BIOMARKERS FOR THE ASSESSMENT OF EXPOSURE AND SUBLETHAL EFFECTS OF PHAHS IN WILDLIFE

With special emphasis on the aquatic food chain

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With special emphasis on the aquatic food chain

Albertinka J Murk

Proefschrift

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STELLINGEN

1 Met behulp van biomarkers voor blootstelling en/of effect is het mogelijk om vast te stellen in welke mate een organisme beïnvloed wordt door toxische stoffen. Voor het voorspellen van de uiteindelijke effecten op in het wild levende organismen is veel additionele informatie nodig over omgevingsvariabelen, kinetiek, en gevoeligheid van levensfasen.
Dit proefschrift

2 Met behulp van een reporter gen assay zoals de CALUX-assay kan op snelle en gevoelige wijze de potentie van een mengsel van stoffen om via een specifiek werkingsmechanisme toxische effecten te veroorzaken direct worden gekwantificeerd. Dit is een groot voordeel boven de conventionele, op chemische metingen gebaseerde methode, gezien de onnauwkeurigheid bij het vaststellen van de toxische equivalentie factoren van met name de minder potente PHAKs.
Dit proefschrift

3 De *in vitro* inductie van de EROD-activiteit is uitsluitend een bruikbare maat voor de kwantificering van de toxische equivalentie van potente Ah-receptor agonisten.
Dit proefschrift

4 Dankzij aanzienlijke emissiereducties worden effecten op populaties niet meer voornamelijk door stoffen veroorzaakt, maar vormen de meer subtiele effecten van stoffen een onderdeel van een complex van antropogene stress factoren, met o.a. fysieke ingrepen en eutrofiëring.

5 Om te voorkomen dat aan PCBs verwante stoffen, zoals gebromeerde difenylethers op termijn vergelijkbare nadelige effecten zullen veroorzaken, moet er op korte termijn veel aandacht worden besteed aan een zorgvuldig produktie-, en hergebruik- of verwerkingssysteem van de produkten die dergelijke stoffen bevatten.

6 In het huidige toelatingsbeleid en waterkwaliteitsbeheer wordt weinig rekening gehouden met vòòrkomen en effecten van transformatieprodukten van bestrijdingsmiddelen. De veel gehoorde opvatting dat deze minder persistent en toxisch zijn dan de uitgangsstoffen kan niet gestaafd worden door de beperkte beschikbare gegevens.
Belfroid AC, Van Drunen M, Van Gestel CAM en Van Hattum B (1996). Relative risks of transformation products of pesticides for aquatic ecosystems. IVM-rapport R-96/09.

7 De bepaling van de toxiciteit van uit milieumatrices geïsoleerde mengsels van stoffen, ligt op het grensvlak tussen de chemie en de toxicologie. Hierdoor ontbreekt in publicaties vaak een goede validatie van de extractiemethoden of juist de bioassay.
Conclusie van de workshop 'Extractie milieutoxiciteit' op 26/9/1996, werkgroep Extractie en bioanalyse van milieutoxiciteit.

8 Het voeren van vismeel aan herbivoren zoals koeien is niet alleen tegennatuurlijk, het leidt bovendien tot een recycling van de in visolie geconcentreerde persistente, lipofiele verbindingen.

9 Voor veel Nederlandse zoutwatersystemen is bekend dat tributyltin concentraties het risiconiveau voor imposex bij Gastropoden ruimschoots overschrijden. Minder bekend is dat ook in zoetwatersystemen vergelijkbare effecten te verwachten zijn, omdat ook daar regelmatig het risiconiveau overschreden wordt.

Mensink BP, Van Hattum B, Vethaak AD en Boon JP (1996). The development of imposex in relation to organotin contamination in the common whelk, Buccinum undatum. NIOZ-rapport 1996-3; Van Hattum B, Ariese F, Van Kesteren J en Freriks I (1996). Organotinverbindingen in sediment uit het Noordzeekanaal. IVM-rapport R-96/10.

10 Hoewel sociaal medeleven en zorgzaamheid leiden tot een grotere overlevingskans van sociale diersoorten, wil dat niet zeggen dat het 'er beter van worden' de individuele drijfveer voor zulk gedrag is.

Naar: Frans de Waal (1996): Van nature goed; over de oorsprong van goed en kwaad in mensen en andere dieren.

11 De terecht stringente voorwaarden die worden opgelegd bij het gebruik van proefdieren, vormt een groot contrast met de schrijnende manier waarop overigens met dieren wordt omgegaan, bijvoorbeeld in de bio-industrie en de visserij.

12 Hoewel soft drugs op zich misschien niet verslavend zijn of gevaarlijk voor de gezondheid, is veelvuldig gebruik ervan door jongeren schadelijk als ze hiermee kunnen vluchten voor de voor hun leeftijd normale onzekerheden en problemen, in plaats van ermee leren om te gaan.

13 Hoewel ook bij de LUW met de mond de wens van grotere participatie van vrouwen in hogere functies wordt beleden, doet de geringe aandacht voor het oplossen van het knelpunt van onvoldoende kinderopvang vermoeden, dat hierbij de belangstelling vooral uitgaat naar vrouwen zonder baarmoeder.

14 Het succes van 'new age'-achtige bewegingen in de zogenaamd moderne en ontwikkelde West-Europese samenleving, biedt de op zich te prijzen 'rationalisten beweging' (rationalist movement) in India, die probeert om de schadelijke rol van het bijgeloof en de afgoderij in hun maatschappij terug te dringen, een weinig rooskleurig vooruitzicht op duurzaam succes.

15 De natuurlijke neiging van jongeren hun grenzen te onderzoeken en zich ten opzichte van elkaar te bewijzen, brengt met zich mee dat vandalisme niet zozeer 'zin'-loos geweld is, maar wel een uitdaging richting maatschappij om duidelijke grenzen te stellen.

16 Zelfs een hobby waarbij de liefde voor de onderwaterwereld centraal staat, kan leiden tot erosie van dit fraais wanneer ze zonder sturende maatregelen door grote aantallen duikers beoefend kan worden.

Stellingen behorend bij het proefschrift *'Biomarkers for the assessment of exposure and sublethal effects of PHAHs in wildlife'* door Tinka Murk, Wageningen, 15 januari 1997.

Voor mijn ouders,
die mij de ruimte gaven om de wereld te ontdekken,
en een vertrouwde basis om op terug te vallen.

ABBREVIATIONS

AhR	aryl hydrocarbon receptor
CALUX	chemical activated luciferase gene expression
CALUX-TEQ	TCDD-equivalence determined using the CALUX assay
CloA50	Clophen A50 (a technical PCB-mixture)
DRE	dioxin responsive element
ELS	early life stage assay
EROD	ethoxy resorufin *O*-deethylase
FT4	free thyroxine
H4IIE	a rat hepatoma cell line
HPLC	high performance liquid chromatography
mo-PCB	mono-ortho substituted PCB
OH-	hydroxylated
P450	cytochrome *P450*
PBDE	polybrominated diphenyl ether
PCB	polychlorinated biphenyl
PCB-77	3,3',4,4-tetrachloro biphenyl
PCDD	polychlorodibenzo-*p*-dioxin
PCDE	polychlorinated diphenyl ether
PCDF	polychlorodibenzofuran
PCT	polychlorinated terphenyl
PHAH	polyhalongenated aromatic hydrocarbon
PHDE	polyhalogenated diphenyl ether
PROD	pentoxy resorufin *O*-deethylase
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TEF	toxic equivalence factor
TEQ	(TCDD) toxic equivalence
TTR	transthyretin
(T)T3	(total) tri-iodothyronine
(T)T4	(total) thyroxine
UDPGT	uridine-5-diphosphate glucuronyltransferase

CONTENTS

CHAPTER 1

The research described in this thesis concerns the effects of polyhalogenated aromatic hydrocarbons (PHAHs) on wildlife species, and the development of biomarkers for exposure and effect based on the mechanism of toxic action of PHAHs. In addition, attention is paid to differences in vulnerability between species with regard to exposure, kinetics and (eco)physiology, and the possible impact of current PHAH levels in The Netherlands on fish-eating wildlife species. The research was performed within the framework of the ecotoxicological research program of the department of Toxicology.

GENERAL INTRODUCTION

Triggered by a large number of case studies during the 1950s and 1960s, and by publications such as Rachel Carson's 'Silent Spring' (Carson, 1962), restrictive measures were taken to prevent the often not anticipated damage on fish and wildlife populations caused by newly introduced chemicals such as pesticides. In most industrialized countries legislation was implemented requiring a number of single species toxicity tests before production and use of a compound (OECD, 1989). Despite their limitations (Van Leeuwen, 1990), the implementation of such tests in addition to curative measures, has led to great improvements in environmental quality. Single species toxicity tests, however, mainly assess acute toxicity but hardly sublethal effects or effects of chemicals on ecosystems. Therefore, especially with persistent compounds, unforseen exposure routes and effects are to be anticipated. The development of irreversible ecological damage can only be prevented if the fate and effects of such chemicals in ecosystems are effectively monitored.

Although restrictive measures have been taken regarding the production and use of certain PHAHs, such as polychlorinated biphenyls (PCBs), a lot of them are still circulating in the environment. In addition, structurally related chemicals are still being produced and appear in wildlife species.

