Stellingen

behorende bij het proefschrift "Tumour promotion by complex mixtures
of polyhalogenated aromatic hydrocarbons (PHAHs) and the applicability
of the toxic equivalency factor (TEF) concept" van Simone Antoinette
van der Plas, te verdedigen op vrijdag 22 december 2000.

1. De tumor-promoverende potentie van PCB's wordt in ruime mate onderschat door het TEF concept, dat wordt toegepast bij de risico-evaluatie van PHAH residuen en concentraties in respectievelijk voedingsmiddelen en milieucompartimenten. (*dit proefschrift*)

2. De verstoring van vitamine A en schildklierhormoon niveau's door PHAH's met een lage dioxine-achtige activiteit kan aanzienlijk versterkt worden bij gelijktijdige blootstelling aan PHAH's met een hoge dioxine-achtige activiteit, hetgeen moet worden toegeschreven aan een verhoogde vorming van OH-PCB's. (*dit proefschrift*)

3. Bij de toepassing van flavonoïden in zogenaamde 'functional foods' wordt teveel nadruk gelegd op de anti-oxidant eigenschappen van deze stoffen.

4. 'Moederlijk gedrag' wordt beïnvloed door een veelheid van genen en lijkt te worden gestimuleerd door de endocriene veranderingen die optreden tijdens zwangerschap en geboorte. (Robert S. Bridges, *Nature* 20, p. 108-109, 1998)

5. Het totale bestrijdingsmiddelen gebruik in de aardappelteelt in Nederland is eerder toe dan afgenomen met de opkomst van biologische aardappelteelt.

6. Het stimuleren vanuit de overheid van nevenactiviteiten (boerencamping, zorgboederij, windmolens etc.) in de landbouw voor het verkrijgen van extra inkomen voor boeren, betekent feitelijk dat de landbouw in Nederland als volwaardige bedrijfstak geacht wordt te verdwijnen.

Tumour Promotion by Complex Mixtures of Polyhalogenated Aromatic Hydrocarbons (PHAHs) and the Applicability of the Toxic Equivalency Factor (TEF) Concept

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
dr. ir. L. Speelman
in het openbaar te verdedigen
op vrijdag 22 december 2000
des namiddags te half twee in de Aula.

The barriers of human achievement
lie only in the mind

from: Master Wang's Laws of Wisdom
(Bubishi)

CONTENTS

CHAPTER 1

General introduction

In the last half century several cases of accidental food and environmental contamination have occurred involving the notorious class of persistent and toxic polyhalogenated aromatic hydrocarbons (PHAHs). For example, large-scale food poisoning with polychlorinated biphenyls (PCBs) and polychlorinated dibenzofurans (PCDFs) due to contamination of cooking oil took place in Japan (1968) and in Taiwan (1979). Exposed individuals suffered from a large number of symptoms, including chloracne, reproductive and developmental disorders, and are still followed up epidemiologically to investigate possible increases in the incidence of several cancer types. In the Seveso accident (1976), an explosion of a reaction vessel in a factory producing organochlorine pesticides caused a massive environmental contamination with 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD) and a consequent syndrome of chloracne and related dioxin-like symptoms. In addition to these accidental contaminations, there have been many cases of environmental contamination with PHAHs, due to e.g., improper industrial processes, waste incineration, bleaching of pulp and paper and spills. In 1989 the so-called "Lickebaert affair" took place in the Netherlands. Relatively high levels of PCDDs and PCDFs were found in milk of cows grazing in the surroundings of waste incinerators with relatively high emissions of PHAHs. During that period of time, farmers were not allowed to sell their dairy products. There was a high and acute demand for PCDD/PCDF analyses to be performed by high resolution-mass spectrometry (HR-MS), while the capacity to perform HR-MS analyses was absolutely insufficient for the demand and the costs were high. In this time period the project called 'Biological Effect Assays for Monitoring' (BEAM) was initiated[1]. The BEAM project was comprised of two main objectives:

1) To study the predictive value of the toxic-equivalency-factor (TEF) concept for the toxicity of complex mixtures of PCBs, PCDFs and PCDDs and in this context, to reveal possible antagonistic or synergistic interactions between congeners; 2) To develop a fast and efficient bio-assay (Ah receptor-dependent luciferase reporter gene or DR-CALUX bio-assay) for the analysis of the toxic potential of complex mixtures of polyhalogenated aromatic hydrocarbons (PHAHs). The importance of such a method was only recently demonstrated (1999) when in Belgium on a large scale chicken meat appeared to be contaminated with high concentrations of PCBs and PCDDs.

In this thesis the predictive value of the toxic equivalency factor (TEF) concept for the toxicity of complex environmental mixtures of PHAHs was studied. Special emphasis was put on the tumour promotion potential as a sub-chronic endpoint for the toxicity of PHAHs.

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General properties of PCBs, PCDDs and PCDFs

Polychlorodibenzo-*p*-dioxins (PCDDs), polychloro-dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs) are members of the group of polyhalogenated aromatic hydrocarbons (PHAHs) and consist of 75, 135 and 219 different isomers respectively. Their chemical structures are shown in figure 1.1. PCDDs and PCDFs are mainly formed as unwanted by-products during incomplete combustion processes in the presence of chlorine, such as municipal waste burning, and the synthesis of a wide range of organohalogens commercial chemicals, as herbicides, fungicides and PCBs (Safe, 1990, 1994). Commercial polychlorinated biphenyls (PCBs) were widely used as industrial products with diverse applications, such as plasticizers, heat transfer fluids, cutting oils, wax extenders, adhesives, flame retardants and dielectric fluids for capacitors and transformers. PCBs were produced in large quantities since 1929 and their total production is estimated on 1.5 million metric tons (De Voogt and Brinkman, 1989; Silberhorn *et al.*, 1990). PCDDs, PCDFs and PCBs are highly lipophilic and the degree of lipophilicity is increasing with increased chlorination. In addition, these compounds are biologically stable and resistant to breakdown by acids, bases and heat. As a result of their lipophilic and persistent nature, PCDDs, PCDFs and PCBs were shown to accumulate in the environment and were reported as contaminants in almost every component of the global ecosystem, including air, water, fish, wildlife and human adipose tissue, milk and serum. PCDDs, PCDFs and PCBs have been shown to elicit a broad spectrum of toxic effects and biochemical changes in both animals and human, e.g. body weight loss, thymic atrophy, impairment of immune responses, hepatotoxicity, teratogenicity,
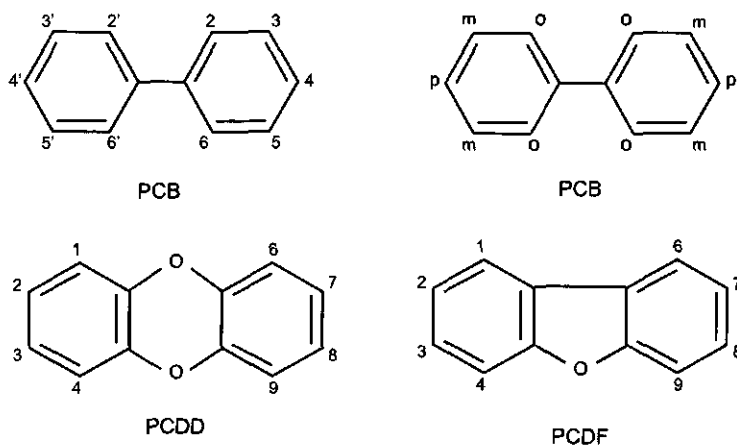

Figure 1.1 The chemical structures of PCBs, PCDDs and PCDFs

carcinogenicity and induction of hepatic cytochrome P450 isoenzymes (reviewed by Safe, 1989, 1990, 1994). For this reason, much effort has been put on emission control and other measures to reduce the introduction of PCDDs and PCDFs into the environment. In addition, from the 1980s onwards PCBs were banned or the production, use and disposal was severely restricted in most industrialised countries (Silberhorn et al., 1990; Brouwer et al., 1998a). Since then, environmental and dietary levels of PCDDs, PCDFs and PCBs have been found to decline in all compartments (CCRX 1993; Liem and Theelen, 1997; Fürst and Wilmers, 1999; Hori et al., 1999; Päpke et al., 1999).

The most toxic PCDDs, PCDFs and PCBs exhibit a planar molecular conformation and most if not all of their toxic responses are thought to be mediated by the aryl hydrocarbon (Ah) receptor (Safe, 1994). The Ah receptor is present in the cell cytosol as a complex with the 90 kDa heat-shock protein (hsp90) (Schmidt and Bradfield, 1996). After binding of TCDD or other congeners to the Ah receptor, hsp90 is dissociated from the Ah receptor-ligand complex, followed by translocation of the complex to the nucleus and dimerization of the Ah receptor with the Ah-receptor translocater (Arnt) protein. The ligand-AhR-Arnt complex binds selectively to dioxin-responsive elements (DRE) on the DNA, thereby inducing the expression of DRE-regulated genes, such as cytochrome P4501A1 and 1A2 (Schmidt and Bradfield 1996). The most potent ligand for Ah receptor binding is 2,3,7,8-TCDD. Binding competition studies with ^{3}H-labeled 2,3,7,8-TCDD showed that the 2,3,7,8-substituted tetra- to hexa CDDs and CDFs are the most competitive ligands for Ah receptor binding. The most competitive PCBs are substituted on both *para* and at least two *meta* positions, the so called non-*ortho* or planar PCBs. Mono-*ortho* substituted PCBs exhibit lower competitive binding affinities for the Ah receptor and they are considered as 'mixed-type inducers', because of their capability to mediate both dioxin-like and non-dioxin-like toxicity (Bandiera et al., 1982; Safe, 1990).

Non-planar PHAH compounds, i.e. di-*ortho* substituted PCBs, exhibit a so called non-dioxin-like toxicity. The toxicity of the non-planar PCBs resembles the toxicity of the barbiturate phenobarbital, characterised by the induction of cytochrome P4502B1 and 1B2 (Safe, 1990, 1994). The mechanism of cytochrome P4502B induction by di-*ortho* PCBs is unknown.

The toxic equivalency factor concept

In environmental matrices and biota PCDDs, PCDFs and PCBs are always present as complex mixtures. The complexity of these mixtures complicates the risk evaluation for humans as well as for fish and wildlife. The toxic equivalency factor (TEF) concept has been developed to aid the risk assessment of complex mixtures of PHAHs and enables the calculation of total toxic potencies, expressed as the toxic equivalent (TEQ), of the mixtures. The TEF approach is based on the assumptions that all toxic PHAH congeners act through the

same dioxin-like Ah receptor-based mechanism of action and that the effects of the individual compounds are additive (Safe, 1994; Ahlborg *et al.*, 1994; Van den Berg *et al.*, 1998). TEF values for the individual PHAH congeners are derived by calculating the relative toxicity of the concerning PHAH congener compared to the toxicity of 2,3,7,8-TCDD. Subsequently, the TEQ of a complex PHAH mixture is calculated using the following equation:

$$TEQ= \Sigma([PCDD_i \times TEF_i]_n) + \Sigma([PCDF_i \times TEF_i]_n) + \Sigma([PCB_i \times TEF_i]_n)$$

In order to harmonise the risk assessment of PHAHs, consensus TEF values for the dioxin-like PHAHs were derived at international expert meetings organised by the World Health Organisation (WHO) (see Table 1.1).

No TEF values are defined for the non-dioxin-like PHAHs. In the recent past TEF values were available for 2,2',3,3',4,4',5-HCB (PCB 170) and 2,2',3,3',4,4',5,5'-OCB (PCB 180) (Ahlborg *et al.*, 1994), but these values were withdrawn because an Ah receptor mediated response of these congeners could not be confirmed (Van den Berg *et al.*, 1998). Although the di-*ortho* substituted PCBs are in general considered to be less toxic and possess no or a negligible Ah receptor mediated activity, i.e. a TEF value close to zero, their concentration in commercial mixtures, in food and environmental matrices is much higher compared to the planar PHAHs. However, till now there is no tool available for risk assessment of this class of PHAHs.

Tolerable Daily Intake and background exposure

At a WHO consultation in 1990, a tolerable daily intake (TDI) of 10 pg TEQ/kg bw/day was established for PCDDs, PCDFs and PCBs, on the basis of the no-observed-adverse-effect level (NOAEL) of 1 ng/kg/day for the carcinogenicity of 2,3,7,8-TCDD in rats (data obtained from Kociba *et al.*, 1978). Because of new epidemiological and toxicological data a revised TDI of 1-4 pg TEQ/kg bw/day was deduced, taking the most sensitive adverse effects in consideration, i.e. hormonal and reproductive effects (Van Leeuwen and Younes, 1998). For the total PCB intake a TDI of 0.4 µg/kg bw/day was suggested, on the basis of the NOEL of 40 µg/kg bw/day in monkeys (CCRX 1993).

Information from food surveys in industrialised countries indicate a daily intake of PCDDs and PCDFs in the order of 1-3 pg TEQ kg/bw/day and if dioxin-like PCBs are also included, the daily TEQ intake will be a factor 2-3 higher (Van Leeuwen and Younes, 1998). In the Netherlands an intake of PCDDs, PCDFs and PCBs of 2.3 pg TEQ/ kg bw/day was reported for the early 1990s (Liem and Theelen, 1997). No information is available on the total intake of PCBs on a weight basis. Because of the complexity of PCB analysis in environmental samples, in most surveys only the so called indicator PCBs (IUPAC NR. 28, 52, 101, 118, 138, 153 and 180) are being analysed. An extensive survey was done in 1994 by

Liem and Theelen (1997), who measured 29 PCB congeners in different food items. They reported a total intake of 20 ng/kg bw/day (Liem and Theelen, 1997) and given the assumption that a level of 20 ng/kg/day in foodstuff covers approximately 20-30% of the total dietary intake, a total PCB intake of 60-100 ng/kg bw/day can be estimated.

Carcinogenicity of PHAHs

Carcinogenicity is one of the toxic endpoints in risk assessment of PHAHs (WHO 1992). An extensive evaluation on the carcinogenic properties of PCDDs and PCDFs was done by the International Agency for Research on Cancer (IARC, 1997; McGregor *et al.*, 1998). They found that there is epidemiological evidence in human for an overall increase in mortality from all cancers combined in the most highly TCDD exposed industrial cohorts,

Table 1.1 PCB TEFs for human intake

Class	IUPAC No.	Structure	old WHO TEFs[a]	new WHO TEFs[b]
Non-*ortho*	77	3,3',4,4'-TeCB	0.0005	0.0001
	81	3,4,4',5-TeCB	-	0.0001
	126	3,3',4,4',5-PeCB	0.1	0.1
	169	3,3',4,4',5,5'-HxCB	0.01	0.01
Mono-*ortho*	105	2,3,3',4,4'-PeCB	0.0001	0.0001
	114	2,3,4,4',5-PeCB	0.0005	0.0005
	118	2,3',4,4',5-PeCB	0.0001	0.0001
	123	2',3,4,4',5-PeCB	0.0001	0.0001
	156	2,3,3',4,4',5-HxCB	0.0005	0.0005
	157	2,3,3',4,4',5'-HxCB	0.0005	0.0005
	167	2,3',4,4',5,5'-HxCB	0.00001	0.00001
	189	2,3,3',4,4',5,5'-HpCB	0.0001	0.0001
Di-*ortho*	170	2,2',3,3',4,4',5-HpCB	0.0001	-
	180	2,2',3,4,4',5,5'-HpCB	0.00001	-

[a]Ahlborg *et al.* (1994)

[b]Van den Berg *et al.* (1998)

while there is less strong evidence of increased risks for cancers of particular sites (IARC, 1997; McGregor *et al.*, 1998). The conclusion of the IARC was that TCDD has to be classified as carcinogenic to humans, but for other PCDDs and PCDFs there is only limited or inadequate evidence (IARC, 1997; McGregor *et al.*, 1998).

PCDDs, PCDFs and PCBs are considered as tumour promoters rather than as initiators of carcinogenesis (Safe, 1989; Silberhorn *et al.*, 1990; Whysner and Williams, 1996; IARC, 1997). In rodents, individual congeners as well as mixtures of PCDDs, PCDFs and PCBs were found to exhibit a distinct tumour promotion activity in various two-stage skin- and hepatocarcinogenicity bioassays (Poland *et al.*, 1982; Hébert *et al.*, 1990; Table 1.2). Although PCDDs, PCDFs and PCBs are considered as non-genotoxic and non-mutagenic (Safe, 1989; Silberhorn *et al.*, 1990; Huff *et al.*, 1994), they may also act as complete carcinogens. In several life time carcinogenicity studies in rats, treatment with TCDD or commercial PCB mixtures showed an increased incidence of neoplastic lesions in for example stomach, intestine, thyroid gland and liver, without prior exposure to an experimental initiator (Kimbrough *et al.*, 1975; Kociba, 1978; Norback and Weltman, 1985; Ward, 1985; Mayes *et al.*, 1998). Female rats appeared to be much more sensitive to the hepatocarcinogenic activity of PCDDs, PCDFs and PCBs than male rats (Kociba *et al.*, 1978; Norback and Weltman, 1985; Mayes *et al.*, 1998;), which implies that the ovarian hormones, presumably estrogens, may influence the carcinogenic response (Lucier *et al.*, 1991).

Proposed mechanisms for the carcinogenic activity of PCDDs, PCDFs and PCBs include alteration of the DNA-damaging potential of endogenous compounds (including estrogens) by increased cytochrome P450-mediated metabolic activation, DNA single-strand breaks resulting from lipid peroxidation, alterations in cell proliferation and differentiation via modifications of growth factor and cytokine pathways, and inhibition of gap-junctional intercellular communication and apoptosis (Silberhorn *et al.*, 1990; Huff *et al.*, 1994; Grassman *et al.*, 1998; Schwarz *et al.*, 2000). It is assumed that the Ah receptor plays an important role in mediating the carcinogenic activity of PHAHs, which was supported by a positive correlation between tumour promotion activity of PHAH compounds and their affinity for the Ah receptor or Ah receptor mediated toxicological effects, such as CYP1A1 induction (Poland and Knutson 1982; IARC, 1997; Schwarz *et al.*, 2000).

Several non-dioxin-like di-*ortho* substituted PCBs were shown to possess a tumour promotion activity as well (for an overview see Table 1.2). However, it is unlikely that the Ah receptor is involved in the tumour promotion activity of di-*ortho* PCBs. Mechanisms possibly involved are for instance the inhibition of intercellular communication and stimulation of cell proliferation (Silberhorn *et al.*, 1990).

Intercellular communication plays an important role in cell growth, differentiation and maintenance of cell homeostasis (Loewenstein, 1990; Budunova, 1994). Inhibition of the gap junctional intercellular communication (GJIC) is suggested to be an important step in carcinogenesis and considered as an *in vitro* indicator of the tumour promotion capacity of chemicals (Mesnil *et al.*, 1993). Both dioxin-like and non-dioxin-like PCBs have been

demonstrated to inhibit the intercellular communication in primary liver cells (Swierenga, *et al.*, 1990) and hepatoma cells (De Haan *et al.*, 1994b, 1996; Bager *et al.*, 1997) *in vitro*. On the basis of both *in vitro* and *in vivo* studies on GJIC, it is hypothesised that different mechanisms are involved for the dioxin-like and the non dioxin-like PCBs (De Haan *et al.*, 1996; Bager *et al.*, 1997).

Interactions between PHAH compounds

There is a growing number of experimental data suggesting non-additive interactions between PHAHs, mostly between planar and non-planar compounds, on several toxicity parameters. Interactive effects were reported on for instance immunotoxicity (Biegel *et al*, 1989; Davis and Safe, 1989), teratogenicity (Zhao *et al.*, 1994) hepatotoxicity (Yao and Safe, 1989; Van Birgelen *et al.*, 1996a) and tumour promotion (Sargent *et al.*, 1991; Bager *et al.*, 1995, Haag-Grönlund *et al.*, 1998). Only a few studies have shown synergistic interactions between PHAHs. Synergism is likely to occur when more than one pathway, i.e. Ah receptor independent, may be involved (Safe, 1997/98) like with liver porphyry (Van Birgelen *et al.*, 1996a) and the promotional effect on the formation of enzyme altered hepatic foci (Bager *et al.*, 1995). A toxicokinetic basis was found for the synergistic effect on the ethoxyresorufin-*O*-deethylase and aryl hydrocarbon hydroxylation activity in mice co-treated with TCDD and 2,2',4,4',5,5'-HxCB (PCB 153) (De Jongh *et al.*, 1993a,b, 1995). Antagonistic interactions between PHAHs are most frequently observed. Antagonists might be weaker Ah receptor agonists or full antagonists. A weaker agonist exhibits a partial agonistic and thus an inhibitory activity. Full antagonists on the other hand, might block the formation of the transcriptionally active nuclear Ah receptor complex when co-administered with a Ah receptor agonist (Aarts *et al.*, 1995). Antagonistic interactions of PHAHs have all been observed at relatively high concentrations of both agonist and antagonist, and the importance of these interactions at lower, environmental levels is not known.

Outline of the present study

The major aim of this project consisted of two main objectives: 1) to examine the tumour promotion potential of complex, environmentally relevant mixtures of polyhalogenated aromatic hydrocarbons (PHAHs) and 2) to evaluate the applicability of the Toxic Equivalency Factor (TEF) concept for the tumour promotion potential of complex PHAH mixtures. In addition, the effect of sub-chronic exposure to these complex PHAH mixtures was determined on endocrine parameters, i.e. the vitamin A and thyroid hormone status, which play an essential role in normal tissue growth and foetal development and are possibly involved in the process of carcinogenesis.

Experimental approach

The tumour promotion potential of PHAH mixtures was studied in young female Sprague Dawley rats, using a two-stage tumour promotion model introduced by Pitot *et al.* (1978). This model makes it possible to make a clear distinction between the initiation and the promotion event during carcinogenesis. The initiation step of the model consisted of a nitrosodiethylamine injection 24 hours after a partial hepatectomy, followed by a promotion treatment of 20 weeks starting 6 weeks after the initiation procedure. For the tumour promotion studies, two environmental relevant mixtures of PHAHs were designed. The first mixture was a synthetic mixture, which consisted mainly of dioxin-like PHAH compounds and covering over 90% of the TEQs present in Baltic herring. The di-*ortho* PCB 153 (2,2',4,4',5,5-HxCB) was added to this mixture to study possible interactive effects with dioxin-like congeners. All PHAH compounds in the synthetic mixture were individually tested in a similar tumour promotion experimental set-up as used here, by Wærn *et al.* (1991), Hemming *et al.* (1993), and Haag-Grönlund *et al.* (1997a,b). In order to study the contribution of non-dioxin-like PCBs to the tumour promotion potential of PHAH mixtures, a second mixture was designed in which the focus was on the 2-4 *ortho* substituted PCBs. For this purpose the commercial PCB mixture Aroclor 1260 was fractionated into a dioxin-like and a non-dioxin-like fraction. The PCB fractions were tested separately and as a reconstituted mixture.

In chapter 2, the toxic potency of the complex PHAH mixtures and the possibility of interactions between PHAH congeners was studied *in vitro*, using the ethoxyresorufin-*O*-deethylase (EROD) and the Ah receptor-dependent Luc-reporter gene (DR-CALUX) bio-assay, both indicator assays for an Ah receptor mediated dioxin-like toxicity. Preliminary results are presented on the inhibition of GJIC by the PHAH mixtures. In chapter 3, the toxicokinetic behaviour of the compounds in the dioxin-like PHAH mixture was investigated *in vivo*, in order to get a better understanding of underlying mechanisms of possible interactive effects between PHAHs. In chapter 4 the tumour promotion study is described, aimed at the predictive value of the TEF concept for the tumour promotion potential of the synthetic dioxin-like PHAH mixture. Chapter 5 describes the study on the contribution of the non-dioxin-like PCBs to the tumour promotion potential of PHAH mixtures. In chapter 6 endocrine effects were determined of sub chronic exposure to PHAH mixtures, i.e. effects on the thyroid hormone and vitamin A status. In chapter 7, the main conclusions and overall impact of the study results are discussed with respect to involved mechanisms, consequences for risk assessment and human and environmental relevance.

Table 1.2A Tumour promotion studies in rats using short-term two stage hepatocarcinogenicity models; individual PHAHs

Compound (IUPAC nr.)	Dose	Treatment	Altered hepatic foci	Rat strain	Author
2,3,7,8-TCDD	**0.01** µg/kg/day	PH/NDEA + 6 months promotion	GST-p positive GGT positive ATP-ase deficient G6Pase deficient	Fischer 344 (f)	Dragan et al., 1991
2,3,7,8-TCDD	**0.14** µg/kg biweekly s.c.	PH/NDEA + 1, 3 or 5 months promotion	GST-p positive GGT positive ATP-ase deficient G6Pase deficient	Charles River (f)	Dragan et al., 1992
2,3,7,8-TCDD	0.07, **0.7** µg/kg bw/week, s.c.	PH/NDEA initiation + 15 or 27 weeks promotion[a]	GGT positive	Sprague Dawley (f)	Flodström and Ahlborg, 1989
2,3,7,8-TCDD 1,2,3,7,8-PeCDD 2,3,4,7,8-PeCDF	**0.044, 0.175, 0.7** **0.088, 0.35, 1.4** 0.16, **0.64, 2.6** µg/kg bw/week, s.c.	PH/NDEA initiation + 20 weeks promotion[a]	GST-p positive	Sprague Dawley (f)	Wærn et al., 1991
2,4,8-TCDF	200, **500** mg/kg, 5 times weekly	NDEA + 10 weeks promotion	GGT positive ATP-ase deficient	Sprague Dawley (f)	Deml et al., 1989
2,3,7,8-TCDD 1,2,3,7,8-PeCDD 2,3,4,7,8-PeCDF 3,3',4,4',5-PeCB (126) 2,3,3',4,4'-PeCB (105)	**0.044, 0.175, 0.7** **0.088, 0.35, 1.4** 0.16, **0.64, 2.6** **10, 100** 500, **1500**, 5000 µg/kg bw/week, s.c.	PH/NDEA initiation + 20 weeks promotion[a]	GST-p positive GGT positive	Sprague Dawley (f)	Flodström and Ahlborg, 1992

Compound (IUPAC nr.)	Dose	Treatment	Altered hepatic foci	Rat strain	Author
2,3,7,8-TCDD 3,3',4,4',5-PeCB (126)	0.1, 0.316, **1** 0.316, 1, 3.16, **10** µg/kg bw/week, s.c.	PH/NDEA initiation + 20 weeks promotion[a]	GGT positive	Sprague Dawley (f)	Hemming et al., 1995
3,3'-DCB (11) 2,2',5,5'-TeCB (52)	**150** **150** µmol/kg bw/week, i.p.	NDEA initiation + 8 weeks promotion	ATP-ase deficient	Wistar (f)	Buchmann et al., 1986
2,2',4,4'-TeCB (47) 2,2',5,5'-TeCB (52)	**100** **100** ppm in the diet	PH/NDEA initiation + 28 weeks promotion	GGT positive	Sprague Dawley (f)	Preston et al., 1985
4-MBP (3) 2,2',4,5'-TeCB (49) 3,3',4,4'-TeCB (77) 2,3,4,4',5-PeCB (118)	150 150 **15, 150** 450 in total[b] µmol/kg bw/week, i.p.	NDEA initiation + 8 weeks promotion	ATP-ase deficient GGT positive	Wistar (f)	Buchmann et al., 1991
3,3',4,4'-TeCB (77) 2,2',4,5,5'-HxCB (153)	**88** **108** mg/kg bw biweekly	NDEA + 8 weeks promotion	GGT positive ATP-ase deficient G6Pase deficient	Sprague Dawley (f)	Berberian et al., 1995
2,3',4,4',5-PeCB (118)	10, 40, 160, 640, 2500, **10000** 40, 640, **10000** µg/kg bw/week, s.c.	PH/NDEA initiation + 20 weeks promotion[a] PH/NDEA initiation + 52 weeks promotion[a]	GST-p positive	Sprague Dawley (f)	Haag-Grönlund et al., 1997a

Compound (IUPAC nr.)	Dose	Treatment	Altered hepatic foci	Rat strain	Author
3,3',4,4',5,-PeCB (126) 2,2',4,4',5,5'-HxCB (153)	1, 3.16, **10** **5000** µg/kg bw/week, s.c.	PH/NDEA initiation + 20 weeks promotion[a]	GST-p positive	Sprague Dawley (f)	Bager *et al.*, 1995
3,3',4,4',5,-PeCB (126) 2,3,3',4,4'-PeCB (105) 2,2',4,4',5,5'-HxCB (153)	**10**, 100 **500, 1500, 5000** **1000, 5000, 20000** µg/kg bw/week, s.c.	PH/NDEA initiation + 20 weeks promotion[a]	GST-p positive GGT positive	Sprague Dawley (f)	Hemming *et al.*, 1993
2,3,3',4,4',5-PeCB (156)	50, 300, **1500, 7500** µg/kg bw/week, s.c.	PH/NDEA initiation + 20 weeks promotion[a]	GST-p positive	Sprague Dawley (f)	Haag-Grönlund *et al.*, 1997b

Bold printed doses showed a statistically significant increase of altered hepatic foci compared to the negative control group. (f)=female

[a] The first dose (week 1 of the promotion period) was a loading dose, which was 5 times the maintenance dose administered in the following 19 weeks
[b] PCB 118 was initially administered at a dose of 150 µmol/kg bw. Because the compound was found to excert strong toxic effects, the dose was reduced to 75 µmol/kg bw for the third and the fourth doses and only four weekly doses were admininstered.

Table 1.2B Tumour promotion studies in rats using short-term two stage hepatocarcinogenicity models; mixtures of PHAHs

Compound (IUPAC nr.)	Dose	Treatment	Altered hepatic foci	Rat strain	Author
2,3,7,8-TCDD + 3,3',4,4',5-PeCB (126)	0.1+1, 0.316+3.16, 1+10 µg/kg bw/week, s.c.	PH/NDEA initiation + 20 weeks promotion[a]	GGT positive	Sprague Dawley (f)	Hemming et al., 1995
3,3',4,4',5,-PeCB (126) + 2,2',4,4',5,5'-HxCB (153)	1+5000, 3.16+5000, 10+5000	PH/NDEA initiation + 20 weeks promotion[a]	GST-p positive	Sprague Dawley (f)	Bager et al., 1995
3,3',4,4',5,-PeCB (126) + 2,3,3',4,4'-PeCB (105) + 2,2',4,4',5,5'-HxCB (153)	126: L=0.13 M=0.93 H=6.6; 105: L=66 M=467 H=3302; 153: L=220 M=1556 H=11003 µg/kg bw/week, s.c. All possible combinations were tested. Significant combinations: **MMM, HLL, HMM, HHL**	PH/NDEA initiation + 20 weeks promotion[a]	GST-p positive	Sprague Dawley (f)	Haag-Grönlund et al., 1998
Clophen A50	**100** mg/kg bw/week, i.g.	NDEA initiation + 1-7 weeks promotion	GGT positive	Sprague Dawley (f)	Oesterle and Deml, 1983
PHAH mix[b] PHAH mix – 2,2',4,4',5,5'-HxCB (153)	**17, 35, 70** **15** mg/kg bw/week, s.c.	PH/NDEA initiation + 20 weeks promotion[a]	GST-p positive	Sprague Dawley (f)	Van der Plas et al., 1999 (Chapter 4)

Compound (IUPAC nr.)	Dose	Treatment	Altered hepatic foci	Rat strain	Author
Aroclor 1260 0-1 *ortho* fraction[c] 2-4 *ortho* fraction 0-4 *ortho* fraction	**10** 1 **1, 3, 9** **10** mg/kg bw/week, s.c.	PH/NDEA initiation + 20 weeks promotion[a]	GST-p positive	Sprague Dawley (f)	Van der Plas et al., 2000a (*Chapter 5*)

Bold printed doses showed a statistically significant increase of altered hepatic foci compared to the negative control group. (f)=female

[a] The first dose (week 1 of the promotion period) was a loading dose, which was 5 times the maintenance dose administered in the following 19 weeks

[b] The PHAH mixture consisted of 2,3,7,8-TCDD, 1,2,3,7,8-PeCD, 2,3,4,7,8-PeCDF, 3,3',4,4',5-PeCB, 2,3',4,4',5-PeCB, 2,3,3',4,4',5-HxCB and 2,2',4,4',5,5'-HxCB in relative concentrations of 1:3.3:17:61:12800:1888:20000.

[c] Aroclor 1260 was fractionated into a 0-1 *ortho* and a 2-4 *ortho* substituted PCB fraction, which were 10% and 90% (weight basis) of Aroclor 1260 respectively. The 0-4 *ortho* fraction is reconstituted of the 0-1 *ortho* and the 2-4 *ortho* substituted PCB fraction and differs from Aroclor 1260 in its degree of contamination with e.g. PCDFs.

CHAPTER 2

In vitro interactive effects between planar and non-planar polychlorinated aromatic hydrocarbons (PHAHs).

Abstract

The aim of this study was to investigate possible interactions between 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and several mono- or di-*ortho* polychlorinated biphenyls (PCBs) for the induction of the ethoxyresorufin-*O*-deethylase (EROD) and the Ah receptor dependent luciferase reporter gene (DR-CALUX) activity *in vitro*. In addition, the DR-CALUX assay was performed to determine the relative toxic potential (REP) of PHAH mixtures designed for tumour promotion experiments *in vivo*. Preliminary results are also presented on the inhibition of gap-junctional intercellular communication (GJIC), which is seen as an *in vitro* parameter for tumour promotion, by the PHAH mixtures and combinations of TCDD with 2,2',5,5-tetrachlorobiphenyl or Aroclor 1254. Both the mono- and the di-*ortho* PCBs inhibited the EROD and CALUX activity induced by TCDD, possibly via competition for Ah receptor binding. No interactions between congeners were seen in a semi-synthetic dioxin-like PHAH mixture designed for *in vivo* tumour promotion studies. To determine the contribution of non-dioxin-like congeners to the tumour promotion activity of a complex mixture of PHAHs, Aroclor 1260 was fractionated into a 0-, 1- and 2-4 *ortho* PCB fraction. The CALUX activity induced by the 0-*ortho* fraction was strongly inhibited by its 1-*ortho* but especially its 2-4 *ortho* substituted PCB fraction. For the inhibition of GJIC, combined exposure of PHAHs indicated that similar non-additive interactions occurred as observed in the EROD and CALUX assay. For the antagonistic interactions, high concentrations (near Ah receptor saturation) of the Ah receptor agonists and a large concentration difference between the agonists and partial- or non-agonists are required. It is concluded that in environmental exposure to PHAHs these conditions will be seldomly reached and therefore antagonistic interactions are not likely to play a role *in vivo* in real life situations. The dioxin-like PHAH mixtures were potent inhibitors of the GJIC. The 2-4 *ortho* fraction appeared to be the most potent inhibiting fraction of Aroclor 1260. The *in vitro* potency of PHAH mixtures to inhibit the GJIC was largely in agreement with the tumour promotion properties seen *in vivo*. Since this were preliminary results, more research is needed to elucidate the predictive value of GJIC inhibition as an early indicator for the tumour promotion potency *in vivo*.

Introduction

Polychlorinated biphenyls (PCBs), polychlorodibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and other polyhalogenated aromatic hydrocarbons (PHAHs) are ubiquitously present in the environment. They elicit a broad spectrum of toxicological and biochemical responses, including dermal toxicity, endocrine disruption, impairment of immune responses, adverse effects on reproduction and development, and carcinogenicity (Kimbrough, 1974; Safe, 1990; Van den Berg *et al.*, 1998; Brouwer *et al.*, 1999).

In environmental matrices and biota, PCBs, PCDDs and PCDFs are always present as complex mixtures. To enable risk assessment of complex mixtures of PHAHs the toxic equivalency factor (TEF) concept has been developed, where the toxicity of PHAHs is being expressed relative to the toxicity of the most toxic congener 2,3,7,8-TCDD. The TEF concept is based on the assumptions that 1) all dioxin-like congeners act through the same Ah receptor based mechanism, 2) that the effects of individual congeners are additive and 3) that the compounds are persistent and accumulate in the environment (Safe, 1990, 1994; Van den Berg *et al.*, 1998). Although there is experimental evidence supporting these assumptions (Safe 1990, 1994), there is also a growing number of studies suggesting non-additive interactions between PHAHs on several toxicity parameters including immunotoxicity (Biegel *et al*, 1989; Davis and Safe, 1989), teratogenicity (Zhao *et al.*, 1994), hepatotoxicity (Yao and Safe, 1989; Van Birgelen *et al.*, 1996a) and tumour promotion (Sargent *et al.*, 1991; Bager *et al.*, 1995, Haag-Grönlund *et al.*, 1998). Antagonistic interactions between PHAHs were most frequently observed. Antagonists might be weak partial or full Ah receptor antagonists. Synergism is likely to occur when more than one pathway, i.e. Ah receptor independent, may be involved in the development of the toxic response, like with liver porphyria and the promotional effect on the formation of enzyme altered hepatic foci (Bager *et al.*, 1995; Van Birgelen *et al.*, 1996a; Safe, 1997/98).

Carcinogenicity is one of the key toxic endpoints in risk assessment of PHAHs. PHAHs may act as complete carcinogens (Kimbrough *et al.*, 1975; Kociba *et al.*, 1978; Mayes *et al.*, 1998) but are generally considered as tumour promoters (Silberhorn *et al.*, 1990). Individual PHAH congeners as well as mixtures of PCDDs, PCDFs and PCBs were found to exhibit a distinct tumour promotion activity in various two-stage hepatocarcinogenicity bioassays (Pitot *et al.*, 1980; Oesterle and Deml, 1983; Waern *et al.*, 1991; Hemming *et al.*, 1993; Bager *et al.*, 1995; Haag-Grönlund *et al.*, 1997a,b, 1998). It is assumed that the Ah receptor pathway plays an important role in mediating the carcinogenic activity of PHAHs (Poland *et al.*, 1982; Silberhorn *et al.*, 1990; Schwarz *et al.*, 2000). However, both *in vitro* and *in vivo* studies suggest also a tumour promotion capacity of several non-dioxin-like di-*ortho* substituted PCBs (Preston *et al.*, 1985; Hemming *et al.*, 1993; De Haan *et al.*, 1996), where it is unlikely that the Ah receptor is involved. One of the mechanisms possibly involved in the carcinogenicity of both the dioxin-like and-non-dioxin-like PCBs is the inhibition of

intercellular communication (Silberhorn *et al.*, 1990). Intercellular communication plays an important role in cell growth, differentiation and maintenance of cell homeostasis (Loewenstein, 1990; Budunova, 1994). Therefore inhibition of the gap junctional intercellular communication (GJIC) is suggested to be an early step in the development of tumours.

The aim of our project was to examine the tumour promotion potential of complex, environmentally relevant mixtures of PCBs, PCDDs and PCDFs containing both dioxin- and non dioxin-like congeners, and, to evaluate the applicability of the Toxic Equivalency Factor (TEF) concept for the tumour promotion potential of complex mixtures of PCBs, PCDDs and PCDFs. In this manuscript *in vitro* data are presented on the interactive effects between planar and non-planar PHAH congeners on the Ah receptor-dependent induction of ethoxyresorufin-*O*-deethylase (EROD) and luciferase, in the EROD and reporter gene (DR-CALUX) bio-assays. In addition, preliminary results are presented on the inhibition of GJIC by combined exposure of individual PHAHs as well as PHAH mixtures originally designed for tumour promotion experiments *in vivo*.

Materials and Methods

Chemicals

Aroclor 1254 and 1260 were kindly provided by Dr. M. Van den Berg (Research Institute of Toxicology, University of Utrecht, The Netherlands). 2,3,7,8-TCDD was obtained from Radian CIL, Inc. (USA). 2,2',4,4'-TeCB (PCB 47), 2,2',5,5'-TeCB (PCB 52), 2,2',4,5,5'-PeCB (PCB 101), 2,3,3',4,4'-PeCB (PCB 105), 2,3,4,4',5-PeCB (PCB 114), 2,3',4,4',5-PeCB (PCB 118), 2,2',3,3',4,4'-HxCB (PCB 128), 2,2',4,4',5,5'-HxCB (PCB 153), 2,3,3',4,4',5-HxCB (PCB 156), and 2,2',3,4,4',5,5'-HeCB (PCB 180) were from Schmidt B.V. (Amsterdam, The Netherlands) and Ultra Scientific (North Kingstown, Ireland). All PHAH stock solutions were dissolved in dimethyl sulfoxide (DMSO 99.9%; Janssen Chimica, Geel, Belgium).

Minimal essential medium (alfa-MEM) and foetal calf serum for cell culture were obtained from Gibco (Rockville, Maryville, USA). Resorufin was obtained from Janssen Chimica (Geel, Belgium) and ethoxyresorufin and penthoxyresorufin were synthesised according to Mayer *et al.* (1977). NADPH (98%) was from Boehringer (Mannheim, Germany). A Bradford Protein Assay kit was obtained from Bio-rad (Hercules, California, USA). Phosphate buffered saline (Dulbecco's 'A', pH 7.3; PBS) was purchased from Oxoid (London, UK), NaCl and Tris were from Merck (Darmstadt, Germany). Luciferin assay mix was from Promega (Madison, Wisconsin, USA). Lucifer Yellow CH (98%) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other compounds were of analytical grade.

