTR: Retinol binding protein-transthyretin-complex; RDH: Retinol dehydrogenase; REH: Retinyl ester hydrolase; REOH: Retinol; RXR: Retinoid X receptor; UGT: UDP-glucuronosyltransferase; Biotransformation; Binding equilibrium. Dashed lines indicate uncertainties in current knowledge.

Thus, it is clear that the changes of the hepatic retinoid system were more pronounced in the female rats and occurred from lower dose levels of HBCD in comparison to male rats, these results are consistent with the more pronounced toxicity profile in the female rats. However, further research is needed to clarify the exact role of hepatic CAR and PXR, in the HBCD-induced modulation of hepatic retinoid system.

3.4.3.3 TBBPA 28d (paper V)

Results from this study, which was performed to provide information about the effect of TBBPA exposure on the retinoid system, are summarized in Table 3. The few retinoid changes observed in paper V are limited and to few, thus regarded as preliminary data since analyses of all four retinoid forms and more genes are needed before final conclusions can be drawn. However, the indications of a less or differently affected retinoid system in the TBBPA exposed animals are consistent with the no observed receptor mediated mechanisms (Germer et al., 2006), which supports the role of those receptors for the other substances.

3.4.3.4 PentaBDE (paper VI)

Results from the pentaBDE study, where one aim was to provide information about the effect of pentaBDE on the retinoid system, are summarized in Table 3. Briefly, pronounced reductions of retinol and retinyl palmitate were observed from low doses in both genders (Table 3). In parallel, CYP1A, 2B and 3A mRNA, proteins (paper VI) as well as associated microsomal enzyme assays EROD, PROD and LBD were highly induced in both genders (Table 6, 7 and 8). Thus, it appears that AhR, CAR and PXR mediated mechanisms could be involved in the decreased levels of both retinol and retinyl palmitate following pentaBDE exposure, whereas complementing studies on polar retinoids and associated gene expressions are needed to conclude the mode of action of retinoid system disturbances.

3.4.4 Compound and gender comparison (paper II and V)

3.4.4.1 Compound comparison

Table 3 and 6 summarizes all hepatic retinoid- and gene expression results from the 28 day studies (TG 407) of this thesis. These data show that the retinoid system is altered in a compound specific manner (Table 7). Taken together with literature from the field of receptor mediated toxicity research, it is suggested that AhR, CAR and/or PXR play significant compound and gender specific roles in BFR-induced retinoid system changes.

Retinyl palmitate and retinol (paper I, II, V, VI)

Retinyl palmitate and retinol were measured for all BFRs, where pentaBDE and HBCD were most potent to cause decreases of retinyl palmitate from low doses in both genders of the pentaBDE study and in females of the HBCD study (Table 3, 6). In contrast retinyl palmitate levels tended to increase in both decaBDE female and TBBPA male rats.

Hepatic retinol levels were only affected in the pentaBDE study where it was reduced from low doses in high magnitudes (Table 3, 6).

Table 6. Hepatic retinoids, selected gene expressions and enzyme activities of the 28 day studies[a].

	28 day studies							
	Males					Females		
Effect	PentaBDE	DecaBDE	HBCD	TBBPA		PentaBDE	DecaBDE	HBCD	TBBPA
Hepatic retinoids									
All-trans RA	b	↓↓	↓	c		b	↓[d]	↓[f]	g
9c-4o-13,14-dh-RA	b		↓	c		b		↓↓	g
Retinol	↓					↓			
Retinyl palmitate	↓↓	(↓)[d]		(↑)		↓↓	↑	↓↓	
Hepatic gene expressions and enzyme activity									
Aldh1a1	b		↑			b		↑↑	
Aox1	b					b		↑	
Cyp1b1	b	↑↑[c]		g		b	↑↑[e]	↓	g
Cyp26a1	b	↑[c]				b	↑[e]	↑	↑
Crabp1	b	↑↑[c]		g		b	↑[e]	(↓)	g
Ahr	b					b			
Arnt	b					b	↑		
Ahrr	b	↑↑[c]	e			b	↑↑[e]	e	
Cyp1a	↑↑	(↑)				↑↑			
EROD -activity	↑↑	(↑)				↑↑	(↑)		
Car	b			g		b	↑↑		g
Cyp2b	↑↑↑	↑	↑			↑↑		↑	
PROD-activity	↑↑	(↑)	(↑)			↑↑			
Pxr	b			g		b			g
Cyp3a	↑↑	g	↑			↑↑	g	↑↑	
Lrat	b		↓			b	↑[e]	↓	
Ugt1a1	b		↑			b		↑	
Ugt1a6	b					b		↑	
Ugt1a9	b					b		↑	