Polyhalogenated aromatic hydrocarbons

The ubiquitous occurrence of PHAHs in highly complex mixtures of varying composition, has been widely documented (e.g. Kimbrough and Jensen, 1980; Firestone, 1984; IPCS, 1993, 1994; Tanabe *et al.*, 1994;). Some widely occurring PHAHs are (Figure 1.1):

- Polychlorinated dibenzo-*p*-dioxins (PCDDs) and -dibenzofurans (PCDFs), which are unwanted byproducts from incomplete combustion in the presence of chlorine, for example during waste incineration, and the production of certain metals, certain pesticides and organic chemicals (Firestone, 1984; Rappe and Buser, 1989; Ahlborg *et al.*,

1992). Despite measures taken to reduce formation and release of PCDDs and PCDFs, these chemicals are still entering the environment.

- Polychlorinated biphenyls (PCBs) -terphenyls (PCTs), and -naphtalenes (PCNs) became industrial useful chemicals from the early 1930s on. Especially PCBs have been used in large quantities, for example in capacitors, transformers, paints, construction materials, lubricant oils, sealing material and fire retardants (Brinkman and De Kok, 1980; Firestone, 1984; De Voogt en Brinkman, 1989; IPCS, 1993, 1994; Pijnenburg *et al.*, 1995). Although these compounds have been banned in most industrialized countries since the early 1980s, they are still entering the environment by leakage from old so-called closed systems, from recycling of old materials, from volatilization and leakage from landfills and sewage sludge, and from continued usage in tropical countries (Ballschmitter *et al.*, 1989; De Voogt and Brinkman, 1989; IPCS, 1993; Tanabe *et al.*, 1994). In 1969 PCBs were reported as environmental pollutants in The Netherlands for the first time (Koeman *et al.*, 1969). By the end of 1980 about 1,054,800 tonnes of PCBs had been produced by OECD countries (IPCS, 1993). It has been estimated that only 16-30% of the PCBs have entered the environment yet, and there is a serious risk that a large part of the rest will ultimately reach the world oceans unless actively retrieved and destroyed (Klamer *et al.*, 1991).
- Polybrominated diphenyl ethers (PBDEs) are still in full use as fire retardants in a wide range of applications such as furniture and electrical and computer components and housing. The use of PBDEs has significantly increased over the last years (IPCS, 1994).
- Polybrominated biphenyls (PBBs) are still in use as flame retardants (Janus *et al.*, 1994). Tetrachlorobenzyltoluenes, such as the technical mixture Ugilec 141 (Figure 1.1), are preventively prohibited in The Netherlands, but are still in use as PCB-substitutes in other European countries (Janus *et al.*, 1994).

In general background levels of PCBs and other PHAHs are especially elevated in highly industrialized areas with historical input from mining and leakage from large scale applications (IPCS, 1993). Atmospheric transport contributes to a major extent to the global distribution of PCBs, including remote areas (Barrie *et al.*, 1992; Falandysz, 1994, Tanabe *et al.*, 1994). Due to the low ambient temperature, PCBs will eventually be deposited in the polar regions, where great levels in particularly fish-eating marine organisms is to be expected (Tanabe et al, 1994). Most of the environmental load of PHAHs has been estimated to be in aquatic sediments, which acts both as an environmental sink and a reservoir (IPCS, 1993).

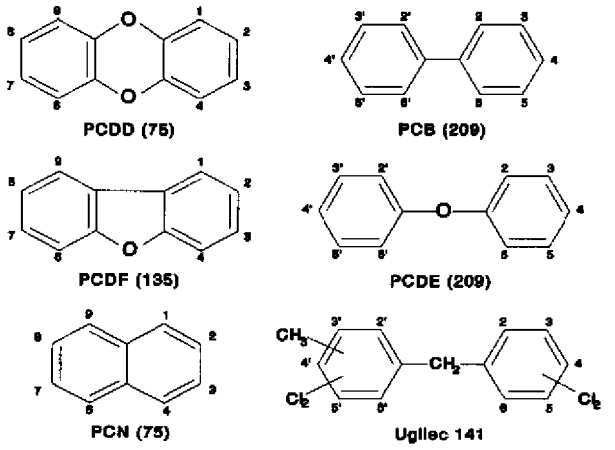


Figure 1.1 General molecular structures and ring positions of PCDD (polychlorinated dibenzo-*p*-dioxin), PCB (polychlorinated biphenyl); PCDF (polychlorinated dibenzofuran), PCDE (polychlorinated diphenylether), PCN (polychlorinated naphtalene) and Ugilec 141 (a technical mixture of tetrachlorobenzyltoluenes). The numbers between brackets indicate the number of possible congeners. For each of these classes, except PCNs, brominated analogues have been produced as well.

Bioaccumulation and metabolism of PHAHs

The fate of PHAHs in the environment, leading to exposure of species, greatly depends on their chemical structure. The lower halogenated PHAHs are generally easier degraded than the higher halogenated ones. Lower chlorinated PHAHs can be degraded by microorganisms under aerobic conditions and higher chlorinated PHAHs under anaerobic conditions (IPCS, 1993; Beurskens, 1995). Photolysis is an important abiotic transformation process for PHAHs in the atmosphere and in the aquatic environment (IPCS, 1993). As sources and routes of environmental contamination differ, the absolute and relative abundance of different PHAH congeners may differ greatly between environmental matrices and sites (Rappe and Buser, 1989; Norstrom *et al.*, 1990; Duinker *et al.*, 1989). Due to their lipophilic character, PHAHs almost exclusively occur in the environment associated with organic compounds and lipids. In general, species that are part of the aquatic food chain contain higher levels of PHAHs than exclusively terrestrial species (Gilbertson, 1989; Kannan *et al.*, 1989b). PHAHs are also passed on to the next generation via deposition into eggs (Biessmann, 1980; Ankley *et al.*, 1991) and via the transplacental transport and the consumption of milk in mammals (Disser and Nagel, 1989;

for review see Brouwer *et al.*, 1995).

PHAH congener patterns differ considerably between the various trophic levels, which is partially due to differences in exposure, but mainly to differences in biotransformation capacity between species (Muir *et al.*, 1988; Boon *et al.*, 1992; Leonards *et al.*, 1994a; Brouwer, 1996). There is an upward trend for bioaccumulation and -magnification with increased halogenation of PHAHs (Tanabe *et al.*, 1988; Duinker *et al.*, 1989; Ahlborg *et al.*, 1992; Leonards *et al.*, 1994a). The rate at which organisms can excrete PHAH congeners is to a large extent determined by the readiness to metabolize them to more polar compounds, which are mainly eliminated via the bile and faeces. Metabolism primarily occurs by the hepatic cytochrome P450 (P450)-dependent mono-oxygenase system, of which different types of biotransformation enzymes can be induced, depending on the possibility of the PHAH to adapt a planar conformation. Planar, non-*ortho*-substituted PHAHs induce in rodents P450 1A1/2 activity, often measured with the model substrate ethoxyresorufin as ethoxyresorufin O-deethylase (EROD) activity, or with benzo(a)pyrene as arylhydrocarbon hydroxylase (AHH) activity. Non-planar, for example di-*ortho*-substituted, PHAHs induce primarily P450 2B1/2 activity, often measured as pentoxyresorufin O-deethylase (PROD) activity. Mono-*ortho*-substituted PHAHs induce both types of P450 isoenzymes, and are therefore called mixed-type induces (Bandeira *et al.*, 1992; Safe, 1989, 1990). However, species differences exist. Fish, for example, have been reported to lack P450 1A2 inducibility (Goksøyr and Förlin, 1992; Stegeman and Kloepper-Sams, 1987) and most fish-eating birds and fish lack P450 2B inducibility (Ronis and Walker, 1989; Bosveld *et al.*, 1995).

Differences in metabolizing capacity will not only have consequences for elimination half-lives of individual PHAH-congeners and resulting differences in PHAH-pattern, but also in the quantity and quality of metabolites formed, which have been shown to induce their own specific set of toxic effects. Elimination half-lives of PCB congeners in rats have been reported to range from less than one day to about 450 days (Nord, 1992). Hydroxylated (OH) products are the major PCB metabolites, but also conjugated metabolites such as methyl sulphonyl metabolites, and partially dechlorinated metabolites have been identified (Lund et al 1985; Klasson-Wehler *et al.*, 1989; Nord, 1992). OH- and methyl-sulphonyl metabolites of PCBs have been reported to accumulate in fetuses, resulting in fetal tissue concentrations which are, in contrast to levels of parent PCBs, higher than corresponding maternal levels (Danerud *et al.*, 1986; Klasson-Wehler *et al.*, 1989; Bergman *et al.*, 1992; Morse *et al.*, 1996).

Ah-receptor mediated effects of PHAHs

For PCBs and other PHAHs species differences in sensitivity and pattern of symptoms

have been identified, but the reasons for this variation in species sensitivity have not yet been (fully) elucidated (Kimbrough, 1987; Safe, 1990; Gallo *et al.*, 1991; Håkansson *et al.*, 1991). Toxic effects commonly observed after acute, subchronic and chronic administration of PHAHs include progressive weight loss; adverse effects retinoid and thyroid hormone homeostasis and male and female reproduction; hepatotoxicity; teratogenicity and developmental toxicity; neurotoxicity; immunotoxicity and tumour promotion (McConnell, 1985, 1989; Goldstein and Safe, 1989; Safe, 1990; Nord, 1992; DeVito and Birnbaum, 1994). Most of the toxic effects induced by PHAHs, such as immunotoxicity and teratogenic effects, are suggested to be mediated by the aryl hydrocarbon receptor (AhR).