Ethoxyresorufin-O-deethylase assay

The induction of ethoxyresorufin-*O*-deethylase (EROD) activity was analysed in the

wild type mouse hepatoma cell line Hepa1c1c7 as described by De Haan *et al.* (1996). Cells were seeded in 96-wells plates (Greiner) and grown in minimal essential medium (alfa-MEM) with 10% foetal calf serum. After culturing for 24 hours under standard conditions (37 °C, 5% CO_2), the cells were exposed to the test compounds during another 24 hours. Then the medium was removed from the cells, the cells were rinsed with 0.5×PBS buffer, lysed with 20 µl distilled water and frozen at -80 °C for at least 10 minutes. For the analysis of the EROD activity cells were thawn at room temperature. Then cells were pre-incubated at 37 °C in the presence of 50 µl Tris-sucrose buffer (50 mM/0.1 M) with dicumarol (0.04 mM) and 25 µl 20 µM 7-ethoxyresorufin solution. Subsequently the reaction was started after addition of 25 µl 1.0 mM NADPH. After incubation at 37 °C for 1 hour, resorufin concentrations were measured with a fluoro spectrophotometric plate reader (CytoFluor 2530, Millipore). The protein content in the wells was analysed with a Bradford Protein Assay kit.

Each concentration of a sample was tested at least in ten wells per experiment and each experiment was repeated twice. DMSO and TCDD were incorporated as a negative and a positive control respectively on each plate. The DMSO concentration in the cell medium did not exceed 0.1%.

Ah receptor-dependent H4IIE-Luc reporter gene assay (DR-CALUX)

To exclude the possibility of competitive inhibition of PHAH congeners with ethoxyresorufin in the EROD assay (as observed by Besselink *et al.*, 1998), which would result in an underestimation of the Ah receptor-based potency of mixtures, combinations of PHAH congeners were also tested in the DR-CALUX assay. The DR-CALUX assay was performed according to Aarts *et al.* (1995) adapted for a 96-wells plate as described by Murk *et al.* (1998). Recombinant mouse hepatoma Hepa1c1c7 cells and rat H4IIE.pGudluc1.1 cells were prepared as previously described (Aarts *et al.*, 1995; Garrison *et al.*, 1996). Cells were seeded in 96-wells plates (Packard, Meriden, CT, USA) and grown in minimal essential medium (alfa-MEM) with 10% heat inactivated foetal calf serum. After culturing for 24 hours under standard conditions (37 °C, 5% CO_2) the cells were exposed to the test compounds. After 24 hours incubation the medium was removed from the cells, the cells were rinsed with 0.5×PBS buffer, treated with lysis buffer (10 mM Tris, 2 mM dithiolthreitol and 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetra-acetic acid, pH 7.8) and after adding the luciferin assay mix the light signal was measured in a Luminometer plate reader (Luminometer, Labsystem). Each concentration of a sample was tested at least in triplicate and each experiment was repeated twice. DMSO and TCDD were incorporated as a negative and a positive control respectively on each plate. The DMSO concentration in the cell medium did not exceed 0.1%.

Analysis of the gapjunctional intercellular communication

The inhibition of gapjunctional intercellular communication (GJIC) was analysed in the wild type mouse hepatoma cell line Hepa1c1c7 as described by De Haan *et al.* (1996). Cells were seeded in petri dishes (3.5 cm²; Greiner) and grown in minimal essential medium (alfa-

MEM) with 10% foetal calf serum. After culturing for 24 hours under standard conditions (37 °C, 5% CO_2), culture medium was replaced by exposure medium. Cells were exposed to the test compounds for 24 hours. Micro-injection experiments were performed in confluent, or nearly confluent cell cultures by a dye transfer technique as described by De Haan *et al.* (1994b). In each petri dish at least 20 injections with a 10% lucifer yellow CH solution in 0.33 M lithium chloride using a vertical injection system (Olympus IMT-2-SYF, PAES Ned.) were done. The injected cells were morphologically examined by phase contrast and fluorescence microscopy, directly after micro-injection. The number of fluorescent (communicating) cells was counted by eye using fluorescence microscopy, 10-15 minutes after lucifer yellow injection. Each individual exposure level was tested in duplicate or triplicate and at least two independent tests were done. The DMSO concentration in the cell medium never exceeded 0.1%.

Test compounds and mixtures

Two sets of experiments were performed. In the first set of experiments the interaction between PCBs and 2,3,7,8-TCDD was analysed in the EROD and DR-CALUX assay. Individual PCB congeners and the commercial PCB mixture Aroclor 1254 in concentration ranges of 0-66 µM, were co-administered with 50 pM TCDD. A concentration of 50 pM TCDD was chosen, because at this concentration nearly 90% of the maximum EROD and CALUX induction by TCDD was achieved. The following mono- and di-*ortho* PCB congeners were tested: PCB 105, PCB 114, PCB 118, PCB 156, PCB 47, PCB 52, PCB 101, PCB 128, PCB 153 and PCB 180. In addition to the combination experiments with 50 pM TCDD, PCB 114 was tested also with 5, 10 and 27 pM TCDD in order to study the inhibitory behaviour of a mono-*ortho* PCB with lower concentrations of TCDD. To study the possibility of interactive effects on the inhibition of GJIC two experiments were performed: 50 pM TCDD was co-administred with increasing concentrations of PCB 52 or Aroclor 1254.

In the second set of experiments induction of the CALUX activity and inhibition of GJIC by complex PHAH mixtures was tested. Because these mixtures were designed for rat experiments (Van der Plas *et al.*, 1999 *Chapter 4*, 2000a *Chapter 5*), the rat hepatoma cell line H4IIE.luc was chosen to determine the toxic equivalency (TEQ) in the CALUX assay. All PHAH mixtures were tested in a concentration range of 0-250 µM.

Two synthetic dioxin-like PHAH mixtures were studied, a PHAH+ and a PHAH- mixture, covering >90% of the TEQ in Baltic herring. The PHAH- mixture contained six congeners; 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 2,3,4,7,8-PeCDF, PCB 126, PCB 118 and PCB 156 in relative concentration ratios of 1, 3.3, 17, 61, 12800 and 1888 respectively. The PHAH+ mixture consisted of the PHAH- mixture to which PCB 153 was added, in order to study possible interactive effects with a di-*ortho*, non-dioxin-like PCB congener. The relative ratio of PCB 153:2,3,7,8-TCDD was 20000:1. The PHAH mixtures are described in detail by Van der Plas *et al.* (1999 *Chapter 4*).

The other tested PHAH mixtures were the commercial PCB mixture Aroclor 1260 and fractions thereof. Aroclor 1260 was fractionated into three fractions according to a method described by Athanasiadou *et al.* (1991) with slight modifications as described by Van der Plas *et al.* (2000a *Chapter 5*). Three fractions were obtained; a 2-4 *ortho* non-dioxin-like fraction (~90.3% of the total mass), a 1- *ortho* fraction (~6.6% of the total mass) containing mono and a trace of di-*ortho* PCBs and a 0- *ortho* dioxin-like fraction (~3.1% of the total mass) containing mainly non-*ortho* PCBs with a trace of mono-*ortho* PCBs.

Statistics

To calculate EC_{50} and IC_{50} values, a dose-response curve was fitted to the data using a the 1-site ligand binding equation $y=a_0x/(a_1+x)$. For all curve fitting the program Slide Write 4.0 was used. The calculation of relative potencies (REPs) for the PHAH mixtures was calculated on the basis of the CALUX data by interpolation of the dose-response curves of the mixtures on a standard curve of TCDD, according to Murk *et al.* (1996).

Data were analysed with the statistical package SPSS-PC 7.5. A Student Newman Keuls (SNK) test was used to analyse statistical significant differences between dose levels.

Results

EROD assay

In figure 2.1 the EROD dose-response curve for TCDD is presented. Data are expressed as percentage relative to the induction at 1 nM, which was 25.5 pmol resorufin/min×mg protein. On the basis of the dose-response curve a concentration of 50 pM was chosen for the combination experiments. A concentration of 1 nM was used as a positive control in the consecutive experiments.

Mono-ortho PCBs

All tested mono-*ortho* PCBs induced the EROD activity up to a plateau level of approximately 40-60% of the maximum induction reached by TCDD, with EC_{50} values ranging between 1 and 33 µM (Table 2.1). When the PCBs were co-administered with TCDD, the TCDD induced EROD activity was partially inhibited by the mono-*ortho* PCBs down to approximately the maximum induction level of the PCB itself. This is clearly illustrated in figure 2.2A, showing the dose-response curves of the EROD induction by PCB 118 alone and in the presence of TCDD. These 'mirror'-like curves as shown for PCB 118 were also observed for the other mono-*ortho* PCBs. IC_{50} values (Table 2.1) showed that PCB 105 and PCB 118 had the greatest inhibitory potency while PCB 156 was the least potent inhibitor of the mono-*ortho* PCBs tested. The order with which the tested mono-*ortho* PCBs were capable of inhibiting the TCDD induced EROD activity reflected their own potency to induce the EROD activity.
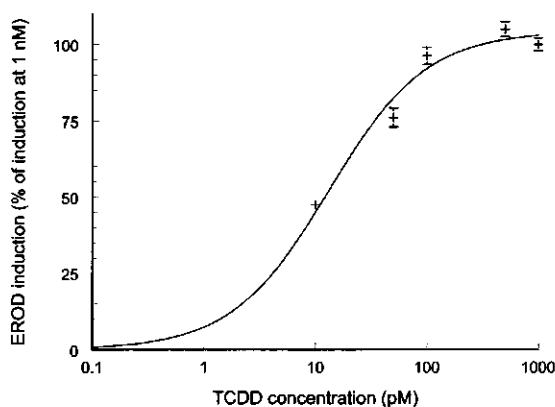
Figure 2.1 Dose effect curve of the EROD induction by 2,3,7,8-TCDD in hepa1c1c7 cells.

Data are presented as mean ± SE.

In order to study interactive effects at lower concentrations of TCDD and thus at lower parts of the EROD dose-response curve, TCDD concentrations ranging from 5 to 50 pM were co-administered with increasing concentrations of PCB 114 (Figure 2.2B). At the lowest concentrations of both TCDD and PCB 114, the effects of the individual compounds appeared to be additive. However, when the concentration difference between PCB 114 and TCDD became larger, the shape of the curve became similar to the curve of individually dosed PCB 114. From 27 pM TCDD onwards, the shape of the curve differed from the response curve of individually dosed PCB 114 and an antagonistic interactive effect was clearly visible.

Di-ortho PCBs

No EROD activity was found after exposure to any of the di-*ortho* PCBs (Table 2.1). However, all di-*ortho* PCBs antagonised the TCDD-induced EROD activity in a dose-dependent manner and with different potencies (Figure 2.2C, Table 2.1). IC_{50} values (Table 2.1) showed that the di-*ortho* substituted PCB 153 was the least potent inhibitor whereas PCB 128 was the most potent inhibitor of TCDD mediated EROD induction.

CALUX assay

To exclude the possibility of competitive inhibition of PHAH congeners with ethoxyresorufin in the EROD assay (as observed by Besselink *et al.*, 1998) some of the combined exposures were also tested in the CALUX assay. In addition, the CALUX assay was used to determine the relative toxic potency of the PHAH mixtures, designed for *in vivo* testing.
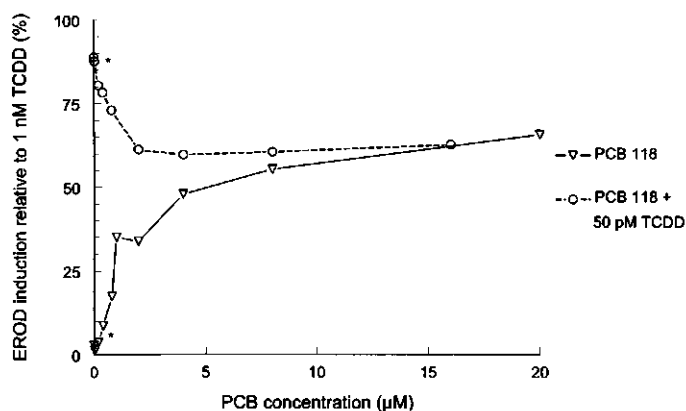
Figure 2.2A Dose effect curve of the EROD induction by individually dosed PCB 118 and co-administered with 50 pM TCDD, in hepa1c1c7 cells.
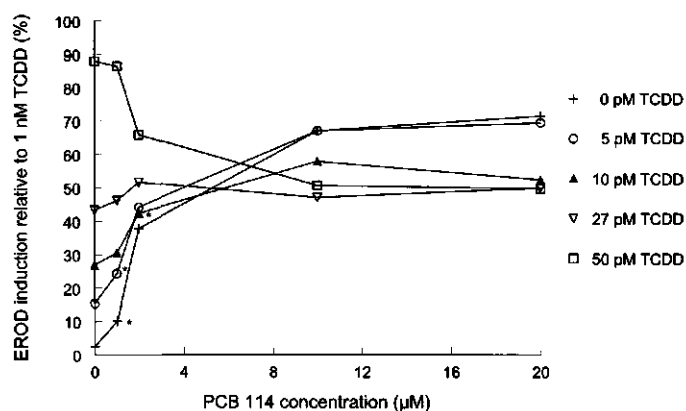
Figure 2.2B Dose effect curves of the EROD induction in hepa1c1c7 cells, after co-administration of different TCDD concentrations with PCB 114.
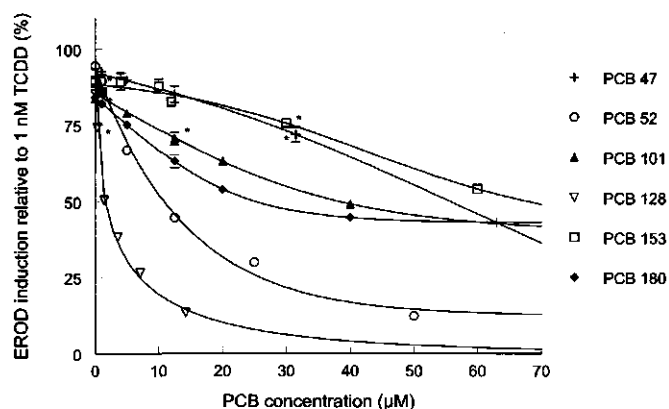
Figure 2.2C Dose effect curves of the EROD induction in hepa1c1c7 cells, after co-administration of 50 pM TCDD with different di-*ortho* PCBs.

Data are presented as mean ± SE. * From this data point onwards significantly different ($p<0.05$, SNK) from 0 μM PCB.

Table 2.1 EC_{50} values of mono-*ortho* and di-*ortho* PCBs for the induction of EROD activity and IC_{50} values for the inhibition of the EROD activity induced by 50 pM TCDD

PCB (IUPAC)	EROD		
	EC_{50} (µM)	$IC_{50,}$ (µM)	[PCB]/[TCDD]
2,3,3',4,4'-PeCB (105)	3	1	2×10^3
2,3,4,4',5-PeCB (114)	2	4	4×10^4
2,3',4,4',5-PeCB (118)	2	1	4×10^3
2,3,3',4,4',5-HxCB (156)	33	22	4×10^5
2,2',4,4'-TeCB (47)	n.d.	60	6.3×10^5
2,2',5,5'-TeCB (52)	n.d.	12	2×10^4
2,2',4,5,5'-PeCB (101)	n.d.	52	2.5×10^5
2,2',3,3',4,4'-HxCB (128)	n.d.	2	6×10^3
2,2',4,4',5,5'-HxCB (153)	n.d.	82	6×10^5
2,2',3,4,4',5,5'-HeCB (180)	n.d.	45	1×10^5
Aroclor 1254	3	2	2×10^4

n.d.=not detectable; n.a.=not analysed; IC_{50} is defined as the concentration inhibiting 50% of the EROD activity induced by 50 pM TCDD; [PCB]/[TCDD] is the concentration difference required for a significant inhibition of TCDD induced EROD activity.

Mono- and di-ortho PCBs

The tested mono-*ortho* PCBs all induced the CALUX activity (Figure 2.3A-C). Although the maximum induction of the CALUX activity reached by PCB 105 and PCB 118 was lower as compared to the maximum EROD activity induced by these congeners, the same trend was observed for the inhibition of the CALUX activity induced by TCDD.

The di-*ortho* PCB 180 induced the CALUX activity dose-dependent up to almost 16% of the maximum activity induced by TCDD, at the highest test dose of 40 µM (not shown). The other di-*ortho* PCBs tested did not show any activity for the CALUX induction. The CALUX activity induced by TCDD was antagonised by all di-*ortho* PCBs tested, with different potencies (Figure 2.3D).

PHAH mixtures

The CALUX activity of the dioxin-like PHAH mixtures (see 'Materials and methods' section for the composition) is shown in figure 2.4. Both the PHAH- mixture, without PCB

Table 2.2 EC_{50} values and relative potencies of the PHAH mixtures, Aroclor 1260 and its fractions for the CALUX activity.

Mixture	EC_{50} ($\mu g/ml$)	CALUX REP	Literature TEF value[a]
PHAH- mixture	9.3×10^{-3}	3.3×10^{-4}	9.7×10^{-4}
PHAH+ mixture	15.0×10^{-3}	2.0×10^{-4}	4.4×10^{-4}
Aroclor 1260	0.9	1.7×10^{-7}	4.6×10^{-6}
0-4 *ortho* fraction	n.d.	n.d.	4.6×10^{-6}
0-1 *ortho* fraction	1.7	8.2×10^{-7}	4.6×10^{-6}
0- *ortho* fraction	0.8	2.5×10^{-6}	0.2×10^{-6}
1- *ortho* fraction	1	2.2×10^{-7}	4.4×10^{-6}
2-4 *ortho* fraction	n.d.	n.d.	-

n.d. = not possible to determine. [a] TEF values for the individual congeners are based on WHO-TEFs of 1997 (Van den Berg *et al.*, 1998). PCB concentrations in Aroclor 1260 are when possible based on Van der Plas *et al.* (2000a *Chapter 5*) and otherwise on Leonards *et al.* (1995).

153, and the PHAH+ mixture, with PCB 153, induced the CALUX activity up to the maximum inducible level by TCDD. The REP_{CALUX} of the PHAH+ and PHAH- mixture was very close to the literature TEF value for these mixtures, within a factor of 3 (Table 2.2). Apparently, there were no interactive effects between the PHAH congeners in the synthetic PHAH mixtures.

Aroclor 1260 induced the CALUX activity to a maximum level of approximately 10% of the maximum activity induced by TCDD (Figure 2.5). No plateau was reached since a further increase of the Aroclor 1260 concentration resulted in cell death. A completely different picture is demonstrated with the separate fractions of Aroclor 1260. The 0-*ortho* fraction showed the highest CALUX activity. At the highest concentration tested the 0-*ortho* fraction showed an activity of 40% of the maximum induced activity by TCDD. The 1-ortho fraction showed an induction of 8% at the highest dose tested, while the 2-4 *ortho* fraction did not show any activity at all. Reconstitution of the fractions to the 0-4 *ortho* fraction, resulted clearly in an inhibitory effect of the CALUX activity by both the 1- and 2-4 *ortho* fraction. The reconstituted 0-4 *ortho* fraction had a similar dose-response curve as compared to Aroclor 1260, except that the induction of the 0-4 *ortho* fraction is a factor of 3 lower. The difference in induction between Aroclor 1260 and the 0-4 *ortho* fraction can possibly be explained by the absence of impurities such as PCDFs, which are lost during the fractionation of Aroclor 1260 (Athanasiadou *et al.*, 1991). Due to its low induction, no relative toxic potency (REP_{CALUX}) could be calculated for the 0-4 ortho fraction. The REP_{CALUX} for Aroclor 1260 and its fractions are shown in table 2.2. The REP values do not correspond with the literature TEF values,

which were based on GCMS-data (Van der Plas *et al.*, 2000a *Chapter 5*). This may be partly explained by the interactions between congeners in the fractions.

Micro-injection

In the microinjection experiments 1 nM TCDD was incorporated as a positive control on the basis of experiments of De Haan *et al.* (1994b, 1996), which were also performed at our laboratory. A GJIC dose-response curve of TCDD is presented in figure 2.6. In our experiments inhibition of the GJIC by TCDD at a dose level of 1 nM, ranged from 37% to 39% as compared to the vehicle control. An exception was the experiment with the 0-4 *ortho* fraction of Aroclor 1260, with an inhibition of the GJIC of only 27% (not shown).

Combination experiments

The inhibition of GJIC by individual PHAH congeners was determined by De Haan *et al.* (1994b, 1996), who observed GJIC inhibition by PCB 77 (3,3',4,4'-TeCB), 126, 169 (3,3',4,4',5,5-HxCB), 105, 114, 118, 156, 52, 128 and 153 (Table 2.3). For the combination experiments 50 pM TCDD was co-administred with increasing concentrations PCB 52 or Aroclor 1254. PCB 52 was chosen because it was shown to be a potent inhibitor of GJIC (De Haan *et al.*, 1996) and a strong antagonist of the EROD activity induced by TCDD. Aroclor 1254 had a weak partial agonisitic activity in the EROD assay (maximum induction of 22% compared to TCDD) and a strong partial antagonistic effect on the EROD activity induced by TCDD.

Individually dosed PCB 52 inhibited the GJIC down to 67% of the vehicle control (Figure 2.7A). Co-administration of PCB 52 and 50 pM TCDD resulted in a further reduction of the GJIC at the highest test concentrations of PCB 52, as compared to individually dosed PCB 52 or TCDD. However, the effect was not additive. Since the concentration difference between PCB 52 and TCDD was not high enough for antagonistic interactions (Table 2.1) between the congeners, it does not seem to be likely that the observed non-additivity is due to competition for Ah receptor binding. Aroclor 1254 appeared to be a weak inhibitor of GJIC (Figure 2.7B) with a maximum inhibition of approximately 20%. Co-administration of TCDD and Aroclor 1254 showed a reduction of the induced inhibition by TCDD, towards the maximum of Aroclor 1254.

PHAH mixtures

Both the synthetic PHAH mixtures were capable of inhibiting the GJIC (Figure 2.8). The PHAH- mixture showed a reduction of the GJIC of almost 40% at a dose level of 1000 nM (~331 μg/ml). The PHAH+ mixture showed a stronger inhibitory effect on the GJIC as compared to the PHAH- mixture and even exceeded the maximum inhibition of the GJIC by TCDD. The PHAH+ mixture inhibited the GJIC down to 55% of the control at the highest test dose of 2000 nM. The IC_{50} values for the PHAH- and the PHAH+ mixture were difficult to

Table 2.3 EC_{50} values and relative potencies of inhibition of GJIC in hepa1c1c7 cells

Congener	IC_{50} (nM)	REP[a]
3,3',4,4'-TeCB (77)[b]	5.5	0.25
3,3',4,4',5-PeCB (126)[c]	0.7	0.21
3,3',4,4',5,5'-HxCB (169)[b]	11	0.013
2,3,3',4,4'-PeCB (105)[c]	100	0.0014
2,3,4,4',5-PeCB (114)[c]	117	0.0011
2,3',4,4',5-PeCB (118)[c]	2.5	0.06
2,3,3',4,4',5-HxCB (156)[c]	28	0.005
2,2',5,5'-TeCB (52)[c]	54	0.003
2,2',3,3',4,4'-HxCB (128)[c]	2.5	0.06
2,2',4,4',5,5'-HxCB (153)[c]	$> 10^4$	$< 2 \times 10^{-4}$
PHAH- mixture	30	0.0016-0.0047 [d]
PHAH+ mixture	30-95	0.00055-0.0047 [d]
0-1 *ortho* fraction	>1 [e]	$< 1.6 \times 10^{-5}$
2-4 *ortho* fraction	2 [e]	$8 \times 10^{-6} - 24 \times 10^{-6}$ [d]
0-4 *ortho* fraction	>6 [e]	$< 2.7 \times 10^{-6}$

[a] Relative potency with regard to TCDD. EC50 values were used for the calculation. [b] Derived from De Haan *et al.* (1994b). [c] Derived from De Haan *et al.* (1996). [d] Based on an EC50 value for TCDD ranging from 0.05-0.15 nM (De Haan *et al.*, 1994b, 1996). [e]μg/ml

determine because no plateau was reached. The IC_{50} for the PHAH- was estimated on 30 nM. For the PHAH+ mixture the IC_{50} was estimated between 30 and 95 nM. Assuming an IC_{50} ranging from 0.05-0.15 nM for TCDD (De Haan *et al.*, 1994b; 1996), REP values can be estimated for the PHAH mixtures ranging from 1.6×10^{-3} to 4.7×10^{-3} for the PHAH- mixture and from 5.5×10^{-4} to 4.7×10^{-3} for the PHAH+ mixture (Table 2.3).

The 0-1 *ortho* fraction (Figure 2.9) of Aroclor 1260 did not inhibit the GJIC at concentrations up to 906 ng/ml (equivalent to 25 μM Aroclor 1260), at which dose level only a marginal CALUX induction was observed (Figure 2.5). The 2-4 *ortho* fraction showed a reduction of the GJIC with 25% at the highest test dose of 17 μg/ml (equivalent to 50 μM Aroclor 1260) and the reconstituted 0-4 *ortho* fraction showed a slight inhibition of 15% as compared to the control at the highest test dose of 6.2 μg/ml (equivalent to 16 μM Aroclor 1260). It is likely that a stronger inhibitory effect could have been found when higher test

doses were been used. However, it is noted that in the CALUX assay with the H4IIE cell line, cytotoxicity was observed > 5 μg/ml 0-4 *ortho* fraction. Although no morphological signs of cytotoxicity were observed in the micro-injection assay, it can not be excluded.


Discussion

The aim of the study presented here was to investigate the interactive effects between PHAHs *in vitro* on the EROD and CALUX activity, both based on an Ah receptor mediated mechanism of action, and on the GJIC, an *in vitro* parameter for tumour promotion. In addition, the relative toxic potency (REP) of complex PHAH mixtures originally designed for tumour promotion experiments (*Chapter 4,5*) was determined, as well as their ability to inhibit GJIC.

Interactions between individual congeners

Ah receptor based interactive effects between individual PHAH congeners were studied in the EROD and CALUX Ah-receptor reporter gene assay. For the interaction experiments 50 pM TCDD was co-administered with different concentrations of individual mono- and di-*ortho* PCBs. All tested mono- and di-*ortho* PCBs were found to inhibit the TCDD induced EROD and CALUX activity, with different potencies. The shape of the dose-response curves at different concentrations of TCDD suggests that antagonistic interactions observed between the mono-*ortho* PCBs and TCDD are based on competition at the Ah receptor level. The occurrence of antagonistic interactions between potent and less potent PHAH congeners was also found by others (Haake *et al.*, 1987; Astroff and Safe, 1989) and is consistent with results obtained for other ligand-induced transcription factors such as the steroid hormone receptors (Safe 1997/1998). Antagonism may only occur under the condition that the receptor is almost saturated with the ligand. This is illustrated by the experiments where different concentrations of TCDD were co-administered with PCB 114. The antagonistic effect between PCB 114 and TCDD is only clearly visible at the highest test dose of 50 pM TCDD, which induces the EROD activity up to 90% of the maximum TCDD inducible activity. *In vivo* antagonistic interactions were also observed at high concentrations of both the agonist and the partial agonist (Bannister *et al.*, 1987a; Haake *et al.*, 1987; Davis and Safe, 1989; Biegel *et al.*, 1989).

All tested di-*ortho* PCBs appeared to be capable of antagonising the EROD and CALUX activity induced by TCDD, despite the fact that they do not induce any activity by themselves. In agreement with our results, Aarts *et al.* (1995) demonstrated that PCB 52 (2,2',5,5'-TeCB) antagonised the *in vitro* luciferase expression (CALUX) and EROD activity induced by PCB 77 (2,2',3,3',4,4'-HxCB). Their results suggest that the antagonist blocks formation of the transcriptionally active nuclear AhR complex (Aarts *et al.*, 1995). Wölfle (1997/98) reported on antagonistic interactions between TCDD and PCB 153 for the
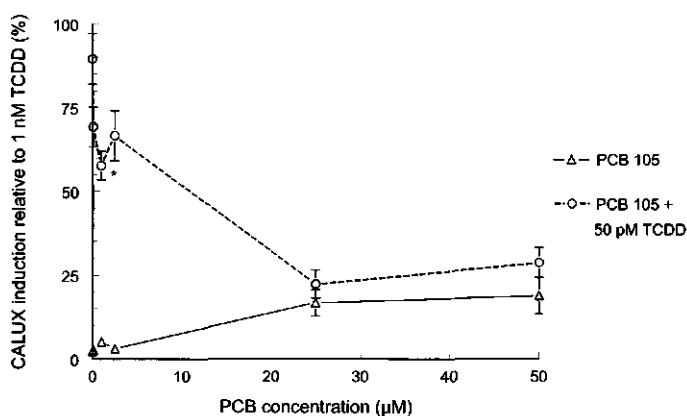
Figure 2.3A Dose effect curve of the CALUX induction by individually dosed PCB 105 and co-administered with 50 pM TCDD in recombinant hepa1c1c7 cells.
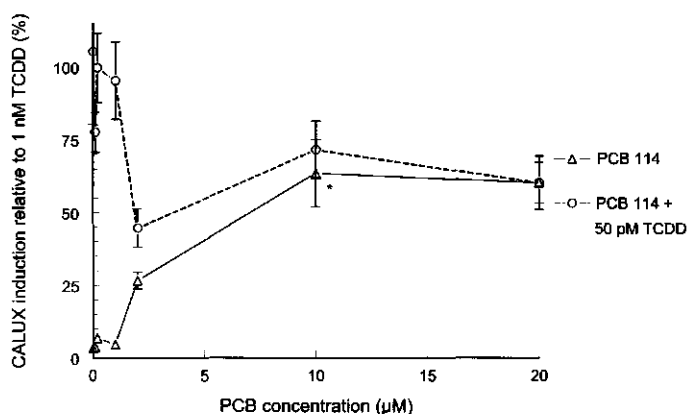
Figure 2.3B Dose effect curve of the CALUX induction by individually dosed PCB 114 and co-administered with 50 pM TCDD in recombinant hepa1c1c7 cells.

Data are presented as mean ± SE. * From this data point onwards significantly different (p<0.05, SNK) from 0 μM PCB.

enhancement *in vitro* (promotion) of malignant transformation of carcinogen-initiated C3H/M2 mouse fibroblasts. In this tumour promotion assay individually dosed TCDD or PCB 153 both induced a maximal effect, probably via Ah receptor dependent and independent mechanisms respectively (Wölfle, 1997/98). The antagonistic interaction between TCDD and PCB 153 in this tumour promotion assay is not understood.

In the microinjection assay for analysing inhibition of GJIC, combined exposure was performed with TCDD and PCB 52. PCB 52 was demonstrated to be a potent inhibitor of the GJIC *in vitro* (De Haan *et al.*, 1996) as well as a tumour promoter *in vivo* (Preston *et al.*, 1985). Combined exposure of PCB 52 and TCDD did result in a further decrease of the GJIC rather than in inhibition of the effect induced by TCDD. Although in the microinjection assay the concentration difference between PCB 52 and TCDD is <2×10^4, which is probably to low for antagonistic interactions between these congeners (see EROD results), it does not seem to be likely that at higher concentrations of PCB 52 the inhibition of GJIC by TCDD will be antagonised. Even at concentration levels where there is competition between TCDD and PCB 52 for Ah receptor binding, the inhibition of GJIC will possibly be at a maximum due to
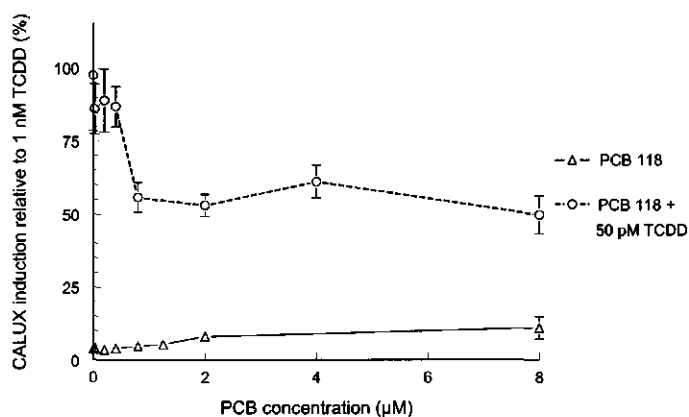
Figure 2.3C Dose effect curve of the CALUX induction by individually dosed PCB 118 and co-administered with 50 pM TCDD in recombinant hepa1c1c7 cells.
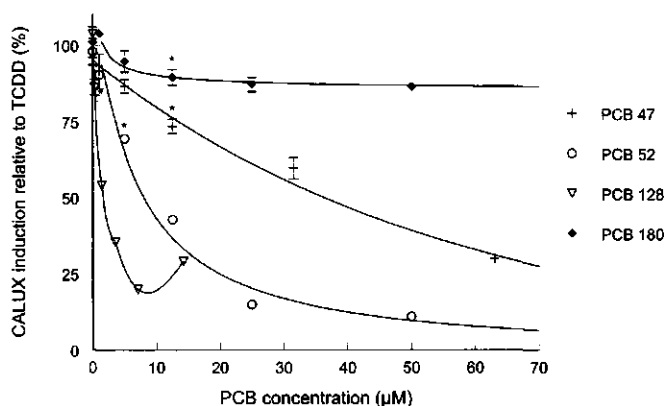

Figure 2.3D Dose effect curves of the CALUX induction in recombinant hepa1c1c7 cells, after co-administration of 50 pM TCDD with increasing concentrations of different di-*ortho* PCBs.

Data are presented as mean ± se. * From this data point onwards significantly different (p<0.05, SNK) from 0 µM PCB.

the GJIC inhibitory potential of PCB 52 itself. Co-administration of Aroclor 1254 and TCDD in the microinjection assay did show an antagonistic interactive effect, probably due to the low inhibitory potency of Aroclor 1254. Antagonising properties of Aroclor 1254 have been found before both *in vitro* and *in vivo* on several parameters, such as enzyme induction, immunotoxicity and teratogenicity (Bannister *et al.*, 1987a; Davis and Safe, 1989; Haake *et al.*, 1987).

Interactions between congeners in mixtures

In the CALUX assay no interactive effects were observed between the compounds in the synthetic dioxin-like PHAH-mixture (for the composition see materials and methods), neither was there an effect of PCB 153 on the CALUX activity induced by the PHAH+ mixture. In addition, it can be calculated on the basis of the minimum concentration difference needed between TCDD and the mono- or di-*ortho* PCB to antagonise the EROD activity induced by
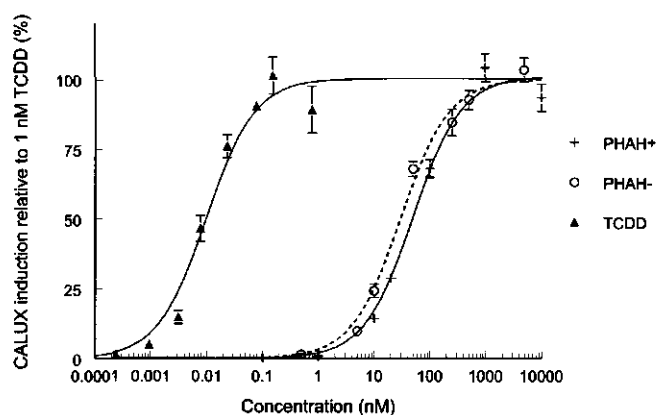
Figure 2.4 Dose effect curve of the CALUX induction in H4IIE.pGudluc1.1 cells by 2,3,7,8-TCDD, a dioxin-like synthetic PHAH-mixture (without PCB 153) or a PHAH+ mixture (with PCB 153).

Data are presented as mean ± std.
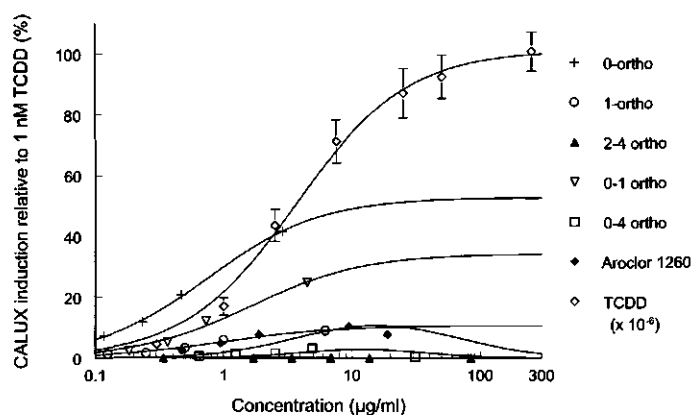
Figure 2.5 Dose effect curve of the CALUX induction in H4IIE.pGudluc1.1 cells by 2,3,7,8-TCDD (standard curve), the commercial PCB mixture Aroclor 1260, its fractions, and combinations thereof.

Data are presented as mean ± std.

TCDD, that antagonistic interactive effects between the partial and full agonists in the PHAH mixtures were not likely to occur. In the microinjection assay the synthetic PHAH+ mixture showed a larger inhibition on the GJIC compared to theoretically equipotent levels of the PHAH- mixture or TCDD. However, PCB 153 was found to be a very weak inhibitor of the GJIC (De Haan *et al.*, 1996) and at the dose levels tested the concentration of PCB 153 in the PHAH+ mixture is certainly not high enough to induce an effect on the GJIC. Also *in vivo* the PHAH+ mixture had a stronger effect on the development of altered hepatic foci as compared to the PHAH- mixture, which could not fully be explained by the tumour promotion potency of PCB 153 itself (Van der Plas *et al.*, 1999 *Chapter 4*). In this *in vivo* experiment PCB 153 was also found to increase the hepatic retention of the PHAH congeners in the mixture, thereby increasing the target dose (Van der Plas *et al.*, 1998b *Chapter 3*, 1999 *Chapter 4*).
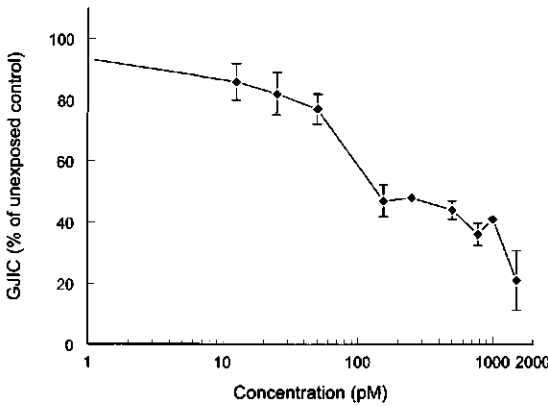
Figure 2.6 Dose effect curve of the inhibition of GJIC in hepa1c1c7 cells, by 2,3,7,8- TCDD. * From this data point onwards significantly different (p<0.05, SNK) from 0 µM 2,3,7,8-TCDD.

Data are presented as mean ± SE.
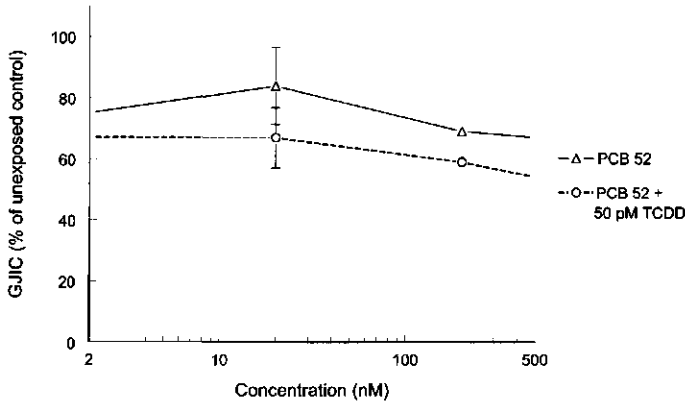
Figure 2.7A Dose effect curve of the inhibition of GJIC in hepa1c1c7 cells, by individually dosed PCB 52 and co-administered with 50 pM 2,3,7,8-TCDD.
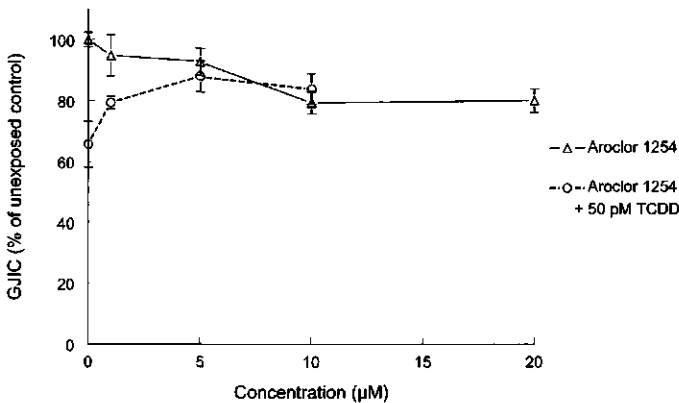
Figure 2.7B Dose effect curve of the inhibition of GJIC in hepa1c1c7 cells, by the commercial PCB mixture Aroclor 1254 and after co-exposure of Aroclor 1254 and 50 pM 2,3,7,8-TCDD.