Blank: No effect; () trend; ↑/↓ effect, ↑↑/↓↓: strong effect; ↑↑↑: very strong effect ALDH: Retinal dehydrogenase; AhR: Aryl hydrocarbon receptor; AhRR: Aryl hydrocarbon receptor repressor; AOX: Aldehyde oxidase; ARNT: Aryl hydrocarbon receptor nuclear translocator; CAR: Constitutive androstane receptor; CRABP: Cellular retinoic acid binding protein; CYP: Cytochrome P450 superfamily; LRAT: Lecithin:retinol acyltransferase; PXR: Pregnane X receptor; UGT: UDP-glucuronosyltransferase.

[a] van der Ven et al., 2006; paper I, paper II, paper V, paper VI, Germer et al., 2006. [b] in progress; [c] ctrl and high dose alone; [d] not significant with ANOVA but with BMD; [e] low efficiency primer; [f] only with internal doses; [g] Not tested.

All-trans RA and 9c-4o-13,14-dh-RA (paper II and V)

In both decaBDE and HBCD studies we observed sensitive all-*trans* RA reductions, much more pronounced in the decaBDE animals where CEDs in the male rats were 100 fold lower in comparison to the HBCD male rats (Table 3). In addition, the two studies differed in the manner all-*trans* RA levels were reduced; where decaBDE caused a dynamic response with 50% reduction of all-*trans* RA from first dose in males, whereas HBCD exposure in male rats altered all-trans RA levels in a dose-response relationship. For both studies all-*trans* RA reductions were explained by increased levels of CYP2B as well as CYP3A for the HBCD study and possibly for the decaBDE study (Table 3, 6 and 7). Other important genes involved in the all-*trans* RA degradation were *Cyp26a1,* which was induced in females of the HBCD study and in both genders for the decaBDE study (Table 6 and 7) and increased *Crabp1* for decaBDE animals, probably to increase availability of all-*trans* RA for degradation (Table 6, 7).

Table 7. Summary of compound specific mode of actions for modulations of the retinoid system

	PentaBDE		**HBCD**		**DecaBDE**		**TBBPA**	
	M	F	M	F	M	F	M	F
Receptors								
AhR	X	X						
CAR	X	X	X	X	X			
PXR	X	X	(X)	X	[b]	[b]		
Enzymes / proteins								
CYP1A	↑	↑			(↑)			
CYP2B	↑↑	↑	↑	↑	↑			
CYP3A	↑	↑	↑	↑	?	**?**		
All-trans RA degrading genes								
CYP26	[a]	[a]		↑	↑	↑		↑
CRABP1	[a]	[a]		(↓)	↑↑	↑		
UGT1A	[a]	[a]	↑	↑				
All-trans RA synthezising genes								
CYP1B1	[a]	[a]			↑↑	↑↑		
ALDH1A	[a]	[a]	↑	↑				
AOX1	[a]	[a]		↑				
Adh1	[a]	[a]		↑				
Mobilization of retinyl palmitate								
LRAT	[a]	[a]	↓	↓		↑		

Blank: No effect; () trend/ indications via literature; ADH: Cytosolic Medium-Chain Alcohol Dehydrogenase; ALDH: Retinal dehydrogenase; AhR: Aryl hydrocarbon receptor; AOX: Aldehyde oxidase; CAR: Constitutive androstane receptor; CRABP: Cellular retinoic acid binding protein; CYP: Cytochrome P450 superfamily; LRAT: Lecithin: retinol acyltransferase; PXR: Pregnane X receptor; UGT: UDP-glucuronosyl-transferase. [a] In progress; [b] PXR not tested but supported by literature (Pacyniak et al., 2007, Lee et al., 2010).

Reduction of hepatic 9-cis-4-oxo-13,14-dh-RA levels was the most pronounced physiological effect following HBCD-exposure (Table 3), while it was not affected in the decaBDE study.

In summary, several lines of evidence suggest that both decaBDE and HBCD reduces all-trans RA levels via CAR and PXR mediated inductions of CYP2B and 3A (Table 7). Other important genes for all-trans RA degradation were Cyp26a1 inductions for HBCD females and decaBDE animals as well as Crabp1 expression in the decaBDE study. Thus it appears that decaBDE and HBCD have similar mechanisms for the observed reduction of all-trans RA with the exception of Crabp1 expression.