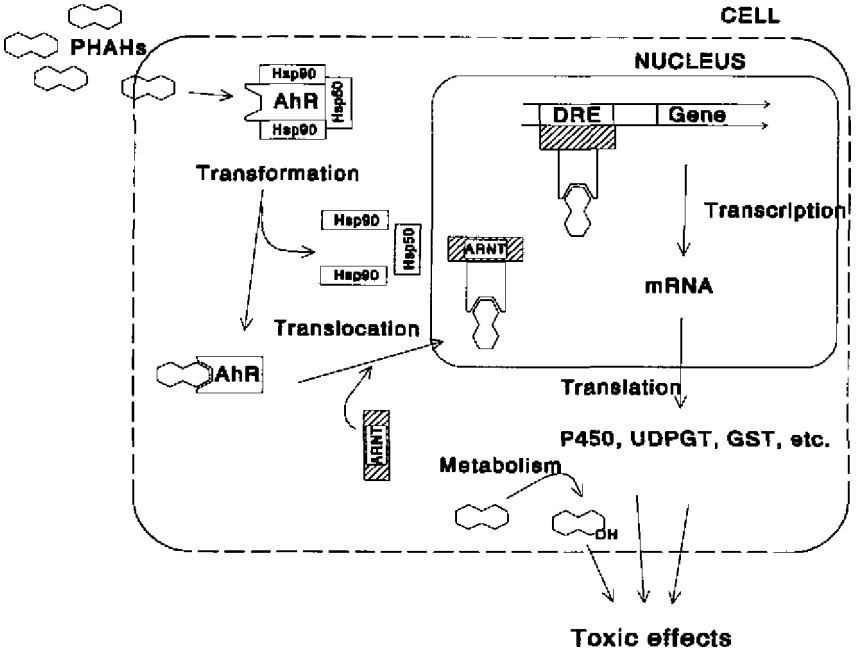


Figure 1.2 Schematic representation of the Ah-receptor mediated mechanism of toxicity. Abbreviations: PHAHs: polyhalogenated aromatic hydrocarbons; AhR: arylhydrocarbon receptor; Hsp: heat shock protein; ARNT: Ah-receptor nuclear translocator; DRE: dioxin responsive enhancer; P450: cytochrome P450 1A; UDPGT: uridine-5-diphosphate-glucuronyltransferase; GST: glutathione *S*-transferase

The AhR resides in the cytoplasm of responsive cells (Figure 1.2) in a soluble complex with two copies of the heat shock protein Hsp90 as chaperones and a, not yet fully characterized, protein designated Hsp50 (Landers and Bunce, 1991; Reyes *et al.*, 1992). Upon binding of a ligand, the AhR-complex dissociates and the ligand-AhR interacts with the Ah-Receptor-Nuclear-Translocator (ARNT). This activated toxicant-AhR-ARNT complex translocates to the nucleus where it binds with high affinity to specific DNA enhancer sequences, the dioxin responsive enhancers (DREs), resulting in increased or inhibited transcription of genes adjacent to the DRE (Nebert *et al.*, 1993; Okey *et al.*, 1994; Weber and Stahl, 1995; Denison and Whitlock, 1995; Okey *et al.*, 1995). To date, at least 26 genes have been shown to be either directly controlled by the AhR or to be responsive to AhR agonist (Sutter and Greenlee, 1992; Okey *et al.*, 1994; Hankinson, 1995). The most investigated AhR-mediated response is increased synthesis of P450 1A and its associated EROD activity (Whitlock, 1990; Safe, 1994). Also the level and activity of some phase II enzymes such as uridine-5-diphosphate-glucuronyltransferases (UDPGT) and glutathione *S*-transferase (GST) have been shown to be induced in an AhR related manner (DeVito and Birnbaum, 1994). Epidermal growth factor (EGF) receptor and estrogen receptor are examples of proteins that are 'down-regulated' by TCDD and these reductions appear to be mediated by the AhR (Goldstein *et al.*, 1990; Line *et al.*, 1991; Zacharewski *et al.*, 1991; Okey *et al.*, 1994). The AhR-binding of PHAHs is dependent on the planarity and presence of lateral chlorines on the molecule. Safe (1990) concluded that the non-*ortho* congeners, which are substituted in both para and at least two meta, and no *ortho* positions, are the most toxic PCB congeners. Chlorination at one or two *ortho* positions further decreases the toxic. The sensitivity for AhR related effects, however, is species specific.

Disruptive effects of PHAHs on retinoid and thyroid hormone homeostasis

Not all toxic effects of PHAHs can be directly attributed to an AhR mediated mechanism. Many symptoms of PCB-exposure resemble those of a vitamin A deficiency (Brouwer, 1991; Nord, 1992), and at least one additional mechanism of action is involved in PHAH-induced effects on the retinoid homeostasis. Although non- and mono-*ortho*-PCB congeners have been shown to reduce retinoid storage in several species (Jensen *et al.*, 1987; Spear *et al.*, 1988, 1989; Bank *et al.*, 1989a,b; Zile *et al.*, 1989; Chen *et al.*, 1992), also non-planar PHAHs decrease serum retinol levels (Brouwer, 1991; Chen *et al.*, 1992). Recent studies have revealed that hydroxy (OH-) metabolites of PCBs and other PHAHs inhibit thyroxine (T4) binding to the T4-plasma transport protein transthyretin (TTR) (Brouwer and Van den Berg, 1986; Lans *et al.*, 1993, 1994). T4 is transported in the blood plasma in a complex of T4-TTR-retinol binding protein(RBP)-retinol. Certain OH-PCBs

directly compete with T4 for the T4-binding site on TTR, resulting in a loss of T4 from the circulation and a reduced TTR-RBP binding (Brouwer *et al.*, 1989). The retinol-RBP unbound to TTR is small enough to be filtered out of the blood by the kidney, and is lost from the body via the urine (Brouwer and Van den Berg, 1986; Brouwer, 1987). TTR-binding by OH-PCBs is, in contrast to AhR-binding by PCBs, not favoured by planarity, and even mono-ring structures such as chlorinated phenols, interact with TTR (Van den Berg *et al.*, 1991; Den Besten *et al.*, 1993; Lans et *al.*, 1994). In addition to interference of OH-PCB with plasma T4 levels, induction of T4- or T3 (triiodothyronine)-glucuronidation may enhance hepatic elimination of thyroid hormones (Barter and Klaassen, 1992; Beetstra et *al.*, 1991; Visser *et al.*, 1993). OH-PCBs have also been shown to competitively inhibit hepatic T4-5'-deiodinase activity, involved in the conversion of T4 to T3 (Adams *et al.*, 1990; Brouwer *et al.*, 1994; Lans et al, 1996).

Indications for toxic effects of PHAHs in wildlife species

Most toxicological studies with PHAHs have been performed with laboratory mammals such as rodents (rats and mice), and to a lesser degree with monkeys. Experimental studies with herbivorous birds, such as chicken, dove and quail dosed with technical PCB-mixtures, indicated that PCBs reduces egg production and hatchability in chickens; extends the courtship period, decreases parental attentiveness and thyroid hormone levels in doves; reduces nest-building activity of pigeons; and delays onset of egg laying and reduces avoidance behaviour in Japanese quail (McArthur *et al.*, 1983; Gilbertson, 1989; Fox, 1993; IPCS, 1993). Experiments performed with birds and mammals feeding on aquatic prey are much more rare. In studies with female mink fed technical PCB mixtures, reduced embryo implantation, kit survival and kit body weight gain were reported (Brunström *et al.*, 1994). Comparable effects were observed after feeding mink with fish from polluted the lakes (Aulerich *et al.*, 1970; Aulerich and Ringer, 1977; Wren et *al.*, 1987; Kihlstrom et al, 1992; Leonards *et al.*, 1995). In a Dutch experimental study harbour seals (*Phoca vitulina*) were fed fish diets from either the greatly polluted Wadden sea or the relatively clean Atlantic ocean. The heavily PHAH-exposed group of seals showed impaired reproductive success (Reijnders *et al.*, 1986) and significantly decreased plasma thyroid hormone and retinol levels, suggesting an impaired physiology (Brouwer et *al.*, 1989). In a comparable study, a significant reduction in immune functions was observed for harbour seals fed on fish from the polluted Baltic sea if compared to seals fed with Atlantic ocean fish (De Zwart *et al.*, 1994; Ross *et al.*, 1995)

Many field observations in the area of the Great Lakes report decreased reproductive success in fish eating birds, such as reduced egg hatchability and chick survival and skeletal deformations, which correlated with residues of PHAHs (Hoffman et *al.*,

1987; Kubiak *et al.*, 1989; Tillitt et al., 1992). In a Dutch study, developmental impairment has been observed for cormorant hatchlings *(Phalacrocorax carbo)* (Van den Berg *et al.*, 1994; Van der Gaag *et al.*, 1993). Field observations on mink, otter and seal populations around the Great Lakes and in the Nordic countries indicated an association between relatively high PCB concentrations in fat and declining populations (Gilbertson 1989; Olsson *et al.*, 1992; Leonards *et al.*, 1995).