Data are presented as mean ± SE.
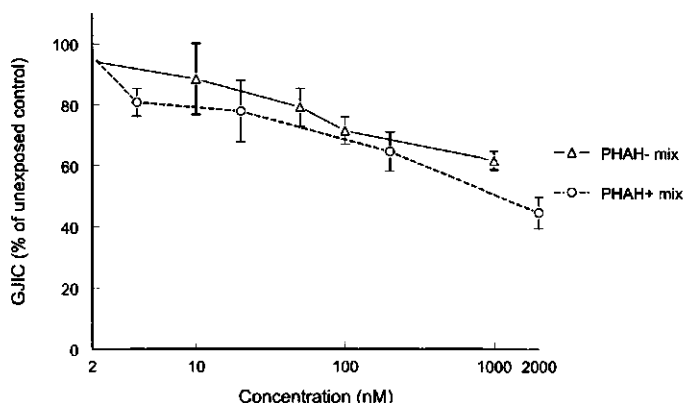
Figure 2.8 Dose effect curve of the inhibition of GJIC in hepa1c1c7 cells by a dioxin-like synthetic PHAH- mixture (without PCB 153) or a PHAH+ mixture (with PCB 153). Data are presented as mean ± SE.
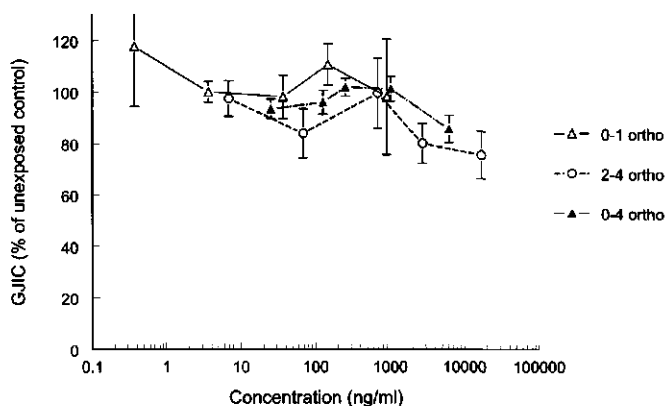
Figure 2.9 Dose effect curve of the inhibition of GJIC in hepa1c1c7 cells by the 0-1 *ortho*, the 2-4 *ortho*, and the 0-4 *ortho* fraction of the commercial PCB mixture Aroclor 1260. Data are presented as mean ± SE.

The 0- and the 1- *ortho* fraction of the commercial PCB mixture Aroclor 1260, both showed a higher induction of the CALUX activity as compared to Aroclor 1260 itself. Reconstitution of the fractions clearly resulted in a strong antagonising effect of the 1- *ortho* and especially of the 2-4 *ortho* fraction on the planar 0- *ortho* fraction. In the microinjection assay no interactive effects were observed between the fractions of the Aroclor 1260. It is concluded that the low maximum response induced in EROD and CALUX assays by Aroclor 1260 *in vitro*, is caused by antagonistic interactions between dioxin-like and non-dioxin-like congeners in the mixture. In a sub-chronic study in rats no such interactions between the different Aroclor 1260 fractions were observed (Van der Plas *et al.*, 2000a,b *Chapter 5, 6*). This was probably because the TEF value of the 0-1 *ortho* fraction and Aroclor 1260 mixture was to low to induce a strong effect on the EROD activity (Van der Plas *et al.*, 2000a *Chapter 5*) and thus to create a condition under which competition between congeners for Ah receptor binding might occur. However, another important observation in this respect is that *in vivo* the hepatic retention as percentage of the given dose differs between PHAH congeners (Abraham

et al, 1989; Wærn *et al*, 1991; Hemming *et al*, 1993; Van der Plas *et al.*, 1998b *Chapter 3*). This is explained by differences in affinity between the PHAH congeners for the hepatic binding protein CYP1A2 (Kedderis *et al.*, 1993; Andersen *et al.*, 1993; Van den Berg *et al*, 1994; DeVito *et al.*, 1998; Diliberto *et al.*, 1999). The consequence of differences in hepatic retention between PHAH congeners is that the initial relative concentrations in a mixture will change in favour of the Ah receptor agonists at the target tissue level. Thus the conditions under which antagonistic interactive effects between PHAHs will occur, are more difficult to reach *in vivo* as compared to the *in vitro* situation.

Inhibition of GJIC by PHAH mixtures

In the microinjection assay both synthetic PHAH mixtures had a strong inhibitory effect on the GJIC, at least 50% inhibition at 1-2 µM concentrations. Although it was difficult to calculate an EC_{50} value, the data suggest that the observed potency to inhibit the GJIC was within a factor of 10 of the theoretical potency, which was predicted on the basis of the TEF concept. In addition, the maximum GJIC inhibition of the PHAH mixtures was close to the maximum effect induced by TCDD. Also *in vivo* the dioxin-like PHAH mixtures were shown to be potent tumour promoters, within a factor of 2 of what was expected on the basis of the TEF concept (Van der Plas *et al.*, 1999 *Chapter 4*).

Only a small inhibitory effect on the GJIC could be found for the 2-4 *ortho* and the 0-4 *ortho* fractions (~25% inhibition), while the 0-1 *ortho* fraction had no effect on the GJIC. This suggests that the observed GJIC inhibition by the 0-4 *ortho* fraction is caused by the 2-4 *ortho* PCBs, which is in agreement with the results observed in a rat tumour promotion experiment performed by Van der Plas *et al.* (2000a *Chapter 5*). Van der Plas and co-workers (2000a *Chapter 5*) showed that the 2-4 *ortho* PCBs were responsible for approximately 80% of the tumour promotive capacity of Aroclor 1260. In the CALUX assay the 0- *ortho* fraction induced the CALUX activity up to 40%, indicating that this fraction and thus also the 0-1 *ortho* fraction contains dioxin-like compounds. It is therefore assumed that at higher doses of the 0-1 *ortho* fraction an effect on the GJIC would have been observed.

Inhibition of GJIC by di-*ortho* substituted PCBs was also demonstrated by others (Swierenga *et al.*, 1990; Hemming *et al.*, 1991; De Haan *et al.*, 1994b, 1996). In hepa1c1c7 cells the relative potency for inhibiting the GJIC of for instance PCB 52 and PCB 128 (2,2',3,3',4,4'-HxCB) was shown to be in the same order as for the mono- and non-*ortho* PCBs (De Haan *et al.*, 1996). In addition, some of the methyl-sulfonyl metabolites of PCBs, which may be formed from the 2-4 *ortho* PCBs, were found to inhibit the intercellular communication *in vitro* (Kato *et al.*, 1998). It is assumed that the Ah receptor pathway is involved in inhibition of the GJIC by TCDD and dioxin-like compounds (De Haan *et al.*, 1994b; Baker *et al.*, 1995). However, there is evidence that the mono- and di-*ortho* PCBs modulate their effect on GJIC via a different, Ah receptor independent, pathway (Swierenga *et al.*, 1990; Hemming *et al.*, 1991; De Haan *et al.*, 1996; Bager *et al.*, 1997). The mechanism of this non-Ah receptor pathway is unclear at the moment. The *in vitro* results are in accordance

with the observation that both dioxin-like and non-dioxin-like PCBs, often referred to as phenobarbital-like, can act as tumour promoters *in vivo* (Preston *et al.*, 1985; Hemming *et al.*, 1993; Bager *et al.*, 1995; Haag-Grönlund *et al.*, 1997a, 1997b, 1998). In addition to data from De Haan *et al.* (1996) on the inhibition of the GJIC by PHAHs, our data suggest that the hepa1c1c7 cell line might be a useful *in vitro* model for studying the capacity of both individual and complex mixtures of PHAHs to act as a tumour promoter *in vivo*. However, the *in vitro* data presented here are only preliminary data and more experiments would be necessary to draw firm conclusions.

In summary, *in vitro* non-additive antagonistic interactions (on the EROD and CALUX activity as well GJIC inhibition) occurred between agonists and partial or non-agonists, possibly at Ah receptor level. These non-additive antagonistic interactions seem to be only possible in case the compound concentrations are high enough to induce at least a sub-maximal effect (i.e. induction of EROD and CALUX activity or inhibition of GJIC) and, when the concentration difference between the potent and less potent agonist is large enough for competition for Ah receptor binding. In environmental exposure to PHAHs these conditions will be seldomly reached and therefore antagonistic interactions are not likely to play a role *in vivo* in real life situations. In case both PHAH congeners are strong inducers of an effect, whether or not via different mechanisms, additivity is more likely to occur. The *in vitro* potency of PHAH mixtures to inhibit the GJIC was largely in agreement with the tumour promotion properties *in vivo*. However, more research is needed to elucidate the predictive value of this parameter for the tumour promotion potency *in vivo*.

Acknowledgements

CHAPTER 3

Toxicokinetics of an environmentally relevant mixture of dioxin-like polyhalogenated aromatic hydrocarbons (PHAHs) with or without a non-dioxin-like PCB in a sub-chronic exposure study in female Sprague Dawley rats

Abstract

Female Sprague Dawley rats were treated subcutaneously for 20 weeks with an environmentally relevant mixture of dioxin-like PHAHs with (PHAH+) or without (PHAH-) 2,2',4,4',5,5'-hexachlorobiphenyl. The hepatic retention (% of given dose) of the various PHAH congeners differed considerably and in the following order: 2,3,4,7,8-pentachlorodibenzofuran (30.5-43.1%), 3,3',4,4',5-pentachlorobiphenyl (12.8-17.6%), 1,2,3,7,8-pentachlorodibenzo-p-dioxin (6.9-10.8%), 2,3,7,8-tetrachlorodibenzo-p-dioxin (3.2-4.5%), 2,3,3',4,4',5-hexachlorobiphenyl (1.0-1.7%), 2,2',4,4',5,5'-hexachlorobiphenyl (0.5-0.8%) and 2,3',4,4',5-pentachlorobiphenyl (0.2-0.4%). A decrease of the hepatic retention of 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD and 2,3,4,7,8-PeCDF was found at increasing doses of the PHAH+ mixture. 2,2',4,4',5,5'-Hexachlorobiphenyl increased the hepatic retention (1.3-2 times) of all congeners in the PHAH+ group, compared to the TEQ equivalent dosed PHAH-group. No interactions were observed on the ethoxyresorufin-O-deethylase activity.

Introduction

Polychlorinated biphenyls (PCBs), polychlorodibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and other polyhalogenated aromatic hydrocarbons (PHAHs) are ubiquitously present in the environment. Their toxicity, which is characterized by, e.g. body weight loss, thymic atrophy, impairment of immune responses, hepatotoxicity, teratogenicity and carcinogenicity (Safe, 1990), was intensively studied over the last 25 years.

PCBs and PCDDs have been considered to be weak complete hepatocarcinogens (Kimbrough *et al.*, 1975; Kociba *et al.*, 1978). More recent studies primarily showed a potent tumour promoter activity for several chlorinated biphenyls, dioxins and furans (Pitot *et al.*, 1980; Silberhorn *et al.*, 1990; Wærn *et al.*, 1991; Hemming *et al.*, 1993; Haag-Grönlund *et al.*, 1997a,b). Most studies performed to date have focussed on the exposure to single congeners with a dioxin-like, Ah receptor mediated toxicity. However, also some of the non-dioxin-like, di-ortho substituted PCBs were found to possess a tumour promoting activity in the liver (Hemming *et al.*, 1993). In addition, interactive effects on tumour promoting activity were reported between non-dioxin-like and planar, dioxin-like PHAH congeners (Sargent *et al.*, 1991; Bager *et al.*, 1995). Interactions between congeners have also been reported by several other investigators with respect to, e.g. immunotoxicity (Biegel *et al.*, 1989; Davis and Safe, 1989), teratogenicity (Zhao *et al.*, 1994) and hepatotoxicity (Yao and Safe, 1989; Van Birgelen *et al.*, 1996a). It is suggested that some of the observed interactive effects may be due to changes in liver deposition as a result of interactions between congeners at the toxicokinetic level (Biegel *et al.*, 1989; De Jongh *et al.*, 1993a,b).

The overall objective of our studies is to determine the tumour promotion capacity of complex mixtures of PCDDs, PCDFs and PCBs, relevant for human exposure. To study the possible interactive effects of di-ortho PCBs with the tumour promotion activity of planar PHAHs, a mixture was designed covering over 90% of the total dioxin-like toxicity (TEQs) in Swedish herring. This dioxin-like PHAH mixture was combined with or without 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153) as a di-ortho PCB following 20 weeks of treatment. Here we will discuss the toxicokinetic data and ethoxyresorufin-O-deethylase (EROD) activity as an indication for dioxin-like effects in the liver.

Materials and Methods

Chemicals

N-nitrosodiethylamin (NDEA) was obtained from Fluka (Fluka Chemie, Buchs, Switzerland). 3,3',4,4',5-Pentachlorobiphenyl (PCB 126), 2,3',4,4',5-pentachlorobiphenyl (PCB 118), 2,3,3',4,4',5-hexachlorobiphenyl (PCB 156) and 2,2'4,4',5,5'-hexachlorobiphenyl (PCB 153) were kindly provided by Prof. Bergman (Department of Environmental Chemistry, Stockholm University, Stockholm, Sweden). 1,2,3,7,8-Pentachlorodibenzo-*p*-dioxin (PeCDD)

was obtained from Wellington Laboratories (Canada), 2,3,4,7,8-pentachlorodibenzofuran
(PeCDF) was a gift from Prof. S.H. Safe (college of Veterinary Physiology and
Pharmacology, Texas, A&M University, USA) and 2,3,7,8-tetrachlorodibenzo-p-dioxin
(TCDD) was purchased from RADIAN CIL, inc. (USA). All compounds had a purity > 99%.
For animal exposure all compounds were dissolved in corn oil (Albert Heijn, the
Netherlands).

2,3,7,8-[^{13}C]-TCDD and 3,3',4,4',5-[^{13}C]-pentachlorobiphenyl ([^{13}C]-PCB 126) were
commercially obtained from Cambridge Isotope Laboratories (Woburn, MS, USA) and
1,2,3,7,8-PeCDF, 2,3',4,4',5,5'-hexachlorobiphenyl (PCB 167) and 2,3,3',5,6-
pentachlorobiphenyl (PCB 112) from Ehrenstorfer (Augsburg, Germany). Hexane, toluene,
dichloromethane and nonane, used for the extraction and clean up procedure of the GC-MS
analysis were of nanograde quality and obtained from J.T. Baker (Deventer, The Netherlands).
Silica gel 60 extra pure 70-230 mesh ASTM, NaOH and Na$_2$SO$_4$ were obtained from E.
Merck (Darmstadt, Germany). Aluminum B super I was bought from ICN Biomedicals
(Eschwege, Germany).

NADPH was obtained from Boehringer Mannheim (Mannheim, Germany), bovine-
serum-albumin (BSA) was from Sigma Chemical Company (St. Louis, MO, USA). Resorufin
was obtained from Janssen Chimica (Geel, Belgium) and ethoxyresorufin was synthesised
according to Mayer *et al.* (1977). A Bradford Protein Assay kit was obtained from Bio-rad
(Hercules, California, USA).

Mixtures

The mixture containing solely the dioxin-like PHAHs is referred to as PHAH-. This
PHAH- mixture was composed of 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 2,3,4,7,8- PeCDF, PCB
126, PCB 118 and PCB 156 in a relative ratio based on Swedish herring (Table 3.1). PCB 153
was added to an aliquot of the PHAH- mixture which is referred to as the PHAH+ mixture
(Table 3.1). Both mixtures covered more than 90% of the TEQ in Swedish herring. The
concentrations of the individual congeners in the mixture were confirmed by gas
chromatography/mass spectrometry (GC-MS) by the State Institute for Quality Control of
Agricultural Products (RIKILT-DLO) Wageningen, the Netherlands.

Animal experiment

The treatment protocol used in this study was based on the altered hepatic foci (AHF)
tumour promotion protocol introduced by Pitot *et al.* (1978). Young female Sprague Dawley
rats (Møllegaard Breeding Centre Ltd., Denmark), about three weeks old at the day of arrival,
were kept in wire bottom, stainless steel cages in groups of four animals under standard
conditions (12 hr light/dark cycle, temperature 22 °C, humidity 55%) and fed ad libitum. After
three weeks of acclimatisation the tumour initiation treatment was started by removing $^2/_3$ of
the liver (partial hepatectomy; PH) under ether anaesthesia followed by an i.p. injection with
NDEA (30 mg/kg) 24 hours after PH.

Table 3.1 Composition of the PHAH mixtures

| Congener | Relative level (weight base); | | TEF[a] |
	PHAH- mixture PCB 153	PHAH+ mixture + PCB 153	
2,3,7,8-TCDD	1	1	1
1,2,3,7,8-PeCDD	3.3	3.3	1
2,3,4,7,8-PeCDF	17	17	0.1
PCB 126	61	61	0.1
PCB 118	12800	12800	0.0001
PCB 156	1888	1888	0.0005
PCB 153	-	20000	0.00005

The composition of the mixtures is based on Swedish herring oil and covers more than 90% of the TEQs in Swedish herring. [a] TEF values for 1,2,3,7,8-PeCD, 2,3,4,7,8-PeCDD, PCB 126 and PCB 153 were based on tumour promotion data (Wærn et al., 1991; Hemming et al., 1993, 1995). TEF values for PCB 118 and PCB 156 were based on a report of a WHO meeting in 1993 (Ahlborg et al., 1994).

The tumour promotion treatment was started six weeks after the initiation treatment and consisted of a weekly subcutaneous (s.c.) injection with the test compounds during twenty weeks. One group (n=18) was dosed with corn oil as a vehicle control (1 ml/kg bw/week) and one group (n=12) with 2,3,7,8-TCDD (1 µg TEQ/kg bw/week) as a positive control. The experimental groups (n=10) were given the PHAH+ mixture at a dose level of 2, 1 or 0.5 µg TEQ/kg bw/week, or the PHAH- mixture at a dosage level of 0.93 µg TEQ/kg bw/week. The first dose was a loading dose which was five times higher than the maintaining dose as indicated before. Bodyweight, food and water consumption were recorded weekly.

One week after the last injection the animals were sacrificed under ether anaesthesia by using orbital punction for blood collection, followed by decapitation. The liver was collected, part of the liver was stored at -80 °C for gas chromatography and mass spectrometry (GC-MS) analysis, whereas the rest of the liver was stored at -80 °C for biochemical analysis or fixed for immunohistochemistry. The thymus, spleen, kidneys, heart, lungs and the brains were collected and stored at -80 °C until further analysis.

Extraction and Clean up procedure
Liver tissue (1 to 2 grams) was freeze dried for 24 hours and Soxhlet extracted for 20 hours. After Soxhlet extraction 3 or 30 ng [13]C 2,3,7,8-TCDD, 120 ng 1,2,3,7,8-PeCDF, 300 ng ^{13}C PCB 126, 1500 ng PCB 167 and 2000 ng PCB 112 were added as internal standards. Lipid content was determined by drying the Soxhlet residue at 100 °C for 30 minutes. This Soxhlet extract was dissolved in 5 ml hexane.

The first clean up column (30 x 0.9 id.) consisted of 1 gram 33% NaOH and 3 gram

22% H_2SO_4 on silica and 1 gram Na_2SO_4. The Soxhlet extract was added to the column and eluted with an additional 30 ml hexane. This hexane fraction was evaporated under a gently nitrogen flow and the residue was again dissolved in a small volume (< 0.5 ml) toluene. This extract was directly used for GC-MS analysis after adding 40 ng PCB 118 as an external standard. Some extracts were not sufficiently clean to directly for GC-MS analysis in which cases a second clean up column was used. This second column (30 x 0.9 id.) consisted of 5 gram aluminum oxide and 1 gram Na_2SO_4. The aluminum oxide was activated by heating at 180 °C during 16 hours. The earlier obtained extract from column 1 was dissolved in 5 ml hexane and added to the column. Subsequently, the PCB fraction was eluted from the column with 20 ml hexane/dichloromethane (95:5 v/v) and the dioxin/dibenzofuran fraction with 50 ml hexane/dichloromethane (60:40 v/v). After adding a few drops of nonane both fractions were allowed to evaporate, 1.9 ml toluene was added to the PCB fraction with PCB 169 (10 ng/µl) used as an external standard. The dioxin and dibenzofuran fraction was dissolved in 50 µl toluene with PCB 154 (10 ng/µl) added as an external standard. Different PCB standards had to be used because of differences in retention time and the time windows selected during GC-MS analysis.

GC-MS analysis.

All compounds were analysed on a Carlo Erba QMD 1000 high resolution gaschromatograph/low resolution quadruple mass spectrometer operating in the electron impact mode (70 eV, dwell time 0.1-0.05 s, span 0.5 amu). Source temperature 225 °C. For each congener the M^+, M^{+2} and M^{+4} were scanned on the selective ion mode. A J&W fused silica capillary column was used with stationary phase DB 5.625 (30 m, 0.32 mm id., film thickness 0.35 microns). The following temperature program was used: T_1 120 °C for 1 minute, rate 20 °C/min, T_2 280 °C for 4 minutes. Helium was used as a carrier gas at 50 Kpa.

Preparation of liver microsomes, protein measurements and EROD activity

Livers were homogenised on ice in a Potter tube (± 10 strokes) with 0.01 M Tris-HCl buffer, pH 7.4. Microsomes were prepared by differential centrifugation of liver homogenates at 9,000 g for 30 minutes, the resulting supernatant was centrifuged at 105,000 g for 90 minutes, and the pellet was resuspended in a 0.01 M Tris-HCl buffer, pH 7.4. Microsomes were stored at -80 °C until further analysis.

Protein concentrations in the microsomes were determined according to the method of Bradford (1976), adapted for 96-well plates. A BSA standard curve was made with protein concentrations ranging from 5 to 40 µg/ml. Hundred sixty microliters of standard solution (blank) or microsomes and 80 µl of a diluted Bradford Protein Assay reagens were pipetted in each well and after 10 minutes the absorbance was read at 595 nm in a Molecular Devices Micro plate Reader.

Ethoxyresorufin-*O*-deethylase (EROD) activity was measured in liver microsomes according to Burke *et al.* (1977), adapted for use with 96-well plates and a fluoro

spectrophotometric plate reader (Cyto Fluor 2350, Millipore) as described by Morse *et al.* (1996b).

Statistical analysis

Data were analysed by one-way ANOVA and the Student-Newman-Keulman test (SNK) to determine the statistical significance of differences among individual group means, with the statistical package SPSS/PC+.

In order to study possible interactive effects of the congeners administered in the mixtures, Partial Least Squares (PLS) Analysis was performed using the Software for Chemometric Analysis (version 1.1, Minitab, State College, PA, USA). Hepatic concentrations of all individual congeners as well as liver fat content and administered dose were used as descriptors. Hepatic retention, expressed as compound in liver as fraction of the dose, was chosen as the dependent variable.

Results

Liver weights and fat content

One animal of the mixture group with PCB 153 (PHAH+) dosed at 2 μg TEQ/kg bw/week, died during the treatment period but this did not seem to be related with the treatment. A significant treatment related effect was observed on body weight gain (26% reduction) for the highest dosed PHAH+ group compared to the corn oil group (data not shown). No other obvious signs of toxicity were observed. The liver weights of all treatment groups were significant increased compared to the corn oil group (Table 3.2) and a dose dependent increase of the liver weight was found in the groups treated with increasing doses of the PHAH+ mixture. The liver fat content in the PHAH treated groups was found to be significantly increased, 1.5-2 times, as compared to corn oil controls. The highest fat concentrations of 76 and 72 mg/g liver were observed in the PHAH+ groups dosed at 1 and 2 μg TEQ/kg bw/week, respectively.

Liver retention of the PHAH congeners

Remarkable differences were observed among the PHAH congeners in liver deposition presented as percentage of the given dose (Table 3.3). The hepatic retention was lowest for the mono- and di-ortho substituted PCBs, 0.2-0.4% for PCB 118, 0.5-0.8% for PCB 153 and 1-1.7% for PCB 156. The planar PCB 126 showed a much higher hepatic retention (12.8-17.6%) as compared to the other PCB congeners and was even higher than the deposition of TCDD (3.2-4.5%) and PeCDD (6.9-10.8%). The highest hepatic retention was observed for the PeCDF congener, 30.5-43.1%.

The PHAH+ mixture was tested in three different doses, 0.5, 1, and 2 μg TEQ/kg bw/week. An increase of the total amount of PHAH congeners in the liver was observed with

increasing doses of PHAH+ mix (Table 3.2). However, a decrease of the hepatic retention as percentage of the given dose was observed for 2,3,7,8-TCDD, 1,2,3,7,8- PeCDD and 2,3,4,7,8-PeCDF with increasing doses of PHAH+ mix (Table 3.3).

A comparison between the equivalent dosed PHAH+ and the PHAH- group (~1 µg TEQ/kg bw/week), showed a 1.2-3 times higher amount of all congeners in the liver in the PHAH+ group compared to the PHAH- group (Table 3.2). The hepatic retention as precentage of the given dose of all PHAH congeners, except for 2,3,7,8-TCDD, was increased significant 1.3-2 times in the group given the PHAH+ mixture compared to the PHAH- group (Table 3.3).

Partial Least Square (PLS) analysis of the data from the individual animals exposed to the PHAH+ mixture confirms the decrease of hepatic retention as % of the given dose of all congeners with increasing doses of 0.5, 1 or 2 µg TEQ/kg/week (Table 3.4). The concentration of PCB 153 in the liver is positively correlated with the hepatic retention of all compounds except PCB 156, on which its effect seems not substantial. PeCDF and PCB 126 concentrations generally show a positive correlation with hepatic retention of all congeners. The correlation of PCB 118 concentrations with hepatic retention of other congeners is also positive but much smaller than observed for PeCDF and PCB 126. In contrast, hepatic concentrations of TCDD and PCB 156 are negatively correlated with hepatic retention of all other congeners. The correlation of PeCDD with hepatic retention varies for each congener in direction as well as strength.

Lipid content of the liver is positively correlated with the hepatic retention for PCBs 118, 126 and 156, but not for TCDD, PeCDF and PeCDD.

EROD activity

EROD activity was increased in all PHAH treated groups as compared to the corn oil control group (Figure 3.1). A 90-fold induction was found for both the PHAH+ and PHAH-treated animals dosed at 1 µg TEQ/kg bw/week. The EROD inducing potency of the PHAH mixtures did not differ significantly from the equipotently dosed 2,3,7,8-TCDD group. The EROD activity in the highest dose PHAH+ group (2 µg TEQ/kg bw/week) was significantly increased compared to the other groups.

In order to evaluate a correlation between the internal TEQ dose in the liver and the EROD activity on an individual animal base, relative potencies for all congeners were calculated based on internal congener liver concentrations and liver microsome EROD activity as reported by Wærn *et al.* (1991), Hemming *et al.* (1993) and Haag-Grönlund *et al.* (1997a,b). There was however only a very weak correlation, $R=0.2$, between the internal TEQ dose in the liver and the EROD activity of the individual PHAH+ exposed animals (results not shown).

Table 3.2 Liver weights, fat contents and deposition of PHAH congeners in the liver of female Sprague Dawley following a twenty week exposure period

Groups dose/kg bw/week	Liver weight (g)[d]	Fat content (mg/g)[e]	Total amount of congeners in the liver						
			TCDD (ng)	PeCDD (ng)	PCDF (µg)	PCB 126 (µg)	PCB 118 (µg)	PCB 156 (µg)	PCB 153 (µg)
Corn oil 1 ml	8.8 ± 1.0	39 ± 18	n.d./n.a.	n.d./n.a.	n.d./n.a.	n.d./n.a.	n.d./n.a.	n.d./n.a.	n.d./n.a.
TCDD 1 µg TEQ	11.0 ± 1.6^a	67 ± 9^a	286.5 ± 71.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PHAH+ 0.5 µg TEQ	$10.5 \pm 0.6^{a,b}$	59 ± 10^a	9.9 ± 2.4	77.8 ± 15.5	1.6 ± 0.2	2.3 ± 0.5	9.0 ± 6.2	5.1 ± 1.4	23.5 ± 8.70
PHAH+ 1 µg TEQ	11.6 ± 1.1^a	$76 \pm 18^{a,c}$	17.7 ± 4.1	128.0 ± 22.1	3.0 ± 3.3	4.7 ± 0.8	22.9 ± 11.5	14.2 ± 3.8	71.8 ± 30.5
PHAH+ 2 µg TEQ	12.2 ± 1.1^a	72 ± 14^a	30.7 ± 6.9	198.9 ± 31.1	5.3 ± 0.6	8.7 ± 1.4	34.0 ± 19.7	24.3 ± 6.2	116.8 ± 39.8
PHAH- 1 µg TEQ	$10.7 \pm 1.2^{a,b}$	66 ± 11^a	14.3 ± 3.0	101.0 ± 14.9	2.3 ± 0.3	3.4 ± 0.5	9.9 ± 3.9	8.6 ± 1.9	n.d.

Data are presented as the mean ± standard deviation, n.d. = not detected, n.a. = not analysed, n.d./n.a. = only some randomly taken samples were analysed.
[a] Significantly different from the Corn oil group ($p<0.05$ SNK test). [b] Significantly different from the mixture group with PCB 153 (PHAH+), dosed at 2 µg TEQ/kg bw/week ($p<0.05$ SNK test). [c] Significantly different from the mixture group with PCB 153 (PHAH+), dosed at 1 µg TEQ/kg bw/week ($p<0.05$ SNK test). [d] Liver wet weight. [e] Per gram liver wet weight

Table 3.3 Liver retention expressed as percentage of the given dose of PHAH congeners in female Sprague Dawley rats

Groups dose/kg bw/wk	TCDD	PeCDD	PeCDF	PCB 126	PCB 118	PCB 156	PCB 153
Corn oil 1 ml	n.d./n.a.	n.d./n.a.	n.d./n.a.	n.d./n.a.	n.d./n.a.	n.d./n.a.	n.d./n.a.
TCDD 1 µg TEQ	4.2 ± 0.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PHAH+ 0.5 µg TEQ	4.5 ± 1.1^a	$10.8 \pm 2.0^{a,b,c}$	$43.1 \pm 5.5^{a,b}$	17.1 ± 3.6^a	0.3 ± 0.2	1.2 ± 0.3^c	0.5 ± 0.2
PHAH+ 1 µg TEQ	4.1 ± 1.0	$9.0 \pm 1.6^{a,b}$	40.6 ± 4.8^a	17.6 ± 3.4^a	0.4 ± 0.2^a	1.7 ± 0.5^a	0.8 ± 0.4
PHAH+ 2 µg TEQ	3.6 ± 0.7	7.0 ± 1.0	36.4 ± 4.0^a	16.6 ± 2.5^a	0.3 ± 0.2	1.5 ± 0.3^a	0.7 ± 0.2
PHAH- 1 µg TEQ	3.2 ± 0.6	6.9 ± 1.0	30.5 ± 4.2	12.8 ± 1.8	0.2 ± 0.1	1.0 ± 0.3	n.d.

Data represent mean ± standard deviation. Doses are given per kg bodyweight per week. PHAH+ is the mixture with PCB 153 given in three different dosages and PHAH- is the mixture given without PCB 153. n.d. = not detected, n.a. = not analysed, n.d./n.a. = only some randomly taken samples are analysed. [a] Significantly different from the mixture group without PCB 153 (PHAH-), dosed at 1 µg TEQ/kg body weight/week (p<0.05 SNK test).
[b] Significantly different from the mixture group with PCB 153 (PHAH+), dosed at 2 µg TEQ/kg body weight/week (p<0.05 SNK test), [c] Significantly different from the mixture group with PCB 153 (PHAH+), dosed at 1 µg TEQ/kg body weight/week (p<0.05 SNK test)

Table 3.4 Partial least square analysis of the liver retention data of the PHAH congeners in female Sprague Dawley rats

Response variable	final R^2	Predictor Importances of the descriptors								
		TCDD	PeCDD	PeCDF	PCB 126	PCB 118	PCB 156	PCB 153	TEQ dose	fat content
TCDD	0.7913	(0.9616)	-0.05917	0.2183	0.8473	0.05042	-0.7764	0.3097	-1.875	0.08163
PeCDD	0.8322	-0.5426	(0.8902)	0.2843	1.096	0.06179	-0.8732	0.4010	-1.878	0.03222
PeCDF	0.8322	-0.6747	-0.1433	(2.346)	0.7382	0.1972	-0.5976	0.2887	-2.509	0.004741
PCB 126	0.8208	-0.8860	0.05257	0.2806	(3.300)	0.1307	-0.9913	0.2276	-2.331	0.2174
PCB 118	0.8646	-0.4247	0.1375	0.2488	0.9507	(0.9302)	-1.080	0.2280	-0.8668	0.3914
PCB 156	0.8623	-0.4762	-0.3631	0.8797	0.4183	0.1274	(1.074)	-0.01552	-1.462	0.2418

Response variable is the hepatic retention expressed as percentage of the given PHAH dose. Used descriptors were the hepatic concentration of all individual compounds (ng/g), the administered TEQ dose (μg TEQ/kg body weight/week) and the liver fat content (mg/g). The predictor importance of the PHAH congener which is the response variable in the equation is given in parenthesis.
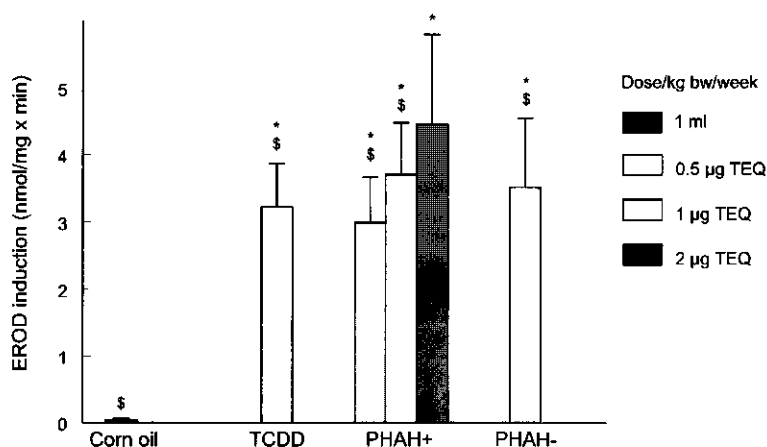
Figure 3.1 Liver microsomal EROD activity in female Sprague Dawley rats after different PHAH treatments; Corn oil (control), TCDD, PHAH+ mixture with PCB 153, PHAH- mixture without PCB 153. Statistical differences were tested with the Student-Newman-Keulman-test. * Significant different from the corn oil control group (p<0.05). $ Significant different from the PHAH+ group dosed at 2 μg TEQ/kg bw/week (p<0.05).

Discussion

This study was focussed on possible interactions between dioxin-like PHAHs and the non-dioxin-like PHAH 2,2',4,4',5,5'-HxCB (PCB 153). In addition it was investigated whether the total toxic potency of a mixture of dioxin-like PHAHs would match the effect of an equipotent dose of 2,3,7,8-TCDD as a single congener. We observed remarkable differences in hepatic retention as percentage of the given dose, among the PHAH congeners of the PHAH mixture groups, with highest retention for 2,3,4,7,8-PeCDF (30.5-43.1%) and lowest for PCB 118 (0.5-0.8%). A decrease was observed in the hepatic retention (% of the given dose) of some of the PHAH congeners with increasing exposure doses of the PHAH+ mixture (with PCB 153). A comparison between the equipotently dosed PHAH+ and PHAH- groups showed an increase of the hepatic deposition of all PHAH congeners after co-exposure with PCB 153. The EROD activity was significantly enhanced in all groups treated with PHAHs and no differences were observed between the equipotent dosed PHAH+, PHAH- and 2,3,7,8-TCDD group.

Differences between single congeners in the hepatic deposition as percentage of the given dose were reported by other authors as well (Wærn *et al.*, 1991; Hemming *et al.*, 1993; Haag-Grönlund *et al.*, 1997b). This phenomenon can probably be explained by differences in affinity between the congeners for hepatic binding sites, e.g. CYP1A2, rather than by liver fat

concentrations (Van den Berg *et al.*, 1994; De Jongh *et al.*, 1995).

In the experimental groups receiving the mixture with PCB 153 (PHAH+) the liver retention, expressed as percentage of the given dose, decreased significantly at increasing exposure levels for TCDD, PeCDD and PeCDF whereas for the PCBs no clear tendency was observed. Analysis of the data with the Partial Least Square (PLS) method confirmed the negative correlation between the given TEQ dose and the hepatic retention of all congeners. In similar exposure studies with individual congeners a slight decrease as percentage of the given dose was observed for 2,3,7,8-TCDD and 2,3,4,7,8-PeCDF whereas an increase was found for 1,2,3,7,8-PeCDD (Wærn *et al.*, 1991), PCB 126 and 2,3,7,8-TCDD (Hemming *et al.*, 1995). A dose dependent increase of TCDD in the liver as percentage of the given dose has been reported by some other authors as well, both after a single injection (Abraham *et al.*, 1989; Kedderis *et al.*, 1993) and sub-chronic exposure (Van der Kolk *et al.*, 1992).

Andersen *et al.* (1993) described a receptor-mediated physiologically based pharmacokinetic (PB-PK) model for the tissue distribution of TCDD, based on data of Abraham *et al.* (1988), and calculated a bell shape curve for the retention of TCDD as percentage of the dose in the liver. This model suggests a slow saturation of the liver with increasing doses of TCDD and consequently a decrease of the percentage of the dose deposited in the liver after a certain maximum. A similar model would fit for other congeners with saturation maxima at different congener concentrations (Andersen *et al.*, 1993). Deposition of PHAHs in the liver depends on the solubility of the congeners in liver fat and the induction of and/or binding to hepatic binding proteins (Leung *et al.*, 1988), of which CYP1A2 is assumed to be an important inducible binding site for PHAHs in the liver (Andersen *et al.*, 1993; Kedderis *et al.*, 1993). Since the animals in our experiment were exposed to relative high amounts of PHAH congeners, the observed decrease of hepatic retention of the congeners in our experiment might probably be explained by saturation of the hepatic binding proteins rather than saturation of the liver fat. However, since CYP1A2 levels were not determined here, a discussion of the presently observed phenomenon must remain speculative and a possible explanation can only be given in the light of previous results on interactive effects on hepatic retention between PCDD/Fs and PCBs in rats and mice (De Jongh *et al.*, 1993a,b, 1995).

A comparison between the mixture group with PCB 153 (PHAH+) and the mixture group without PCB 153 (PHAH-) both dosed at ~1 µg TEQ/kg bw/week, resulted in a significantly higher retention of all PHAH congeners in the PHAH+ group except for 2,3,7,8-TCDD. Interactive effects of PCB 153 on the liver retention of other PHAH congeners have been observed by other authors as well. De Jongh *et al.* (1993b) found an increase of about 8% of the liver retention after a single exposure of 1,2,3,7,8- PeCDD co-administered with PCB 153 in C57BL/6J mice. A combination of PeCDD, 1,2,3,6,7,8-HxCDD, 2,3,4,7,8-PeCDF and PCB 153 resulted even in a stronger increase on deposition of PeCDD in the liver. In a similar experiment the retention of PCB 156 co-administered with PCB 153 was twice the retention of PCB 156 single dosed (De Jongh *et al.*, 1993a). PCB 153 was found to be

capable of inducing both CYP1A2 activity and protein levels in C57BL/6J mice (De Jongh *et al.*, 1995). Although all congeners in this study are CYP1A2 inducers, PCB 153 is the only one which induces this protein without binding to it and this may readily explain its effect on hepatic retention of the other compounds. All other congeners competitively bind to CYP1A2 and complex interactive effects in binary, ternary and quaternary mixtures of PCB 153, TCDD, PeCDF, PeCDD or PCB 156 have been reported (De Jongh *et al.*, 1993a,b, 1995).

The EROD activity in the liver was enhanced in all PHAH treated groups. No differences were observed between the equipotent dosed PHAH+, PHAH- and 2,3,7,8-TCDD groups. The EROD activity in the highest dose PHAH+ group (2 μg TEQ/kg bw/week) was significantly increased compared to the other groups. Since it was observed that the proportion (% of the given dose) of the indvidual PHAH congeners in the liver differed considerably, TEFs based on liver tissue concentrations were used to calculate internal TEQ values. However, there was only a very weak correlation (R=0.2) between the internal TEQ dose in the liver and the EROD activity of the individual PHAH+ exposed animals, probably due to a maximum induction of CYP1A1 in the PHAH treated groups. In addition, no correlation was found between the volume fraction of the liver occupied by altered hepatic foci and the EROD activity, while there was a weak correlation (R=0.38) between the volume fraction and the internal TEQ dose in the liver (Van der Plas *et al.*, 1999 *Chapter 4*).