Mobilization and synthesis of all-trans RA

The major difference between HBCD and decaBDE mode of actions for retinoid system modulations appears to be mobilization mechanisms for keeping all-*trans* RA levels in steady-state, where HBCD exposure resulted in the activation of all-*trans* RA synthesizing mechanisms via the following genes: *Aldh1, Adh1 and Aox1* (Table 3, 6). In contrast, decaBDE seemed to only synthesize all-*trans* RA via induction of *Cyp1b1*, since no further mobilization mechanisms were observed.

Thus, it appears that a major difference in HBCD and decaBDE induced modulation of the retinoid system can be linked to degraded all-*trans* RA levels, where it is clear that decaBDE animals despite the major reductions of all-*trans* RA levels does not mobilize other hepatic retinoid forms to restore all-*trans* RA levels up to control levels, whereas HBCD do mobilize retinyl esters to synthesise more all-*trans* RA.

Overall conclusion of the compound comparison is that the retinoid system was altered in a compound specific manner where it appears that a compound specific activation of AhR, CAR, PXR and perhaps AhRR for the decaBDE study plays significant roles.

Gender differences

Apart from the compound specific effects on the retinoid system it can also be concluded that there is a gender-specific effect (Table 3, 6). For decaBDE, male rats were more highly affected where reductions of the all-*trans* RA levels were observed at 10-fold lower CED values than for female rats (Table 3) in line with more pronounced toxicity effect outcomes observed in the male rats (Table 8). In the pentaBDE study females and rats were equally sensitive in decreased levels of retinol and retinyl palmitate (Table 3), while for other toxicity effect outcomes, males tended to be more sensitive (paper VI). In contrast to decaBDE, females were the more sensitive gender after HBCD exposure both in the 28 day study (Table 3, paper I), as well as in the 1-generation study (Table 4). These gender differences can probably be explained by a gender specific activation of AhR-regulated CYP1A1, CAR-regulated CYP2B and PXR-regulated CYP3A activity in male and female rats respectively. Furthermore, kinetic differences may also have contributed to the gender differences since internal concentrations of the individual compounds tended to differ in-between the genders (paper I, VI and van der Ven et al., 2006).

Thus, the observed gender differences in retinoid system disturbances following exposure to HBCD and decaBDE are probably explained by differences in metabolism and subsequently AhR, CAR and PXR mediated mechanisms of action are altered in a gender specific manner (Table 7).

3.4.5 Relevance of retinoid system modulations in comparison to other observed effects

3.4.5.1 DecaBDE 28d (paper I and II)

From the various endpoints studied in paper and II, such as endocrine parameters, clinical chemistry, immunology, haematology, hepatic drug metabolism together with organ-, and body weights and histology, bone and hepatic retinoids, only a few were altered by decaBDE exposure (paper I). The most sensitive endpoint in males was a transient dose-response for increased weight of the seminal vesicle/coagulation gland, and in females a decreased adrenal activity of CYP17 (paper I). Moreover, hepatic CYP1A and CYP2B activities and corresponding mRNA expressions were altered from low CEDs, most pronounced for *Cyp2b* mRNA in male rats (paper I). Thus, it can be concluded that after decaBDE exposure for 28 days in Wistar rats, decreased all *trans*-RA was the most sensitive endpoint in both genders with CEDs of 0.02 and 0.31 mg/kg bw/day in males and females respectively, compared to the range of CEDs of 0.5-417 mg/kg bw/day (paper I) for other sensitive toxicological observations.

3.4.5.2 HBCD (paper V and van der Ven et al., 2006)

HBCD treated animals showed several toxicity effect outcomes, especially in female rats where the following effects were most sensitively affected; increased mineral density of trabecular bone in femur and tibia methaphysis, decreased T4, increased pituitary weight, increased thyroid weight, liver weight induction, reduction of plasma alkaline phosphatase, increased plasma cholesterol (van der Ven et al., 2006). In addition, CYP2B induction was observed in both female and male rats, while CYP3A was induced mainly in females (Germer et al., 2006). Effects on total levels of retinyl esters and retinol and bone parameters from this 28 day study are consistent with the observations in the 1-generation HBCD study, where females also were the more sensitive gender and bone and corresponding retinoids were considered as two of the more sensitive effects (paper III).

Summarizing effects on the retinoid system from 28 day HBCD papers (van der Ven et al., 2006 and paper V), decreased hepatic 9-cis-4-oxo-13,14-dh-RA levels was the most pronounced physiological effect following HBCD-exposure in paper V, while reduction of hepatic retinyl palmitate and all-*trans* RA levels were the most sensitive physiological effects in terms of CEDs, in female and male rats, respectively. For comparison, CEDs of 3.1 and 3.0 mg/kg bw/day, respectively for reductions of 9c-4o-13,14-dh-RA and retinyl palmitate in the females, was low in comparison to CEDs of 3.4-76 mg/kg bw/day for other sensitive toxicological observations in the adult study (Van der Ven et al., 2006).