Establishing causal relationships for PHAHs under field conditions

Currently, most ecotoxicological risk assessments are based on chemical residue analysis of abiotic matrices correlating them with determinations of toxic effects in wildlife species. It is, however, often not known whether these correlations are due to a causal relationship. The possible detection of direct effects in response to release of specific pollutants from point sources (such as industrial effluent or spills) is relatively easy. The detection, however, of chronic effects caused by mixtures such as PHAHs which are released over long time periods and from diffuse sources, is much more difficult. In addition, the response of populations to pollutants is often non-specific and hard to distinguish from responses to natural influences, such as nutritional status and physical stresses. Even if adverse effects observed under field conditions are found to be correlated with certain PHAH-concentrations, it remains unclear whether these contaminants play a role as etiologic agents, since most study areas are polluted with a cocktail of contaminants. It can never be excluded that the suggested causal relationship is due to co-correlation with other pollutants present in the exposure matrix that are not included in the exposure assessment or below the detection limit.

Especially for compounds, such as PHAHs, that are present in complex mixtures and act via a common mechanism of toxicity, exposure analysis would be significantly improved if the toxic potency of the total mixture could be quantified instead of only single representatives. For this purpose the TCDD or toxic equivalency factor (TEF) concept was introduced by Safe (1987), allowing conversion of a chemical data set into the AhR-related toxic potency of the mixture of PHAHs. Concentrations of individual PHAHs are multiplied by their respective TEF-values and added up to give the TCDD- or toxic equivalency (TEQ) value (Safe, 1990). Although this TEF-approach is an improvement compared to sole chemical analysis, it is limited by the fact that it assumes all effects to be mediated by the AhR, and only assumes additive, and not synergistic or antagonistic effects (Brouwer, 1991; Ahlborg *et al.*, 1992, 1995; Safe, 1994). In addition, it is not yet possible to fully quantify the AhR-related toxic potency of environmental mixtures of PHAHs because TEF-values exist for only part of the PHAHs and levels of many PHAHs are not measured as they are unexpected, not looked for, or present in

levels below detection limits.

In addition to a more toxicologically relevant exposure analysis, the role of PHAHs as etiologic agent for observed adverse ecotoxicological effects would be strengthened, if mechanism-based information was available, about biochemical or physiological changes known to be early precursors of PHAH-induced effects.

Biomarkers

The need for more mechanism-based exposure and effect analyses link up with the rapidly developing use of biomarkers in ecotoxicological research. Biomarkers are biochemical, physiological or cellular responses, and can be used (a) to identify the toxicologically effective *exposure*, or (b) indicate early signs of *effects*. A very important advantage of biomarkers is the short response time which is indicative of (sub)lethal effects that may eventually lead to impaired population success, integrating the toxicological (inter)action induced by complex mixtures of chemicals. Additionally, biomarkers can be developed to (c) assess the *sensitivity* of a species or individual animal for a certain class of chemicals, for example based on the presence or absence of specific receptors or enzymes. The usefulness of biomarkers in ecotoxicology and strategies of using them to assess environmental effects are described in a number of recent reviews (McCarthy and Shugart, 1990; Peakall, 1992; Peakall and Shugart, 1993; Fossi and Leonzio, 1994).

Commonly used biomarkers often require the analysis of tissues and organs, such as liver, kidney or brain, involving the destruction of living organisms. This may be undesirable in many situations, for ethical and practical reasons. Therefore, it is important to develop and validate nondestructive biomarkers, i.e. biochemical, cellular, physiological or behavioural parameters that can be measured in an organism or population, which provide evidence of exposure and/or effects of one or more chemical pollutants without causing damage or prolonged stress to the organism or population (Fossi and Leonzio, 1994). Nondestructive biomarkers may include the use of *in vitro* bioassays with cell lines or with tissues from animals that died by accident, or measurement of biochemical changes in blood samples.

Outline of the thesis

This thesis concerns the development and validation of biomarkers to identify causal relationships between exposure to PHAHs and adverse effects, even at low levels of exposure. Of course such biomarkers have to be validated in experimental and field studies. Using these tools the question can be addressed whether at present fish eating wildlife species in the Netherlands are exposed to toxic levels of PHAHs.

Two research questions are derived from these objectives:

1 Do natural fish-eating bird species exhibit toxic effects upon PHAH-exposure, comparable to the effects observed in laboratory species;
2 What is the perspective for the use of an receptor-mediated reporter gene assay to assess the total AhR related potency of environmental pollutants?

The first question will be addressed in Chapters 2-5. Chapters 2-4 describe a semi-field and a field study, respectively with eider ducklings (*Somateria mollissima*) and common tern (*Sterna hirundo*) chicks. The eiders were dosed with the planar PCB-77 congener or the technical PCB mixture Clophen A50. The toxic and biochemical effects and parameters were recorded (Chapter 2). Comparable parameters were measured in common tern chicks that hatched from artificially incubated eggs, originating from 3-egg clutches in colonies differing in degree of pollution. The biochemical results were compared with PHAH-levels and incubation parameters of the same animals (Chapter 3). In the field, the breeding biology of the two remaining eggs and natural influences, such as weather and predation, were monitored and compared with the data from the artificially incubated egg (Chapter 4). As part of PHAH toxicity is caused by metabolites, the capacity of some wildlife species to metabolize the model planar PCB-77 (3,3',4,4'-tetrachlorobiphenyl) congener into hydroxylated metabolites, is qualitatively and quantitatively compared with that of a laboratory rat species (Chapter 5)

To answer the second question, a novel reporter gene assay was further developed for use as an *in vitro* bioassay for measuring the total, integrated potency of mixtures of PHAHs for AhR-mediated toxicity in biotic and abiotic environmental matrices. This assay is based on chemical induced luciferase gene expression, and is applied and validated for measuring the toxic potency of PHAHs, expressed as TCDD equivalents, in sediments and pore water (Chapter 6) and in small aliquots of blood plasma (Chapter 7).

In Chapter 8, the implications of the presented work are discussed, with emphasis on the use of the mechanism-based responses as biomarkers for PHAHs under field conditions; differences in sensitivity between species and life-stages; perspectives for the prediction of population effects of PHAHs; and the possible ecotoxicological effects of current and future PHAH levels in the Dutch situation.

CHAPTER 2

TOXIC AND BIOCHEMICAL EFFECTS OF 3,3',4,4'-TETRACHLOROBIPHENYL (PCB-77) AND CLOPHEN A50 ON EIDER DUCKLINGS *(Somateria mollissima)* IN A SEMI-FIELD EXPERIMENT*

ABSTRACT

In this study the possible toxic and biochemical effects of one intra peritoneal dose of 5 or 50 mg·kg^{-1} 3,3',4,4'-tetrachlorobiphenyl (PCB-77) or 50 or 200 mg·kg^{-1} Clophen A50 (CloA50) on 28-day-old eider ducklings (*Somateria mollissima*) were investigated. After ten days no significant differences could be observed in any of the toxic and biochemical parameters studied apart from ethoxyresorufin *O*-deethylase (EROD) activity, when comparing group average values of the dosed and control animals. However, significant correlations were observed at day 10 after exposure between the internal PCB concentration and body weight gain and beak length growth (negative correlation in the CloA50 groups); relative liver weight and cytochrome P4501A activity (positive correlation in PCB-77 and CloA50 groups); plasma thyroid hormone and hepatic retinoid levels (negative correlations in PCB-77 groups) and plasma retinol levels and the ratio plasma retinol/hepatic retinyl palmitate (positive correlations in PCB-77 groups only). Animal activity was significantly reduced in the group that received 50 mg·kg^{-1} PCB-77. These observations indicate that eider ducks are a sensitive species to PCB toxicity and may be at risk for development of adverse health effects in relatively high contaminated areas such as the Dutch Wadden Sea.

Based on: Murk AJ, Van den Berg JHJ, Fellinger M, Rozemeijer MJC, Swennen C, Duiven P, Boon JP, Brouwer A and Koeman JH (1994): Toxic and biochemical effects of 3,3',4,4'-tetrachlorobiphenyl (CB-77) and Clophen A50 on eider ducklings (Somateria mollissima) in a semi-field experiment. Environ. Pollut. ***86****: 21-30*

INTRODUCTION

Polychlorinated biphenyls (PCBs) are ubiquitous environmental toxicants which accumulate especially in aquatic food chains. Of the total world production of PCBs about 60% is still in use and it is calculated from literature data that 11-17 tonnes of PCBs still enter the North Sea each year (Klamer *et al.*, 1991). Becker *et al.* (1991) found that PCB levels in eggs of various shore birds were increased 2-6-fold in 1987 compared with levels in 1981.

PCBs are known to induce a wide spectrum of toxic effects in laboratory animals (Goldstein and Safe, 1989; McConnell, 1989). In fish-eating birds exposure to PCBs and related compounds has been associated with effects such as impaired reproduction (Hoffman *et al.*, 1986,1987; Kubiak *et al.*, 1989; Van den Berg *et al.*, 1994), morphological abnormalities (Hoffman *et al.* 1986,1987), thymus atrophy and immunotoxicity (Nikolaidis *et al.*, 1988; Andersson *et al.*, 1991), hypovitaminosis A and/or hypothyroidy (Moccia *et al.*, 1986; Spear *et al.*, 1989; Van den Berg *et al.*, 1994), behavioural changes (Kubiak *et al.*, 1989; McArthur *et al.*, 1983; Tori and Peterle, 1983), and loss of body weight (Koeman *et al.*, 1973a; Gilbertson, 1989). Most of these studies are, however, field work in which it is impossible to make a distinction between effects caused by PCBs and other substances such as polycyclic aromatic hydrocarbons (PAHs). Some of the results of these field studies have been supported by experimental observations.