In summary, we observed a decrease of the liver retention of TCDD, PeCDD and PeCDF at increasing dose of the PHAH+ mixture. In addition, the results of the PLS analysis suggest that hepatic lipids are more important for hepatic retention of PCBs than for hepatic retention of either TCDD, PeCDF or PeCDD. Thus, a possible explanation for the observed decrease of the hepatic retention of TCDD, PeCDF and PeCDD might be a saturation of hepatic binding proteins, of which CYP1A2 seems to be an important representative. PCB 153 was found to increase the liver retention for all PHAH congeners in the PHAH+ mix group compared to the TEQ equivalent dosed PHAH- group. However, this was not reflected in the EROD activity in the liver microsomes, probably because a maximum EROD effect was already achieved.

Acknowledgements

CHAPTER 4

Induction of altered hepatic foci by a mixture of dioxin-like compounds with and without 2,2',4,4',5,5'-hexachlorobiphenyl in female Sprague-Dawley rats

Abstract

The hepatic tumour promoting activity of a mixture of polyhalogenated aromatic hydrocarbons (PHAHs) was studied in a medium term two-stage initiation/promotion bioassay in female Sprague-Dawley rats. The PHAH mixture contained 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (PeCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 3,3',4,4',5-pentachlorobiphenyl (PCB 126), 2,3',4,4',5-pentachlorobiphenyl (PCB 118), 2,3,3',4,4',5-hexachlorobiphenyl (PCB 156), 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153) and covered > 90% of the total toxic equivalents (TEQ) present in Baltic herring. To determine possible interactive effects of di-*ortho* substituted PCBs, the PHAH mixture was tested with (PHAH+) and without (PHAH-) PCB 153. Rats were initiated by a diethylnitrosamin injection (i.p. 30 mg/kg body weight) 24 hours after a partial $^2/_3$ hepatectomy. Six weeks after initiation the PHAH mixtures were administered once a week by subcutaneous injections for 20 weeks. Treatment with the PHAH mixtures caused liver enlargement and an increased activity of the hepatic cytochrome P4501A1/2 and P4502B1/2. All PHAH exposure groups exhibited an increased occurrence of hepatic foci positive for the placental form of glutathione-S-transferase. In the PHAH- group dosed 1 μg TEQ/kg body weight/week, the volume fraction of the liver occupied by foci was significantly lower compared to the TEQ equivalent dosed TCDD group (3.8% v.s. 8.7%). The volume fraction was significantly increased in the groups treated with 0.5, 1 or 2 μg TEQ/kg body weight/week of the PHAH+ mixture (4.5%, 5.2% and 6.6% respectively) compared to the corn oil group (2.0%), but to a lower extent than expected on basis of the TEQ doses. Overall, the TEQ-based administered dose overestimated the observed tumour promoting effects of this PHAH mixture. The applicability of the TEF concept, the role of differences in toxicokinetic properties and interactive effects of PCB 153 on hepatic deposition of the dioxin-like congeners are discussed.

Introduction

Polychlorinated biphenyls (PCBs), polychlorodibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and other polyhalogenated aromatic hydrocarbons (PHAHs) are ubiquitously present in the environment. Residues have been detected in fish, wildlife species and human adipose tissue, milk and serum (Safe, 1990). The most toxic PHAH compounds exhibit a planar molecular conformation and most of their toxic responses are believed to be mediated by the aryl hydrocarbon (Ah) receptor (Safe, 1994). PCBs, PCDDs and other PHAHs elicit a broad spectrum of toxic effects and biochemical changes, e.g. body weight loss, thymic atrophy, impairment of immune responses, hepatotoxicity, teratogenicity, carcinogenicity and induction of hepatic cytochrome P450 isoenzymes (Safe, 1990, 1994).

In environmental matrices and biota PCBs, PCDDs and PCDFs are always present as complex mixtures. The toxic equivalency factor (TEF) concept has been developed to aid the risk assessment of complex mixtures of PHAHs and enable the calculation of total toxic potencies, expressed as the toxic equivalent (TEQ), of the mixtures. The TEF approach is based on the assumptions that PHAH congeners act through the same dioxin-like Ah receptor-based mechanism of action and that the effects of the individual compounds are additive (Safe, 1994; Ahlborg *et al.*, 1994).

Carcinogenicity is one of the main toxic endpoints in risk assessment of PCBs, PCDDs and PCDFs (WHO, 1992). A no observed adverse effect level (NOAEL) of 1 ng 2,3,7,8-TCDD has been established after re-evaluation of the 2 year carcinogenicity study as reported by Kociba *et al.* (1978). In a recent WHO/ICPS-expert meeting (summarised by Van Leeuwen and Younes, 1998) it was concluded that the use of TCDD alone as a measure of exposure to PCDDs, PCDFs and PCBs would severely underestimate the risk from exposure to these compounds. Therefore it was proposed to express the daily intake (TDI) of PCDDs, PCDFs, non-*ortho* and mono-*ortho* PCBs in TEQs, i.e. allowing the TEF-concept as a method to predict the total carcinogenic potency of PHAHs. However, there is current debate on the role of the Ah receptor and thus the relevance of the TEF concept for risk assessment of carcinogenicity caused by PHAH mixtures. Both *in vitro* and *in vivo* data suggest a tumour promotion activity of several di-*ortho* substituted PCBs, although they possess no or a negligible Ah receptor mediated activity i.e. a TEF value close to zero. Both 2,2',3,3',4,4'-hexachlorobiphenyl (PCB 128) and 2,2',5,5'-tetrachlorobiphenyl (PCB 52) (De Haan *et al.*, 1996) as well as 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153) (Bager *et al.*, 1997) were shown to be potent inhibitors of the gapjunctional intercellular communication *in vitro*, an indicator of tumour promotion capacity. *In vivo*, 2,2',5,5'-tetrachlorobiphenyl (PCB 52) and 2,2',4,4'-tetrachlorobiphenyl (PCB 47) were shown to be tumour promoters in female Sprague-Dawley rats (Preston *et al.*, 1985) as was 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153) (Hemming *et al.*, 1993), one of the major PHAH congeners in the human diet. In addition there are some data suggesting both synergistic and antagonistic interactions between dioxin-like Ah receptor

agonists and some non-planar PCB congeners on several toxicity parameters, e.g. immunotoxicity (Biegel *et al*, 1989), teratogenicity (Zhao *et al.*, 1994, 1997), and hepatotoxicity (Yao and Safe, 1989; Van Birgelen *et al.*, 1996a) as well as tumour promotion (Sargent *et al.*, 1991; Bager *et al.*, 1995, Haag-Grönlund *et al.*, 1998). Also antagonistic effects of several complex PCB mixtures on immunotoxicity (Bannister *et al.*, 1987a; Davis and Safe, 1989) and teratogenicity have been observed (Haake *et al.*, 1987).

However, most toxicity studies have been performed with individual PHAH congeners or with combinations of two compounds, whereas humans are exposed to complex mixtures of PHAHs in the diet. Therefore, we performed a study on the tumour promotion capacity of a complex mixture of PHAHs resembling the composition of the PHAH mixture present in fish, which is one of the main contributors of PHAHs to the human diet (WHO, 1992). The mixture contained 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (PeCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 3,3',4,4',5-pentachlorobiphenyl (PCB 126), 2,3',4,4',5-pentachlorobiphenyl (PCB 118) and 2,3,3',4,4',5-hexachlorobiphenyl (PCB 156) and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153) in relative concentrations as found in fish (Table 4.1) covering >90% of the total TEQs present in Baltic herring (personal communication, Cynthia De Wit, Institute of Applied Environmental Research, Stockholm University, Sweden). To determine the interactive effect of the non-planar, di-*ortho* substituted PCB 153, the PHAH mixture was also tested without this congener. A medium-term two-stage initiation/promotion protocol (Pitot *et al.*, 1978) was used to study the tumour promotion potential of these PHAH mixtures, measuring the development of glutathione S-transferase-p (GST-p) positive altered hepatic foci (AHF) as being one of the most efficient markers of AHF (Maronpot *et al.*, 1989). The tumour promotion potential as well as the predictability by the TEF concept of the total tumour promotion potential of these PHAH mixtures is discussed.

Materials and methods

Chemicals

N-nitrosodiethylamin (NDEA) was obtained from Fluka (Fluka Chemie, Buchs, Switzerland). 3,3',4,4',5-Pentachlorobiphenyl (PCB 126), 2,3',4,4',5-pentachlorobiphenyl (PCB 118), 2,3,3',4,4',5-hexachlorobiphenyl (PCB 156) and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153) were kindly provided by Prof. Bergman (Department of Environmental Chemistry, Stockholm University, Sweden). 1,2,3,7,8-Pentachlorodibenzo-*p*-dioxin (PeCDD) was obtained from Wellington Laboratories (Canada), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) was a gift from Prof. S.H. Safe (College of Veterinary Physiology and Pharmacology, Texas, A&M University, USA) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was purchased from RADIAN CIL, inc.(U.S.A.). All compounds had a purity > 99%.

The primary antibody for glutathione-S-transferase-p (GST-p) staining was a GST Yp rabbit anti-rat serum, purchased from Biotrin International (Dublin, Ireland). The second antibody was a peroxidase conjugated swine anti-rabbit immunoglobulin (P217) from Dako (Glostrup Denmark). Bovine serum albumin (BSA) was obtained from Sigma Chemical Company (St. Louis, MO, USA), diaminobenzidin tetrahydrochloride (DAB) from Sigma (St. Louis, U.S.A.) and NADPH was from Boehringer Mannheim (Mannheim, Germany). Phosphate buffered saline (Dulbecco's 'A', pH 7.3; PBS) was bought from Oxoid (London, U.K.), NaCl and Tris were from Merck (Darmstadt, Germany).

Resorufin was obtained from Janssen Chimica (Geel, Belgium) and ethoxyresorufin and penthoxyresorufin were synthesised according to Mayer *et al* (1977). A Bradford Protein Assay kit was obtained from Bio-rad (Hercules, California, USA). A commercial ELISA kit for analysis of cytochrome P450 2B1/2 was from Amersham (Buckinghamshire, England). A commercial kit from Roche diagnostic Ltd. (Basel, Switzerland) was used for analysis of alanine aminotransferase (Unimate 5 ALT) and aspartate aminotransferase (Unimate 5 AST).

2,3,7,8-[^{13}C]-TCDD and [^{13}C]-PCB 126 were commercially obtained from Cambridge Isotope Laboratories (Woburn, MS, USA) and 1,2,3,7,8-PeCDF, 2,3',4,4',5,5'-hexachlorobiphenyl (PCB 167) and 2,3,3',5,6-pentachlorobiphenyl (PCB 112) from Ehrenstorfer (Augsburg, Germany). Hexane, toluene, dichloromethane and nonane, used for the extraction and clean-up procedure of the GC-MS analysis were of nanograde quality and obtained from J.T. Baker (Deventer, The Netherlands). Silica gel 60 extra pure 70-230 mesh ASTM, NaOH and Na$_2$SO$_4$ were obtained from E. Merck (Darmstadt, Germany). Aluminum B super I was bought from ICN Biomedicals (Eschwege, Germany).

Animal mixtures

Two PHAH mixtures, a PHAH- mixture without the di-*ortho* PCB 153 and a PHAH+ mixture with PCB 153 were composed, containing 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 2,3,4,7,8-PeCDF, PCB 126, PCB 118 and PCB 156 in relative concentration levels as shown in table 4.1. For calculations of the relative concentration levels of the congeners in the PHAH mixtures, concentration data in six-year-old Baltic herring caught close to the town of Karlskrona in southern Sweden were used. The congeners in the PHAH mixtures covered > 90% of the TEQ in Baltic Herring. For the TEQ calculation of the PHAH mixtures, TEF values based on tumour promotion data were used if available. Because these are not officially published WHO-ECEH TEF values, the term 'relative potency' (REP) will be used instead. The reason for this is that a WHO consultation (Bilthoven, 9-10 August, 1996) recommended not to use the term TEF for estimations based on a single study, but only for consensus value based on all available literature. Exposure levels were chosen on the basis of earlier performed tumour promotion experiments (Waern *et al.*, 1991; Hemming *et al.*, 1993, 1995) such, that no severe liver toxicity was expected at the highest concentrations tested and the expected effects on tumour promotion of the lower test concentrations were increased compared to the control.

Table 4.1 Composition of the PHAH mixtures based on the relative levels in Baltic herring

Congener	(Cl substitution)	Relative level (weight base)		
		PHAH- mixture	PHAH+ mixture	Potency factor
TCDD	(2,3,7,8)	1	1	1
PeCDD	(1,2,3,7,8)	3.3	3.3	1[a]
PeCDF	(2,3,4,7,8)	17	17	0.1[a]
PCB 126	(3,3',4,4',5)	61	61	0.1[b]
PCB 118	(2,3',4,4',5)	12800	12800	0.0001[c]
PCB 156	(2,3,3',4,4',5)	1888	1888	0.0005[c]
PCB 153	(2,2',4,4',5,5')	-	20000	0.00005[d]

[a] REP value based on a tumour promotion study performed by Waern *et al.* (1991)

[b] REP value based on a tumour promotion study performed by Hemming *et al.* (1995)

[c] TEF value as proposed by the World Health Organization (Ahlborg *et al.* 1994)

[d] REP value based on a tumour promotion study performed by Hemming *et al.* (1993)

The concentrations of the compounds in the mixture were reconfirmed by gas chromatography/mass spectrometry (GC-MS) by RIKILT-DLO (Wageningen, the Netherlands).

For animal exposure the mixtures were dissolved in food-grade corn oil.

Animal experiment

Young female Sprague-Dawley rats (Møllegaard Breeding Centre Ltd., Denmark), about three weeks old at the day of arrival, were kept in wire bottom, stainless steel cages in groups of three or four animals under standard conditions (12 hr light/dark cycle, temperature 22 °C, humidity 55%). They were fed ad libitum with standard semi-synthetic rodent chow (RMH-B, Hope Farms Woerden, The Netherlands) and had free access to tap water. After three weeks of acclimatisation the initiation treatment was started by removing two thirds of the liver (partial hepatectomy; PH) under ether anaesthesia followed by an i.p. injection with NDEA (30 mg/kg) 24 hours after PH. About five weeks after PH the animals were divided in treatment groups; a negative control group (n=18), a positive control group (n=12) and four experimental groups (n=10). The promotion treatment was started six weeks after the initiation procedure and consisted of a weekly s.c. injection with the test compounds during twenty weeks. The vehicle control group was dosed with corn oil (1 ml/kg bw/week), the positive control group with 2,3,7,8-TCDD (1 µg/kg bw/week) and the experimental groups with the PHAH+ mixture at a dose level of 0.5 µg TEQ/kg bw/week, 1 µg TEQ/kg bw/week or 2 µg TEQ/kg bw/week or the PHAH- mixture at a dosage level of 1 µg TEQ/kg bw/week (composition shown in Table 4.1). The first dose was a loading dose which was five times higher than the maintenance dose (Flodström and Ahlborg, 1989; Krowke *et al.*, 1989) as indicated before. Body weight, food and water consumption were recorded weekly.

One week after the last exposure the animals were euthanised under ether anaesthesia by orbita puncture for blood collection (heparinised), followed by decapitation. The liver was taken out and a part of the lateral and caudal liver lobe were fixed in ice-cold acetone (4 °C), and imbedded in paraffin 24 hours after fixation. A part of the lateral liver lobe was stored at -80 °C for gas chromatography and mass spectrometry (GC-MS) analyses, whereas the rest of the liver was homogenised on ice in a Potter tube with 0.01 M Tris-HCl-buffer and 0.25 M sucrose (pH 7.4). The homogenate was stored at -80 °C until preparation of microsomes.

Thymus, spleen, lungs, heart, brain, kidneys and some adipose tissue were collected and stored at -80 °C until further analysis.

Staining and analyses of altered hepatic foci

Sections (4 µm) of acetone fixed tissue were stained for gluthatione-S-transferase-p (GST-p) positive foci as described by Haag-Grönlund et al.(1997a). Slices were deparaffinised in xylene and air dried. The endogenous peroxidase activity was blocked with a fresh solution of 0.5% H_2O_2 in methanol (10 min). Unspecific binding sites were then blocked with a 2.5% BSA solution in PBS (30 min). Slices were incubated overnight at room temperature with the GST-p primary antibody (dilution 1:2000) followed by incubation with peroxidase conjugated secondary antibody (dilution 1:100) for 2½ hours. GST-p positive cells were stained for 5 minutes with DAB. Foci were analysed in the right lateral lobe of the liver using a Leica Aristoplan microscope connected to a Quantimet 570 Image Processing and Analysis system (Leica Cambridge Ltd. England). The methods used for stereological evaluation of altered hepatic foci have been reported previously (Flodström et al., 1988). A total sectional area of approximately 3 cm² was analysed for each animal. The smallest group of GST-p positive cells scored as a focus had a radius of 35 µm (cut off limit).

Preparation of microsomes

Microsomes were prepared by differential centrifugation of liver homogenates at 9,000 g for 30 minutes, the resulting supernatant was centrifuged at 105,000 g for 90 minutes, and the pellet was resuspended in a 0.01 M Tris-HCl buffer, pH 7.4. Microsomes were stored at -80 °C until further analysis.

Protein and cytochrome P450 enzyme assays

The protein concentration in the microsomes was measured using the commercial available Bradford Protein Assay reagens with crystalline BSA as a standard.

The total cytochrome P450 content in liver microsomes was analysed according to the method of Omura and Sato (1964) with modifications of Rutten et al. (1987). The difference spectrum was measured of microsome samples (protein concentration ±1 mg/ml) saturated with CO (60 seconds) followed by dithionite reduction of P450.

Activities of ethoxyresorufin-O-deethylase (EROD) and penthoxyresorufin-O-depenthylase (PROD) were measured in liver microsomes according to Burke et al. (1977),

adapted for use with 96-well plates and a fluoro spectrophotometric plate reader (CytoFluor 2530, Millipore) as described by Morse *et al.* (1996b). A preincubated mixture of microsomes (protein concentrations 100-400 µg/ml), BSA (4 mg/ml) and ethoxy- or penthoxyresorufin (8 µM) was incubated for 5 minutes at 37 °C after adding of 0.4 mM NADPH. Resorufin concentrations were measured after the reaction was stopped with 1 M NaOH.

Cytochrome P4502B protein was measured by an ELISA method using a commercially available kit.

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using Roche Cobas Mira as equipment and a commercial kit based on the measurement of the oxidation of NADH into NAD+. The oxidation can be followed by a spectrophotometer at 340 nM.

GC-MS analysis

The individual PHAH congener concentrations in the liver were analysed by gas chromatography and mass spectrometry as described in more detail by Van der Plas *et al.*(1998b *Chapter 3*). Freeze dried liver tissue was soxhlet extracted and dissolved in hexane after adding 2,3,7,8-[^{13}C]-TCDD, 1,2,3,7,8-PeCDF, [^{13}C]-PCB 126, PCB 167 and PCB 112 as internal standards. A two step clean-up procedure was performed using a silica column and an aluminium oxide column. After evaporation the fractions were dissolved in toluene and analysed on a Carlo Erba QMD 1000 high resolution gaschromatograph/low resolution quadruple mass spectrometer. A J&W fused silica capillary column was used with a stationary phase DB 5.625 (30 m, 0.32 mm id., film thickness 0.35 microns)

Statistical analysis

Data were analysed with the statistical package SPSS-PC. Foci data were log transformed in order to obtain a normal distribution of the data and a homogeneous variance between the groups. A One-Way Anova and a Tukey's Honestly Significant Difference test was used to perform a multiple comparison on statistical differences between groups. One outlier was removed from the group treated with 1 µg TEQ/kg bw/week of the PHAH+ mixture, because it exceeded abundantly the mean volume fraction plus two times the standard deviation. Differences between congener concentrations in the liver of the PHAH+ (1 µg TEQ/kg bw/week) and the PHAH- group were analysed with an independent t-test.

Results

Clinical observations

No obvious PHAH related clinical signs of toxicity were observed throughout the study. One animal died during the treatment period in the group that received the highest dose, 2 µg TEQ/kg bw/week, of the PHAH mixture with PCB 153 (PHAH+) but this did not seem to be

Table 4.2 Body, liver and thymus weights of female Sprague-Dawley rats following sub-chronic exposure to PHAH mixtures

Groups dose/kg bw/wk	Body weight[a] g	Body weight gain g	Relative liver weight g/kg bw	Relative thymus weight g/kg bw
Corn oil (n=18) 1 ml	314.0 (6.1)	73.5 (4.0)	27.9 (0.6)	0.58 (0.04)
TCDD (n=12) 1 µg TEQ	314.0 (6.8)	65.5 (3.4)	34.9 (0.9)[b,c]	0.35 (0.05)
PHAH- (n=10) 1 µg TEQ	318.8 (5.8)	71.8 (2.7)	33.6 (1.0)[b,c]	0.37 (0.05)
PHAH+ (n=10) 0.5 µg TEQ	314.1 (5.6)	70.2 (1.8)	33.5 (0.3)[b,c]	0.40 (0.05)
PHAH+ (n=10) 1 µg TEQ	306.8 (5.8)	61.2 (4.5)	38.0 (1.2)[b]	0.35 (0.04)
PHAH+ (n=9) 2 µg TEQ	299.0 (4.5)	54.7 (3.8)[b]	40.8 (1.4)[b]	0.34 (0.08)

Data are given as arithmetic mean ± SE. Doses are given per kg body weight per week. PHAH+ is the mixture with PCB 153 given in three different dosages and PHAH- is the mixture without PCB 153 (see Table 4.1 for the composition). [a] Body weight one week after the last exposure. [b] Statistically different from the corn oil group (p<0.05). [c] Statistically different from the mixture group with PCB 153, dosed with 2 µg TEQ/kg bw/week and 1 µg TEQ/bw/week (p<0.05).

related to the treatment.

Compared to the corn oil control group, a slight treatment and dose dependent decrease in body weight gain was observed in all treatment groups, which was only significantly different for the 2 µg TEQ/kg bw/week PHAH+ group (Table 4.2). No statistical differences in body weights were found at the end of the treatment period between the groups. All treated groups showed an increased relative liver weight, which was almost up to 150% of the control in the 2 µg TEQ/kg bw/week PHAH+ group. The relative thymus weights in the treated groups were reduced compared to the corn oil control levels. However, due to high interindividual variability the observed thymus weight reduction was not statistically different from the corn oil control group. No effects on organ weights were found on the other organs examined (data not shown).

Liver toxicity parameters

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were

measured in the plasma as indicators for liver toxicity. Compared to the corn oil control group a slight, but not significant, increase of the ALT activity was found in the group treated with TCDD and the highest dose of the PHAH+ mixture (Table 4.3). A dose dependent increase of the AST activity was measured in the PHAH+ mixture groups. The AST activity of the PHAH+ mixture group dosed at 2 μg TEQ/kg bw/week was significantly, 2-fold increased compared to the corn oil control group.

A preliminary histopathological evaluation of the liver slides did reveal only small pathological changes in the PHAH exposed groups compared to the corn oil group. In addition, no obvious differences in morphology were observed between the different exposure groups.

Liver cytochrome P450 enzyme induction

The total cytochrome P450 concentration (Table 4.3) in liver microsomes of all PHAH treated groups showed a statistically significant at least two fold increase as compared to the corn oil treated controls. The increase in total cytochrome P450 was lowest in the TCDD treated group and was dose dependent in the groups treated with the PHAH+ mixture. The total cytochrome P450 concentration was also significantly increased in the PHAH+ group dosed with 2 μg TEQ/kg bw/week compared to the TCDD treated, positive control group.

The EROD activity (Table 4.3) was highly increased, 75-112 fold, in all PHAH treated groups compared to the corn oil controls. The PHAH+ mixture groups showed a dose dependent increase with the highest activity, 112-fold induction, at 2 μg TEQ/kg bw/week. This was significantly different from the TCDD group and the group exposed to 0.5 μg TEQ/kg bw/week of the PHAH+ mix.

PROD was used as an indicator of CYP2B activity. The PROD activities were significantly increased in all treated groups compared to the control. The increase was dose-dependent in the PHAH+ mixture groups. An increased PROD activity of three times the control level was also measured for TCDD, which is normally not considered to induce CYP2B. To check if this increased PROD activity corresponded to an increased enzyme protein level, the P4502B1/2 protein concentration was measured. No induction of the protein was found in the group exposed to TCDD as compared to corn oil treated controls whereas an almost two-fold, significant, increase was found in the highest PHAH+ dose group (Table 4.3). A slight, dose dependent increase of the P4502B1/2 protein was found in the lower PHAH dose groups, which however did not differ significantly from the corn oil group.

Foci induction

Altered hepatic foci (AHF) data are presented in figure 4.1A to 4.1C. All groups showed large numbers, 3500-5000/cm³, of GST-p positive foci in the liver with a slight, but not significant increase in the TCDD exposed group compared to the control (Figure 4.1A). The mean foci volume (Figure 4.1B) as well as the volume fraction of the liver occupied by GST-p positive foci (Figure 4.1C) was significantly increased in all treated groups compared to the

Table 4.3 Cytochrome P450 enzyme activities and concentrations in the liver and plasma activities of alanine aminotransferase and aspartate aminotransferase of female Sprague-Dawley rats following sub-chronic exposure to PHAH mixtures

Groups dose/kg bw/wk	Total P450 nmol/mg protein	EROD activity nmol resorufin/ min/mg protein	PROD activity pmol resorufin/ min/mg protein	P4502B1/2 µg/mg protein	ALT U/l	AST U/l
Corn oil 1 ml	0.69 ± 0.03	0.04 ± 0.005	24.60 ± 1.21	1.41 ± 0.07	0.82 ± 0.06	1.96 ± 0.15
TCDD 1 µg TEQ	$1.37 \pm 0.10^{a,b}$	$3.22 \pm 0.19^{a,b}$	73.08 ± 5.92^{a}	1.37 ± 0.12^{b}	1.04 ± 0.08	2.38 ± 0.15^{b}
PHAH- 1 µg TEQ	1.57 ± 0.11^{a}	3.51 ± 0.33^{a}	86.55 ± 5.63^{a}	1.61 ± 0.08^{b}	0.95 ± 0.07	2.38 ± 0.21^{b}
PHAH+ 0.5 µg TEQ	1.41 ± 0.11^{a}	$2.99 \pm 0.21^{a,b}$	74.90 ± 5.73^{a}	1.57 ± 0.09^{b}	0.95 ± 0.06	2.39 ± 0.19^{b}
PHAH+ 1 µg TEQ	1.56 ± 0.19^{a}	3.70 ± 0.25^{a}	95.71 ± 12.99^{a}	1.84 ± 0.15^{b}	0.98 ± 0.04	2.66 ± 0.23^{b}
PHAH+ 2 µg TEQ	1.83 ± 0.13^{a}	4.46 ± 0.45^{a}	100.41 ± 8.81^{a}	2.60 ± 0.28^{a}	1.16 ± 0.19	3.94 ± 0.70^{a}

Data are given as arithmetic mean ± SE. Doses are given per kg body weight per week. PHAH+ is the mixture with PCB 153 given in three different dosages and PHAH- is the mixture without PCB 153 (see Table 4.1 for the composition). [a] Significantly different from the corn oil group (p<0.05).
[b] Significantly different from the mixture group with PCB 153, dosed at 2 µg TEQ/kg body weight/week (p<0.05)

Table 4.4 Concentration of PHAH congeners per gram liver as determined by gas chromatography and mass spectrometry analysis

| Groups dose/kg bw/week	Concentration of congeners per gram liver (wet weight)						
	TCDD ng/g	PeCDD ng/g	PeCDF µg/g	PCB 126 µg/g	PCB 118 µg/g	PCB 156 µg/g	PCB 153 µg/g
Corn oil 1 ml	n.d./n.a.	n.d./n.a.	n.d./n.a.	n.d./n.a.	n.d./n.a.	n.d./n.a.	n.d./n.a.
TCDD 1 µg TEQ	25.9 ± 1.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PHAH- 1 µg TEQ	1.3 ± 0.1	9.5 ± 0.4^{a}	0.21 ± 0.01^{a}	0.32 ± 0.01^{a}	0.93 ± 0.12^{a}	0.81 ± 0.06^{a}	n.d.
PHAH+ 0.5 µg TEQ	0.9 ± 0.1	7.4 ± 0.5	0.15 ± 0.00	0.22 ± 0.02	0.85 ± 0.19	0.48 ± 0.04	2.25 ± 0.28
PHAH+ 1 µg TEQ	1.5 ± 0.1	11.0 ± 0.5	0.26 ± 0.01	0.40 ± 0.02	1.93 ± 0.28	1.23 ± 0.12	6.21 ± 0.91
PHAH+ 2 µg TEQ	2.5 ± 0.2	16.3 ± 0.8	0.44 ± 0.02	0.71 ± 0.04	2.74 ± 0.50	2.00 ± 0.18	9.61 ± 1.18

Data are given as arithmetic mean ± SE. Doses are given per kg body weight per week. PHAH+ is the mixture with PCB 153 given in three different dosages and PHAH- is the mixture without PCB 153 (see Table 4.1 for the composition). n.d. = not detected, n.a. = not analysed, n.d./n.a. = only some randomly taken samples were analysed. [a] Significantly different from the mixture group with PCB 153 (PHAH+), dosed at 1 µg TEQ/kg bw/week (p<0.05).
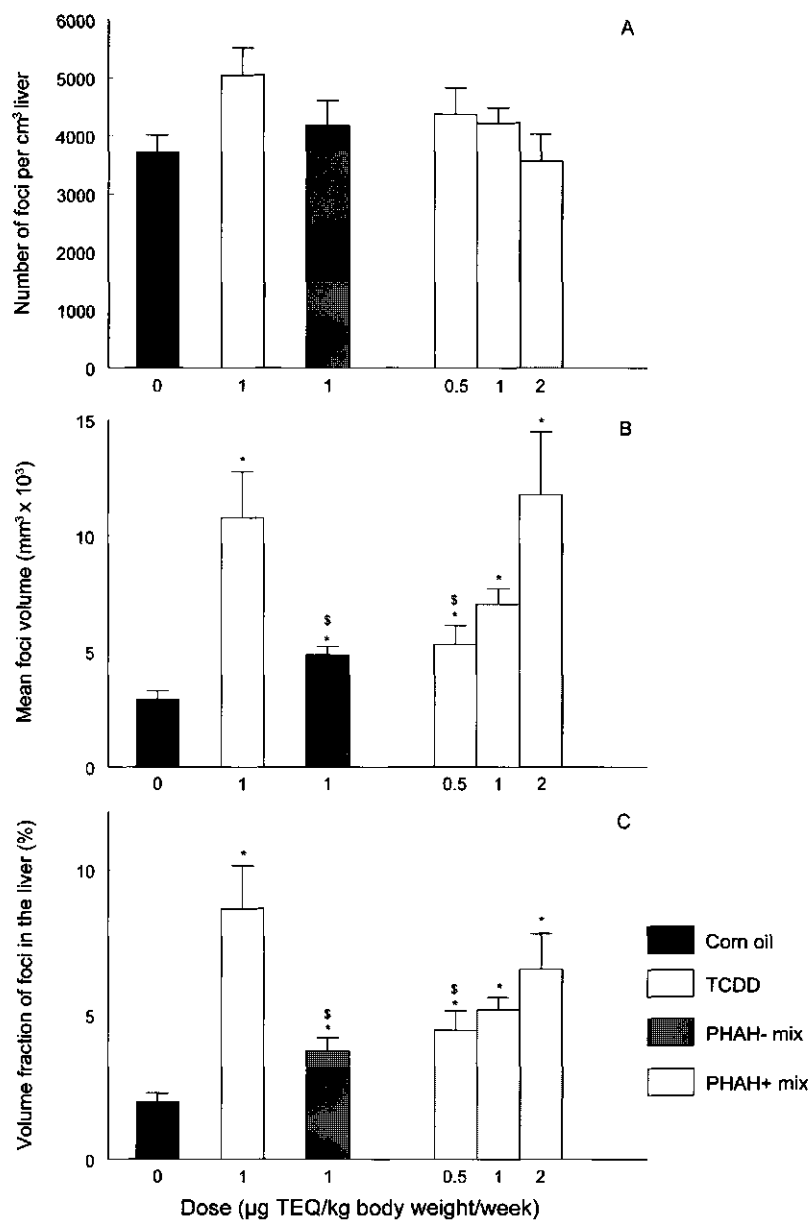
Figure 4.1A-C Occurrence of GST-*p* positive foci in liver tissue of female Sprague Dawley rats after 20 weeks of exposure to corn oil (negative control), 2,3,7,8-TCDD (positive control), the PHAH-mixture (without PCB 153) or different doses of of the PHAH+ mixture (with PCB 153). **A.** Number of foci per cm^3 liver. **B.** Mean foci volume (mm^3×10^3). **C.** Volume fraction of the liver occupied by foci (%). * Statistically different (Tukey, p<0.05) from the corn oil group (negative control). $ Statistically different (Tukey, p<0.05) from the TCDD exposed group.

corn oil control group. 2,3,7,8-TCDD caused a 3.7 fold and a 4.3 fold increase in the mean foci volume and the volume fraction respectively. The increase in mean foci volume and the volume fraction for the PHAH- was significantly smaller than in the TEQ equivalent dosed TCDD group. Addition of PCB 153 to the PHAH mixture (PHAH+) caused higher, though not significant mean foci volume and volume fraction as compared to the PHAH- group. However the increase caused by the PHAH+ mix was still somewhat lower than the effect of 1 µg TCDD/kg bw/week alone. Thus, the foci growth response was not identical between groups given TEQ equivalent doses. The observed differences in foci induction between the TCDD and the TEQ equivalent dosed PHAH mixture groups was not reflected in the EROD activity (Table 4.3).

Liver retention of the congeners

The concentrations of the PHAH congeners in the liver are shown in table 4.4. These results have been discussed in detail elsewhere (Van der Plas *et al.*, 1998b *Chapter 3*). Considerable differences in hepatic concentration were observed, due to large differences in relative levels of the congeners in the administered PHAH mixture. However, the relative levels of congeners in the liver was different compared to the originally given mixture (Table 4.1). The relative hepatic levels of PeCDD, PeCDF and PCB 126 to TCDD increased approximately 2, 10 and 4 times respectively compared to the relative levels in the original mixtures. Opposite effects were found for PCB 118, PCB 156 and PCB 153, their relative levels decreased on average 12, 3 and 6 times respectively. An interesting result was that the concentrations of all congeners, except TCDD, were significantly higher in the PHAH+ group dosed at 1 µg TEQ/kg bw/week, compared to the TEQ equivalent dosed PHAH- group.

Discussion

The major aim of this study was to determine the integral tumour promotion potential of complex mixtures of PHAHs, and to evaluate the applicability of the TEF concept in terms of predicting the effects of PHAH mixtures as present in the human diet. For this purpose the development of altered hepatic foci (AHF) as a measure of the tumour promotion capacity of a PHAH mixture covering >90% of the TEQ in Baltic herring was studied and compared to the effects of a TEQ equivalent dose of 2,3,7,8-TCDD alone. The PHAH mixture used consisted of 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 2,3,4,7,8-PeCDF, PCB 126, PCB 118, PCB 156 and PCB 153 in relative concentrations as indicated in table 4.1. The PHAH mixture was also studied at one dose level without the di-*ortho* substituted PCB 153 (PHAH-) in order to determine possible interactive effects of this congener. The PHAH mixtures used in this study significantly enhanced the development of GST-p positive AHF, compared to corn oil treated rats, but to a lower extent than in the TEQ equivalent dosed TCDD group.

Selective proliferation of initiated cells may indirectly be stimulated by cytotoxic events,

therefore it is important to consider cytotoxic properties of promotive substances (Butterworth *et al.*, 1992). In this study the plasma alanine aminotransferase (ALT) and the plasma aspartate aminotransferase (AST) levels were only slightly, not significantly, increased for all PHAH treated groups. A statistically significant two-fold increase of the AST level in the plasma was found only in animals treated with the highest dose of the PHAH+ mixture. The histopathologic evaluation revealed only minor pathological changes in the exposure groups. Therefore it was concluded that there was no severe hepatotoxicity at the administered PHAH doses.

With respect to the foci data several explanations could be discussed for the observed difference between the treatment with TCDD and the TEQ equivalent PHAH+ and PHAH-mixture. The TEQ value calculated for the mixtures could have been overestimated by using conservative TEF values. The REPs for PeCDD, PeCDF, PCB 126 and PCB 153 used for the calculation of doses in this study were based on tumour promotion studies performed by Waern *et al.* (1991) and Hemming *et al.* (1993; 1995). The REPs for PCB 118 and PCB 156 based on tumour promotion data were not available until recently (Haag-Grönlund *et al.*, 1997a,b), therefore WHO-ECEH TEF values were used for PCB 156 and PCB 118. PCB 118 was found to be less potent than expected on the basis of the official TEF of 0.0001 proposed by WHO-ECEH and IPCS (Ahlborg *et al.*, 1994), which was used for the TEQ calculations in this study. Based on tumour promotion data a REP for PCB 118 was estimated to be less than 0.00002 (Haag-Grönlund *et al.*, 1997a) and for PCB 156 a range was derived of 0.0001-0.001 (Haag-Grönlund *et al.*, 1997b). A recalculation of the toxic potency of the PHAH mixture using the REPs based on only tumour promotion data would lower the TEQ value of the PHAH mixtures only slightly with 0.5 to 12%. Using the international TEF values for all congeners in the mixture as proposed by the Nordic Council (Ahlborg *et al.*, 1988) and WHO-ECEH and IPCS (Ahlborg *et al.*, 1994) would result in a 27% higher TEQ value for the PHAH mixtures thus a larger overestimation of the tumour promotion effect. The conservative TEFs and the uncertainty in the REPs used for dose setting could at least partly explain the difference in foci occurrence between TEQ equivalent groups.

Secondly, the hepatic deposition of the PHAH congeners in the liver as percentage of the given dose differed considerably (Table 4.4), which was observed by others as well (Abraham *et al.*, 1989; Wærn *et al.*, 1991; Hemming *et al.*, 1993; De Jongh *et al.*, 1993b). This highly influences the concentration ratios between the PHAH congeners and possibly the toxic potency of the mixture in the liver. In addition, several authors (De Jongh *et al.*, 1993a,b; Rozman *et al.*, 1995) reported on small kinetic interactions between congeners after administration of PHAH mixtures. Also in this experiment the possibility of kinetic interactions influencing the toxicity of the mixture in the liver can not be excluded. However, differences in deposition may play a role in the toxic potency of the mixture in the liver, which is illustrated by the following calculation of the TEQ of the hepatic mixture. Because international TEF values are intake values and may not be appropriate for body burden assessments (Ahlborg *et al.*, 1994), internal REPs based on hepatic concentrations (IREPs)
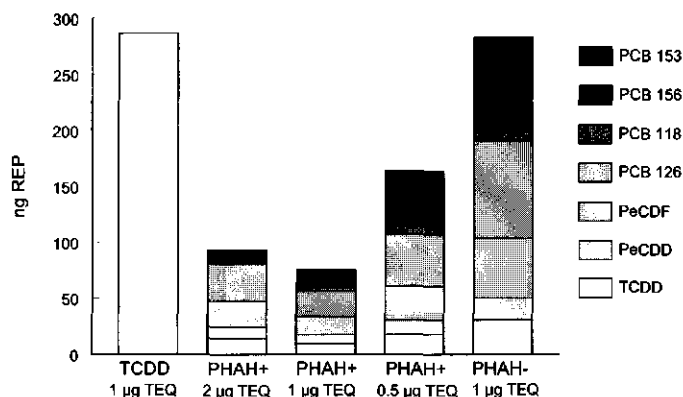
Figure 4.2 Hepatic dose of congeners expressed as internal toxic equivalency (ITEQ) per total liver. Dose levels are per kg body weight per week.

were used to calculate an internal toxic equivalent (ITEQ). IREPs based on tumour promotion data for PeCDD (0.1), PeCDF (0.01), PCB 126 (0.01), PCB 156 (0.0002-0.002) and PCB 153 (0.0001-0.001) were calculated by Wærn *et al.*(1991), Hemming *et al.* (1993) and Haag-Grönlund *et al.* (1997b). The IREP for PCB 118 (<0.0003) was deduced on basis of a study of Haag-Grönlund *et al.* (1997a) (unpublished data). The ITEQs were calculated by multiplying the congener liver concentration with the IREP values. In case a range was given for the IREP, the middle of the range was used. Figure 4.2 shows the ITEQs calculated as indicated above. The calculations suggest that although the groups administered 1 μg TEQ/kg bw/week were TEQ equivalent dosed, the ITEQ is lower in the groups exposed to the PHAH mixtures compared to the TCDD group and is more in line with the foci induction than the externally administered TEQ dose. In addition, the ratio between the congener levels in the PHAH mixture seems to be changed. In the original PHAH+ mixture, PeCDD was responsible for approximately 22% of the total TEQ but in the hepatic mixture only for about 8% of the total ITEQ. The TEQ for PCB 153 turned out to be less in the original mixture than in the hepatic mixture; 7% of the total TEQ v.s. 16-22% of the total ITEQ. For PCB 126 the contribution to the TEQ changed from 40% externally to approximately 30% internally and for PeCDF from 11% externally to 19% internally.