3.4.5.3 TBBPA (paper IV and V)

Not many effects were observed in rats exposed to TBBPA for 28 days (paper IV). Among the few observations were alterations of plasma T3 and T4 levels in male rats. Increased testis weight and male pituitary (CEDs 1.4 and 2.2 mg/kg bw/day, respectively), were observed in the one-generation study. Alterations of plasma total T3 and T4 levels were also observed at CEDs of 13-100 mg/kg/day in male and female offspring. Other effects in the reproduction study were decreased pup mortality (CED 41-131 mg/kg bw/day) (paper IV). The increased levels of retinyl palmitate observed in male rats (preliminary results) (Table 3), suggest that these may occur from lower doses in comparison to plasma T3 and T4 levels. However, more studies are needed for a full characterization of TBBPA-induced retinoid system changes.

3.4.5.4 PentaBDE 28d (paper VI)

In this thesis, highest number of effects were observed after pentaBDE exposure (paper VI). Decreased levels of plasma T4, induction of hepatic CYP2B1, CYP1A2 and CYP3A1 expressions, proteins and activities, as well as increased liver weight occured at CEDs on the same range (0.2-26 mg/kg/bw/day) as hepatic retinol and retinyl palmitate: 2-22 mg/kg bw/day.

An overall conclusion of this section is that the retinoid system is altered from low doses in comparison to other sensitive effects after exposure to decaBDE, HBCD, pentaBDE and perhaps TBBPA.

3.4.6 Human risk characterization

3.4.6.1 Margin of exposure (MOE)

In this thesis it has been shown that decaBDE, pentaBDE, HBCD and (TBBPA) results in disturbances of hepatic retinoids that were more or equally sensitive effect endpoints for the individual BFR exposures than plasma T3 and T4 levels, as well as other sensitive endpoints such as reproductive organ weights (paper I and II, IV and VI). Thus it was considered relevant to evaluate the relevance of using such effects for human risk characterization by estimating retinoid system based MOE values (Table 8). Briefly, MOE-values based on hepatic retinoid levels and plasma thyroid hormones for decaBDE and pentaBDE exposures among E-waste exposed male and female groups showed that they exceed the acceptable estimated risk (MOE<25) (EFSA, 2011 a,b). In addition, also high fish consuming men from the lake Mjösa exceeded the risk for pentaBDE exposure, whereas breastfeeding mothers in Sweden or Spain, and women in Norway eating high amounts of fish from lake Mjösa, were not at risk for any of the compounds (Table 8).

For HBCD, MOEs for hepatic retinyl palmitate concentration was the most physically relevant endpoint, along with the thyroid weight (van der Ven et al., 2006, paper V). HBCD exposure in humans were measured in fetal livers (Rawn et al., 2014), where MOE-values from median exposure levels for these endpoints did not represent a risk as MOEs were well above 25.

Table 8 – Margin of exposure (MOE) values based on hepatic all-*trans* retinoic acid, 9c-4o-13,14-dh-RA, retinyl palmitate, retinol, plasma T3 and T4 for DecaBDE, PentaBDE and HBCD studies.

Scenarios	Exposure (ng/g lipid in plasma)	Margin of exposure values					
		At-RA	CORA	RePA	ReOH	Plasma T4	Plasma T3
DecaBDE							
Women							
Mothers (Uppsala) [l]	5,9[d]	1095					15005
Mothers (Asturias) [m]	8,7[d]	743					10175
Fish consumption (Lake Mjøsa) [h]	14[e]	462					6320
Proximity electronic components factory (Laizhou Bay) [i]	1690[f]	**3,8**					52
Electronic waste recycling plant (Guiyu) [j]	4500[e]	**1,4**					**20**
Men							
Fish consumers (Lake Mjøsa) [h]	14[a]	114					
Proximity electronic components factory (Laizhou Bay) [i]	335[b]	**4,8**					
Electronic waste recycling plant (Guiyu) [j]	4500[a]	**0,4**					
PentaBDE							
Women							
Mothers (Uppsala) [l]	3,0[d]			1606	3631	3882	
Mothers (Asturias) [m]	11[d]			425	960	1027	
Fish consumption (Lake Mjøsa) [h]	66[e]			74	167	178	
Proximity electronic components factory (Laizhou Bay) [i]	259[f]			**19**	42	45	
Electronic waste recycling plant (Guiyu) [j]	661[e]			**7,3**	**17**	**18**	
Men							
Fish consumers (Lake Mjøsa) [h]	117[a]			**10**	**17**	**8,3**	
Proximity electronic components factory (Laizhou Bay) [i]	180[b]			**6,4**	**11**	**19**	
Electronic waste recycling plant (Guiyu) [j]	661[a]			**1,8**	**3,0**	**1,5**	
HBCD							
Healthy fetus (Canada) livers[k]	29[c]		922	560		7255	
	1028[g]		26	**16**		205	