PCDDs, PCDFs and some PCBs initiate their effects, at least partially, through binding to the arylhydrocarbon (Ah)-receptor followed by the induction of specific gene products such as cytochrome P4501A1 and 1A2. The Ah-receptor mediated effects are induced by the parent compounds. However, reports have been published recently that indicate that toxicity of PCBs may partly be due to Ah-receptor mediated production of hydroxylated PCB-metabolites which interfere with thyroid hormone and vitamin A transport system (Brouwer, 1991).

Blue mussels (*Mytilus edulis*) are the main source of PCB-exposure for Eider ducks (Broman *et al.*, 1990). During the growth period a single bird consumes every 24 hr more than 3000 specimens of Mytilus of 15-25 mm in size. Among other substances, extreme PCB levels of 890-2400 $\mu g \cdot g^{-1}$ lipid were measured in blue mussels close to the Dutch shore (Klamer *et al.*, 1991). From earlier research it is known that female eider ducks (*Somateria mollissima*) are especially at risk for development of PCB toxicity because they do not feed during incubation, leading to a strong increase in blood concentration (Koeman and Van Genderen, 1972). A reduction of about 65% in the size of the breeding population of eiders on Vlieland (an island in the north of The Netherlands) was observed in the period from 1988-1990. The question addressed in this study

concerns whether the eiders are sensitive to PCB toxicity.

Eider ducks were experimentally exposed to a single i.p. dose of a planar PCB congener, 3,3',4,4' tetrachlorobiphenyl (CB-77) or to a commercial PCB mixture, Clophen A50 (CloA50) under semi-field conditions. Several toxic and biochemical parameters were investigated.

Materials and Methods

Animals

Newly hatched eider ducklings were caught on the isle of Vlieland, in the Dutch Wadden Sea. They were kept on the isle of Texel at The Netherlands Institute for Sea Research (NIOZ), in two large open-air cages. Each cage consisted of a seawater section of 49 m^2, 0.6 m deep that was flushed through with filtered seawater, and a terrestrial part of 9.75 m^2 with a shelter and heat lamp. The animals were walked with daily to keep them in a good condition.

After 14 days, the eiders were individually numbered with an aluminum leg ring. Their weight and beak length (culmen midline) were measured daily before feeding. Food (Poultry pellets, Koopmans BV, Leeuwarden) was available *ad libitum* during daytime until 10 pm. Fresh water was available at all times.

Treatment

After an acclimation period of 27 days, the animals were divided among 5 groups and received an extra plastic leg ring with their group colour. Ten animals were dosed once by i.p. injection with corn oil (5 $ml \cdot kg^{-1}$ body weight (bw)) as a vehicle, six animals with 5, ten with 50 $mg \cdot kg^{-1}$ PCB-77, six animals with 50, and ten with 200 $mg \cdot kg^{-1}$ bw CloA50 dissolved in 5 $ml \cdot kg^{-1}$ bw corn oil. For practical reasons, the animals from the low-dose groups were dosed one day later than the animals in the other groups.

Blood was collected from the superficial plantar metatarsal vein at days 0, 1, and 7 after exposure. At day 10, the animals were killed under ether anaesthesia through heart puncture. Wing length was measured, and blood, liver, thymus, brain and adipose tissue collected. Liver, thymus and brain weight were recorded immediately. After decapitation, the skull length was measured from the tip of the beak to the basioccipital condyle. Blood was centrifuged at 1000*g* for 10 min and plasma was stored at -20°C. Liver was immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

PCB analyses in adipose tissue

For adipose tissue analyses, a piece of (± 60 mg) abdominal fat was used to extract and measure PCB-77 levels and to determine the pattern of PCB congeners (CloA50 groups) by GC-ECD (analytical CPSil 8 column, 50 m x 0.25 mm i.d., Chrompack, The Netherlands), as described by Everaarts *et al.* (1991). Concentrations of PCB-77 are expressed as $\mu g \cdot g^{-1}$ lipid. Total concentrations of CloA50 congeners are converted into toxic equivalences (TEQs) using international toxic equivalence factors (TEFs) as described by Safe (1990).

Not all congeners can be separated on the analytical CPSil 8 column used. As a consequence, TEQ estimates have to be based on a limited number of congeners. None of the non-*ortho*-substituted PCBs (PCBs -77, -126 and -169) can be determined. Of these congeners, only PCB-126 is measurably present in CloA50 (Schulz *et al.*, 1989). Of the mono-*ortho*-substituted PCBs present in CloA50, PCB-105 and PCB-118 can be determined unequivocally. PCB-156, however, is not separated from PCB-171. The ratio between PCB-156 and PCB-171 in CloA50 is 1.43 : 0.50. As the height of the combined peak showed a constant ratio with respect to PCB-180 in the eider ducklings in this experiment (Rozemeijer *et al.*, 1991), it is assumed that the original ratio in the CloA50 mixture is maintained during the experimental period of 10 days. The measured concentrations of PCB-156 will therefore be multiplied by a factor of 0.74 for TEQ calculations. The mono-*ortho*-substituted PCBs have TEF values of 0.001 (Safe, 1990). Of the di-*ortho*-substituted congeners, only PCB-128, PCB-138 and PCB-170 are present in relatively high concentrations in Clo A50 (Schulz *et al.*, 1989). PCB-128 is well separated. PCB-138 co-elutes with PCB-158, but since they have identical TEFs (0.00002) the concentrations measured as PCB-138 equivalents are applied for the calculation of TEQs. PCB-170 co-elutes with PCB-190 (Schulz *et al.*, 1989). The same reasoning as for PCB-156/-171 is valid for these two pairs of congeners. The correction factor for measured concentrations of PCB-170 is 0.65:0.70 = 0.93. In total 62.4% of the total amount of TEQs present in CloA50 (Schultz *et al.*, 1989) can be measured. To obtain an estimate of the total TEQ body burden of the CloA50-dosed animals, the observed amount will be multiplied by 1.6.

EROD and PROD activities

Hepatic microsomes were prepared according to the method of Gibson and Skett (1986), and stored in a 0.1 M Na-K phosphate buffer pH 7.4, containing 20% (v/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol (DTT) in liquid nitrogen. EROD and PROD activities were determined according to the method of Burke and Mayer (1983).

Extractions and HPLC analysis of retinoids

Aliquots of 50 μl of plasma were spiked with retinyl acetate as an internal standard and extracted with 50 μl of methanol and 100 μl of diisopropyl ether. The ether phase was filtered over a 0.45 μm Millipore filter, dried under a gentle stream of nitrogen gas and dissolved in 50 μl of methanol. Aliquots of 20 μl extract were analysed isocratically on a reverse phase silica RP C_{18} column (Perkin Elmer, Pecosphere 3*3) with methanol/water (85:15) as eluent with a flow rate of 1 ml.min^{-1} and data collection for 10 min according to the method described by Morse *et al.* (1995a). Aliquots of 50 μl of liver homogenate were extracted according to the same procedures as described for plasma, but elution was by methanol/water (85:15) for 1.5 min, followed by a gradient to 100% methanol for 2.5 min, and subsequent elution of the retinyl esters at 100% methanol for 12 minutes. Retinoids were detected at 326 nm and quantified using standard curves of retinol or retinyl palmitate. The column was then re-equilibrated at methanol/water (85:15) for 6 min. Further details are described by Morse *et al.* (1995a).

Thyroid hormones

Total thyroxin (T4) and total triiodothyronine (T3) levels were determined in, respectively, 10 and 25 μl aliquots of plasma by chemiluminescence immunoassay using commercially available kits (Amerlite assay kits, Amersham International plc, Amersham, UK).

Animal activity

Swimming and eating activities were scored in the control, the CloA50 200 mg·kg^{-1} and the PCB-77 50 mg·kg^{-1} groups, by counting the number of times that the eiders crossed the footboard to the water or went eating. Activities were scored with a frequency of five times a day, for 10 min, starting three days before dosing and continuing until the end of the experiment.

Statistics

Statistical analysis of dose-effect relationships was performed by unweighted least-squares linear regression analysis. PCB levels, expressed either as mass or TEQs, and EROD activity are presented on a log scale to obtain better distribution of the values over the axes. Differences between group means were tested using the Mann-Whitney test. Differences in activity were tested with ANOVA. The acceptance level was set at $P<0.05$.