Non-additivity due to partial receptor agonism of some congeners in the mixture could be another explanation for the observed difference between the treatment with TCDD alone and the TEQ equivalent PHAH+ and PHAH- mixture groups. High concentrations of *ortho*-substituted PCBs, e.g. PCB 118 and PCB 156, which have a relatively low affinity for the receptor and generally a lower toxic potency and maximum effect than the dioxin-like PCBs, could compete with PCDDs, PCDFs and the dioxin-like non-*ortho* PCBs for receptor binding (Van den Berg *et al.*,1994) and thus lowering the observed toxic or biochemical effect. Partial

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antagonistic effects of both individual PCBs and commercial PCB mixtures on TCDD induced effects e.g. EROD activity (Biegel *et al*, 1989; Aarts *et al*, 1995), immunotoxicity (Davis and Safe, 1989; Davis and Safe, 1990) and teratogenicity (Haake *et al*, 1987) were reported by several authors. Also some furans were found to partially antagonise TCDD induced EROD activity (Bannister and Safe, 1987; Astroff and Safe, 1989). The Ah receptor affinity of compounds determined in *in vitro* competitive binding studies showed a correlation between the competitive displacement of TCDD from the Ah receptor and *in vivo* activity of congeners as partial antagonists (Astroff and Safe, 1989; Aarts *et al*, 1995). However, it is not clear whether or not this effect plays a role in our observations.

PCB 153 increased the hepatic congener concentrations of all congeners, except for TCDD, present in the mixture (Table 4.4). An increase of the liver retention of several PHAH congeners when co-administered with PCB 153, was reported also by De Jongh *et al.* (1993a,b; 1995). The interaction of PCB 153 with the hepatic deposition of PCDDs, PCDFs and some PCBs has been explained by its capacity to induce Ah-receptor levels in the liver (Denomme *et al.*, 1986; Bannister and Safe, 1987; De Jongh *et al.* 1993a) and to induce indirectly the activity and protein level of CYP1A2 (Kuroki *et al.*, 1986; Voorman and Aust, 1987, 1989; De Jongh *et al.*, 1995), which is assumed to be an important hepatic binding site for PHAH congeners (Kedderis *et al.*, 1993; Andersen *et al.*, 1993). This PCB 153 correlated change in congener-retention may explain the small difference in AHF level between the PHAH+ group compared to the TEQ equivalent dosed PHAH- group. A second explanation for the observed difference in AHF development between the PHAH+ and the PHAH- group is the tumour promoting activity of PCB 153 itself, which has been demonstrated by several others (Hemming *et al.*, 1993; Bager *et al.*, 1995; Berberian *et al.*, 1995) even at such a low dose of PCB 153 (Hemming *et al.*, 1993) as used in our study. It should be noted that the additive effects of PCB 153 on AHF development by the dioxin-like compounds in rats does not preclude that different effects may occur in different species.

The proposed mechanisms of partial antagonism and toxicokinetic interactions to explain the differences between the TEQ equivalent dosed groups on the AHF development are not supported by the observed EROD activities. The EROD activity in the PHAH+ and PHAH- group was even slightly, although not significant, higher compared to the TEQ equivalent dosed TCDD group. No correlation between volume fraction and EROD induction was found. A potentiation of the EROD activity by PCB 153 when co-administered with other congeners has been observed by others and could be explained by biochemical and/or toxicokinetic interactions (Bannister *et al.*, 1986, 1987b; Leece *et al.*, 1987; De Jongh *et al.*, 1993a,b). In our study however, no significant increase of the EROD activity in the PHAH+ group compared to the TEQ equivalent PHAH- group was observed. A possible reason could be that at the exposure levels utilised in this study, maximum EROD activity was already nearly achieved. A higher exposure of the animals may therefore result in a relatively small increase of the EROD activity.

The PROD activity was increased in all treatment groups including TCDD, which was

somewhat surprising. However, the increase in PROD activity did not correspond to an increase in enzyme protein levels. In our laboratory we have observed a similar increasing effect of PCB 126 on the PROD activity without any induction on CYP2B protein or mRNA levels. This TCDD and PCB 126 induced PROD activity possibly reflects induction of CYP1A as penthoxyresorufin is also moderately metabolised by CYP1A (Burke *et al.*, 1994).

Based on the weekly administered dose a REP for the PHAH+ and the PHAH- mixture of approximately 0.00026 and 0.00033 respectively was deduced. This is somewhat lower than the expected REPs of 0.00044 and 0.00097 for the PHAH+ and PHAH- mixture. The TEF concept was developed to facilitate risk assessment of complex mixtures of PCDDs, PCDFs and dioxin-like PCBs. Information from food surveys in industrialised countries indicate a daily intake of PCDDs, PCDFs and dioxin-like PCBs in the order of 100-600 pg TEQ/person/day (Van Leeuwen and Younes, 1998), which is a factor 40-1000 lower compared to the levels used in this study. Baltic herring and other fish species are among the main contributors of these substances to the human diet, the major source of human background exposure. This study confirmed that the international TEFs are conservative for evaluation of tumour promoting effects of mixtures similar to that in Baltic herring, resulting in risk assessments with large safety margins. REPs derived from tumour promotion studies would result in better, although still conservative, risk assessments of tumour promotive effects of these mixtures.

In conclusions, the dioxin-like PHAH mixtures used in this study enhanced the promotion of GST-p positive AHF significantly in female Sprague-Dawley rats but to a lower extent than expected based on the TEF concept. The conservative international TEFs (WHO 1993) and the uncertainty in the REPs used for the calculation of the composition and doses of the PHAH mixtures, may partly explain the observed differences in foci induction between the rats exposed to TCDD and the PHAH- mixture. However, differences in toxicokinetic properties of the congeners and interactive effects on deposition of the congeners may have influenced the predicted toxic potency of the mixture as well. Overall our study indicates that the TEF approach predicts the tumour promotion potency for dioxin-like dietary relevant mixtures of PHAHs quite well, i.e. within a factor of two.

Acknowledgements

CHAPTER 5

Contribution of planar (0-1 *ortho*) and non-planar (2-4 *ortho*) fractions of Aroclor 1260 to the induction of altered hepatic foci in female Sprague-Dawley rats

Abstract

The hepatic tumour promoting activity of the planar 0-1 *ortho* (~9.7% w/w) and the non-planar 2-4 *ortho* (~90.3% w/w) fraction of the commercial PCB mixture Aroclor 1260 was studied using a medium term two-stage initiation/promotion bioassay in female Sprague-Dawley rats. Fractionation was carried out on an activated charcoal column. The composition of the effluent from the column was tested by GC-ECD. The absence of planar compounds in the 2-4 *ortho* fraction was confirmed by GC-MS analysis. The dioxin-like toxic potency of the fractions was determined with the DR-CALUX assay. The animal experiment was started with the initiation procedure (i.p. diethylnitrosamin injection, 30 mg/kg body weight, 24 hours after $^2/_3$ hepatectomy), followed six weeks later by the promotion treatment which consisted of a weekly subcutaneous injection during 20 weeks. Exposure groups (n=10) received the following treatments (dose/kg body weight/week): Aroclor 1260 (10 mg), 0-1 *ortho* fraction (0.97 mg), 2-4 *ortho* fraction (1, 3 or 9 mg), a reconstituted 0-4 *ortho* fraction (9.97 mg), 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153; 1 or 9 mg), 2,3,7,8-TCDD (1 μg; positive control) or corn oil (1 ml; vehicle control). One group did not receive a promotion treatment. All exposure groups exhibited a significantly increased volume fraction of the liver occupied by hepatic foci positive for the placental form of glutathione-S-transferase-*p* compared to the corn oil control, except for the groups treated with 0-1 *ortho* fraction and 1 mg PCB 153/kg bw/week. Approximately 80% of the total tumour promoting capacity of the reconstituted 0-4 *ortho* fraction, could be explained by the 2-4 *ortho* PCB fraction while the 0-1 *ortho* fraction had only a negligible contribution. These results suggest that the majority of the tumour promotion potential of PCB mixtures resides in the non-dioxin like fraction, which is not taken into account in the Toxic Equivalency Factor (TEF) approach for risk assessment of PCBs. This may result in an underestimation of the tumour promotion potential of environmental PCB mixtures.

Introduction

Polychlorinated biphenyls (PCBs), polychlorodibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and other polyhalogenated aromatic hydrocarbons (PHAHs) are ubiquitously present in the environment. Their toxicity, which is characterised by effects such as body weight loss, endocrine disruption, impairment of immune responses, hepatotoxicity, reproductive effects, teratogenicity and carcinogenicity (Kimbrough, 1974; Safe, 1990; Brouwer *et al.*, 1999), has been intensively studied over the last 25 years. The most toxic PHAH compounds exhibit a planar molecular conformation with lateral chlorine substitution. Most if not all of their toxic responses are thought to be mediated by the aryl hydrocarbon (Ah) receptor. However, for the in general less toxic non-planar PCBs, other till now unknown mechanisms have been suggested (Safe, 1994)

In environmental matrices and biota, PCBs, PCDDs and PCDFs are always present as complex mixtures. The toxic equivalency factor (TEF) concept has been developed to aid in the risk assessment of complex mixtures of PHAHs and to enable the calculation of total dioxin-like toxic potencies, expressed as the TCDD equivalence (TEQ) of a mixture. The TEF approach is based on the assumptions that all PCBs, PCDDs and PCDFs that can assume a coplanar conformation act through the Ah receptor-based mechanism of action similar to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). In addition, the TEF concept assumes that the effects of the individual compounds are additive (Safe, 1994; Ahlborg *et al.*, 1994). Carcinogenicity is one of the toxic endpoints considered in risk assessment of PCBs, PCDDs and PCDFs (WHO, 1992). The TEF concept is therefore also considered to be a valid method to estimate the total carcinogenic potency of mixtures of PHAHs as they occur in food or environmental matrices.

PCBs, PCDDs and PCDFs have been found to be potent promoters of liver tumour formation in rodents (Safe, 1989; Silberhorn *et al.*, 1990; Whysner and Williams, 1996). Individual congeners as well as mixtures of PCBs, PCDDs and PCDFs have been reported to promote the development of enzyme altered hepatic foci in rats (Pitot *et al.*, 1980; Oesterle and Deml, 1983; Waern *et al.*, 1991; Hemming *et al.*, 1993; Bager *et al.*, 1995; Haag-Grönlund *et al.*, 1997a,b, 1998; Van der Plas *et al.*, 1999 *Chapter 4*). In addition, several PCBs, PCDDs and PCDFs have been shown to inhibit the gap junctional intercellular communication (GJIC) *in vitro* in the mouse hepa1c1c7 liver tumour cell line (De Haan *et al.*, 1996). Inhibition of GJIC has been suggested to be an *in vitro* indicator of the tumour promotion capacity of chemicals. Although the exact mechanism by which tumour promotion of these PHAHs occurs is unclear, it is assumed that the Ah receptor plays an important role (Safe, 1989, 1994).

However, both *in vitro* and *in vivo* studies suggest a tumour promotion capacity of several non-dioxin-like di-*ortho* substituted PCBs as well. 2,2',3,3',4,4'-Hexachlorobiphenyl (PCB 128), 2,2',5,5'-tetrachlorobiphenyl (PCB 52) (De Haan *et al.*, 1996) and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153) (Bager *et al.*, 1997) (IUPAC PCB nos., Ballschmiter et al.,

1992) have been shown to be potent inhibitors of the GJIC *in vitro*. 2,2',5,5'-Tetrachlorobiphenyl (PCB 52), 2,2',4,4'-tetrachlorobiphenyl (PCB 47) and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153) have been found to stimulate the growth of altered hepatic foci (AHF) in female Sprague-Dawley rats (Preston *et al.*, 1985; Hemming *et al.*, 1993). In addition, some studies have suggested both synergistic and antagonistic interactions between PHAHs and some non-planar di-*ortho* PCB congeners, or PCB mixtures on several toxicity parameters, including immunotoxicity (Biegel *et al*, 1989; Davis and Safe, 1989), teratogenicity (Zhao *et al.*, 1994), hepatotoxicity (Yao and Safe, 1989; Van Birgelen *et al.*, 1996a) and tumour promotion (Sargent *et al.*, 1991; Bager *et al.*, 1995, Haag-Grönlund *et al.*, 1998). Although di-*ortho* chlorinated PCBs are generally considered to be less toxic and possess no or negligible Ah receptor mediated activity, and thus have no TEF value, their concentration in commercial mixtures, food and environmental matrices is much higher compared to the planar PCBs.

In a previous experiment the tumour promotion capacity (AHF induction) of a PHAH mixture covering >90% of the TEQ in Baltic herring was determined (Van der Plas *et al.*, 1999 *Chapter 4*). It was shown that the TEF approach predicted the tumour promotion potency for this mixture quite well, i.e. within a factor of two. There was however some evidence for an interactive effect on the liver retention of dioxin-like congeners by addition of the di-*ortho* PCB 153 (Van der Plas *et al.*, 1998b *Chapter 3*), an effect which was also observed by De Jong *et al.* (1993a,b; 1995). The aim of the study presented here, was to determine the relative contribution of the dioxin-like PCBs and non-dioxin-like PCBs to the tumour promotion potential of a complex PCB mixture. As a surrogate mixture for the human diet the commercial PCB mixture Aroclor 1260 was chosen, on the basis of the congener pattern of the higher chlorinated, non-planar congeners. Aroclor 1260 was fractionated in a 0-1 *ortho* fraction and a fraction containing all 2-4 *ortho* substituted PCBs. The tumour promotion capacity of the separate fractions and their relative contribution to the effect of the complete mixture was studied in the rat liver tumour promotion model of Pitot *et al.* (1978).

Material and Methods

Chemicals

N-nitrosodiethylamine (NDEA) was obtained from Fluka (Fluka Chemie, Buchs, Switzerland).

Aroclor 1260 was kindly provided by Dr. M. Van den Berg (Research Institute of Toxicology, University of Utrecht, The Netherlands) and 2,2',4,4',5,5'-Hexachlorobiphenyl (PCB 153) was synthesised by Prof. Å. Bergman (Department of Environmental Chemistry, Stockholm University, Sweden) and was > 99% pure.

Minimal essential medium (alfa-MEM) and foetal calf serum for cell culture were obtained from Gibco (Rockville, Maryville, USA). 96-Well culture view-plates were from

Packard (Meriden, CT, USA) and luciferin assay mix was from Promega (Madison, Wisconsin, USA).

GST Yp rabbit anti-rat serum was used as the primary antibody for gluthatione-S-transferase-p (GST-p) staining and was purchased from Biotrin International (Dublin, Ireland). The secondary antibody was peroxidase conjugated swine anti-rabbit immunoglobulin (P217) (Dako, Glostrup, Denmark). Bovin serum albumin (BSA) and diaminobenzidine tetra hydrochloride (DAB) were obtained from Sigma Chemical Company (St. Louis, MO, USA) and NADPH was from Boehringer Mannheim (Mannheim, Germany). Phosphate buffered saline (Dulbecco's 'A', pH 7.3; PBS) was purchased from Oxoid (London, UK), NaCl and Tris were from Merck (Darmstadt, Germany).

Resorufin was obtained from Janssen Chimica (Geel, Belgium) and ethoxyresorufin and penthoxyresorufin were synthesised according to Mayer *et al.* (1977). Protein was assessed using a Bradford Protein Assay kit obtained from Bio-rad (Hercules, California, USA). All other compounds were of analytical grade.

Animal mixtures

The commercial PCB mixture Aroclor 1260 was fractionated into three fractions according to the method described by Athanasiadou *et al.* (1991) with slight modifications. The method is based on a charcoal column procedure where the PCB congeners are eluted strictly according to the number of *ortho* chlorine's, i.e. higher *ortho* chlorinated congeners eluting first. Fractions are obtained containing two to four, one and no chlorine's substituted in the *ortho* positions. An additional fraction was obtained containing PCDFs and PCNs. Gas chromatography (GC) was performed according to Athanasiadou *et al.* (1991), but in addition to the DB-5 column a BPX5 column (25m × 0.22 i.d., SGE international Pty. Ltd) was used in order to get a full separation between two critical pairs of congeners that co-elute on the DB-5 column. These pairs are PCB 149 (2,2',3,4',5',6) from PCB 118 (2,3',4,4',5) and PCB 132 (2,2',3,3',4,6') from PCB 105 (2,3,3',4,4').

5.0 g Aroclor 1260 was dissolved in 170 ml of a heptane:benzene mixture (95:5) and transferred to a wet packed charcoal column (72 g charcoal) at a speed of 10 ml per hour followed by the same solvent mixture at 30 ml per hour up to 3500 ml sampled in 500 ml sub-fractions. The first 1500 ml eluate from the charcoal column contained 90.3% of the total PCB mass and the next 2000 ml 6.6%. The charcoal column was rinsed for planar PCBs not eluted by the heptane:benzene mixture. This was done in a Wallenberg-extractor (described by Athanasiadou *et al.*, 1991) using three 150 ml portions of recycling boiling toluene for 5 hours each. The combined extracts from the Wallenberg extractor contained 3.1% of the total PCB mass. Finally the composition of the different fractions was qualitatively analysed by GC-ECD. Three fractions were obtained with a total recovery of 99.1%; a 2-4 *ortho* non-dioxin-like fraction (~90.3% of the total mass), a 1-2 *ortho* fraction (~6.6% of the total mass) containing mono and di-*ortho* PCBs and a 0-1 *ortho* dioxin-like fraction (~3.1% of the total mass) containing mainly non-*ortho* PCBs with a trace of mono-ortho PCBs.

Table 5.1 Test compounds and treatment groups used in the tumour promotion experiment

Test compound	number of animals per group	maintenance[a] dose per kg bw/week	µg TEQ per kg bw/week	equivalent to amount of Aroclor 1260
- (untreated)	10	-	-	-
Corn oil	18	1 ml	no activity	-
2,3,7,8-TCDD	10	1 µg	1	-
0-1 *ortho* fraction	10	1 mg	0.0008	10 mg
2-4 *ortho* fraction	10	1 mg	no activity	1.1 mg
	10	3 mg	no activity	3.3 mg
	10	9 mg	no activity	10 mg
0-4 *ortho* fraction	10	10 mg	0.0008	10 mg
Aroclor 1260	10	10 mg	0.0017	10 mg
PCB 153	10	1 mg	no activity	-
	10	9 mg	no activity	-

[a] The first dose was a loading dose which was five times higher than the maintenance doses indicated here

For the animal experiment, the 0-1 *ortho* and the 1-2 *ortho* PCB fractions were combined into one fraction. Although this combined fraction contained approximately 5% of the total di-*ortho* PCB mass in Aroclor 1260, it will be further referred to as 0-1 *ortho* fraction. This was done in order to make a clear distinction between the fraction containing all PCB congeners with a dioxin-like activity on the one hand and the fraction strictly containing non-dioxin-like congeners on the other hand.

The 0-1 *ortho* and the 2-4 *ortho* fractions were studied separately as well as a reconstituted mixture, the 0-4 *ortho* fraction. The combination of the 0-1 *ortho* and the 2-4 *ortho* fractions was based on the relative concentrations of these congeners in Aroclor 1260 (Table 5.1). A small amount of the fractions was dissolved in DMSO to test the total dioxin like potency of the fractions in the Ah receptor-dependent H4IIE-Luc reporter gene assay (DR-CALUX). For animal exposure, all fractions were dissolved in corn oil and the following exposure groups were included: corn oil as a negative control; TCDD as a positive control (1 µg/ml); a 2-4 *ortho* PCB fraction of Aroclor 1260 (1, 3 and 9 mg/ml); a 0-1 *ortho* PCB fraction of Aroclor 1260 (1 mg/ml); a reconstituted 0-4 *ortho* fraction (10 mg/ml); Aroclor 1260 (10 mg/ml); and, PCB 153 (1 and 9 mg/ml) (Table 5.1).

GC-ECD and GC-MS analysis

The PCB congener composition and the concentrations of 16 selected chlorobiphenyls in corn oil samples of Aroclor 1260, the Aroclor 1260 fractions, as well as rat liver samples of groups treated with the 0- 1 *ortho* (n=3), the 0-4 *ortho* (n=3) and the highest dose of the 2-4 *ortho* fraction (n=3) were analysed by the Netherlands Institute for Fisheries Research (RIVO).

Extraction and clean-up prior to the GC determination of the PCBs were similar for *ortho* and non-*ortho* PCBs (De Boer, 1988), but an additional HPLC step was added to separate the non-*ortho* PCBs from the other PCBs. About 1 g of corn oil and 1 g of rat liver was homogenised, mixed with Na_2SO_4 and after ca. 3 hours drying time Soxhlet extracted for 6 hours with dichloro methane/pentane (1:1, v/v). A spike of ^{13}C labelled PCBs 77, 126 and 169 was added prior to extraction. After extraction, iso-octane was added as a keeper and the dichloro methane was removed by means of a rotary evaporator. Pentane was added to a volume of 50 ml. A gravimetric extractable lipid determination was carried out on this residue. The extracts were eluted with n-pentane over 15 g alumina columns (80% H_2O) with a capacity of 250 mg lipid. On average ca. 40-120 mg of lipid per rat liver was transferred to the alumina columns. Subsequently, the extracts were concentrated to 2 ml and eluted over 1.6 g silica columns (2% H_2O). The first fraction of 11 ml contained all PCBs. The mono- and di-ortho PCBs 101 (2,2',4,5,5'-PeCB), 105 (2,3,3',4,4'-PeCB), 118 (2,3',4,4',5-PeCB), 128 (2,2',3,3',4,4'-HxCB), 138 (2,2',3,4,4',5'-HxCB), 153 (2,2',4,4',5,5'-HxCB), 156 (2,3,3',4,4',5-HxCB), 180 (2,2',3,4,4',5,5'-HeCB), 187 (2,2',3,4',5,5',6-HeCB), 189 (2,3,3',4,4',5,5'-HeCB), 195 (2,2',3,3',4,4',5,6-OcCB) and 206 (2,2',3,3',4,4',5,5',6-NoCB) were analysed by GC-ECD according to a method of De Boer (1988). PCB 112 was used as an internal standard. An internal reference material, cod liver, a blank and a recovery standard were analysed in parallel. Two GC columns were used under conditions described in table 5.2.

The elution order and retention times of the PCBs mentioned above were all carefully checked as well as possible co-elution with other PCBs. This was done by injecting three standard solutions containing in total 94 PCBs on both columns and comparing the chromatograms with available information on retention times of ca. 50 PCBs previously individually injected on the same columns and with literature data (Larsen *et al.*, 1992, Larsen, 1995). Another part of this fraction was concentrated and transferred to an HPLC column. The non-*ortho* PCBs 77 (3,3',4,4'-TeCB), 81 (3,4,4',5-TeCB), 126 (3,3',4,4',5-PeCB) and 169 (3,3',4,4',5,5'-HxCB) were separated from the *ortho* PCBs by HPLC using a graphitised (Hypercarb) carbon column according to a method of De Boer *et al.* (1992). The non-*ortho* PCB fraction was concentrated under nitrogen to 0.2 ml in toluene, after addition of a recovery standard (PCB 101). Eel was analysed in parallel as an internal reference. The extracts were analysed by GCMS, under conditions as described in table 5.2 according to De Boer *et al.* (1992).

Table 5.2 Analytical conditions used for GC/ECD and GC/MS

Parameter	GC/ECD	GC/ECD	GC/MS
Instrument	HP 6890	PE Autosystem	HP 5890 (GC) HP 5988A (MS)
Column	CP Sil 19, 50 m, 0.15 mm, 0.2 µm	HT-8, 50 m, 0.20 mm, 0.2 µm	DB-5, 20 m, 0.25 mm, 0.25 µm
Carrier gas	Hydrogen, 200kPa	Hydrogen, 180 kPa	Helium, 100 kPa
Ionisation method			NCI, 150 eV
Oven temp.	3 min 90 °C, 30 °C/min to 215 °C, 40 min 215 °C, 5 °C/min to 270 °C, 20 min 270 °C	2 min 90 °C, 30 °C/min to 170 °C, 3 °C/min to 215 °C, 15 min 215 °C, 2 °C/min to 280 °C	3 min 90 °C, 30 °C/min to 180 °C, 10 °C/min to 280 °C, 45 min 280 °C
Reagent gas			methane, 0.65 torr
Source temp.			175 °C
Analyser temp.			100 °C (front) 75 °C (back)
Detector temp.	300 °C	375 °C	
Injector temp.	250 °C	270 °C	290 °C
Interface temp.			285 °C
Injection	Splitless (2 min)	Splitless (1 min)	Splitless (2 min)
Injection volume	1 µl	0.5 µl	2 µl

The detection limit (ng/kg) of the congeners was calculated as:

$$\frac{\text{lowest calibration concentration (ng/ml) x volume (ml) x 1000}}{\text{sample amount (g)}}$$

The lowest concentration of the calibration curve was chosen at a signal-to-noise ratio of ca. 5. The relative standard deviations of the analysed PCB congeners were as follows: PCB 77 2.6%; PCB 126 3.4%; PCB 169 16%; PCB 28 16%; PCB 52 9.4%; PCB 101 7.5%; PCB 105 8.5%; PCB 118 7.7%; PCB 138 7.5%; PCB 153 6.9%; PCB 156 10%; and, PCB 180 7.0%.

Ah receptor-dependent H4IIE-Luc reporter gene assay (DR-CALUX)

The DR-CALUX assay was performed to test the dioxin-like potency of the PHAH mixtures according to Aarts *et al.* (1995) adapted for a 96-wells plate as described by Murk *et al.* (1998). Rat H4IIE.pGudluc1.1 cells were prepared as previously described (Aarts *et al.*, 1995; Garrison *et al.*, 1996). Cells were seeded in 96-wells plates and grown in minimal essential medium (alfa-MEM) with 10% heat inactivated foetal calf serum. After culturing for 24 hours under standard conditions (37 °C, 5% CO_2) the cells were exposed to the test compounds for another 24 hours. Then the medium was removed from the cells, the cells were rinsed with 0.5×PBS buffer, lysed with lysis buffer (10 mM Tris, 2 mM dithiolthreitol and 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetra-acetic acid, pH 7.8) and after adding the luciferin assay mix the signal was measured with a Luminometer plate reader (Luminometer, Labsystem). Each concentration of a sample was tested in triplicate. Dose-response curves of the samples were interpolated on a standard curve of TCDD to obtain a relative toxic potency (REP_{CALUX}) for the sample.

Rat liver tumour promotion experiment

The animal experimental protocols were approved by the Animal Welfare Committee before starting the experiments. Young female Sprague Dawley rats (Møllegaard Breeding Centre Ltd., Denmark), about three weeks old at the day of arrival, were kept in wire bottom, stainless steel cages in groups of three or four animals under standard conditions (12 hr light/dark cycle, temperature 22 °C, humidity 55%) and fed ad libitum (Hope Farms Woerden, The Netherlands). After three weeks of acclimatisation, the initiation treatment was started by removing two thirds of the liver (partial hepatectomy; PH) under ether anaesthesia followed by an i.p. injection with NDEA (30 mg/kg) 24 hours after PH. Five weeks after PH the animals were divided in treatment groups; a vehicle control group (n= 18), a TCDD exposed positive control group (n= 10) and eight experimental groups (n=10). One group of animals did not receive a promotion treatment at all. The promotion treatment was started six weeks after the initiation procedure and consisted of a weekly s.c. injection with the test compound in corn oil (1 ml/kg body weight per week) for twenty weeks. Exposure concentrations were as indicated in table 5.1. The first dose was a loading dose, which was five times higher than the maintenance dose in order to achieve steady state conditions within a shorter period of time (Flodström and Ahlborg, 1989; Krowke *et al.*, 1989). Body weight, food and water consumption were recorded weekly.

One week after the last exposure the animals were sacrificed under ether anaesthesia by orbita puncture for blood collection (heparinised), followed by decapitation. The liver was sampled and a part of the lateral and caudal liver lobe was fixed in ice-cold acetone (4 °C), and embedded in paraffin 24 hours after fixation. A part of the lateral liver lobe was stored at -80 °C for gas chromatographic and mass spectrometric (GC-MS) analyses whereas the rest of the liver was homogenised on ice in a Potter tube with 0.01 M Tris-HCl-buffer and 0.25 M sucrose (pH 7.4). The homogenate was stored at -80 °C until preparation of microsomes.

Thymus, spleen, lung, heart, brain, kidneys and some adipose tissue were collected, weighed and stored at -80 °C for possible future studies.

General histopathology of liver samples

General histopathology was performed on liver tissue of the groups treated with corn oil, TCDD, Aroclor 1260, 0-1 *ortho* fraction, 9 mg/kg bw/week 2-4 *ortho* fraction and the 0-4 *ortho* fraction. Sections (5 µm) of formalin fixed tissue (n=5) were processed routinely for paraffin embedding, stained with haematoxylin and eosin (H&E) and examined by light microscopy.

Staining and analysis of altered hepatic foci

Sections (4 µm) of acetone fixed tissue were stained for GST-p positive foci as described by Haag-Grönlund *et al.* (1997a). Sections were deparaffinised in xylene and air dried. The endogenous peroxidase activity was blocked with a fresh solution of 0.5% H_2O_2 in methanol (10 min). Aspecific binding sites were then blocked with a 2.5% BSA solution in PBS (30 min). Sections were incubated overnight at room temperature with the GST-p primary antibody (dilution 1:2000) followed by incubation with peroxidase conjugated secondary antibody (dilution 1:100) for $2^1/_2$ hours. GST-p positive cells were stained for 5 minutes with diaminobenzidine tetra hydrochloride. Foci were analysed in the right lateral lobe of the liver using a Leica Aristoplan microscope connected to a Quantimet 570 Image Processing and Analysis system (Leica Cambridge Ltd. England). The methods used for stereological evaluation of altered hepatic foci have been reported previously (Flodström *et al.*, 1988). A total sectional area of approximately 3 cm^2 was analysed whereby the smallest group of GST-p positive cells scored as a focus had a radius of 35 µm (cut off limit).

Preparation of microsomes

Microsomes were prepared by differential centrifugation of liver homogenates at 9000 g for 30 minutes, the resulting supernatant was centrifuged at 105,000 g for 90 minutes, and the pellet was resuspended in a 0.01 M Tris-HCl buffer, pH 7.4. Microsomes were stored at -80 °C until further analysis.

Cytochrome P450 and other liver enzyme activity and protein measurements

The protein concentration in the liver microsomes was measured using the commercial available Bradford Protein Assay reagent with crystalline BSA as a standard.

The total cytochrome P450 content in liver microsomes was analysed according to the method of Omura and Sato (1964) with modifications of Rutten *et al.*, (1987). The difference spectrum was measured of microsome samples (protein concentration ±1 mg/ml) saturated with CO (60 seconds) followed by dithionite reduction of P450.

Ethoxyresorufin-*O*-deethylase (EROD) and penthoxyresorufin-*O*-deethylase (PROD) activity were measured in liver microsomes according to Burke *et al.*, (1977), adapted for use

with 96-well plates and a fluoro spectrophotometric plate reader (CytoFluor 2530, Millipore) as described by Morse *et al.*, (1996b). A pre-incubated mixture of microsomes (protein concentrations 100-400 µg/ml), BSA (4 mg/ml) and ethoxy- or penthoxyresorufin (8 µM) was incubated for 5 minutes at 37 °C after adding of 0.4 mM NADPH. Resorufin concentrations were measured after the reaction was stopped with 1 M NaOH.

Alanine-aminotransferase (ALT) and aspartate-aminotransferase (AST) were measured using a commercially available kit (Boehringer Mannheim, Germany), adapted for 96-wells plates. 200 µl Buffer (37 °C) was incubated for several minutes at 37 °C with 20 µl plasma where after 20 µl substrate was added. The absorbency change was measured at 365 nm with a spectrophotometer (Spectramax 340, Molecular Devices) during 5 minutes with intervals of 11 seconds.

Statistical analysis

Data were analysed with the statistical package SPSS-PC 7.5. Foci and total P450 data were log transformed in order to obtain a homogeneous variance between the groups. A Tukey's Honestly Significant Difference (HSD) test was used to perform a multiple comparison on statistical differences between groups.

Results

CALUX activity of the Aroclor 1260 fractions

The Ah receptor-mediated induction of luciferase (DR-CALUX) activity of the Aroclor 1260 fractions was measured in order to determine the dioxin-like activity in these fractions. The 0-1 *ortho* fraction showed CALUX activity up to 25 % of the maximum effect induced by TCDD (Figure 5.1). However, the 0-4 *ortho* fraction showed a maximum CALUX activity of only 3% and cytotoxicity at the highest dose level tested. The 2-4 *ortho* fraction did not demonstrate any CALUX activity at all indicating the absence of Ah receptor agonists. Using the CALUX, the dioxin-like activity for the different mixtures could be deduced (Table 5.1). The low maximum activity of the 0-4 *ortho* fraction is possibly a consequence of antagonistic interactions between the 2-4 *ortho* substituted PCBs and the planar congeners, as was observed by Aarts *et al.* (1995) and Van der Plas *et al.* (1998a *Chapter 2*). Because no reliable dioxin-like potency could be calculated from the 0-4 *ortho* curve, the curve of the 0-1 *ortho* fraction was used for this purpose.

GC-ECD and GC-MS analysis of the Aroclor 1260 fractions

Qualitative GC-ECD analysis of the Aroclor 1260 sub-fractions was performed prior to the final fraction preparation, to ensure that the 2-4 *ortho* fraction contained no 0- and 1-*ortho* PCB congeners. The 0-*ortho* PCB concentrations were below detection level and therefore the analysis was focussed on the 1-*ortho* PCBs, i.e. when 1-*ortho* PCBs are absent consequently
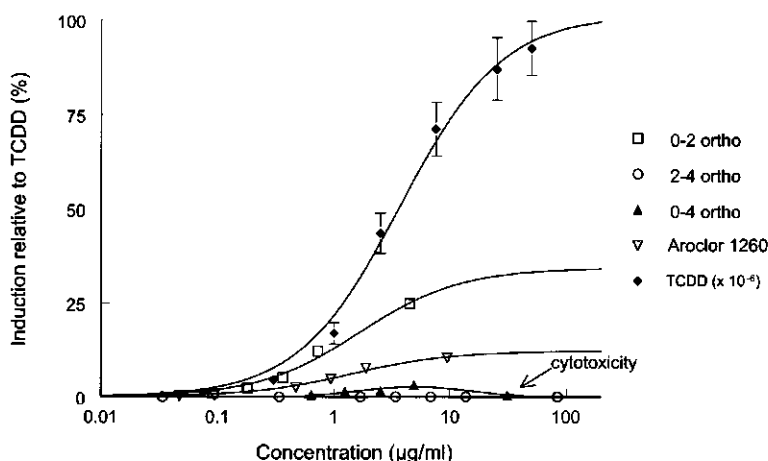
Figure 5.1 CALUX activity of 2,3,7,8-TCDD (concentration in $\mu g/ml \times 10^{-6}$) and the Aroclor 1260 fractions, expressed as % of the maximum induction by 2,3,7,8-TCDD

the 0-*ortho* PCBs are absent as well. No traces of 1-*ortho* congeners were found in the 2-4 *ortho* sub-fractions whereas seven 1-*ortho* PCBs were found in the 0-1 *ortho* sub-fractions. The following 1-*ortho* PCBs could be measured: PCB 105 (2,3,3',4,4'), 118 (2,3',4,4',5), 122 (2',3,3',4,5), 156 (2,3,3',4,4',5), 157 (2,3,3',4,4',5'), 167 (2,3',4,4',5,5') and 189 (2,3,3',4,4',5,5').

Concentrations of sixteen PCB congeners were quantitatively measured in Aroclor 1260 and the final Aroclor 1260 fractions (Table 5.3). No 0- and 1-*ortho* PCBs could be detected in the 2-4 *ortho*-substituted fraction, which is in accordance with the results obtained from the GC-ECD analysis of the sub-fractions. However, subtraction of the measured levels in the reconstituted 0-4 *ortho* and the 0-1 *ortho* fraction suggests relatively low levels of PCB 118, PCB 156 and PCB 189 in the 2-4 *ortho* fraction compared to the 0-1 *ortho* fraction. In the 0-1 *ortho* fraction low levels of several di-ortho PCBs were found (Table 5.3). The sum of PCB congener concentrations in the 0-1 and the 2-4 *ortho* fraction corresponds quite well to the PCB levels measured in the reconstituted mixture 0-4 *ortho* fraction. PCB levels in the 0-4 *ortho* fraction are slightly lower compared to PCB levels found in Aroclor 1260.

GC-MS analysis of rat liver tissue

Large differences between PCB concentrations per kg liver tissue (ww) were observed (Table 5.4) which is largely in accordance with concentration differences as found in the given PCB mixtures (Table 5.3). With the exception of PCB 126 in the livers of 0-1 *ortho* exposed animals, no planar compounds could be detected in either of the liver samples. Low levels of both 1- and 2-*ortho* PCBs could be detected. Liver retention of the PCB congeners was below 1% of the given dose for most congeners (Table 5.4). For PCB 126, a retention of 6% was observed in the 0-4 *ortho* group but this was only a single and possibly extreme observation.

Table 5.3 PCB congener concentrations in Aroclor 1260 and Aroclor fractions.

IUPAC nr.	Cl substitution	Aroclor µg/ml corn oil	0-1 *ortho* µg/ml corn oil	2-4 *ortho* µg/ml corn oil	0-4 *ortho* µl/ml corn oil
77	3,3',4,4'	0.02	0.07	<0.0001	0.05
81	3,4,4',5	0.003	0.004	<0.00008	0.002
101	2,2',4,5,5'	363	26	243	288
105	2,3,3',4,4'	<9	2.1	<6	<7
118	2,3',4,4',5	53	31	<18	39
126	3,3',4,4',5	0.02	0.023	<0.00005	0.014
128	2,2',3,3',4,4'	43	12	23	34
138	2,2',3,4,4',5'	594	62	396	477
153	2,2',4,4',5,5'	914	40	729	756
156	2,3,3',4,4',5	41	28	<7	32
169	3,3',4,4',5,5'	<0.01	0.004	<0.00007	<0.003
180	2,2',3,4,4',5,5'	1013	41	801	810
187	2,2',3,4',5,5',6	625	3.2	531	522
189	2,3,3',4,4',5,5'	13	9	<2	10
195	2,2',3,3',4,4',5,6	96	<2	78	81
206	2,2',3,3',4,4',5,5',6	64	<1	55	53

Animals received 1 ml corn oil/kg body weight/week for 20 weeks. The first dose was a loading dose, which was 5 times the PCB concentration of the maintaining dose. The total dose given to the animals is therefore equivalent to 24 ml corn oil/kg body weight ~21.6 gram corn oil/kg body weight. 0-1 *ortho*= 0-1 *ortho* substituted PCB fraction of Aroclor 1260 with a trace of 2 *ortho* substituted PCBs; 2-4 *ortho*= 2-4 *ortho* substituted PCB fraction of Aroclor 1260; 0-4 *ortho*= the reconstituted mixture of the 2-4 and the 0-1 *ortho* fraction. Absolute lowest reliable detection limits for the planar PCBs were 0.16 pg for PCB 77, 0.08 pg for 126, 0.12 pg for PCB 169 and 0.14 pg for PCB 81.

PCB 126 levels in the other analysed liver samples of the 0-4 *ortho* group were below the detection limit.

Clinical observations

No obvious treatment related signs of toxicity were observed throughout the study. Three animals were sacrificed during the promotion treatment period because of a poor condition: one animal of the TCDD treatment group (week 4); one in the group treated with the 0-1 *ortho* fraction (week 19); and, one animal in the group treated with the highest dose of the 2-4 *ortho* fraction (week 19). None of them was considered to be related to the treatment.