At-RA: All-*trans* retinoic acid; CORA: 9c-4o-13,14-dh-RA; RePA: Retinyl palmitate; ReOH: Retinol; T3: Triiodothyronine. Exposure and benchmark doses lower bound of the 95th confidence interval (CEDLs) are provided in ng/g lipid in plasma. Hepatic at-RA CEDLs were computed using internal liver lipid doses. CEDLs were converted to ng/g lipid in plasma using 0,23% as average lipid contents in plasma and then using distribution ratio between liver and plasma of 0,075 according to Huwe (Huwe and Smith 2007) for the decaBDE study and 9.5 for the pentaBDE study (Sanders et al., 2006). Human exposures for the substances were represented with: [a] maximum exposure value detected; [b] mean exposure plus two standard deviations; [c] median; [d] 95th percentile; [e] maximum exposure value detected; [g] mean of maximum exposure values detected each year; [h] fish consumers (41 men and 25 women) from the highly contaminated lake Mjøsa in Norway (Thomsen et al., 2008); [i] i volunteers (71 male and 85 female) residing in the south coast area of Laizhou Bay China (Jin et al., 2009); [j] workers (18 men and 8 women) from an electronic waste recycling site in South China in Guiyu (Bi et al., 2007); [k] Rawn et al., 2014, 52 liver samples collected from elective pregnancy terminations from 6.5-19.5 weeks aged healthy fetus. Samples were taken from 1998-2002 and 2008-2010. Oral dose-based CEDLs for T3 and T4 were transformed into internal dose-based CEDLs using regression equation from van der Ven (2006). Regression for females: concentration liver lipid=33.377*Ce0.5587, and for males: 61.01*Ce0.6276. [l] serum samples from 24 Swedish mothers in Uppsala (Sahlström et al., 2014); [m] serum samples from 325 pregnant women collected during the first trimester of gestation in Asturias, Spain (Vizcaino et al., 2014). MOEs lower than 25 suggest a non-acceptable risk (EFSA, 2011).

However, it was considered that the highly exposed 95th percentile of the population are at or below 25 for the retinoid endpoints and for circulating levels of T4-glucuronide (Table 8), suggesting that this segment of the population is at risk of having modulated retinoid and thyroid hormone systems.

Taken together, based on the effects on the retinoid system as well as thyroid hormone systems, highly exposed E-waste workers from China as well as individuals living nearby E-waste recycling activities or electronic components factories exposed to decaBDE, and pentaBDE, exceeded the risk for altered hepatic retinoid levels as well as thyroid hormones.

3.5 OVERALL CONCLUSIONS

In this thesis it can be concluded that:

- the four selected hepatic retinoid forms were sensitive to BFR exposure and therefore well suited for detecting compound and gender specific (homeostatic) modulations of retinoid signaling and metabolism.

- the 25 selected (target) genes were suitable for explaining the compound and gender specific changes of the analysed hepatic retinoid forms in this study.

- quantitative analyses of data, including CED and MOE calculations and PLS analyses, support the use of retinoid system markers for toxicological evaluation of various categories and chemical compounds.

3.6 FUTURE STUDIES

For the inclusion of retinoid system modulations in the test battery of EDCs, further research is needed to identify suitable markers for retinoid system disturbances in (human) observational studies and to identify AOPs:

Additional retinoid measurements:

- All-*trans* RA metabolites for excretion via UGTs
- All-*trans* RA metabolites with signalling properties

- Measurements of retinoids also in serum as well as other tissues such as skin?

Retinoid related genes:

- Measure more functional genes related to toxicological pathways, such as REHs, and receptors which dimerize with RXRs: PPARs, LXR, FXR, Vitamin D receptor.
- Measure complete set of genes also for other organic pollutants as well as other chemicals to verify MOA.
- Measure complete set of retinoid related genes in other life stages as well as in vitro also to better understand MOA.

Regulatory purposes:

- Evaluate how to make use of data on retinoid system for regulatory purposes.
- What tissues and which retinoid formes/ genes are most relevant to measure in humans.

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