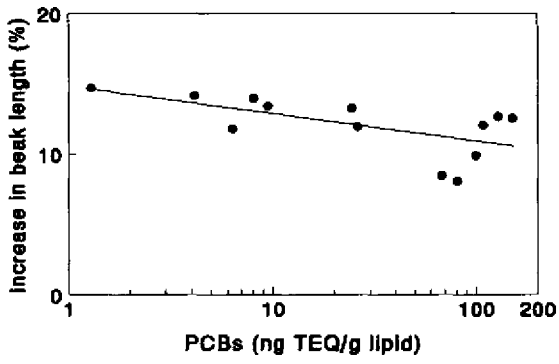
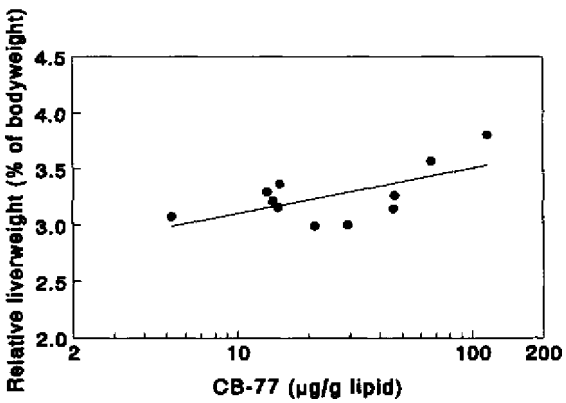
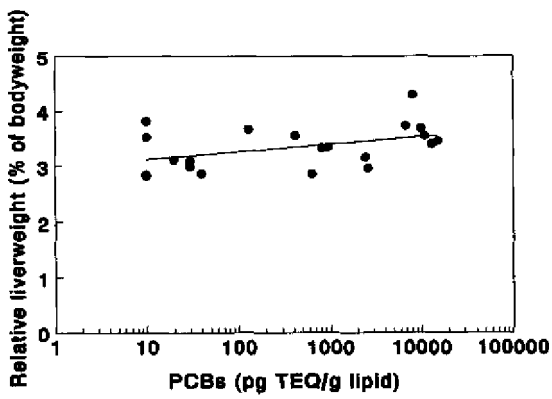


Figure 2.1 Increase in beaklength in CloA50-dosed animals between day 0 and day 10 of the experiment plotted against the PCB concentration, expressed as ng TEQs/g body lipid ($r = 0.62$, $P < 0.05$).



(a)



(b)

Figure 2.2 Relative liver weight of the (a) CB-77-dosed ($r = 0.63$, $P < 0.05$) and (b) CloA50-dosed and control eiders ($r = 0.43$, $P < 0.05$), plotted against internal PCB concentration expressed as, respectively, µg CB-77/g or pg TEQ/g body lipid.

Table 2.1 Internal PCB levels in the five exposure groups of eider duck.

Parameter	Control	CB-77		CloA50	
		5 mg/kg	50 mg/kg	50	200 mg/kg
TEQ (ng*)	0.3 ± 0.2	140 ± 52	875 ± 715	23 ± 17	129 ± 86
CB-77 (μg*)	n.d.	14.0 ± 5.2	87.5 ± 71.5	n.d.	n.d.

*Data are expressed as means·g^{-1} lipid ± S.D.
Abbreviation: n.d., not determined.

Results

Internal concentration of PCBs in body lipids

The mean internal PCB concentrations expressed as ng TEQ·g^{-1} lipid and μg·g^{-1} (PCB-77 only) are presented in Table 2.1. The average TEQ values of the high-dose CloA50 group (129 ng·g^{-1} lipid) is similar to that of the low-dose PCB-77 group (140 ng TEQ·g^{-1} lipid). The individual variation is very large, up to 82% of the average value in the 50 mg·kg^{-1} bw PCB-77 group.

Morphological measurements

The total body weight of the eider ducklings increased from 246-492 g (14 days old) to 949-1443 g (end of the experiment, 38 days old). This is similar to the body weight gain of eider ducks in the wild (Swennen, 1991). The beak length increased in the same period from 26.3-33.9 mm to 40.1-49.1 mm. No significant differences in group averages of total body weight gain nor increase in beak length were observed between the PCB-exposed and control groups at day 10 of exposure. In the CloA50-dosed groups, however, body weight gain in 10 days (%increase = 45 - 3.0 [TEQ]) and increase in beak length in 10 days (Figure 2.1) were significant negatively correlated with internal PCB concentrations expressed as pg TEQ·g^{-1} body lipid. This correlation was not seen in the PCB-77-dosed eiders.

The relative liver weights (group average values) were not significantly different between PCB-exposed groups and controls. However, when relative liver weight of each individual animal was plotted against internal PCB-77 level (μg·g^{-1} lipid) or CloA50 (ng TEQ·g^{-1} lipid) significant positive correlations were observed (Figure 2.2 a,b).

No significant alterations were observed for relative thymus weight, relative brain weight, wing length and skull length, either compared as group averages or individually correlated with internal PCB concentrations.

Table 2.2 Plasma total thyroxine (T4) and total thyronine (T3) levels in control and PCB-exposed eider ducklings.

Parameter	Control	PCB-77		CloA50	
nmol/l		5 mg/kg	50 mg/kg	50 mg/kg	200 mg/kg
T4, day 1	7.5 ± 2.6	9.1 ± 3.3	7.7 ± 2.2	9.7 ± 3.2	6.9 ± 1.9
T4, day 7	9.7 ± 2.8	8.3 ± 1.9	10.4± 1.5	7.5 ± 2.5	10.3± 3.4
T4, day 10	11.7± 4.2	13.3± 2.5	12.7± 3.5	13.9± 5.7	9.9 ± 3.2
T3, day 1	1.5 ± 0.3	1.4 ± 0.2	1.4 ± 0.4	1.6 ± 0.4	1.3 ± 0.3
T3, day 7	1.9 ± 0.4	2.5 ± 0.3*	1.6 ± 0.3	2.7 ± 0.1*	1.3 ± 0.1
T3, day 10	0.8 ± 0.2	0.9 ± 0.1	0.9 ± 0.2	1.1 ± 0.3*	1.3 ± 0.5*

Data are expressed as means ± S.D.
*Significantly different from control with $P<0.05$.

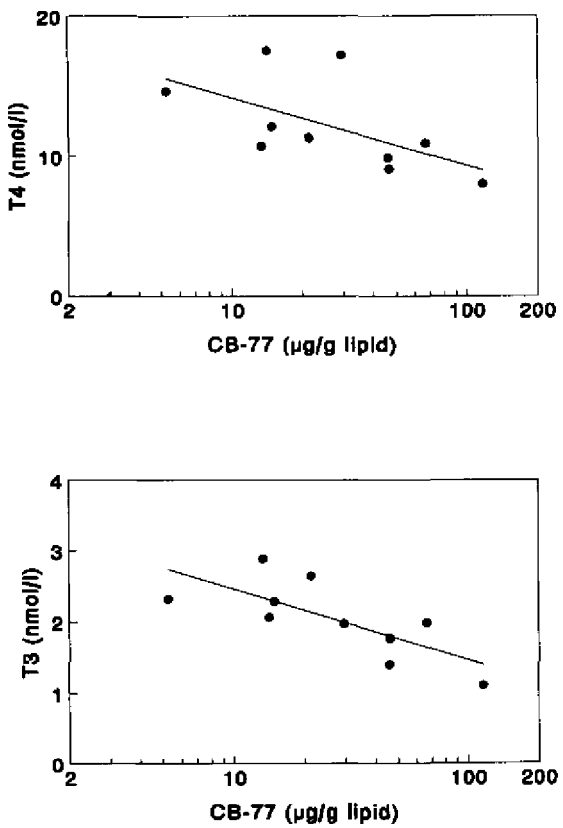


Figure 2.3 Correlations between (a) plasma total thyroxin levels (T4, $r=0.69$, $P<0.05$) 10 days after dosing or (b) plasma triiodothyronine (T3, $r=0.74$, $P<0.005$) levels 7 days after CB-77 dosing plotted against PCB levels (µg CB-77/g body lipid).

Hepatic EROD activity

Cytochrome P4501A induction measured as EROD activities were dose-dependently increased in the PCB-77 groups (respectively 60.2 ± 13.4 and 502.8 ± 412.9 nmol·mg^{-1}·min^{-1}) compared with control values (8.8 ± 4.1 nmol·mg^{-1}·min^{-1}). No significant differences were found for both CloA50-dosed groups (respectively 9.7 ± 11.7 and 17.4 ± 14.0 nmol·mg^{-1}·min^{-1}) compared with the control group. However, when correlated on an individual basis, the EROD activities in both the PCB-77 and the CloA50 groups were significant positively correlated with internal PCB levels expressed (respectively log EROD = 1.19 + 0.48 log [CB-77], $r = 0.70$, $P < 0.001$ and log EROD = 0.46 + 0.41 log [TEQ], $r = 0.67$, $P < 0.005$).

Thyroid hormone measurements

The average total thyroxine (T4) levels in the control group increased from 6.1 ± 1.2 nmol·l^{-1} plasma at day 0 to 11.7 ± 4.2 nmol·l^{-1} at day 10 of the experiment. Plasma total triiodothyronine (T3) levels decreased from 1.8 ± 0.6 to 0.9 ± 0.2 nmol·l^{-1} over the same period. There were no significant differences in group average plasma values for T4 levels at any time point of exposure (Table 2.2). Group average values of plasma total T3 levels of the low-dose PCB-77 and the low-dose CloA50 groups were significantly greater than control levels (respectively 132% and 142 %) at day 7 of exposure (Table 2.2). At the same time point a reduced plasma total T3 level compared with the control group (respectively 84% and 68%) was observed in both high-dose groups, but this difference was significant only in the CloA50 200 mg·kg^{-1} group. At day 10, plasma total T3 levels in both PCB-77-dosed groups did not differ from the controls. In the low and high-dose CloA50 groups total T3 levels were significantly greater compared with controls (138% for the 50 mg·kg^{-1} and 163% for the 200 mg·kg^{-1} CloA50 group).