No changes were observed in body weight and body weight gain in the treatment groups (Table 5.5). The relative thymus weight in the TCDD treated group was slightly but not statistically significant reduced compared to the corn oil treated control group. The relative liver weights were significantly increased in the TCDD group, the group treated with 9 mg/kg

Table 5.4 PCB congener concentrations in the liver of female Sprague Dawley rats following sub-chronic exposure to PCB mixtures

IUPAC	Chlorine substitution	PCB content in the liver (µg/kg)			Liver retention (% of given dose)		
		0-1 *ortho* (0.97 mg)	2-4 *ortho* (9 mg)	0-4 *ortho* (9.97 mg)	0-1 *ortho* (0.97 mg)	2-4 *ortho* (9 mg)	0-4 *ortho* (9.97 mg)
77	3,3',4,4'	<0.5	<0.5	<0.5	-	-	-
81	3,4,4',5	<0.4	<0.4	<0.4	-	-	-
101	2,2',4,5,5'	15 ± 1	39 ± 22	32 ± 9	0.07 ± 0.001	0.03 ± 0.005	0.02 ± 0.005
105	2,3,3',4,4'	6 ± 1	<45	43 ± 3	0.39 ± 0.06	-	-
118	2,3',4,4',5	90 ± 8	0.025	127 ± 6.4	0.37 ± 0.03	-	0.47 ± 0.05[a]
126	3,3',4,4',5	0.18 ± 0.01	<0.6	0.19	0.99 ± 0.04	-	5.96[b]
128	2,2',3,3',4,4'	34 ± 4	39 ± 2	58 ± 7	0.38 ± 0.04	0.25 ± 0.007	0.24 ± 0.03
138	2,2',3,4,4',5'	190 ± 17	1830 ± 33	2230 ± 318	0.40 ± 0.03	0.68 ± 0.01	0.67 ± 0.09
153	2,2',4,4',5,5'	130 ± 12	3000 ± 115	3600 ± 557	0.43 ± 0.03	0.60 ± 0.04	0.68 ± 0.10
156	2,3,3',4,4',5	80 ± 9	<24	160 ± 19	0.38 ± 0.03	-	0.71 ± 0.08
169	3,3',4,4',5,5'	<0.3	<0.3	<0.3	-	-	-
180	2,2',3,4,4',5,5'	140 ± 19	2900 ± 88	3770 ± 601	0.43 ± 0.05	0.52 ± 0.02	0.66 ± 0.10
187	2,2',3,4',5,5',6	30 ± 3	1900 ± 88	2130 ± 318	1.03 ± 0.11	0.51 ± 0.03	0.58 ± 0.09
189	2,3,3',4,4',5,5'	30 ± 4	<6	53 ± 8	0.42 ± 0.04	-	0.76 ± 0.12
195	2,2',3,3',4,4',5,6	<15	350 ± 14	420 ± 55	-	0.65 ± 0.008	0.74 ± 0.10
206	2,2',3,3',4,4',5,5',6	<6	260 ± 6	320 ± 45	-	0.69 ± 0.03	0.86 ± 0.12

Data are given as arithmetic mean ± the standard error (n=3). Doses are given per kg bodyweight per week. - = not possible to calculate
0-1 *ortho*= 0-1 *ortho* substituted PCB fraction of Aroclor 1260 with a trace of 2 *ortho* substituted PCBs; 2-4 *ortho*= 2-4 *ortho* substituted PCB fraction
of Aroclor 1260; 0-4 *ortho*= the reconstituted mixture of the 2-4 and the 0-1 *ortho* fraction. *ᵃ* n=2, standard error is equal to the standard deviation. *ᵇ* n=1,
standard error could not be calculated

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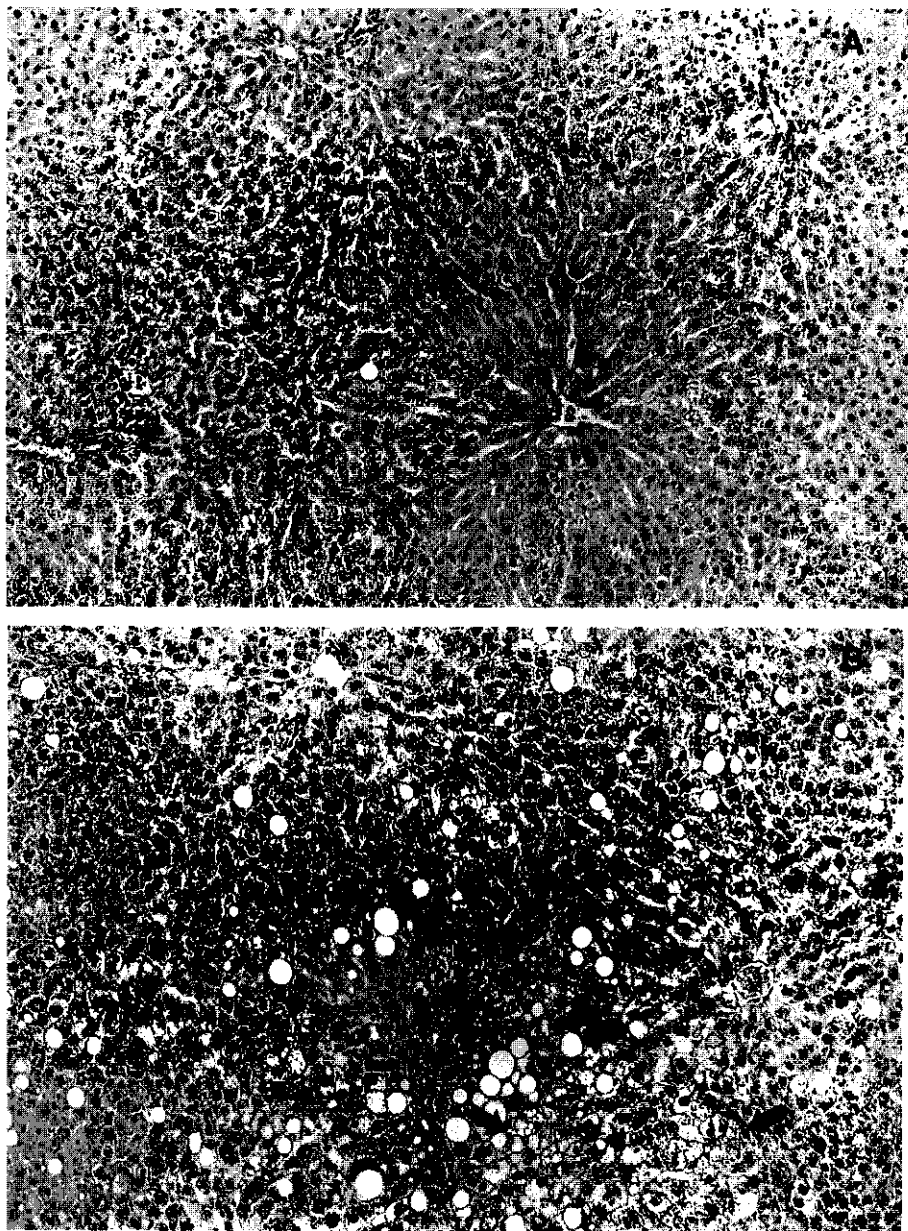
Figure 5.2A-B Liver tissue (HE stained) of female Sprague Dawley rats after 20 weeks of promotion treatment (magnifiction 10x). A Corn oil control; no evident changes in the morphology but some hepatocellular heterogeneity and clear cell foci were present. B TCDD; multinucleate cells, diffuse (non-zonal) lipid-laden cells (arrowhead) and an increased number of clear cell foci (arrow) were observed.
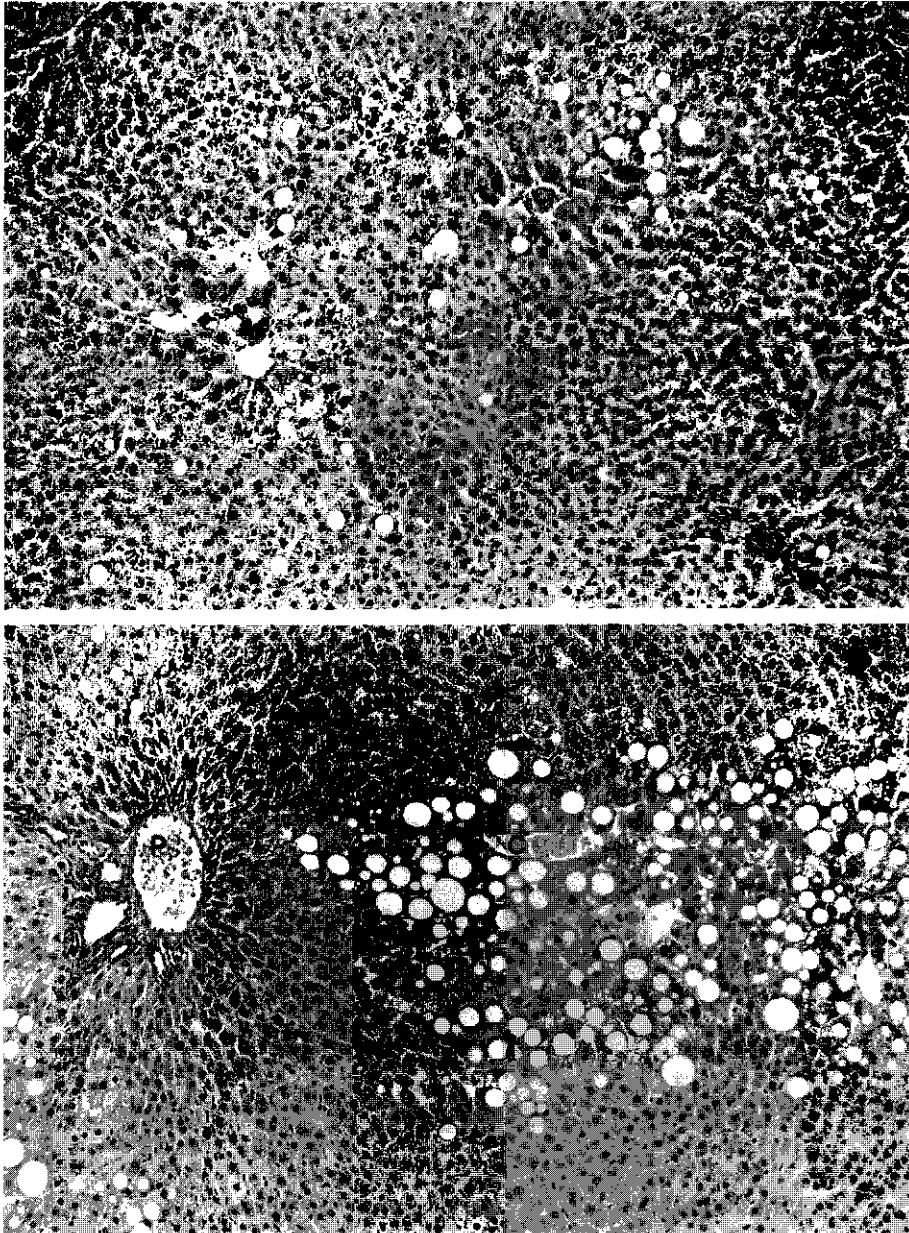
Figure 5.2C-D Liver tissue (HE stained) of female Sprague Dawley rats after 20 weeks of promotion treatment (magnifiction 10x). **C** 2-4 *ortho* fraction; changes were mainly centrilobular and consisted of prominent hepatocellular hypertrophy with morphological evidence for SER proliferation and fatty vacuolated cells. **D** 0-4 *ortho* fraction; pathology was similar to the 2-4 *ortho* fraction group (C=central vein, indicating centrilobular area; P=portal area). In none of the groups clear signs of liver necroses were found.

bw/week PCB 153, the 0-4 *ortho* fraction and Aroclor 1260 as compared to the corn oil group. A slight dose dependent increase of the relative liver weights was observed in the 2-4 *ortho* treated animals, this was however not statistically significant. No changes were found in the weights of the other organs examined (data not shown).

Liver toxicity parameters

Aspartate transaminase (AST) and alanine transaminase (ALT) activities were measured in the plasma as indicators for liver toxicity and are presented in table 5.5. No significant changes were observed in AST and ALT levels, indicating no overt liver damage in the various treatment groups.

Histopathology of the liver

In the corn oil control animals, some hepatocellular heterogeneity and clear cell foci were present but no evident changes in the morphology were observed (Figure 5.2A). Similar histology was observed in the group treated with the 0-1 *ortho* fraction (not shown), with the exception of a small increase in lipid-laden cells. In the other PHAH treatment groups, a more obvious change in morphology was observed in the liver tissue compared to the corn oil control group. In the TCDD treatment group, multinucleate cells, diffuse (non-zonal) lipid-laden cells and an increased number of clear cell foci were observed (Figure 5.2B). However, the observed changes in the 2-4 *ortho* (Figure 5.2C), the 0-4 *ortho* (Figure 5.2D) and the Aroclor 1260 (not shown) group were different from the TCDD treatment group. The most remarkable difference was that the changes seen in the 2-4 *ortho*, the 0-4 *ortho* and the Aroclor 1260 group were mainly centrilobular; these changes consisted of prominent hepatocellular hypertrophy with morphological evidence for SER proliferation and fatty vacuolated cells. No clear differences between the 2-4 *ortho*, the 0-4 *ortho* and the Aroclor 1260 group could be seen (not shown). No clear signs of liver necroses were found in any of the treatment groups.

Liver Cyp450 enzyme induction

The total cytochrome P450 content (Table 5.6) was significantly increased compared to the corn oil group in all PHAH treated groups except in the 0-1 *ortho* PCB group and the 1 and 3 mg/kg bw/week 2-4 *ortho* PCB treated groups. A dose dependent increase in the total P450 content was found for both the 2-4 *ortho* PCB and the PCB 153 treated groups.

The EROD activity was increased by 146 fold in the TCDD treated group compared to the corn oil and the untreated group (Table 5.6). None of the PCB treated groups reached similar activity levels. The EROD activity in the groups treated with Aroclor 1260 was significantly increased (8 fold) compared to the corn oil group and was slightly, but not significantly, higher than the group treated with the 0-4 *ortho* reconstituted PCB fraction. However, a 3-fold difference in EROD activity between the 0-1 *ortho* and the 0-4 *ortho* fraction was found, although both PCB fractions contained the same amount of dioxin-like

congeners. The groups treated with the 2-4 *ortho* fraction showed a slight, dose dependent increase of EROD activity which was only significantly different from the control group for the highest treatment dose.

The PROD activity was significantly increased in all exposure groups compared to the corn oil and the untreated control group, except in the groups treated with the 0-1 *ortho* fraction and the lowest dose of the 2-4 *ortho* fraction (Table 5.6). An increased PROD activity of 4 times the control level was also measured for TCDD, which is normally not considered to induce CYP2B.

Hepatic foci induction

Altered hepatic foci (AHF) development (Table 5.6) was expressed as the number of foci per cm^3 exceeding a cut off limit of 35 μm, the mean foci volume and the volume fraction of the liver occupied by foci.

All groups showed large numbers (5000-8000 foci/cm^3) of GST-p positive foci in the liver with a significant increase in the TCDD and the 0-4 *ortho* PCB exposed group compared to the corn oil control. The number of foci in the untreated control group represents the foci that are found after initiation treatment only (partial hepatectomy followed by a NDEA injection) without further promotion treatment and therefore represents a control for the initiation event.

The mean foci volume was significantly increased compared to the corn oil group in the groups treated with TCDD, 9 mg/kg bw/week of 2-4 *ortho* fraction and 9 mg/kg bw/week of PCB 153, the 0-4 *ortho* fraction and Aroclor 1260. A slight but non-significant increase was found in the groups treated with the lowest and the middle dose of the 2-4 *ortho* PCBs and PCB 153. No effect was found in the group treated with the 0-1 *ortho* fraction. The group treated with the reconstituted 0-4 *ortho* fraction showed a slight but non-significant lower mean foci volume compared to Aroclor 1260 treated group.

The volume fraction of the liver occupied by GST-p positive foci (VF) is considered the best parameter reflecting the promoting potential of a compound (Pitot *et al.*, 1980, 1987). The VF was significantly increased in most treated groups compared to the corn oil control and the untreated group but not in the groups treated with the lowest dose of PCB 153 and the 0-1 *ortho* PCB fraction. No clear dose-response of the 2-4 *ortho* fraction was observed on the VF. However it was striking that 1 mg/kg bw/week 2-4 *ortho* fraction showed a statistically significant effect on the VF while the same concentration 0-1 *ortho* fraction did not. The highest dose of the 2-4 *ortho* fraction and the reconstituted 0-4 *ortho* fraction caused a 2.6 and a 3.1 fold induction respectively of the VF, whereas the concentration equivalent 0-1 *ortho* fraction caused a mere 1.3 fold induction. Again, the induction in the group treated with Aroclor 1260 was slightly higher compared to the 0-4 *ortho* fraction. PCB 153 had a similar effect on both the mean foci volume and the VF compared to the 2-4 *ortho* fraction.

The sum of the individual effects of the 0-1 and the 2-4 *ortho* fraction on the mean foci volume and the VF was found to be equal to the effect of the 0-4 *ortho* fraction (Figure 5.3),
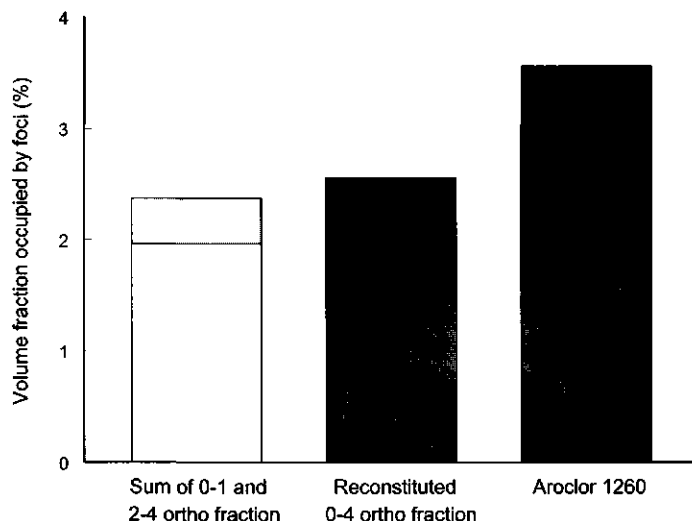
Figure 5.3 The volume fraction of the liver occupied by foci as: the sum of the individual 0-1 *ortho* (upper part) and the 2-4 *ortho* fraction (lower part), induced by the reconstituted 0-4 *ortho* fraction and induced by Aroclor 1260.

suggesting that possibly no interactive effects occurred between the 0-1 and the 2-4 *ortho* fraction. Interestingly, the 2-4 *ortho* fraction contributed approximately 80% to the effect on the VF by the reconstituted 0-4 *ortho* fraction (Figure 5.3).

Discussion

Most studies on tumour promotion by PCBs have investigated the potency of planar, dioxin-like congeners, with the presumption that the Ah-receptor pathway is also involved in mediating the tumour promoting effects of PHAHs. In addition, in most experiments the tumour promotion potential of individual compounds was studied (Flodström and Ahlborg, 1989; Wærn *et al.*, 1991; Hemming *et al.*, 1993; Haag-Grönlund *et al.*, 1997a,b) or at most combinations of two or three congeners (Sargent *et al.*, 1991; Bager *et al.*, 1995; Haag-Grönlund *et al.*, 1998). In this study, we have focused on complex mixtures of PCBs more closely resembling the mixtures present in the human diet. In a previous experiment it was shown that the TEF approach predicted the tumour promotion potency for a complex, dioxin-like PHAH mixture quite well, *e.g.* within a factor of two (Van der Plas, *et al.*, 1999 *Chapter 4*). The major aim of this study was to determine the contribution, if any, of the 2-4 *ortho* substituted PCBs as compared to the dioxin-like, 0-1 *ortho* PCBs. A secondary goal was to

Table 5.5 Body, liver and thymus weights and plasma activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) of female Sprague Dawley rats following sub-chronic exposure to PHAHs

Groups dose/kg bw/wk		Body weight[a] g	Body weight gain g	Relative liver weight g/kg bw	Relative thymus weight g/kg bw	ALT U/l	AST U/l
Untreated	- (n=9)	326.9 ± 12.9	225.9 ± 11.0	26.0 ± 1.0	0.61 ± 0.05	59.4 ± 6.5	142.2 ± 23.3
Corn oil	1 ml (n=18)	322.4 ± 5.3	245.2 ± 7.8	28.0 ± 0.6	0.62 ± 0.04	59.8 ± 3.7	140.4 ± 12.7
TCDD	1 μg (n=9)	308.7 ± 9.7	213.2 ± 12.0	34.6 ± 1.6[b]	0.45 ± 0.06	53.6 ± 4.0	87.6 ± 12.7
PCB 153	1 mg (n=10)	321.1 ± 4.3	226.7 ± 10.5	27.0 ± 0.8	0.68 ± 0.03	42.6 ± 3.2	87.5 ± 11.2
PCB 153	9 mg (n=10)	322.8 ± 6.5	261.6 ± 9.8	33.8 ± 0.6[b]	0.69 ± 0.05	48.6 ± 3.2	121.1 ± 13.6
2-4 *ortho*	1 mg (n=10)	329.4 ± 9.5	264.5 ± 12.5	28.5 ± 0.7	0.64 ± 0.07	61.7 ± 6.4	173.1 ± 24.4
2-4 *ortho*	3 mg (n=10)	321.9 ± 9.1	252.3 ± 14.9	29.6 ± 1.0	0.80 ± 0.09	40.4 ± 2.9	102.0 ± 12.5
2-4 *ortho*	9 mg (n=9)	328.6 ± 10.5	244.6 ± 15.1	30.8 ± 0.7	0.78 ± 0.10	51.9 ± 3.0	140.5 ± 19.4
0-1 *ortho*	1 mg (n=9)	322.1 ± 6.9	247.2 ± 15.5	27.1 ± 0.5	0.67 ± 0.06	50.9 ± 6.2	110.1 ± 14.8
0-4 *ortho*	10 mg (n=10)	323.7 ± 7.2	247.9 ± 15.4	32.2 ± 0.7[b]	0.67 ± 0.04	50.5 ± 5.9	117.0 ± 23.6
Aroclor 1260	10 mg (n=10)	320.8 ± 3.5	228.7 ± 9.4	32.3 ± 1.1[b]	0.59 ± 0.03	52.5 ± 3.1	133.5 ± 21.9

Data are given as arithmetic mean ± the standard error. Doses are given per kg bodyweight per week. 2-4 *ortho*= 2-4 *ortho* substituted PCB fraction of Aroclor 1260; 0-1 *ortho*= 0-1 *ortho* substituted PCB fraction of Aroclor 1260 with a trace of 2 *ortho* substituted PCBs; 0-4 *ortho*= the reconstituted mixture of the 2-4 and the 0-1 *ortho* fraction. [a] Body weight one week after the last exposure. [b] Significantly different from the corn oil group (p<0.05 Tukey HSD test).

Table 5.6 Cytochrome P$_{450}$ enzyme activity in the liver and foci development in female Sprague Dawley rats following sub-chronic exposure to PHAHs

Groups		Total P450	EROD activity	PROD activity	Number of foci	Mean foci volume	Volume fraction
dose/kg bw/wk		nmol/mg protein	nmol RR/min/ mg protein	pmol RR/min/ mg protein	foci/per cm^3	mm$^3 \times 10^3$	%
Untreated	- (n=9)	0.49 ± 0.04	24.0 ± 2.8	23.8 ± 0.8	5631.1 ± 318.3	2.5 ± 0.2	1.4 ± 0.2
Corn oil	1 ml (n=18)	0.46 ± 0.02	25.8 ± 2.0	22.3 ± 0.7	5132.9 ± 292.3	2.3 ± 0.2	1.2 ± 0.1
TCDD	1 µg (n=9)	1.05 ± 0.07^a	$3594.2 \pm 230.6^{a,b}$	91.6 ± 7.8^a	8406.1 ± 663.2^a	$7.2 \pm 1.4^{a,b}$	$6.3 \pm 1.5^{a,b}$
PCB 153	1 mg (n=10)	0.63 ± 0.05^a	32.5 ± 4.6^b	34.4 ± 3.0^a	6154.0 ± 734.0	3.2 ± 0.3	2.1 ± 0.4
PCB 153	9 mg (n=10)	1.11 ± 0.11^a	35.4 ± 3.4	161.1 ± 21.7^a	7352.6 ± 357.4	$4.7 \pm 0.5^{a,b}$	3.4 ± 0.3^a
2-4 ortho	1 mg (n=10)	0.56 ± 0.03	30.1 ± 2.1^b	25.1 ± 0.9	7098.5 ± 638.7	3.5 ± 0.4	2.5 ± 0.4^a
2-4 ortho	3 mg (n=10)	0.60 ± 0.03	34.4 ± 3.1	48.9 ± 3.1^a	7527.9 ± 673.8	3.4 ± 0.3	2.5 ± 0.3^a
2-4 ortho	9 mg (n=9)	0.94 ± 0.06^a	54.0 ± 6.3^a	137.3 ± 9.2^a	7021.2 ± 591.3	4.4 ± 0.6^a	3.2 ± 0.5^a
0-1 ortho	1 mg (n=9)	0.49 ± 0.04	59.7 ± 8.5^a	25.8 ± 0.9	6568.3 ± 778.2	2.5 ± 0.3	1.6 ± 0.2
0-4 ortho	10 mg (n=10)	1.11 ± 0.09^a	$176.8 \pm 19.0^{a,b}$	159.9 ± 13.1^a	8041.2 ± 817.4^a	$4.5 \pm 0.5^{a,b}$	3.7 ± 0.5^a
Aroclor 1260	10 mg (n=10)	1.00 ± 0.08^a	$218.9 \pm 29.8^{a,b}$	142.3 ± 16.3^a	7551.3 ± 706.4	$6.9 \pm 1.5^{a,b}$	$4.8 \pm 0.8^{a,b}$

Data are given as arithmetic mean ± the standard error. Doses are given per kg bodyweight per week. 2-4 *ortho*= 2-4 *ortho* substituted PCB fraction of Aroclor 1260; 0-1 *ortho*= 0-1 *ortho* substituted PCB fraction of Aroclor 1260 with a trace of 2 *ortho* substituted PCBs; 0-4 *ortho*= the reconstituted mixture of the 2-4 and the 0-1 *ortho* fraction. [a] Significantly different from the corn oil group (p<0.05 Tukey HSD test).
[b] Significantly different from the 0-1 *ortho* group (p<0.05 Tukey HSD test).

evaluate the predictability of the TEF concept for the tumour promoting potency of complex PHAH mixtures as surrogates for the actual mixtures present in the human diet. Separate fractions of 2-4 and 0-1 *ortho* substituted PCB mixtures were obtained by sub-fraction of the technical PCB mixture Aroclor 1260.

The 2-4 *ortho* substituted PCB fraction induced the formation of altered hepatic foci (AHF) to the same extent as the reconstituted 0-4 *ortho* fraction and Aroclor 1260 (Table 5.6). Surprisingly, approximately 80% of the total tumour promoting capacity of the reconstituted 0-4 *ortho* fraction, was explained by the effects induced by the 2-4 *ortho* PCB fraction. The contribution of the 0-1 *ortho* PCB fraction had a negligible effect on the induction of AHF. At the dose levels used, reconstitution of the 0-1 and the 2-4 *ortho* fraction into the 0-4 *ortho* mix, resulted in additive rather than interactive effects on AHF formation. This is an astonishing and important result, particularly because the toxicity of 2-4 *ortho* substituted PCBs is not taken into account in any kind of risk analysis of complex environmental PHAH mixtures.

To analyse the tumour promoting capacity of the tested PCB mixtures, the development of enzyme altered, glutathione-S-transferase-p positive foci was used. Foci are commonly seen as an early predictor of tumour promotion. Because selective proliferation of initiated cells may indirectly be stimulated by cytotoxic events, it is important to consider cytotoxic properties of promotive substances (Butterworth *et al.*, 1992). In this study, no biochemical signs of severe liver cytotoxicity were observed in any of the exposure groups. Histopathology of the liver slices showed no clear signs of necrosis. Therefore, we are confident that the observed foci induction is due to specific interaction of the test compounds and not a result of an aspecific toxicological response. Furthermore, a similar histological appearance was observed in the 2-4 *ortho* treatment group as in the groups treated with Aroclor 1260 and the 0-4 *ortho* fraction. The pathological changes observed in the liver tissue of the group treated with the 2-4 *ortho* fraction, were different from the changes observed in the TCDD treated group and resembled the morphology seen after phenobarbital treatment i.e. centrilobular hypertrophy and SER proliferation (Buchmann *et al.*, 1991; Popp and Cattley, 1991). This supports the observation that the liver effects are mainly caused by the 2-4 *ortho* substituted PCBs in the commercial mixture.

To be certain that the observed effect on tumour promotion of the 2-4 *ortho* fraction was really an effect of the non dioxin-like PCBs and not a result of contamination with planar compounds, several analyses were performed. After fractionation of Aroclor 1260 a qualitative GC analysis was done. On the basis of the GC chromatograms it was concluded that it was highly unlikely that planar compounds were present in the 2-4 *ortho* fraction. A GC-MS analysis of the Aroclor fractions was performed to quantify a selection of PCB congeners. No planar PCBs could be detected in the 2-4 *ortho* fraction. In addition to the GC-MS analysis, the DR-CALUX assay was used to test the total dioxin-like potency of the Aroclor fractions. No CALUX activity was observed in the 2-4 *ortho* fraction, indicating the absence of dioxin-like compounds. Another indicator for the presence of dioxin-like

compounds is the EROD activity in the liver of treated animals. Although there was a tendency towards a dose dependent increase of EROD activity induced by the 2-4 *ortho* fraction, EROD activity levels were still below the observed activity after exposure to the 0-1 *ortho* fraction. Based on the combined results of the GC and GC-MS analysis, the DR-CALUX and EROD assay, it can be concluded that the observed effects on tumour promotion by the 2-4 *ortho* fraction can be truly attributed to the non-dioxin-like PCBs. Consequently, the 2-4 *ortho* PCBs present in the reconstituted 0-4 *ortho* fraction were mainly responsible for the effects observed. Furthermore, GC-MS analysis showed that, with the exception of PCB 126, no planar congeners were detected in liver tissue of animals exposed to Aroclor 1260, the 0-1 or the 0-4 *ortho* fraction, which also suggests a minor role for the planar PCBs on the induction of AHF.

The hepatic retention (% of the given dose) was calculated based on the measured PCB concentrations in the Aroclor fractions and liver samples of the exposed animals. The hepatic retention tended to be quite low for e.g. PCB 156 (2-4 fold) but especially for PCB 126 (3-18 fold) as compared to retention levels seen in earlier experiments reported by Hemming *et al.* (1995) and Van der Plas *et al.* (1998b *Chapter 3*). Abraham *et al.* (1988) described a dose-dependent hepatic retention of TCDD, which did not change significantly between 1 and 10 ng TCDD/kg body weight but rised sharply between doses of 10 and 300 ng TCDD/kg body weight. In addition a linear relationship between the TCDD liver concentration and the EROD induction was observed (Abraham *et al.*, 1988). Deposition of PHAHs in the liver depends on the solubility of the congeners in liver fat and the induction of and/or binding to hepatic binding proteins (Leung *et al.* 1988) of which CYP1A2 is assumed to be an important inducible binding site in the liver for TCDD and related compounds (Kedderis *et al.*, 1993; Andersen *et al.*, 1993; DeVito *et al.*, 1998; Diliberto *et al.*, 1999). From the observed EROD induction and the GC-MS analysis of both Aroclor fractions and liver tissue it is evident that the animals in our study were exposed to low levels of dioxin-like PCBs. Low induction of hepatic binding proteins due to low exposure levels of dioxin-like PCBs might therefore explain the low hepatic retention of some of the PCB congeners observed in this study.

The EROD activity in the reconstituted 0-4 *ortho* group was approximately twice the sum of the activity observed after exposure to the separate 0-1 *ortho* and 2-4 *ortho* fractions. This observed non-additivity might be explained by the low EROD activity induced by the individual 0-1 *ortho* and 2-4 *ortho* fraction, below the steep part of the dose response curve. A low, statistically significant EROD induction was observed in the 9 mg/kg bw/week dose of the 2-4 *ortho* fraction. However, no Ah-receptor activity in the 2-4 *ortho* fraction was found with the *in vitro* CALUX assay.

An increased PROD activity was observed in the groups exposed to PCB 153, the 2-4 *ortho* fraction, the reconstituted 0-4 *ortho* fraction and Aroclor 1260. An increase of 4 times the control level was also measured for TCDD, which is normally not considered to induce CYP2B. An increased PROD activity induced by TCDD, or PCB 126 has been reported before and was shown not to correspond with increased enzyme P4502B1/2 protein or mRNA

levels (Van Birgelen *et al.*, 1996b; Van der Plas *et al.*, 1999 *Chapter 4*; Schuur *et al.*, 1998). This TCDD and PCB 126 induced PROD activity possibly reflects induction of CYP1A as penthoxyresorufin is also moderately metabolised by CYP1A (Burke *et al.*, 1994).

From these results it is clear that the 2-4 *ortho* substituted PCBs play an important role in tumour promotion by the commercial PCB mixture Aroclor 1260. This challenges the hypothesis that the Ah receptor route is the only mechanism involved in tumour promotion by PHAHs. Other mechanisms, different from the Ah receptor route may play an important role in the tumour promotive effects of PCBs. There are only very limited studies that focus on mixtures of di-tetra *ortho* substituted PCBs. Kihlström *et al.* (1992) studied the reproductive effects of Clophen A50, Aroclor 1254 and fractions of these mixtures in mink. From this study it was concluded that the 2-4 *ortho* PCBs increased the toxicity of the other fractions but did not show a significant activity on its own. Furthermore they suggest that from this 2-4 *ortho* fraction the major methyl-sulfonyl-substituted PCBs are formed which can be very persistent in some wildlife animals (Bergman *et al.*, 1992; Kihlström *et al.*, 1992). Interestingly, some of the methyl-sulfonyl-substituted PCBs were found to inhibit the intercellular communication *in vitro* (Kato *et al.*, 1998), which is seen as an important parameter for tumour promotion. Mayes *et al.* (1998) recently published an extensive chronic study on the comparative carcinogenicity of the commercial mixtures Aroclor 1016, 1242, 1254 and 1260. They concluded that the tumour incidence differed between Aroclor mixtures in a manner that paralleled the differences between Aroclors in TEQs (Mayes *et al,.* 1998). Our study however suggests that the group of non-Ah-receptor agonists may contribute considerably to the total tumour promotion potential of PCB mixtures. Another factor of importance in the tumour promotion capacity of complex mixtures may be the presence of readily metabolising PCBs like 2,3',4,4',5,-pentachlorobiphenyl (PCB 118). However, in an earlier study performed by Haag-Grönlund *et al.* (1997a) it was shown that the highly metabolised PCB 118 is a very weak tumour promoter. Not much is known yet about the carcinogenic potency of hydroxy PCB metabolites *in vivo*. However, it was shown *in vitro* that several hydroxy PCB metabolites inhibit intercellular communication (De Haan *et al.,* 1994a).

PCB 153 showed tumour promotion activity comparable to the 2-4 *ortho* fraction, suggesting that this congener may act as a model compound for tumour promotion of non-dioxin-like PCBs. However, nothing is known yet about the mode of action as well as the similarity between the tumour promotion potential of PCB 153 and complex 2-4 *ortho* PCB mixtures with a different composition. This information may be valuable because several studies have been performed, using PCB 153 as a model compound for the group of non-dioxin-like PCBs (Buchmann *et al.*, 1986; Hemming *et al.*, 1993; Bager *et al.*, 1995). The lowest dose of the 2-4 *ortho* PCB fraction (1 mg/kg bw/week) used in this experiment showed a significant effect on the AHF development after sub-chronic exposure. In risk management little or no attention is paid to this category of PCBs. From GC-MS analysis of environmental PCB mixtures it is known that over 90% consist of 2-4 *ortho* substituted

congeners (De Boer *et al.*, 1992). This means that an intake of 160 μg PCBs/kg bw/day, of which 90% is 2-4 *ortho* substituted, corresponds with an intake level of 2-4 *ortho* PCBs found to be carcinogenic in this study. This is approximately 50 times the Acceptable Daily Intake (ADI) of 3 μg PCBs/kg bw/day.

In conclusion, the 2-4 *ortho* PCBs were shown to be potent tumour promoters and were responsible for approximately 80% of the observed effect on foci development by the 0-4 *ortho* PCBs present in Aroclor 1260. No interactive effect on tumour promotion between the 0-1 and 2-4 *ortho* PCBs was observed. This study indicates that the tumour promotion potential of 2-4 *ortho* substituted PCBs is more important in the total toxicity of a complex PCB mixture than dioxin-like PCB congeners. This should seriously be taken into account in risk estimation of the tumour promotion potential of environmental mixtures of PHAHs. There is, however, at this moment no risk management tool (e.g. like the TEF concept for dioxin-like PCBs) available for these non-dioxin-like PCBs. In this study PCB 153 was shown to possess a similar tumour promotion activity as the 2-4 *ortho* fraction of Aroclor 1260 and thus may serve as a model compound for this group of congeners.

Acknowledgements

CHAPTER 6

Effects of sub-chronic exposure to complex mixtures of dioxin-like and non-dioxin-like polyhalogenated aromatic compounds on thyroid hormone and vitamin A levels in female Sprague-Dawley rats

Abstract

The aim of this study was to determine the effects of sub-chronic exposure to complex mixtures of polyhalogenated aromatic hydrocarbons (PHAHs) on the thyroid hormone and retinoid status in female Sprague-Dawley rats and to investigate the predictability of these effects by the toxic equivalency factor (TEF) concept. In the first experiment the focus was on a complex dioxin-like PHAH mixture, which covered > 90% of the total toxic equivalents (TEQ) present in Baltic herring. In the second experiment the contribution of non-dioxin-like polychlorinated biphenyls (PCBs) was investigated by testing the commercial PCB mixture Aroclor 1260, its 0-1 *ortho* and 2-4 ortho fractions and the reconstituted 0-4 *ortho* fraction. Hepatic retinoid levels were severely decreased (~70%) after treatment with the dioxin-like PHAH mixture, similar to the effect of a TEQ equivalent dose of 1 µg 2,3,7,8-TCDD/kg bw/week. However, the TEF concept failed to predict the effect on plasma retinol since a decrease (21%) was observed after treatment with the PHAH mixture, while an increase (21%) was found after treatment with TCDD. A more severe decrease of total thyroid hormone in plasma was observed after exposure to the PHAH mixture compared to treatment with TCDD (~60% vs. 38%). The discrepancy found between the predicted and observed effects on plasma retinol and thyroid hormone is possibly due to an additional effect of hydroxylated PCBs, formed from metabolisable PCBs present in the PHAH mixture. Aroclor 1260 and its fractions did not significantly alter the retinoid and thyroid hormone status at the dose levels tested, indicating that in case of exposure to complex PCB mixtures at environmental levels, no or at best only marginal effects can be expected on the retinoid and thyroid hormone status.

Introduction

Polychlorinated biphenyls (PCBs), polychlorodibenzo-p-dioxins (PCDDs), poly-chlorinated dibenzofurans (PCDFs) and other polyhalogenated aromatic hydrocarbons (PHAHs) elicit a broad spectrum of toxic effects and biochemical changes, e.g. body weight loss, thymic atrophy, hepatotoxicity, carcinogenicity, induction of hepatic cytochrome P450 isoenzymes and alterations of the retinoid status and thyroid hormone metabolism (Safe, 1990, 1994).

Most if not all of the toxic responses of PHAHs are suggested to be mediated by the aryl hydrocarbon (Ah) receptor (Ahlborg et al., 1994; Safe, 1994). The most toxic PHAH compounds exhibit a planar molecular conformation with lateral chlorine substitution thereby expressing a so-called dioxin-like toxicity. Di-*ortho* substituted PCBs are in general less toxic and due to their non-planar conformation they do not exhibit Ah-receptor agonistic activity but have been shown to possess a phenobarbital-like toxicity (Safe, 1994).

In environmental matrices and biota PCBs, PCDDs and PCDFs are always present as complex mixtures. The toxic equivalency factor (TEF) concept has been developed to aid the risk assessment of complex mixtures of PHAHs. Based on *in vivo* and *in vitro* studies the relative toxic potencies of individual PHAHs have been determined relative to 2,3,7,8-TCDD, as being the most toxic congener. The TEF concept is based on the assumptions that all non-*ortho* and mono-*ortho* chlorine substituted PCB congeners and related dibenzo-*p*-dioxins and dibenzofurans act through the Ah receptor-based mechanism of action and that the effects of the individual compounds in a mixture, expressed as toxic equivalencies (TEQs), are additive (Safe, 1990, 1994; Ahlborg et al., 1994). However, interactive effects have been observed between PHAHs and some non-planar di-*ortho* PCB congeners, or PCB mixtures (Aarts et al, 1995; Bager et al., 1995; Biegel et al, 1989; Davis and Safe, 1989; Haag-Grönlund et al., 1998; Sargent et al., 1991; Yao and Safe, 1989; Zhao et al., 1994). In addition, several di-*ortho* PCBs have been shown to possess toxic properties both *in vivo* and *in vitro*, e.g. disturbance of the vitamin A and thyroid hormone status, development of altered hepatic foci and inhibition of intercellular communication (Van Birgelen et al., 1992; Bager et al., 1997; De Haan et al., 1996).

Several toxicity studies have been performed with complex PCB mixtures (Kimbrough et al., 1975; Ward 1985; Ahlborg et al., 1987; Abraham et al., 1989; Kihlström et al., 1992; Morse et al., 1996a). However, in most studies the focus was on individual PHAH congeners, or combinations of two or three congeners (Bager et al., 1995; Van Birgelen et al., 1994a,b; Haag-Grönlund et al., 1998; Sargent et al., 1991). Our major aim of this project was to evaluate the applicability of the TEF concept for the tumour promotion potential of complex environmentally relevant PHAH mixtures (Van der Plas et al. 1999 *Chapter 4*, 2000a *Chapter 5*). In addition, the effect of sub-chronic exposure to these PHAH mixtures was determined on endocrine parameters, in particular on the vitamin A and thyroid hormone system. Vitamin A and thyroid hormone are both essential for normal tissue growth, differentiation and foetal

development and are possibly involved in carcinogenesis (Blomhoff, 1994; Dunn, 1989; Guernsey, 1993).

Alterations in both vitamin A and thyroid hormone levels are a well known effect of PHAHs in rodents, following single dose, or sub-chronic treatment (Brouwer *et al.*, 1988b; Chen *et al.*, 1992; Van Birgelen *et al.*, 1992, 1994a,b, 1995). In short, retinol and thyroxin (T4) were both reduced in rat plasma following exposure to metabolizable planar (PCB 77), mono- and di-*ortho* PCBs (Brouwer *et al.* 1988a; Morse *et al.* 1996a). However, TCDD and relatively stable PCBs (PCB 126, 169, 156) were found to increase plasma retinol, while T4 was still reduced (Van Birgelen 1994a,b, 1995). The reasons for the effects of PHAHs on plasma levels of retinol and T4 are partly due to a) direct effects of hydroxylated PHAH metabolites on the plasma transport protein complex of T4 and retinol (Brouwer and Van den Berg, 1986; Brouwer *et al.*, 1988a,b) b) effects on hepatic metabolism of T4 and retinol by parent compounds, i.e. mainly increased T4 glucuronidation and reduced formation of retinyl esters and retinol metabolism (Zile, 1992; Brouwer *et al.*, 1998b).

In this manuscript the effects on vitamin A and thyroid hormone status in female Sprague Dawley rats was studied after sub-chronic exposure to complex PHAH mixtures. The possible interactive effects between congeners and the involvement of different mechanisms are discussed. Data were obtained from two independent experiments. In the first experiment the focus was on a complex synthetic mixture of dioxin-like, planar compounds and possible interactive effects with the non-dioxin-like PCB 153 (2,2',4,4',5,5'-HxCB). The composition of this mixture was based on the presence and relative ratio's of PHAH in fish, as being one of the main contributors of PHAHs to the human diet. In the second experiment the main focus was on complex mixtures containing only di-*ortho* PCBs, or planar (non- and mono-*ortho*) PCBs. These mixtures were obtained by fractionation of the commercial PCB mixture Aroclor 1260. The planar and di-*ortho* PCBs were individually tested and after reconstitution.

Materials and methods

Chemicals

N-nitrosodiethylamine (NDEA) was obtained from Fluka (Fluka Chemie, Buchs, Switzerland).

3,3',4,4',5-Pentachlorobiphenyl (PCB 126), 2,3',4,4',5-pentachlorobiphenyl (PCB 118), 2,3,3',4,4',5-hexachlorobiphenyl (PCB 156) and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153) were kindly provided by Prof. Å. Bergman (Department of Environmental Chemistry, Stockholm University, Sweden). 1,2,3,7,8-Pentachlorodibenzo-*p*-dioxin (PeCDD) was obtained from Wellington Laboratories (Canada), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) was a gift from Prof. S.H. Safe (College of Veterinary Physiology and Pharmacology, Texas, A&M University, USA) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was purchased from RADIAN CIL, inc.(U.S.A.). All compounds had a purity > 99%. Aroclor 1254 and 1260

were kindly provided by Dr. M. Van den Berg (Research Institute of Toxicology, University of Utrecht, The Netherlands).

Retinoid standards (retinol, retinyl palmitate and retinyl acetate) were all obtained from Fluka Chemie (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands). Amerlite chemiluminescence assay kits for thyroid hormone analysis were obtained from Ortho-clinical Diagnostics (Amersham, UK).

All other compounds were of analytical grade.

Animal experiments

Two sub-chronic animal experiments were performed, both approved by the animal welfare committee before starting the experiments. The treatment protocol used for these experiments was based on the altered hepatic foci (AHF) tumour promotion protocol introduced by Pitot *et al.* (1978) and described in detail by Van der Plas *et al.* (1999 *Chapter 4*, 2000a *Chapter 5*). In short, an initiation step, consisting of a diethylnitrosamine injection (i.p. 30 mg/kg body weight) 24 hours after a partial $^2/_3$ hepatectomy, is followed by a promotion treatment of 20 weeks starting six weeks after the hepatectomy.

For these experiments, juvenile female Sprague-Dawley rats (Møllegaard Breeding Center Ltd., Denmark) of about six weeks old at the start of the experiment were used. The rats were kept in wire bottom, stainless steel cages in groups of four animals under standard conditions (12 hr light/dark cycle, temperature 22 °C, humidity 55%) and fed ad libitum (pellets, Hope Farms Woerden). Test compounds were administered once a week by subcutaneous injections for 20 weeks in concentrations as indicated in table 6.2. A corn oil group (1 ml/kg body weight/week) as a negative vehicle control and a TCDD group (1 µg/kg body weight/week) as a positive control were incorporated in both experiments. The first dose was a loading dose, which was 5 times the concentration of the maintenance dose given for the following 19 weeks.

One week after the last injection the animals were sacrificed under ether anaesthesia, using orbital puncture for blood collection (heparinised), followed by decapitation. The liver was collected, of which a part was stored at -80 °C for gas chromatography and mass spectrometry (GC-MS) analysis, a part was placed in formaldehyde and acetone fixative for immunohistochemistry purposes whereas the rest of the liver was homogenised on ice in a Potter tube with 0.01 M Tris-HCl-buffer and 0.25 M sucrose (pH 7.4). Plasma was collected by centrifugation of the blood at 500 *g* for 10 minutes.

PHAH exposure mixtures

Two different environmentally relevant PHAH mixtures were tested in separate sub-chronic animal experiments in groups and concentrations as presented in table 6.1.

The composition of the PHAH mixtures tested in experiment 1 was based on PHAH contamination found in Baltic herring and covered over 90% of the TEQs present in these fish. The PHAH mixture contained the following congeners: 2,3,7,8-TCDD, 1,2,3,7,8-

Table 6.1 Dose levels of the PHAH mixtures and Aroclor 1260 fractions used in the animal studies

Mixture (experiment)	Group size	Dose	Equivalent to Aroclor 1260	TEQ
	n	/kg bw/week	amount (mg)	/kg bw/week
Corn oil (1&2)	18	1 ml	-	no activity
2,3,7,8-TCDD (1&2)	12&10	1 µg	-	1 µg
PHAH+ (1)	12	1.1 mg	-	$0.5\ µg^a$
		2.3 mg	-	$1\ µg^a$
		4.5 mg	-	$2\ µg^a$
PHAH- (1)	12	1.0 mg	-	$1\ µg^a$
0-1 *ortho* fraction (2)	10	1 mg	10	$0.8\ ng^b$
2-4 *ortho* fraction (2)	10	1 mg	1.1	no activity^b
		3 mg	3.3	no activity^b
		9 mg	10	no activity^b
0-4 *ortho* fraction (2)	10	10 mg	10	$0.8\ ng^b$
Aroclor 1254 (1)	12	7.5 mg	-	$0.2\ µg^c$
Aroclor 1260 (2)	10	10 mg	10	$1.7\ ng^b$

[a] TEQ values based on literature data (see Van der Plas *et al.* 1999 *Chapter 4*). [b] TEQ values determined using the DR-CALUX assay (Van der Plas *et al.*, 2000a *Chapter 5, Chapter 2*). [c] TEQ value based on WHO TEF values (Van den Berg *et al.*, 1998) and PCB concentrations in Aroclor 1254 (Leonards *et al.*, 1995).

PeCDD, 2,3,4,7,8- PeCDF, PCB 126, PCB 118, PCB 156 and PCB 153, in relative ratios as indicated in table 6.2. In order to investigate possible interactive effects between planar and non-planar congeners, the mixture was also tested without the non-dioxin-like PCB 153. The PHAH mixture containing solely dioxin-like PHAHs is referred to as PHAH- , the mixture including PCB 153 is referred to as PHAH+. A more detailed description of the mixture preparation and the experimental set up is given in Van der Plas *et al.* (1999 *Chapter 4*). In addition to the PHAH mixtures, the commercial PCB mixture Aroclor 1254 was tested. Calculation of the TEQs of the PHAH mixtures was based on TEF values as proposed by the WHO (Ahlborg *et al.*, 1994) and relative potency (REP) values obtained from tumour promotion studies (Wærn *et al.*, 1991; Hemming *et al.*, 1993, 1995).

In experiment 2, the focus was on the non-dioxin-like di- to tetra-*ortho* substituted PCBs. For that purpose the commercial PCB mixture Aroclor 1260 was chosen as the experimental mixture, since approximately 90% of Aroclor 1260 consists of non-dioxin-like

Table 6.2 Congener composition of the PHAH mixtures of experiment 1

| Congener | Relative level (weight base); | |
	PHAH- mixture - PCB 153	PHAH+ mixture + PCB 153
2,3,7,8-TCDD	1	1
1,2,3,7,8-PeCDD	3.3	3.3
2,3,4,7,8-PeCDF	17	17
PCB 126	61	61
PCB 118	12800	12800
PCB 156	1888	1888
PCB 153	-	20000

The composition of the mixtures is based on Swedish herring oil and covers over 90% of the TEQs in Swedish herring.

congeners. Aroclor 1260 was fractionated into a 0-1 *ortho* dioxin-like fraction (~9.7 % of the total mass) containing mainly 0 and 1-*ortho* substituted PCBs and a trace of 2-*ortho* PCBs, and a 2-4 *ortho* non-dioxin-like fraction (~90% of the total mass). This was done according to a method described by Athanasiadou *et al.* (1991) with slight modifications described in more detail by Van der Plas *et al.* (2000a *Chapter 5*). The 0-1 *ortho* fraction and the 2-4 *ortho* fraction were tested separately and as a reconstituted 0-4 *ortho* mixture. Aroclor 1260 and PCB 153 were incorporated as an extra positive control and a model compound for the di-*ortho* PCBs respectively. The composition of PCB congeners in the various fractions was tested by GC-MS analysis and confirmed that no 2-4 *ortho* PCBs were present in the 0-1 *ortho* fraction (Van der Plas *et al.*, 2000a *Chapter 5*). The TEQs of the Aroclor 1260 fractions were determined using the DR-CALUX bio-assay (Van der Plas *et al.*, 2000a *Chapter 5*).

Vitamin A analysis

Retinol levels were measured in plasma and retinol and retinyl palmitate levels in liver homogenates according to Brouwer *et al.* (1989) with some modifications. Plasma, or liver homogenate (50 µl) was extracted with methanol containing a 0.1% BHT as an anti oxidant and an internal standard (0.5 µg retinyl acetate/ml for plasma, 1 µg retinyl acetate/ml for liver homogenate), and diisopropyl ether in a 1:1:2 concentration. Samples were vortexed for 30 sec., kept overnight at -20 °C, vortexed again and centrifuged for 10 minutes at 5000 rpm in an eppendorf centrifuge. The diisopropyl-ether phase was collected and filtered over a 0.45 µm filter (Millipore, Etten Leur, The Netherlands) evaporated under N_2 and resuspended in 50 µl methanol (plasma extracts) or 200 µl 1:3 ethylacetate/methanol (liver extracts) with 0.1% BHT. Extractions were carried out in duplicate. Extraction efficiencies were routinely above 80%. Twenty µl aliquots of resuspended extracts were analysed with HPLC using a C18 analytical reversed phase column (Pecosphere, 3 µm particle size, 3.3 cm length and 4.6 mm

internal diameter, Perkin Elmer) and a wavelength of 326 nm for detection of retinoids. A Merck-Hitachi HPLC system was used consisting a L-6200 Intelligent pump, L-4200 UV-VIS detector, AS-2000 Autosampler and a D-2500 Chromato Integrator. Plasma extracts were analysed isocratically with 86% methanol and 14% water with a flow rate of 1 ml/min and data collection for 10 minutes. Hepatic retinoids were analysed by 86% methanol and 14% water for 1.5 minutes, followed by a gradient to 100% methanol for 2.5 minutes, and subsequent elution of the retinyl esters for 12 minutes. The column was then re-equilibrated at 86% methanol and 14% water for 6 minutes.

Plasma thyroid hormone analysis

Total thyroxin (TT4), total triiodothyronine (T3) and free thyroxin (FT4) levels were determined in plasma using commercially available chemiluminescence kits, according to the protocol of the supplier with the following modifications: the T4 assay buffer was diluted 5 times in demineralised water. The standard curve for TT4 ranged from 0-120 nmol/liter, for T3 from 0-6 nmol/liter and for FT4 from 0-106 pmol/liter. Thyroid hormone levels were calculated from the luminescence data with the Elia-Securia II software program of Canberra Packerd.

Statistical analysis

Data were analysed with the statistical package SPSS-PC 7.5. A Tukey's Honestly Significant Difference test was used to perform a multiple comparison on statistical differences between groups.

Results

Vitamin A levels

Experiment 1 (Table 6.3): Plasma retinol levels were significantly increased by 21% in the TCDD treatment group compared to the corn oil group. In contrast, significantly decreased plasma retinol levels were observed for all dioxin-like PHAH mixture groups as well as for Aroclor 1254. A dose dependent decrease up to 32% reduction was observed in the groups treated with the PHAH+ mixture. The largest decrease in plasma retinol was found after treatment with Aroclor 1254 (38%), which is interesting since in this group no significant effects were observed on the hepatic retinyl palmitate levels. In all other treatment groups, hepatic retinyl palmitate levels were strongly decreased (60 to 80%) as compared to the corn oil control group. The lowest dose of the PHAH+ mixture, 0.5 µg TEQ/kg/week, still gave rise to significant plasma retinol and in particular hepatic retinyl palmitate reductions, indicating that this is a very sensitive response of the PHAH mixture.

Table 6.3 Retinoid levels in plasma and liver tissue of female Sprague-Dawley rats following sub-chronic exposure to dioxin-like PHAHs (Experiment 1)

Treatment dose/kg bw/week	Retinol in plasma (ng/ml)	Retinol in liver tissue (µg/g liver)	Retinyl palmitate in liver tissue (µg/g liver)	Retinol/Retinyl Palmitate (µg/mg)
Corn oil 1 ml	172.3 ± 6.8^{b}	4.75 ± 0.33	1402.8 ± 43.6^{b}	3.5 ± 0.3
TCDD 1 µg	208.3 ± 12.4^{a}	3.63 ± 0.44	357.4 ± 17.7^{a}	10.4 ± 1.4
Aroclor 1254 7.5 mg	$106.8 \pm 5.5^{a,b}$	5.67 ± 0.55	1258.4 ± 51.7^{b}	4.8 ± 0.5
PHAH- 1 µg TEQ	$124.3 \pm 6.5^{a,b}$	3.37 ± 0.32	$471.6 \pm 20.8^{a,b}$	7.3 ± 0.7
PHAH+ 0.5 µg TEQ	155.9 ± 8.1^{b}	4.06 ± 0.43	569.9 ± 37.4^{a}	7.4 ± 0.9
PHAH+ 1 µg TEQ	$136.6 \pm 11.2^{a,b}$	4.04 ± 0.87	$436.9 \pm 16.1^{a,b}$	9.1 ± 1.8
PHAH+ 2 µg TEQ	117.1 ± 9.2^{a}	3.50 ± 0.40	$303.4 \pm 16.2^{a,b}$	11.5 ± 1.0

Data are given as arithmetic mean ± the standard error. Doses are given per kg bodyweight per week.
[a] Significantly different from the corn oil group (p<0.05 Tukey HSD test). [b] Significantly different from the TCDD group (p<0.05 Tukey HSD test).

Table 6.5 Thyroid hormone levels in plasma of female Sprague-Dawley rats following sub-chronic exposure to dioxin-like PHAHs (Experiment 1)

Treatment dose/kg bw/week	Total T3 in plasma (nmol/l)	Total T4 in plasma (nmol/l)	Free T4 in plasma (pmol/l)	Total T4 /Free T4 (nmol/pmol)
Corn oil 1 ml	1.56 ± 0.07	34.39 ± 1.52^{b}	13.75 ± 0.89^{b}	2.6 ± 0.11
TCDD 1 µg	1.46 ± 0.09	21.46 ± 1.34^{a}	8.80 ± 0.60^{a}	2.5 ± 0.15
Aroclor 1254 7.5 mg	1.37 ± 0.09	19.96 ± 1.75^{a}	12.33 ± 1.07	$1.6 \pm 0.05^{a,b}$
PHAH- 1 µg TEQ	1.40 ± 0.07	15.71 ± 1.22^{a}	10.24 ± 0.76	$1.5 \pm 0.06^{a,b}$
PHAH+ 0.5 µg TEQ	1.22 ± 0.09	15.50 ± 1.67^{a}	8.31 ± 0.93^{a}	$1.9 \pm 0.10^{a,b}$
PHAH+ 1 µg TEQ	1.32 ± 0.11	$11.73 \pm 1.32^{a,b}$	8.14 ± 0.91^{a}	$1.5 \pm 0.06^{a,b}$
PHAH+ 2 µg TEQ	1.18 ± 0.11	$8.28 \pm 0.81^{a,b}$	7.34 ± 0.64^{a}	$1.1 \pm 0.08^{a,b}$

Data are given as arithmetic mean ± the standard error. Doses are given per kg bodyweight per week.
[a] Significantly different from the corn oil group (p<0.05 Tukey HSD test). [b] Significantly different from the TCDD group (p<0.05 Tukey HSD test).

Table 6.4 Retinoid levels in plasma and liver tissue of female Sprague-Dawley rats following sub-chronic exposure to non-dioxin and dioxin-like fractions of Aroclor 1260 (Experiment 2)

Treatment dose/kg bw/week		Retinol in plasma (ng/ml)	Retinol in liver tissue (µg/g liver)	Retinyl palmitate in liver tissue (µg/g liver)	Retinol/Retinyl Palmitate (µg/mg)
Untreated	-	176.6 ± 6.9	8.22 ± 0.86	2052.3 ± 111.3	4.0 ± 0.3
Corn oil	1 ml	171.7 ± 6.6	7.37 ± 0.73	1733.1 ± 70.4	4.2 ± 0.4
TCDD	1 µg	223.1 ± 16.0[a]	4.05 ± 0.39[a]	474.5 ± 32.0[a]	9.1 ± 1.3[a]
Aroclor 1260	10 mg	162.1 ± 6.6	4.65 ± 0.31	1280.8 ± 46.3[a]	3.7 ± 0.3
0-4 *ortho*	10 mg	167.0 ± 6.3	5.73 ± 0.68	1400.0 ± 62.3	4.1 ± 0.5
0-1 *ortho*	1 mg	143.4 ± 9.9	6.72 ± 0.76	1731.5 ± 175.7	4.1 ± 0.6
2-4 *ortho*	1 mg	177.3 ± 11.8	5.58 ± 0.72	1688.6 ± 71.9	3.3 ± 0.4
2-4 *ortho*	3 mg	168.3 ± 12.7	4.59 ± 0.43	1529.2 ± 64.3	3.0 ± 0.2
2-4 *ortho*	9 mg	174.0 ± 11.0	5.14 ± 0.58	1476.1 ± 66.9	3.5 ± 0.4
PCB 153	1 mg	168.4 ± 8.6	6.04 ± 0.85	1770.4 ± 89.8	3.4 ± 0.4
PCB 153	9 mg	184.6 ± 12.6	4.56 ± 0.71	1233.9 ± 77.0[a]	3.6 ± 0.5

Data are given as arithmetic mean ± the standard error. Doses are given per kg bodyweight per week. 2-4 *ortho* = 2-4 *ortho* substituted PCB fraction of Aroclor 1260; 0-1 *ortho* = 0-1 *ortho* substituted PCB fraction of Aroclor 1260 with a trace of 2 *ortho* substituted PCBs; 0-4 *ortho* = the reconstituted mixture of the 2-4 and the 0-1 *ortho* fraction. [a] Significantly different from the corn oil group (p<0.05 Tukey HSD test).

Retinol levels in the liver were not affected in the PHAH treatment groups compared to the corn oil group. As a consequence the retinol/retinyl palmitate ratios were increased in all treatment groups as compared to corn oil controls.

Experiment 2 (Table 6.4): Similar to results from the first experiment, the plasma retinol level was significantly increased in the TCDD treatment group, by 30%, compared to the corn oil control group. However, no significant changes in plasma retinol levels were observed after exposure to any of the PCB fractions, Aroclor 1260 or PCB 153. Hepatic retinoid levels in the corn oil group were slightly, but non-significantly lower compared to the untreated control group. Analysis of the hepatic ethoxyresorufin-*O*-deethylase activity (Van der Plas *et al.*, 2000a *Chapter 5*) and luciferase induction by hepatic microsomes (unpublished results) in the AhR-dependent H4IIE-Luc reporter gene (DR-CALUX) assay did not indicate that this may be due to a PHAH contamination of the corn oil. Therefore it is concluded that the observed difference between the untreated and the corn oil group in hepatic retinoid levels is more likely related to the treatment procedure. In the group treated with TCDD the hepatic

retinyl palmitate level was significantly decreased to 55% of the corn oil control group. Aroclor 1260 and PCB 153, 9 mg/kg bw/week, also significantly decreased the hepatic retinyl palmitate levels to 62% of the corn oil group. The Aroclor 1260 fractions and PCB 153, 1 mg/kg bw/week, did not affect retinyl palmitate levels in the liver significantly, although there was a tendency for a dose-dependent reduction in the 2-4 *ortho* fraction. Retinol levels in the liver after PCB exposure were all non-significantly decreased compared to the corn oil control. Except for TCDD, none of the PHAH compounds changed the hepatic retinol/retinyl palmitate ratio.

Thyroid hormone levels

Experiment 1 (Table 6.5): A small statistically non-significant decrease was observed in the total plasma T3 levels after PHAH treatment compared to the corn oil control. However, plasma total and free T4 levels were decreased in most PHAH treatment groups. A dose dependent decrease of the total T4 levels compared to the corn oil group was observed after treatment with different doses of the PHAH+ mixture up to 76% reduction in the highest dose of 2 µg TEQ/kg bw/week. At the lowest dose of 0.5 µg TEQ/kg bw/week of the PHAH+ mixture the plasma total T4 levels was still reduced by >50% as compared to corn oil controls. The total T4 level was much less reduced, 38%, after exposure to 1 µg/kg bw/week TCDD as compared to the TEQ equivalent dose of the PHAH+ mixture. Free T4 levels were decreased in all treatment groups but the ratio of total T4 over free T4 (illustrating the effect on the T4 fraction bound to its transport protein TTR) differed between the treatment groups. The total T4/free T4 ratio was not changed in the TCDD exposure group compared to the corn oil control. However a dose-dependent decrease of the total T4/free T4 ratio was observed for the PHAH mixture groups. Although the PHAH- and PHAH+ groups at 1 µg TEQ/kg bw/week are equipotent in theory, the effects on T3 and T4 levels were stronger in the latter.

Experiment 2 (data not shown): No changes were found in thyroid hormone levels after exposure to Aroclor 1260, any of its fractions or PCB 153. A slight, non-significant decrease in total T4 was observed after treatment with TCDD. The total T4/free T4 ratio was significantly decreased (24%) after treatment with the 0-4 *ortho* fraction.

Discussion

The effects on endocrine parameters, i.e. thyroid hormone and retinoid status, by complex mixtures of PHAHs are part of a larger study aimed at investigating the predictability of sub-chronic effects, as tumour promotion and biochemical effects, by the toxic equivalency factor (TEF) concept. The focus in this paper is on the impact of dioxin-like and non-dioxin-like PHAH mixtures on thyroid hormone and retinoid levels in plasma and liver. Both

parameters have been suggested to be implicated in the expression of sub-chronic effects, such as tumor promotion and, reproductive and developmental effects. Till now, much research has been performed on studying mainly short-term effects of individual congeners of PCBs and dioxins on vitamin A and thyroid hormone metabolism. Here, the impact of complex PHAH mixtures following sub-chronic exposure is discussed and attention is given to the contribution of strictly dioxin-like and non-dioxin-like PCB congeners, the involvement of different mechanisms and the predictive value of the TEF concept.

Effects on hepatic retinoids

A statistically significant decrease of the retinyl palmitate but not of the hepatic retinol levels was observed after treatment with TCDD (experiment 1), the PHAH+ and the PHAH-mixture, PCB 153 and the commercial PCB mixture Aroclor 1260. In experiment 2, TCDD decreased both hepatic retinol and retinyl palmitate. The effects of TCDD on vitamin A status have been extensively studied in many species and have been shown to alter the vitamin A status in all species examined so far (Zile, 1992). A severe decrease of the hepatic vitamin A levels was also observed after exposure to e.g. PCB 77 (Brouwer and Van den Berg, 1986; Chen et al., 1992), PCB 126 (Chen et al., 1992; Van Birgelen et al., 1994b), PCB 156 (Van Birgelen et al., 1994a), and to a lesser extent after treatment with e.g. PCB 153 (Van Birgelen et al., 1992) and Aroclor 1254 (Morse and Brouwer, 1995). The decrease of hepatic retinyl ester levels induced by TCDD and related PHAHs, may be due to a combination of increased mobilisation of hepatic stores of vitamin A and inhibition of the storage of newly ingested vitamin A in the liver (Håkansson and Ahlborg, 1985; Håkansson et al., 1988; Zile, 1992; Kelley et al., 1998). TCDD was found to severely decrease the lecithin:retinol acyltransferase (LRAT) activity in the hepatic stellate cells (Nilsson et al., 1996), an enzyme involved in the conversion of retinol into retinyl esters. Increased mobilisation of hepatic retinyl esters could be the result of a direct effect of TCDD on hepatic enzyme activities or by an up regulation of a signal controlling release of vitamin A stores into circulation (Zile, 1992; Kelley et al., 1998).

The decrease of the hepatic retinyl palmitate concentration appeared to be a rather sensitive effect of PHAH exposure. Even at the lowest dose of 0.5 µg TEQ/kg bw/week of the PHAH+ mixture, equivalent to ~70 ng TEQ/kg bw/day, a reduction of 60% of the hepatic retinyl palmitate level was found. In addition, the effect of the dioxin-like PHAH mixtures appeared to be quite well predicted by the TEF concept, i.e. an almost equal decrease of hepatic retinyl palmitate levels was observed after treatment with TEQ equivalent doses of PHAH mixture and TCDD (68% vs. 75%). The effect of the PHAH+ mixture on the hepatic retinoid level is within the same range as observed for the TEQ equivalent dose of the PHAH-mixture, whereas both mixtures have a slightly, but significant, smaller effect on the hepatic retinoid level as compared to the equipotent TCDD group. The Lowest Observed Adverse Effect Level (LOAEL) for the PHAH mixtures from this study is close to the Lowest

Observed Adverse Effect Level (LOAEL) of 14 ng TEQ/kg bw/day of TCDD reported by Van Birgelen *et al.* (1995). In contrast to the PHAH mixtures, Aroclor 1254 (estimated dose 0.2 µg TEQ/kg bw/week) induced only a slight decrease of the hepatic retinyl palmitate level as compared to the corn oil group. A possible explanation might be that PCB 118 and PCB 156 are the main contributors (~60%) to the TEQ value of Aroclor 1254 (Leonards *et al.*, 1995), while for the TEQ of the PHAH mixtures these congeners are only of minor importance (14%). Håkansson *et al.* (1994) reported that PCB 118 had no effect on the hepatic vitamin A content at dietary levels up to 2000 µg/kg. Also for exposure to PCB 156 the loss of hepatic retinoids was shown to be a less sensitive parameter than for instance reduction of plasma T4 or CYP1A2 induction (Van Birgelen *et al.*, 1994a).

Aroclor 1260 reduced the hepatic retinyl palmitate concentration by 30% compared to the corn oil control, while its 0-4 *ortho* PCB fraction decreased the retinyl palmitate levels, non-significantly, by 20%. The most likely explanation for this difference in effect is the loss of impurities, i.e. PCDFs, during the fractionation of Aroclor 1260 (Athanasiadou *et al.*, 1991). In fact, a slightly lower ethoxyresorufin-*O*-deethylase (EROD) activity was observed after exposure to the 0-4 *ortho* PCB fraction as compared to Aroclor 1260 (Van der Plas *et al.* 2000a *Chapter 5*), which underscores the former explanation.

The effect of the 2-4 *ortho* PCB fraction on the hepatic retinoid concentration was somewhat lower but close to the effect of the di-*ortho* PCB 153. PCB 153 is one of the dominant di-*ortho* congeners in environmental PHAH mixtures and is often used as a representative for the group of 2-4 *ortho* PCBs. In this study a statistically significant effect of PCB 153 on the retinoid levels in the liver was observed at the highest dose of 9 mg/kg bw/week (~1.2 mg/kg bw/day). This is in agreement with the results of a 13 week feeding study in female rat, in which PCB 153 was shown to decrease the hepatic retinoid level from 10 ppm (~0.72 mg/kg bw/day) onwards (Van Birgelen *et al.*, 1992). Since the toxicity of di-*ortho* PCBs is not mediated by the Ah receptor, no TEF values are available and consequently risk assessment is not possible for this category of PCBs. However, the observed effects on the hepatic retinoid level occurred at very high doses and it is not likely that the non-dioxin-like PCBs will effect retinoid status at environmental exposure levels.

Effects on plasma retinol

The plasma retinol level was increased after TCDD exposure in both experiments, compared to the corn oil control. In rat, an increase of plasma retinol after TCDD treatment has been reported before (Håkansson *et al.*, 1988; Van Birgelen *et al.*, 1992, 1994a,b, 1995; Kelley *et al.* 1998) and a similar effect was seen after exposure to 3,3',4,4',5,5'-hexachlorobiphenyl (PCB 169) or 2,3,3',4,4',5-hexachlorobiphenyl (PCB 156) (Chen *et al.*, 1992; Van Birgelen *et al.*, 1994). An increase of the plasma retinol level after PHAH exposure may be the result of an enhanced mobilisation of hepatic vitamin A (Zile, 1992). However, the mechanism by which hepatic mobilisation of retinol is increased is still unknown, as TCDD

and related compounds either inhibit or have no effect on the activity of hepatic retinyl ester hydrolase (REH) (Chen *et al.*, 1992).

In contrast to the TCDD treatment groups, a decrease of the plasma retinol levels was observed in the animals treated with the PHAH mixtures and Aroclor 1254. A decrease of plasma retinol levels was also reported after exposure to e.g. 3,3',4,4'-TCB (PCB 77), 2',3,3',4,5-PeCB (PCB 122), 3,3',4,4',5-PeCB (PCB 126), 2,2',3,3',5,5'-HxCB (PCB 133) and the commercial PCB mixture Aroclor 1254 (Brouwer and Van den Berg, 1986; Chen *et al.*, 1992; Morse *et al.*, 1996a). It has been suggested that a decrease in plasma retinol levels, as seen after exposure to the PHAH mixtures, may be caused by a decrease of hepatic REH activity, involved in the mobilisation of hepatic vitamin A stores (Zile, 1992). However, such a decrease of the REH activity by PHAHs has not been observed before.

Another mechanistic explanation for a decrease of plasma retinol concentrations by PHAHs has been reported by Brouwer *et al.* (1988a). The 4-hydroxy metabolite of PCB 77 was found to displace thyroxin (T4) from its transport protein transthyretin (TTR), leading to a destabilisation of the RBP-TTR complex and subsequently to a decrease in plasma retinol and thyroxin concentrations (Brouwer and Van den Berg, 1986; Brouwer *et al.*, 1988a). A number of other hydroxylated PHAHs were found to possess a high binding affinity for TTR as well, including hydroxylated PCBs likely to be formed of congeners which are present in Aroclor 1254 and/or the PHAH mixtures, i.e., PCB 105 (2,3,3',4,4-PeCB), 118, 126 and 156, (Lans *et al.*, 1993, 1995a; Bergman *et al.* 1994; Brouwer *et al.*, 1998b). Disruption of plasma transport of retinol and thyroxin does not play a role in the case of TCDD exposure because only low amounts of hydroxy-metabolites are formed from TCDD *in vivo* (Lans *et al.*, 1995b). From the data presented here it became clear that there was no correlation between the exposure in TEQs and the effect on plasma retinol. It is therefore concluded that the TEF approach is not applicable to the prediction of effects on plasma retinol levels after exposure to complex PHAH mixtures.

Effects on plasma thyroid hormone

The plasma T3 concentrations were not significantly decreased after PHAH treatment compared to the corn oil control. However, severe reductions in both plasma TT4 and FT4 levels were observed. This phenomenon is in accordance with other reports on effects of PHAHs on thyroid hormones (Van Birgelen *et al.*, 1992, 1994a,b; Lans *et al.* 1995a). Interestingly, the decrease of the TT4 levels in the PHAH mixture and the Aroclor 1254 treatment groups was considerably stronger compared to the effect observed in the TEQ equivalent TCDD group, while less or equal effects were observed for free T4. Consequently, in the Aroclor 1254 and the PHAH groups the TT4/FT4 ratio was decreased as compared to both the TCDD and the corn oil group. This indicates a lower proportion of protein bound T4 after exposure to Aroclor 1254 or the PHAH mixtures, which is in line with a disturbance of the plasma protein transport system of T4 due to T4-TTR binding competition by hydroxylated PCB metabolites (Brouwer *et al.*, 1998b). Lans *et al.* (1995a) demonstrated that

in the blood of rats exposed to a single dose of Aroclor 1254, the hydroxylated PCB metabolite 4-OH-2,3,3',4'5-PeCB competitively inhibited T4 binding to TTR. It might be concluded that the TEF concept failed in its prediction for the effect of PHAH exposure on the thyroid hormone status, since the TEF concept does not account for additional toxicity of hydroxylated PCBs as possibly observed here.

In the PHAH+ group the TT4/FT4 ratio was similar to the TEQ equivalent dosed PHAH- group, but the absolute decrease of the TT4 and FT4 concentrations was stronger in the first. This is probably an indirect effect of the non-dioxin-like PCB 153, which was added to the PHAH+ mixture. PCB 153 was shown to increase the hepatic deposition of all dioxin-like congeners, which resulted in an increased internal exposure (Van der Plas *et al.*, 1998b *Chapter 3*, 1999 *Chapter 4*) and possibly enhancement of hepatic T4 glucuronidation. A direct effect of PCB 153 on hepatic T4 glucuronidation was considered less likely since the amount of PCB 153 in the PHAH mixture was below the concentration where effects of PCB 153 on the thyroid hormone status might be expected (see experiment 2; Van Birgelen *et al.*, 1992).

In the second experiment no statistically significant effects on the thyroid hormone levels were observed, except for a decrease of the total T4/free T4 ratio after treatment with the reconstituted 0-4 *ortho* PCB fraction and a minor non-significantly, decrease of the total T4/free T4 ratio after treatment with Aroclor 1260. Van der Plas *et al.* (2000a *Chapter 5*) reported a seven to eight fold increase of the EROD induction after treatment with the 0-4 *ortho* fraction and Aroclor 1260. Although the EROD activity observed after treatment with the 0-4 *ortho* fraction and Aroclor 1260 is relatively low as compared to the more than 100 times increase which can be observed after treatment with TCDD, the CYP1A induction might be high enough to stimulate formation of hydroxylated PCBs. Based on the exposure in TEQs of the 0-4 *ortho* fraction and Aroclor 1260 (see Table 6.2), no effects were expected since a NOEL of TCDD for decreasing plasma TT4 levels was estimated on 26 ng/kg bw/day (Van Birgelen *et al.*, 1995).

On the basis of these studies it was concluded that the effect of complex PHAH mixtures on hepatic retinyl palmitate was quite well predictable by the TEF concept. However, the TEF concept failed in its prediction for the effects on plasma retinol and underestimated the effect on plasma thyroid hormone concentrations, possibly because the additional toxicity by hydroxylated PCBs is not taken into account. Treatment with the 0-4 *ortho* fraction at a TEQ level more than 100 times below the NOEL for TCDD (estimated by Van Birgelen *et al.*, 1995) still induced a significant decrease of the total T4/free T4 ratio. The non-dioxin-like PCBs did not significantly alter the retinoid and thyroid hormone status at the dose levels tested, indicating that in case of exposure to these PCBs at environmental levels, no or at best only marginal effects can be expected on the retinoid and thyroid hormone status.

Acknowledgements

CHAPTER 7

Summary

The aim of the project described in this thesis consisted of two main objectives, first, to examine the tumour promotion potential of complex, environmentally relevant mixtures of polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzo-*p*-furans (PCDFs) and secondly, to evaluate the applicability of the Toxic Equivalency Factor (TEF) concept for the tumour promotion potential of complex mixtures of PCBs, PCDDs and PCDFs. In addition, the effect of sub-chronic exposure to these complex mixtures was determined on endocrine parameters, i.e. the vitamin A and thyroid hormone status, which play an essential role in normal tissue growth and fetal development and are possibly involved in the process of carcinogenesis.

Carcinogenicity is one of the toxic endpoints in risk assessment of PCBs, PCDDs and PCDFs (WHO, 1992). PCBs, PCDDs and PCDFs are considered as tumour promoters rather than as initiators of carcinogenicity (Safe, 1989; Silberhorn *et al.*, 1990; Whysner and Williams, 1996). So far, most studies on tumour promotion by PCBs have investigated the potency of single, mostly planar dioxin-like congeners, based on the presumption that the Ah-receptor pathway is also involved in mediating the tumour promoting effects of PHAHs (Safe, 1989; Silberhorn *et al.*, 1990). There is much less information available on the tumour promoting effects of complex mixtures of PHAHs after sub-chronic exposure.

The approach in this thesis was to focus on mixtures of polyhalogenated hydrocarbons (PHAHs), both dioxin-like and non-dioxin-like, relevant for the human intake. To reveal underlying mechanisms of possible interactions between PHAH congeners and to determine the toxic potential of the PHAH mixtures, the ethoxyresorufin-*O*-deethylase (EROD) and the AhR-dependent luciferase reporter gene (DR-CALUX) bio-assays were performed. Both assays are indicators for an Ah receptor mediated, dioxin-like toxicity. The tumour promotion potential of complex PHAH mixtures *in vivo*, was studied in female Sprague Dawley rats, using the development of altered hepatic foci (AHF) as a parameter in a two-stage initiation/promotion bio-assay introduced by Pitot *et al.* (1978).

Chapter 2

In chapter 2, the results of *in vitro* experiments are described. Interactions between individual mono- or di-*ortho* PCB congeners and 2,3,7,8-TCDD were studied in the EROD and the DR-CALUX bio-assay, using mouse and rat hepatoma cell lines. In addition, the dioxin-like potential of the PHAH mixtures, designed for the animal experiments, and possible interactions between congeners within the mixtures, was determined in the CALUX assay. Preliminary data are presented on the inhibition of the gap junctional intercellular communication (GJIC), which is seen as an *in vitro* parameter for tumour promotion, by the

PHAH mixtures.

When individually dosed, the mono-*ortho* PCBs induced both the EROD and CALUX activity but to a lower maximum and at higher concentrations as compared to TCDD. Co-administration of mono-*ortho* PCBs and TCDD, decreased the TCDD induced EROD and CALUX activity dose-dependently, with increasing concentrations of the partially antagonistic mono-*ortho* PCBs. The residual level of the EROD and CALUX induction in case of co-administration, was equal to the maximum inducible activity level of the individual mono-*ortho* PCB congener. None of the tested di-*ortho* PCBs was capable of inducing the EROD or CALUX activity. However, all di-*ortho* PCBs antagonised the TCDD-induced EROD and CALUX activity in a dose-dependent manner and with different potencies. A couple of combined exposures were tested for the inhibition of the GJIC. The results indicated that similar non-additive interactions, as observed in the EROD and CALUX assay, were seen here.

The PHAH mixtures designed for the first animal experiment (*Chapter 3,4*), induced the CALUX activity up to the maximum activity level as induced by TCDD. These PHAH mixtures were also potent inhibitors of the GJIC. No interactions between individual congeners in the PHAH mixture could be observed, neither in the CALUX assay or on the inhibiton of the GJIC. Interactive effects were shown in the CALUX assay between the PCB fractions designed for the second animal experiment (*Chapter 5*). The 0- *ortho* and the 1-*ortho* substituted PCB fraction induced the CALUX activity up to 40% and 9% of the maximum level induced by TCDD respectively, while the 2-4 *ortho* fraction did not show any induction of the CALUX activity. Co-administration of the fractions inhibited the CALUX activity down to 3% of the maximum level induced by TCDD. The GJIC was only slightly inhibited by the 2-4 *ortho* and the reconstituted 0-4 *ortho* fractions.