Individual plasma total T4 levels were significant negatively correlated with internal PCB-77 levels at day 10 of exposure only (Figure 2.3a). Plasma total T3 levels were significant negatively correlated with PCB-77 levels at day 7 (Figure 2.3b). No significant correlations of thyroid hormone levels with internal PCB concentrations were found in both CloA50-dosed groups.

Plasma retinol and hepatic retinoids

During the experimental period, the average plasma retinol levels in the control eider ducks were 2.0 ± 0.22 μg·ml^{-1}. Plasma retinol levels of the PCB-dosed groups at days 1, 7 and 10 expressed as a percentage of the control values, are shown in Figure 2.4. In the PCB-77-exposure groups, a significant decrease to 84 and 82% of the control values is observed at day 1, of exposure, followed by a return to control levels at day 7, and a

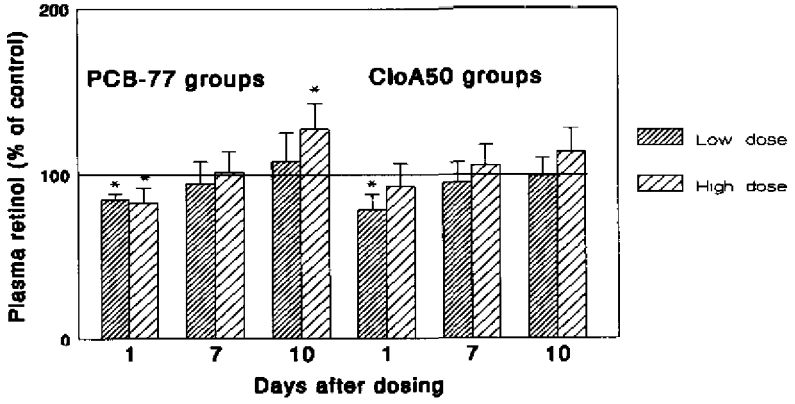


Figure 2.4 Plasma retinol levels on 1, 7 and 10 days after dosing PCBs, expressed as a percentage of the control values (absolute values can be found in Table 2.3). For CB-77 and Clophen A50-dosed groups, low doses are respectively 5 and 50 mg/kg bw, high doses respectively 50 and 200 mg/kg bw.
*Significantly different from control values ($P<0.05$).

subsequent increase at day 10 to 108% for the low-dose and 127% for the high-dose group. In the PCB-77 groups, plasma retinol levels were significantly increased with internal PCB-dose, ten days after dosing (Figure 2.5a).

Hepatic retinol, retinyl palmitate and retinyl stearate levels showed large inter-individual variations (up to 95%; Table 2.3). The only significant difference in group averages from control values was a reduction to 65% of the retinyl palmitate level in the high-dose PCB-77 group. Hepatic retinoid levels were also correlated on an individual basis with the PCB body burdens. In the PCB-77-exposure groups a clear, significant, negative correlation between internal PCB levels and hepatic retinol and both retinyl ester concentrations was observed. Figure 2.5b shows this relationship for hepatic retinyl palmitate. In the CloA50-dosed animals no such relationships were found.

The average ratio between plasma retinol and hepatic retinyl palmitate is significantly different from the controls in the PCB-77 50 $mg \cdot kg^{-1}$ group (Table 2.3). The ratio hepatic retinol over hepatic retinyl palmitate was not significantly correlated with the internal PCB concentration. The ratio plasma retinol to hepatic retinyl palmitate ($g \cdot g^{-1}$), however, is significant positively correlated with the internal PCB-77 concentration ($\mu g \cdot g^{-1}$ lipid) (ratio = -15 + 24.7 log [CB-77]; $r=0.74$, $P<0.05$). In the CloA50-dosed animals this ratio did not change with the PCB body burden (expressed as TEQs).

Table 2.3 Hepatic and plasma retinoid levels in control and PCB exposed eider ducklings.

Parameter	Control	CB-77		CloA50	
		5 mg/kg	50 mg/kg	50 mg/kg	200 mg/kg
H. retinol (μg/g)[a]	5.1 ± 3.6	9.7 ± 8.2	9.8 ± 5.1	11.0 ± 10.6	7.9 ± 3.4
H. ret.palmitate (μg/g)	17.0 ± 6.0	20.0 ± 8.2	11.1 ± 5.2*	19.2 ± 8.5	20.0 ± 4.0
H. ret.stearate (μg/g)	6.9 ± 2.5	8.6 ± 3.8	6.2 ± 2.2	8.7 ± 4.9	9.5 ± 2.1
Pl. retinol day 1 (μg/ml)	6.9 ± 2.5	8.6 ± 3.8	6.2 ± 2.2	8.7 ± 4.9	9.5 ± 2.1
Pl. retinol day 7 (μg/ml)	8.7 ± 4.9	9.5 ± 2.1	6.2 ± 2.2	9.5 ± 2.1	6.2 ± 2.1
Pl. retinol day 10 (μg/ml)	8.7 ± 4.9	9.5 ± 2.1	6.2 ± 2.2	9.5 ± 2.1	6.2 ± 2.1
Retinol/ret.palmitate[b]	0.11 ± 0.04	0.12 ± 0.07	0.24 ± 0.11*	0.11 ± 0.06	0.11 ± 0.03

Data are expressed as means ± S.D.
*Significantly different from control with $p<0.05$.
[a]μg/g liver.
[b]Ratio of (plasma retinol)/(hepatic retinyl palmitate).
Abbreviations: H. = hepatic; Pl. = plasma; ret. = retinyl.

Behavioural activity

In the control group swimming and eating activity gradually increased over the experimental period from 10 times per animal per day (five observations of 10 min per day for both activities) to, respectively, 17 and 19 times per animal per day. This increase was significantly less in the PCB-77-dosed group. Swimming activity in the PCB-77 group was 79% and eating activity 77% of the control values. In the CloA50 200 mg·kg^{-1} group these activities were, respectively, 91% and 98% of the control group, but these differences were not significantly different from the control group.

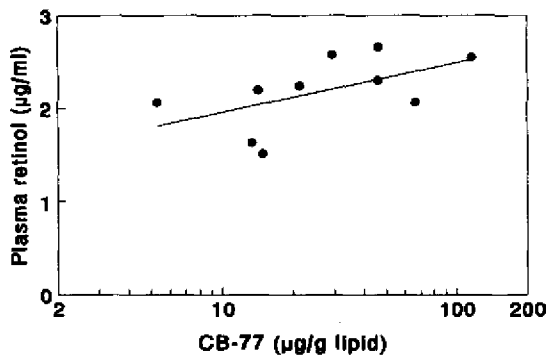
Discussion

In this semi-field study eider ducklings were found to be responsive with respect to Ah-receptor mediated responses, such as cytochrome P4501A induction and hepatomegaly. Some changes were observed in vitamin A and thyroid hormone levels that may be partially mediated by the Ah-receptor. Reduced growth and behavioural activity were recorded.

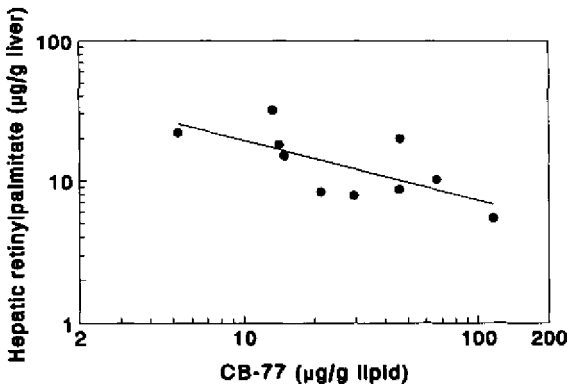
Variation in internal dose

The average internal PCB concentrations in the body lipid of the eiders dosed with 5 mg·kg^{-1} PCB-77 was 140 ± 52 ng TEQ·g^{-1} lipid, of the 200 mg·kg^{-1} CloA50-dosed group

129.4 ± 85.9 ng TEQ·g^{-1}, and of the 50 mg·kg^{-1} CloA50 group 23 ± 17 ng TEQ·g^{-1} lipid. These levels were similar to the levels found in yolksacs of eggs from fish eating birds in recent bird studies in The Netherlands. In relatively polluted cormorant chicks (Biesbosch), average PCB levels of 136 ng TEQ·g^{-1} yolksac lipid were found (Van den Berg *et al.*, 1994), and in common tern (*Sterna hirundo*) yolksacs from a relatively polluted colony (Slijkplaat) 40 ng TEQ·g^{-1} lipid (Murk *et al.* 1993, Bosveld *et al.*, 1995). In our semi-field experiment, as well as in both field studies mentioned above, the large variation in internal dose and measured parameters was obvious. This is mainly due to large intrinsic differences between individuals from a natural species compared with laboratory animals. Even when the diet is identical for all individuals and exposure is controlled, as in our semi-field experiment, correlation of the measured parameters with the individual internal PCB concentration is essential for studying effects.



(a)



(b)

Figure 2.5 Correlation between (a) plasma retinol concentrations ($r = 0.56$, $P < 0.01$) 10 days after exposure or (b) hepatic retinyl palmitate concentrations ($r = 0.69$, $P < 0.01$) and PCB levels in the CB-77-dosed eider ducks.