Chapter 3 & 4

In the first animal experiment (*Chapter 3,4*), the development of AHF by a complex synthetic mixture of dioxin-like compounds was studied. The composition of this mixture was based on the presence of and relative ratio's between the six most relevant PHAHs in Baltic herring and covered over 90% of the TEQs present. To study possible interactive effects, PCB 153 (2,2',4,4',5,5'-HxCB) was added to the mixture as a representative of the non-dioxin-like, di-*ortho* substituted PCBs.

In chapter 3, the toxicokinetic properties of the PHAH congeners are presented. Gas-chromatography and mass-spectrometry (GC-MS) analysis of PHAH concentrations in the liver showed considerable differences in hepatic retention (as percentage of the given dose) between congeners, thereby changing the relative ratios of congeners between external and target dose in favor of the planar compounds. Further, it was shown that addition of PCB 153 to the PHAH mixture increased the hepatic retention of all dioxin-like PHAH congeners in the mixture. This observation is explained by the capacity of PCB 153 to induce Ah receptor levels in the liver, and consequently increase the hepatic level of CYP1A2, which is known to

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possess a high binding affinity for planar PHAHs.

In chapter 4, the AHF data are shown. The promotion of AHF was significantly increased after exposure to the PHAH mixtures, but to a lower extent than expected on the basis of the TEQs calculated from the TEF values as proposed by the WHO (Ahlborg *et al.*, 1994). A difference between the WHO TEF values (Ahlborg *et al.*, 1994) used for the calculation of the TEQ of the PHAH mixture, and the relative potency (REP) values of the individual congeners that are actually based on AHF data, may partly explain the observed differences in AHF induction between the rats exposed to the equipotent doses of TCDD and the PHAH mixtures. In addition, differences in toxicokinetic properties of the congeners and interactive effects on deposition of the congeners (*Chapter 3*) may have influenced the predicted toxic potency of the PHAH mixture as well. No interactive effects of PCB 153 on the AHF development or EROD induction could be observed.

It is concluded that the TEF approach predicted the tumour promotion potency of the investigated PHAH mixtures quite well, within a factor of two. An interactive effect between PCB 153 and the planar PHAHs occurred at the kinetic level.

Chapter 5

Chapter 5 describes the second animal experiment, in which the contribution of non-dioxin-like as well as dioxin-like PCB congeners to the total induction of AHF by a complex PCB mixture was studied. For this purpose the commercial PCB mixture Aroclor 1260 was fractionated into a 0-1 *ortho* and a 2-4 *ortho* PCB fraction, which were tested separately and as a reconstituted 0-4 *ortho* PCB mixture.

GC-MS analysis of the Aroclor 1260 fractions confirmed that there were no planar, dioxin-like compounds present in the 2-4 *ortho* PCB fraction. In addition, the 2-4 *ortho* PCB fraction did not show luciferase induction in the *in vitro* DR-CALUX bio-assay, indicating that this fraction had no dioxin-like potential (*Chapter 2*). A remarkable finding in the rat study was that the 2-4 *ortho* PCB fraction explained approximately 80% of the total observed effect on the development of AHF by the 0-4 *ortho* PCBs present in Aroclor 1260. In contrast to what is generally accepted, the dioxin-like PCB congeners did not significantly contribute to the effect on AHF development. No interactive effect on AHF development or the toxicokinetics was observed for the 0-1 and the 2-4 *ortho* PCB fraction. PCB 153, incorporated as additional treatment, showed a similar potential to induce AHF development as the 2-4 *ortho* PCB fraction in Aroclor 1260.

It was concluded that the TEF concept largely underestimates the tumour promotion effect of complex PCB mixtures, since the tumour promotion potential of the non-dioxin-like PCBs is not taken into account.

Chapter 6

In chapter 6, the results are shown on the vitamin A and the thyroid hormone status of the rats of the first and second tumour promotion experiment (*Chapter 4,5*).

From the first experiment it appeared that hepatic retinyl palmitate is a rather sensitive parameter for exposure to dioxin-like PHAHs, as the retinyl palmitate levels were severely decreased after treatment with the PHAH mixtures and to a similar extent as compared to TCDD treatment. However, an opposite effect was observed on the plasma retinol concentration after treatment with the PHAH mixture and TCDD, respectively. In addition, the PHAH mixture caused a relatively strong decrease of the thyroid hormone levels in plasma and decreased the ratio of total thyroxin and free thyroxine as compared to TCDD. The most likely reason for these observations is the formation of hydroxy-metabolites of PCB 118, present in the PHAH mixture, which are known to disrupt the transport-protein complex (RBP-TTR) of retinol and thyroxine and thereby drastically reducing plasma levels of both vitamin A and thyroxine. This situation does not occur in the case of TCDD exposure, which effects the vitamin A and thyroid hormone status mainly via interference with liver metabolism.

In the second experiment, the retinoid and thyroid hormone levels were not affected significantly. This indicates that in case of exposure to PCBs at environmental levels, no or at best only marginal effects can be expected on the retinoid and thyroid hormone status.

It was concluded on the basis of these observations that the effects on plasma retinol and thyroxine by complex mixtures of PHAHs are not well predicted by the TEF concept, due to involvement of several different mechanisms and mechanistic interactions depending on the composition of the PHAH mixture.

Concluding remarks

Applicability of the TEF concept
The most striking finding of this thesis work is that the non-dioxin-like PCB fraction in the commercial mixture Aroclor 1260 explained over 80% of the observed effect on AHF development (*Chapter 5*). On the basis of these results it was concluded that the TEF approach was inadequate in its prediction for the tumour promotion potential of a complex PCB mixture as used in the second animal experiment. However, the tumour promotion potential of the complex dioxin-like PHAH mixture used in the first animal experiment (*Chapter 4*) was quite well predicted by the TEF approach, e.g. within a factor of two. The observed kinetic interaction between the congeners (*Chapter 3*), had apparently no significant consequence for the tumour promotion potential of the PHAH mixture.

Further it was apparent that the TEF concept failed to predict the effect of the dioxin-like PHAH mixture on plasma retinol and underestimated the effect on the thyroid hormone concentrations (*Chapter 6*). The lack of predictability by the TEF approach for these endocrine effects is possibly due to additional toxicity of hydroxylated PCBs, formed of PCB congeners present in the dioxin-like PHAH mixture.

Interactive effects

The *in vitro* experiments (*Chapter 2*) indicated the possibility of interactive effects between dioxin-like PHAHs and mono- and di-*ortho* PCBs, at the level of Ah receptor binding. However, competition between compounds is only likely to occur under conditions of Ah receptor saturation and a large concentration difference between the dioxin-like PHAH and the mono- and/or di-*ortho* PCBs. These conditions can be easily reached *in vitro* but will be seldomly observed *in vivo*. In the animal experiments the major interaction was observed at the kinetic level, namely, PCB 153 enhanced the hepatic deposition of the dioxin-like PHAHs (*Chapter 3*), most likely by induction of CYP1A2 which is known to have a high binding affinity for dioxin-like PHAHs. No interactive effects, at any level, were observed between the dioxin-like and the non-dioxin-like PCB fraction of Aroclor 1260. This may be explained by the low hepatic retention of the congeners, possibly due to the low TEQ level of the dioxin-like fraction in Aroclor 1260, i.e. no or a marginal induction of hepatic binding proteins. At such a low level of hepatic retention interactive effects will not be seen. In terms of TEQs the dioxin-like fraction was certainly more close to environmental levels of exposure as occurs in wildlife and human, leading to the conclusion that kinetic interactions do not play a role at environmental exposure levels.

Implications for risk assessment

A remaining question is if, on the basis of these results, it can be concluded whether the current approach for risk estimation of complex mixtures of PCDDs, PCDFs and PCBs is appropriate or not. On the basis of a chronic carcinogenicity study performed by Kociba *et al.* (1978) for TCDD a no-observed-adverse-effect level (NOAEL) of 1 ng/kg/day was derived. A NOAEL for the synthetic dioxin-like PHAH mixture might be close to the NOAEL of TCDD (*Chapter 3,4*), if it is assumed that the dose-response curves for the carcinogenic potential of the PHAH mixtures and TCDD have a similar shape. This indicates that the TEF approach sufficiently predicts the potential risk of exposure to dioxin-like PHAHs.

For the non-dioxin-like PCBs it is more complicated, since the TEF concept is not applicable for the risk estimation of non-dioxin-like PCBs nor is there another tool available for this purpose. In addition, the total PCB intake is not well known since no congener specific analysis of the occurrence of PCBs in foodstuff is available as well as any information about differences in congener patterns between food items. An extensive survey was done in the Netherlands by Liem and Theelen (1997), who reported an intake of 20 ng/kg/day (sum of 29 PCBs) in 1994, based on a Dutch diet. Given the assumption that a level of 20 ng/kg/day in foodstuff covers approximately 20-30% of the total dietary intake, a daily intake of PCBs of 60-100 ng/kg/day can be estimated of which >90% consists of 2-4 *ortho* PCBs. In the tumour promotion experiment, a NOAEL for the 2-4 *ortho* PCBs in Aroclor 1260 was not achieved; the lowest experimental dose of 1 mg/kg bw/week (~140 μg/kg bw/day, *Chapter 5*) still enhanced the development of AHF two-fold. Using a factor of 5 for extrapolation from the lowest-observed-adverse-effect-level (LOAEL) to NOAEL, a NOAEL

of approximately 30 µg/kg/day can be deduced. For the calculation of a Tolerable Daily Intake (TDI) for dioxin-like compounds, the WHO uses a safety margin of 100. When the same margin is used for the non-dioxin-like compounds a TDI of 300 ng/kg/day can be calculated, which is a factor 3-5 above the presumable daily intake. Although there are many uncertainties in this calculation, there is probably no reason for immediate concern as large safety margins were applied. However, the results of this thesis work demonstrate the necessity for risk assessment to look at both the dioxin-like and non-dioxin-like PCBs.

Overall conclusions

Overall, the most important conclusion which can be drawn from this thesis work is that the majority of the effect on tumour promotion by PCBs is caused by a non-dioxin-like mechanism of action. Therefore the TEF approach, although useful to predict effects of dioxins and similar compounds, does not predict the tumour promotion potential of complex mixtures of PCBs as being present in the environment. This may have important implications for the risk assessment of complex mixtures of PHAHs as occur in e.g. foodstuff.

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Samenvatting

Het doel van dit promotie onderzoek was tweeledig; 1. Onderzoek naar de tumor-promoverende potentie van complexe mengsels van polygehalogeneerde aromatische koolwaterstoffen (PHAH's), welke relevant zijn voor het milieu en 2. Evaluatie van de toepasbaarheid van het toxisch equivalenten factoren (TEF) concept voor de tumor-promoverende potentie van die complexe mengsels. Daarnaast is het effect bestudeerd van sub-chronische blootstelling aan complexe mengsels van PHAH's op een tweetal endocriene parameters, te weten de vitamine A en schildklierhormoonstatus. Zowel vitamine A als het schildklierhormoon spelen een vitale rol in de normale weefselgroei en foetale ontwikkeling en zijn waarschijnlijk betrokken bij het proces van de carcinogenese.

Carcinogeniteit is één van de toxische eindpunten in de risicobeoordeling van polychloorbiphenylen (PCB's), polychloordibenzo-*p*-dioxines (PCDD's) en de polychloordibenzofuranen (PCDF's) (WHO, 1992). PCB's, PCDD's en PCDF's worden beschouwd als tumor promotors en niet zozeer als initiators van de carcinogenese (Safe, 1989; Silberhorn *et al.*, 1990; Whysner and Williams, 1996). Tot nu toe werd de tumor-promoverende potentie van vooral individuele, dioxine-achtige congeneren onderzocht, gebaseerd op de aanname dat de Ah-receptorroute ook betrokken is bij de tumor-promoverende werking van PHAH's (Safe, 1989; Silberhorn *et al.*, 1990). Veel minder onderzoek is gedaan naar de effecten op tumorpromotie van complexe mengsels van PHAH's na subchronische blootstelling.

Als uitgangspunt voor dit proefschrift is daarom gekozen voor complexe mengsels van PCDD's, PCDF's en PCB's, relevant voor de humane inname van deze stoffen. Om onderliggende mechanismen van mogelijke interacties tussen PHAH congeneren te bestuderen en om de toxische potentie te meten van de gebruikte PHAH mengsels, zijn de ethoxyresorufine-*O*-deethylase (EROD) en de Ah-receptor afhankelijke Luc-reporter-gen (DR-CALUX) bio-assay gebruikt. Beide assays worden beschouwd als indicatoren voor een Ah-receptor gemedieerde, dioxine-achtige toxiciteit *in vitro*. De tumor-promoverende potentie van de complexe PHAH mengsels, is *in vivo* bestudeerd in vrouwelijke Sprague Dawley ratten, waarbij gebruik gemaakt is van een twee-fasen initiatie/promotie bio-assay (geïntroduceerd door Pitot *et al.*, 1978).

In hoofdstuk 2 zijn de resultaten beschreven van de *in vitro* experimenten. Interacties tussen de individuele mono- of di-*ortho* PCB congeneren en 2,3,7,8-TCDD zijn bestudeerd in de EROD en CALUX assay, in zowel een muizen- als rattenhepatomacellijn. Bovendien zijn van complexe PHAH mengsels ontworpen voor de dierexperimenten, de dioxine-achtige potentie en het optreden van mogelijke interacties tussen congeneren in die mengsels onderzocht in de CALUX assay. Ook worden voorlopige resultaten gepresenteerd met

betrekking tot de remming van de intercellulaire communicatie via gap junctions (GJIC), door de PHAH mengsels. Remming van de GJIC wordt gezien als een *in vitro* parameter voor tumorpromotie.

Individueel gedoseerde mono-*ortho* PCB's induceerden zowel de EROD als de CALUX activiteit, maar bereikten een lagere maximum inductie bij hogere concentraties dan het 2,3,7,8-TCDD. Gelijktijdige toediening van mono-*ortho* PCB met 2,3,7,8-TCDD, resulteerde in een dosis afhankelijke verlaging van de door 2,3,7,8-TCDD geïnduceerde EROD en CALUX activiteit, door de partieel agonistische PCB's. Het uiteindelijke niveau van de EROD en CALUX inductie bij gelijktijdige toediening van mono-*ortho* PCB's met 2,3,7,8-TCDD, was gelijk aan het maximale inductieniveau van de individuele PCB congeneren. Geen van de getestte di-*ortho* PCB's bezat enige potentie met betrekking tot inductie van de EROD of CALUX activiteit. Echter, alle di-*ortho* PCB's bleken in staat de door 2,3,7,8-TCDD geïnduceerde EROD of CALUX activiteit dosis-afhankelijk en met verschillende potenties te antagoneren. Een tweetal combinaties is eveneens getest op remming van de GJIC. De resultaten suggereren dat in deze assay vergelijkbare, non-additieve interacties optreden als in de EROD en CALUX assay.

De PHAH mengsels ontworpen voor het eerste dierexperiment (*Hoofdstuk 3,4*), induceerden de CALUX activiteit tot hetzelfde maximum niveau als 2,3,7,8-TCDD. Deze PHAH mengsels bleken eveneens potente inhibitoren te zijn van de GJIC. Er werden geen interacties gevonden tussen de individuele congeneren in het PHAH mengsel in zowel de CALUX assay als in de GJIC assay. In tegenstelling hiermee, werden er tussen de PCB fracties bedoeld voor het tweede dierexperiment wél interacties gevonden (*Hoofdstuk 5*). De 0- *ortho* en de 1- *ortho* gesubstitueerde PCB fracties induceerden de CALUX activiteit tot een maximum van respectievelijk 40% en 9% ten opzichte van de maximum inductie die gevonden wordt na blootstelling aan 2,3,7,8-TCDD. De 2-4 *ortho* PCB fractie bleek niet in staat tot inductie van de CALUX activiteit. Gelijktijdige blootstelling aan alle fracties resulteerde in remming van de CALUX activiteit van de 0-1 en de 1-2 *ortho* PCB fractie, tot 3% van het maximale inductieniveau van 2,3,7,8-TCDD. GJIC was slechts marginaal verlaagd na blootstelling aan de 2-4 *ortho* en de gereconstitueerde 0-4 *ortho* PCB fractie.

In het eerste dierexperiment (*Hoofdstuk 3,4*) is de tumor-promoverende potentie van een complex, synthetisch mengsel van dioxine-achtige stoffen bestudeerd. De samenstelling van het mengsel was gebaseerd op het voorkomen van en de onderlinge verhouding tussen de zes meest relevante PHAH's in haring van de Baltische zee en dekte meer dan 90% van de totale TEQ's. Om de mogelijkheid van interacties te bestuderen, was PCB 153 (2,2',4,4',5,5'-HxCB) aan het mengsel toegevoegd. PCB 153 diende hierbij als een representant van de niet-dioxine-achtige di-*ortho* gesubstitueerde PCB's.

In hoofdstuk 3 zijn de toxicokinetische eigenschappen van de PHAH congeneren beschreven. Analyse van concentraties van PHAH's in de lever m.b.v. gaschromatografie en massaspectrometrie (GC-MS) wees uit, dat er aanzienlijke verschillen bestaan in de

leveraccumulatie (uitgedrukt als percentage van de gegeven dosis) tussen de verschillende PCB congeneren. Hierdoor verandert de onderlinge verhouding van de PHAH's in het mengsel. Verder werd aangetoond dat door toevoeging van PCB 153 aan het mengsel, accumulatie van de dioxine-achtige congeneren in de lever werd verhoogd. Dit wordt mogelijk verklaard door het vermogen van PCB 153 om het Ah-receptorniveau in de lever te verhogen, met als logisch gevolg een verhoogde inductie van b.v. CYP1A2, dat een hoge bindingaffiniteit bezit voor planaire PHAH's.

In hoofdstuk 4 zijn de tumorpromotie gegevens weergegeven. De promotie van de zogenoemde 'Altered Hepatic Foci' (AHF) was significant verhoogd na blootstelling aan de PHAH mengsels, maar enigszins minder dan verwacht op basis van de TEQ. De TEQ van het PHAH mengsel was berekend aan de hand van de TEF waarden, vastgesteld door de WHO (Ahlborg *et al.*, 1994). Een gedeeltelijke verklaring voor het gevonden verschil in de ontwikkeling van AHF tussen ratten blootgesteld aan de PHAH mengsels en de equipotente dosis 2,3,7,8-TCDD, is een verschil tussen de TEF waarden van de WHO (Ahlborg *et al.*, 1994) die gebruikt zijn voor de berekening van de TEQ enerzijds en de relatieve potenties (REP's) van de individuele congeneren die feitelijk op tumorpromotie gegevens zijn gebaseerd anderzijds. Bovendien kunnen mogelijk de gevonden verschillen in toxicokinetische eigenschappen en interactieve effecten op de depositie van de congeneren (*Hoofdstuk 3*), de voorspelde toxische potentie van PHAH mengsels beïnvloeden. Er zijn geen interactieve effecten van PCB 153 gevonden op de ontwikkeling van AHF of the EROD inductie.

De conclusie op basis van deze resultaten is, dat met behulp van het TEF-concept de tumor-promoverende potentie van de onderzochte dioxine-achtige PHAH mengsels redelijk te voorspellen was, de afwijking bedroeg minder dan een factor 2. Interactie van PCB 153 met de planaire PHAH's is waargenomen op kinetisch niveau.

Hoofdstuk 5 beschrijft het tweede dierexperiment, waarbij de bijdrage van zowel de dioxine-achtige als de niet-dioxine-achtige PCB congeneren aan de totale tumor-promoverende potentie van een complex PCB mengsel is bestudeerd. Voor dit doel is het commerciële PCB mengsel Aroclor 1260 gefractioneerd in een 0-1 *ortho* en een 2-4 *ortho* PCB fractie. De fracties zijn afzonderlijk getest en als een gereconstitueerd 0-4 *ortho* PCB mengsel.

Met behulp van GC-MS analyse kon worden bevestigd dat er geen planaire, dioxine-achtige stoffen aanwezig waren in de 2-4 *ortho* PCB fractie. Bovendien bleek de 2-4 *ortho* fractie niet in staat CALUX activiteit te induceren *in vitro*, wat impliceert dat deze fractie geen dioxine-achtige potentie bezit (*Hoofdstuk 2*). Een opmerkelijke bevinding was dat de 2-4 *ortho* PCB fractie ongeveer 80% van het totale effect van de 0-4 *ortho* fractie op de AHF ontwikkeling kon verklaren. In tegenstelling tot wat algemeen aangenomen wordt, droegen de dioxine-achtige PCB congeneren niet significant bij aan het effect op de ontwikkeling van AHF. Er zijn geen interactieve effecten waargenomen tussen de 0-1 of de 2-4 *ortho* PCB

fractie op de AHF ontwikkeling of de toxicokinetiek. PCB 153, toegevoegd als extra experimentele behandeling, vertoonde een vergelijkbare potentie voor de inductie van AHF als de 2-4 *ortho* fractie van Aroclor 1260.

Op basis van deze resultaten is geconcludeerd dat het TEF-concept het tumor-promoverende effect van complexe PCB mengsels fors onderschat, omdat geen rekening wordt gehouden met het effect op tumorpromotie van de niet-dioxine-achtige PCB's.

In hoofdstuk 6 zijn de vitamine A en schildklierhormoon gegevens gepresenteerd van de dieren uit zowel het eerste als het tweede dierexperiment (*Hoofdstuk 4,5*).

Uit het eerste dierexperiment bleek dat het retinyl-palmitaat gehalte in de lever een zeer gevoelige parameter is voor de blootstelling aan dioxine-achtige PHAH's. De retinyl-palmitaatniveau's in de lever waren fors verlaagd na behandeling met de PHAH mengsels en vergelijkbaar met de verlaging die gevonden werd na blootstelling aan 2,3,7,8-TCDD. Echter, op het niveau van plasma retinol werd na behandeling met respectievelijk het PHAH mengsel en 2,3,7,8-TCDD een tegengesteld effect gevonden. Bovendien veroorzaakten de PHAH mengsels in vergelijking met 2,3,7,8-TCDD een relatief sterke verlaging van schildklierhormoonniveau's en, in tegenstelling tot 2,3,7,8-TCDD, een verlaging van de ratio 'totaal thyroxine/vrij thyroxine'. De meest waarschijnlijke oorzaak hiervoor is de vorming van hydroxy-metabolieten van bijvoorbeeld PCB 118, aanwezig in het PHAH mengsel. Van hydroxy-metabolieten is bekend dat zij het transporteiwit complex (RBP-TTR) van retinol en thyroxine kunnen verstoren met als gevolg een drastische verlaging van plasma niveau's van zowel vitamine A als thyroxine. Dit effect treedt niet op bij blootstelling aan 2,3,7,8-TCDD, dat de vitamine A en schildklierhormoonstatus voornamelijk beïnvloed via een verstoring van het lever metabolisme van deze stoffen.

In het tweede dierexperiment waren de vitamine A en schildklierhormoonniveau's niet significant veranderd. Dit geeft aan dat in geval van PCB blootstelling aan een milieu-relevante concentratie, geen of slechts marginale effecten kunnen worden verwacht.

Op basis van deze resultaten kan worden geconcludeerd dat het TEF-concept een zeer beperkte voorspellende waarde heeft met betrekking tot de effecten van complexe mengsels van PHAH's op retinol en schildklierhormoonniveau's in plasma. Dit is het gevolg van de betrokkenheid van verschillende mechanismen en interacties tussen mechanismen, afhankelijk van de congeneer samenstelling van het PHAH mengsel.

Slotopmerkingen

Toepasbaarheid van het TEF-concept

Het meest opzienbarende resultaat van dit onderzoek is, dat de niet-dioxine-achtige PCB fractie van het commerciële PCB mengsel Aroclor 1260 verantwoordelijk bleek voor meer dan 80% van het gevonden effect op de ontwikkeling van AHF (*Hoofdstuk 5*). Op grond van dit resultaat is geconcludeerd dat het TEF-concept inadequaat is in zijn voorspelling voor de tumor-promoverende potentie van het complexe PCB mengsel, gebruikt in het tweede dierexperiment. Echter, de tumor-promoverende potentie van het dioxine-achtige PHAH mengsel gebruikt in het eerste experiment, was redelijk goed voorspeld met behulp van het TEF-concept: met een afwijking kleiner dan een factor twee (*Hoofdstuk 4*). De gevonden kinetische interacties tussen de congeneren (*Hoofdstuk 3*) hadden geen significante consequenties voor de tumor-promoverende potentie van het PHAH mengsel.

Een andere bevinding is dat het TEF-concept faalde in zijn voorspelling van het effect van het dioxine-achtige PHAH mengsel op het retinolniveau in plasma. Bovendien gaf het TEF-concept een onderschatting van het effect op het schildklierhormoonniveau in plasma. De gebrekkige voorspelling door het TEF-concept is waarschijnlijk te verklaren door een additionele toxiciteit van gehydroxyleerde PCB's, welke kunnen worden gevormd uit congeneren in het PHAH mengsel.

Interactieve effecten

De *in vitro* experimenten (*Hoofdstuk 2*) suggereren de mogelijkheid van interacties tussen dioxine-achtige PHAH's en mono- en di-*ortho* PCB's op basis van competitie voor Ah-receptor binding. Echter, competitie tussen stoffen is alleen waarschijnlijk onder omstandigheden van Ah-receptor verzadiging en een groot concentratie verschil tussen de dioxine-achtige PHAH's en de mono- en/of di-*ortho* PCB's. Deze condities kunnen makkelijk worden gecreëerd *in vitro*, maar zullen zelden optreden *in vivo*. In de dierexperimenten zijn de voornaamste interacties gevonden op kinetisch niveau. PCB 153 verhoogde de depositie van dioxine-achtige PCB's in de lever (*Hoofdstuk 3*), meest waarschijnlijk door inductie van CYP1A2, waarvan bekend is dat het een grote bindingaffiniteit vertoont voor dioxine-achtige PHAH's. Er zijn geen interacties aangetoond, op welk niveau dan ook, tussen de dioxine-achtige en niet-dioxine-achtige PCB fractie van Aroclor 1260. Dit kan mogelijk worden verklaard door de lage accumulatie van de congeneren in de lever, waarschijnlijk veroorzaakt door de lage TEQ waarde van de dioxine-achtige fractie, wat betekent dat er geen of slechts een marginale inductie van CYP1A2 in de lever heeft plaatsgevonden. Op een dergelijk laag niveau van accumulatie zullen interactieve effecten dan ook niet zichtbaar zijn. In termen van TEQ's was de dioxine-achtige fractie zeker dichter bij de blootstellingniveau's zoals deze voorkomen in het milieu, dan het PHAH mengsel van het eerste experiment. Hieruit kan worden geconcludeerd dat kinetische interacties geen rol zullen spelen bij milieu relevante blootstellingniveau's.

Implicaties voor risicoschatting

Een belangrijke vraag is nu of, op basis van de hier gepresenteerde resultaten, kan worden geconcludeerd dat de huidige benadering voor risicoschatting van complexe mengsels van PCDD's, PCDF's en PCB's toereikend is of niet. Op basis van een chronische carcinogeniteit studie, uitgevoerd door Kociba e.a. (1978), is voor 2,3,7,8-TCDD een 'No-Observed-Adverse-Effect-Level' (NOAEL) bepaald van 1 ng/kg/dag. Een NOAEL voor de synthetische dioxine-achtige PHAH mengsels ligt waarschijnlijk dicht bij de NOAEL voor 2,3,7,8-TCDD (*Hoofdstuk 3,4*), als wordt aangenomen dat de dosis-response curven voor de carcinogene potentie van de PHAH mengsels en 2,3,7,8-TCDD hetzelfde verloop hebben. Dit impliceert dat de TEF benadering in voldoende mate het potentiële risico voorspelt van blootstelling aan dioxine-achtige PHAH's.

Voor de niet-dioxine-achtige PCB's is het meer gecompliceerd. Het TEF-concept is niet toepasbaar voor de risicoschatting van niet-dioxine-achtige PCB's en evenmin is er een alternatief beschikbaar voor dit doel. Bovendien is de totale inname van niet-dioxine-achtige PCB's niet bekend, omdat er geen congeneer-specifieke analyses beschikbaar zijn met betrekking tot het voorkomen van PCB's in voedingsmiddelen en informatie met betrekking tot verschillen in congeneer patronen tussen voedingsbestanddelen ontbreekt. Een uitgebreid onderzoek is uitgevoerd in Nederland door Liem en Theelen (1997). Zij rapporteerden een inname van 20 ng/kg/dag (som van 29 PCB's) in 1994, gebaseerd op een Nederlands dieet. Gegeven de aanname dat een niveau van 20 ng/kg/dag in voedsel ongeveer 20-30% van de totale inname via voedsel bedraagt, kan een dagelijkse inname voor PCB's worden geschat op 60-100 ng/kg/dag. Hiervan bestaat meer dan 90% uit 2-4 *ortho* gesubstitueerde PCB's. In het tumorpromotie experiment was een NOAEL voor de 2-4 *ortho* PCB's uit Aroclor 1260 niet bereikt, de laagste test-dosis van 1 mg/kg/week (~140 µg/kg/dag, *Hoofdstuk 5*) gaf nog steeds een verdubbeling in de ontwikkeling van AHF. Als een factor 5 gebruikt wordt om te extrapoleren van de 'Lowest-Observed-Adverse-Effect-Level' (LOAEL) naar een NOAEL, kan een NOAEL van ongeveer 30 µg/kg/dag worden afgeleid. Voor de berekening van een 'Tolerable-Daily-Intake'(TDI) voor dioxine-achtige stoffen hanteert de WHO een veiligheidsmarge van 100. Als dezelfde veiligheidsmarge wordt gebruikt voor de niet-dioxine-achtige PCB's kan een TDI van 300 ng/kg/dag worden berekend. Dit is een factor 3-5 boven de veronderstelde dagelijkse inname. Hoewel er veel onzekerheden zitten in deze berekening, is er waarschijnlijk geen reden tot onmiddellijke ongerustheid omdat ruime veiligheidsmarges zijn gebruikt. Echter, de resultaten van dit onderzoek onderstrepen de noodzaak om voor de risicobeoordeling te kijken naar zowel de dioxine-achtige als de niet-dioxine-achtige PCB's.

Conclusie

De belangrijkste conclusie van het onderhavige onderzoek is dat het overgrote deel van het effect op tumorpromotie door PCB's, wordt veroorzaakt door een niet-dioxine-achtig mechanisme. Daarom voorspelt de TEF benadering, hoewel bruikbaar voor de voorspelling van effecten van dioxine-achtige stoffen, de tumor-promoverende potentie van complexe mengsels van PHAH's zoals deze voorkomen in het milieu niet. Deze conclusie kan belangrijke gevolgen hebben voor de risicobeoordeling van complexe PHAH mengsels in bijvoorbeeld onze voeding.

ABBREVIATIONS

AHF	altered hepatic foci
Ah-receptor	arylhydrocarbon receptor
bw	body weight
CALUX	chemical activated luciferase gene expression
CALUX-TEQ	TCDD-equivalence determined using the CALUX assay
CYP	cytochrome P450
EC_{50}	concentration causing 50% of the maximum effect
EROD	ethoxyresorufin-O-deethylase
FT4	free thryroxine
GJIC	gap junctional intercellular communication
GST-p	gluthatione-S-transferase-p
IC_{50}	concentration causing 50% inhibition
LOAEL	lowest observed adverse effect level
NDEA	N-nitrosodiethylamine
NOAEL	no observed adverse effect level
OH-	hydroxylated
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo-p-dioxin
PCDF	polychlorinated dibenzofuran
PHAH	polyhalogenated aromatic hydrocarbon
PROD	penthoxyresorufin-O-deethylase
RBP	retinol binding protein (transport protein of retinol)
RE	retinol (transport form of vitamin A)
REP	relative potency
RP	retinyl palmitate (storage form of vitamin A)
SER	smooth endoplasmatic reticulum
TDI	tolerable daily intake
TEF	toxic equivalence factor (relative to TCDD)
TEQ	(TCDD) toxic equivalence
(T)T3	(total) tri-iodothyronine (active form of thyroid hormone)
(T)T4	(total) thyroxine (transport form of thyroid hormone)
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TTR	transthyretin (transport protein of thyroxine)
VF	volume fraction of the liver occupied by foci
WHO	World Health Organization

Numbering system of chlorobiphenyls

No.	Structure	No.	Structure	No.	Structure	No.	Structure
	Monochlorobiphenyls		**Tetrachlorobiphenyls**		**Pentachlorobiphenyls**		**Hexachlorobiphenyls**
1	2	52	2,2',5,5'	105	2,3,3',4,4'	161	2,3,3',4,5',6
2	3	53	2,2',5,6'	106	2,3,3',4,5	162	2,3,3',4',5,5'
3	4	54	2,2',6,6'	107	2,3,3',4',5	163	2,3,3',4',5,6
		55	2,3,3',4	108	2,3,3',4,5'	164	2,3,3',4',5',6
	Dichlorobiphenyls	56	2,3,3',4'	109	2,3,3',4,6	165	2,3,3',5,5',6
4	2,2'	57	2,3,3',5	110	2,3,3',4',6	166	2,3,4,4',5,6
5	2,3	58	2,3,3',5'	111	2,3,3',5,5'	167	2,3',4,4',5,5'
6	2,3'	59	2,3,3',6	112	2,3,3',5,6	168	2,3',4,4',5',6
7	2,4	60	2,3,4,4'	113	2,3,3',5',6	169	3,3',4,4',5,5'
8	2,4'	61	2,3,4,5	114	2,3,4,4',5		
9	2,5	62	2,3,4,6	115	2,3,4,4',6		**Heptachlorobiphenyls**
10	2,6	63	2,3,4',5	116	2,3,4,5,6	170	2,2',3,3',4,4',5
11	3,3'	64	2,3,4',6	117	2,3,4',5,6	171	2,2',3,3',4,4',6
12	3,4	65	2,3,5,6	118	2,3',4,4',5	172	2,2',3,3',4,5,5'
13	3,4'	66	2,3',4,4'	119	2,3',4,4',6	173	2,2',3,3',4,5,6
14	3,5	67	2,3',4,5	120	2,3',4,5,5'	174	2,2',3,3',4,5,6'
15	4,4'	68	2,3',4,5'	121	2,3',4,5',6	175	2,2',3,3',4,5',6
		69	2,3',4,6	122	2',3,3',4,5	176	2,2',3,3',4,6,6'
	Trichlorobiphenyls	70	2,3',4',5	123	2',3,4,4',5	177	2,2',3,3',4',5,6
16	2,2',3	71	2,3',4',6	124	2',3,4,5,5'	178	2,2',3,3',5,5',6
17	2,2',4	72	2,3',5,5'	125	2',3,4,5,6'	179	2,2',3,3',5,6,6'
18	2,2',5	73	2,3',5',6	126	3,3',4,4',5	180	2,2',3,4,4',5,5'
19	2,2',6	74	2,4,4',5	127	3,3',4,5,5'	181	2,2',3,4,4',5,6
20	2,3,3'	75	2,4,4',6			182	2,2',3,4,4',5,6'
21	2,3,4	76	2',3,4,5		**Hexachlorobiphenyls**	183	2,2',3,4,4',5',6
22	2,3,4'	77	3,3',4,4'	128	2,2',3,3',4,4'	184	2,2',3,4,4',6,6'
23	2,3,5	78	3,3',4,5'	129	2,2',3,3',4,5	185	2,2',3,4,5,5',6
24	2,3,6	79	3,3',4,5'	130	2,2',3,3',4,5'	186	2,2',3,4,5,6,6'
25	2,3',4	80	3,3',5,5'	131	2,2',3,3',4,6	187	2,2',3,4',5,5',6
26	2,3',5	81	3,4,4',5	132	2,2',3,3',4,6'	188	2,2',3,4',5,6,6'
27	2,3',6			133	2,2',3,3',5,5'	189	2,3,3',4,4',5,5'
28	2,4,4'		**Pentachlorobiphenyls**	134	2,2',3,3',5,6	190	2,3,3',4,4',5,6
29	2,4,5	82	2,2',3,3',4	135	2,2',3,3',5,6'	191	2,3,3',4,4',5',6
30	2,4,6	83	2,2',3,3',5	136	2,2',3,3',6,6'	192	2,3,3',4,5,5',6
31	2,4',5	84	2,2',3,3',6	137	2,2',3,4,4',5	193	2,3,3',4',5,5',6
32	2,4',6	85	2,2',3,4,4'	138	2,2',3,4,4',5'		
33	2',3,4	86	2,2',3,4,5	139	2,2',3,4,4',6		**Octachlorobiphenyls**
34	2',3,5	87	2,2',3,4,5'	140	2,2',3,4,4',6'	194	2,2',3,3',4,4',5,5'
35	3,3',4	89	2,2',3,4,6	141	2,2',3,4,5,5'	195	2,2',3,3',4,4',5,6
36	3,3',5	89	2,2',3,4,6'	142	2,2',3,4,5,6	196	2,2',3,3',4,4',5,6'
37	3,4,4'	90	2,2',3,4',5	143	2,2',3,4,5,6'	197	2,2',3,3',4,4',6,6'
38	3,4,5	91	2,2',3,4',6	144	2,2',3,4,5',6	198	2,2',3,3',4,5,5',6
39	3,4',5	92	2,2',3,5,5'	145	2,2',3,4,6,6'	199	2,2',3,3',4,5,5',6'
		93	2,2',3,5,6	146	2,2',3,4',5,5'	200	2,2',3,3',4,5,6,6'
	Tetrachlorobiphenyls	94	2,2',3,5,6'	147	2,2',3,4',5,6	201	2,2',3,3',4,5',6,6'
40	2,2',3,3'	95	2,2',3,5',6	148	2,2',3,4',5,6'	202	2,2',3,3',5,5',6,6'
41	2,2',3,4	96	2,2',3,6,6'	149	2,2',3,4',5',6	203	2,2',3,4,4',5,5',6
42	2,2',3,4'	97	2,2',3',4,5	150	2,2',3,4',6,6'	204	2,2',3,4,4',5,6,6'
43	2,2',3,5	98	2,2',3',4,6	151	2,2',3,5,5',6	205	2,3,3',4,4',5,5',6
44	2,2',3,5'	99	2,2',4,4',5	152	2,2',3,5,6,6'		
45	2,2',3,6	100	2,2',4,4',6	153	2,2',4,4',5,5'		**Nonachlorobiphenyls**
46	2,2',3,6'	101	2,2',4,5,5'	154	2,2'4,4',5,6'	206	2,2',3,3',4,4',5,5',6
47	2,2',4,4'	102	2,2',4,5,6'	155	2,2',4,4',6,6'	207	2,2',3,3',4,4',5,6,6'
48	2,2',4,5	103	2,2',4,5',6	156	2,3,3',4,4',5	208	2,2',3,3',4,5,5',6,6'
49	2,2',4,5'	104	2,2',4,6,6'	157	2,3,3',4,4',5'		
50	2,2',4,6			158	2,3,3',4,4',6		**Decachlorobiphenyl**
51	2,2',4,6'			159	2,3,3',4,5,5'	209	2,2',3,3',4,4',5,5',6,6'
				160	2,3,3',4,5,6		

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CURRICULUM VITAE

Simone Antoinette van der Plas was born in Alphen aan de Rijn, the Netherlands, on April 6, 1968. In 1987 she graduated (VWO) from the Pieter Groen College in Katwijk aan Zee and at the end of that summer she started to study Human Nutrition at Wageningen Agricultural University (WAU), the Netherlands, with a specialisation on 'Nutrition & Health'. During her study she worked for several months at the department of Sociology (WAU), the department of Toxicology (WAU), the department of Human Nutrition (WAU) and in collaboration with the department for laboratory animals at the University of Utrecht, and at the department of Biological Toxicology of TNO Zeist (the Netherlands). In August 1993 she graduated for her M.Sc. in Human Nutrition. At the end of the same year she started on a project for 3 months as a research assistant at the department of Toxicology (WAU). After finishing this project she started in April 1994 as a Ph.D. student on a research project investigating the tumour promotion potential of complex, environmentally relevant mixtures of polyhalogenated aromatic hydrocarbons and the applicability of the Toxic Equivalency Factor concept, at the department of Toxicology (WAU) under supervision of dr. A. Brouwer, and in collaboration with RIKILT-DLO (The Netherlands) and the institute for Environmental Medicine (Karolinska Institute Stockholm, Sweden). In June 1999 she started to work as toxicological assessor of preclinical studies of human medicine dossiers at the RIVM in Bilthoven (The Netherlands). In July 2000 she left the RIVM to move to Zeeuws-Vlaanderen (The Netherlands). This thesis is based on research that was conducted between 1994 and 1999.

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