Ah-receptor mediated effects

A strong P450 induction was observable in the PCB-77 groups, while no significant induction was observed in the CloA50 groups (Rozemeijer *et al.*, 1991). We nevertheless found a PCB-related EROD induction in both PCB-77 and CloA50 groups. In both CloA50 and PCB-77-dosed eiders, we also found a significant positive correlation between relative liver weight and PCB body burden. This usually coincides with P450 induction.

For a number of reasons it is difficult to compare the relationship PCB body burden and EROD activity that we found here with the results of other studies; firstly, this is because wild birds always are exposed to various chemicals which may induce or inhibit P450 activity; secondly, it was because most of the birds used were adults, often caught during breeding season (Walker, 1990), when the mixed-function oxidase activity has its peak (Fossi *et al.*, 1989). We therefore only compared our results with that of one-day-old chicks of common tern and cormorant. In our experiment, the slope of the correlation of log (EROD activity) to log [PCB-77] internal dose was 0.49, with the corresponding slope to log TEQ internal dose in the CloA50-dosed groups of 0.41. In one-day-old cormorants (Van den Berg *et al.*, 1994), the log (EROD activity) to log [mono-*ortho*-PCBs] slope was 0.49 and in one-day-old common terns 0.50 was the ratio to log [TEQ]. The eider ducklings therefore showed an P4501A-inducing potency similar to that of the cormorant and the common tern.

The only study reported of the effects of PCBs on eiders was performed with eggs by Brunström *et al.* (1990). They concluded from egg injection experiments that eider embryos were rather insensitive to PCB-77 and 3,3',4,4',5-pentachlorobiphenyl. They did not find significant embryonic mortality at a dose of 1 mg PCB-77·kg^{-1} egg, but they did not measure biochemical parameters such as EROD activity or retinoid levels. It is difficult to compare the results of their egg experiment with our results with eider ducklings because no internal doses were measured in the eggs. A very rough estimation of the internal dose per gram lipid after injection of 2 mg PCB-77·kg^{-1} egg, based on the assumption that 4% of the egg is lipid, as in cormorant eggs (Van Schaik and De Voogt, 1989) and that all PCBs will be present in the lipid, results in a PCB level of 50 mg PCB-77·kg^{-1} lipid. In our experiment, such a body burden resulted in large reductions in hepatic retinoid stores and increase of EROD activity.

Morphology

We did not detect any morphological aberrations as the ones related to PCBs in field studies with the black-crowned night herons (*Nycticorax nycticorax*) (Hoffman *et al.* 1986) and Forster's tern (*Sterna fosteri*) (Hoffman *et al.*, 1987). One possible explanation is that, under natural circumstances, exposure already takes place *in ovo*, during organ

formation. The eider ducklings in our experiment were dosed at the age of 28 days. They were growing rapidly (36% in 10 days), but tissue differentiation had already taken place before dosing. On the other hand, important differences between field studies and more controlled experimental studies, like our eider study, are the presence in the field of various other pollutants and confounding factors such as parasites, which can cause additional effects that may be attributed to PCBs.

We did find ±45% difference in body weight gain between the animals with low and high PCB body burdens in the ClоA50-dosed groups. The mechanism of body weight loss and reduced body weight gain is not yet elucidated.

Thyroid hormones

In the unexposed eider ducklings, plasma T3 levels decreased by about 50% during the experimental period and T4 levels increased by 90%. This is in accordance with Decuypere and Kühn (1988), who described an increase in T4 concentrations with age and weight in chicks after hatching, up to 128 days. In those chicks, plasma T3 levels increased in the first 2 weeks after hatching and then declined with age.

In both ClоA50- and PCB-77-dosed groups there was a significant decrease in plasma T3 level at day 7 related to PCB body burdens, but not at day 10. Plasma T4 levels were only significantly negatively correlated with internal PCB-77 concentrations at day 10 of exposure. Under normal circumstances reductions in thyroid hormone levels will be compensated by additional secretion of thyroid hormone by the thyroid gland. We nevertheless found significant plasma T3 and T4 reductions at, respectively, 7 and 10 days after a single PCB-77 dose. A reduction of thyroid hormone levels in birds by PCBs has been demonstrated before in experiments with black-backed gull (*Larus fuscus*), pigeons (Jefferies and French, 1971) and Japanese quail (*Coturnix coturnix*; Grässle and Biessmann, 1982). In field studies a correlation between PCB body burdens and thyroid hormone levels has been demonstrated in common tern (Murk *et al.*, 1994b) and cormorant (Van den Berg *et al.*, 1994) chicks.

PCBs have been reported to influence thyroid hormone levels at two levels at least, firstly interference of hydroxylated metabolites with the T4 transportation system (Brouwer *et al.*, 1990, Lans *et al.*, 1993) and secondly by induction of T4-glucuronidation, thus enhancing hepatic elimination of thyroid hormone (Barter and Klaassen, 1992; Beetstra *et al.*, 1991; Visser *et al.*, 1993). Arguments that both mechanisms may actually be involved in eiders and other fish-eating birds have been obtained recently. From *in vitro* metabolism experiments (Murk *et al.*, 1994c) it was apparent that hepatic microsomes of eiders from the ClоA50 and the PCB-77 groups, are able of producing especially 5OH-metabolites of PCB-77. These metabolites are very potent in interfering

with the T4 transport system (Lans et *al.*, 1993). In addition a significant positive correlation between T4-glucuronidation and PCB body burdens was demonstrated in common tern (Murk *et al.*, 1994b).

Thyroid hormones are important regulators of physiological functions, such as the maintenance of body temperature (Falconer, 1984), energy metabolism, growth and differentiation of epithelia, and synchronization of reproduction and migration of birds with the seasons (Nicholls *et al.*, 1985, Kar and Chandola, 1985; Sharp and Klandorf, 1985; Dawson, 1989a,b). However, at present it is unknown whether thyroid hormone-related health effects may develop in fish eating birds upon exposure to environmental levels of PCBs.

Retinoids

One day after exposure a significant decrease of plasma retinol levels in the CloA50-50 $mg \cdot kg^{-1}$ group and in both PCB-77-dosage groups was observed to $\pm 80\%$ (Figure 2.4). The plasma retinol levels probably are influenced by two processes at least: an increase by enhanced mobilization from the liver and a decrease through interference of the hydroxylated metabolites with the plasma transport. In laboratory animals such as mice, a reduction in plasma retinol concentration can be observed within a day after a single dose of PCB-77, followed by a recovery, and sometimes overcompensation, in the following days (Murk *et al.*, 1991).

The hepatic retinyl palmitate levels were more than 4-fold less in the animals with great PCB-77 body burdens than in the animals with smaller PCB-77 concentrations (Figure 2.5b). At the same time (day 10 of the experiment) plasma retinol levels were significantly positively correlated with PCB-77-body burden (Figure 2.5b). These results were not observed for the CloA50-dosed animals.

In laboratory studies with rats dosed 40 $mg \cdot kg^{-1}$ 3,3',4,4',5,5'-hexabromobiphenyl (HBB). Spear *et al.* (1988) observed after 28 days, elevated serum retinol levels, increased liver weight, and decreased liver retinol and retinyl palmitate levels. In rats dosed 20 $mg \cdot kg^{-1}$ HBB only liver retinol and retinyl palmitate levels concentrations declined. They found *in vitro* elevated hydroxylation and conjugation by UDP-glucuronyl transferase of retinoic acid in liver microsomes that corresponded with increased activities of P4501A. Spear *et al.* (1985, 1990) observed reduced liver retinol and retinyl palmitate levels in natural populations of herring gulls (*Larus argentatus*) from contaminated colonies compared to relatively clean colonies. Also in our field study with common terns chicks we observed reduced yolksac retinyl palmitate levels and increased plasma retinol concentrations (Murk *et al.*, 1994b).

If the processes mentioned above would continue for a longer period of time,

depletion of the retinoid store is to be expected. This may have consequences for vitamin A-mediated physiological functions such as reproduction, differentiation of epithelia and good skin condition (Sporn and Roberts, 1983),and resistance against infections (Sijtsma *et al.*, 1989).

Behaviour

In our experiment we found a significant decrease in swimming and eating activity for the PCB-77-50 $mg.kg^{-1}$ dosed animals. This effect was not found for the CloA50-200 $mg{\cdot}kg^{-1}$ group. PCBs have been shown to influence bird behaviour, such as parental attentiveness (Kubiak *et al.*, 1989, McArthur *et al.*, 1983), courtship behaviour (Tori and Peterle, 1983) and avoidance behaviour (Kreitzer and Heinz, 1974). The mechanism through which behaviour is influenced is not yet clear. For eider ducklings it is very important to react on a distress call of the females by congregating into as compact a group as possible for protection against predators (Swennen, 1989). Under natural circumstances, less active reaction could decrease the chance of survival.

Conclusions

From this experiment we can conclude that eider ducks are vulnerable to the toxic action of PCBs. This effect is at least partially Ah-receptor related. Whether PCBs actually have adverse health effects on eiders in natural populations is not known. In The Netherlands, eider populations are not only exposed to various chemicals, but they are also seriously affected by mechanical destruction of mussel banks by fishing activity. This forces the eiders to eat more crabs, which contain the parasite *Polymorphus botulus*, leading to increased incidence of infection (Swennen, 1991).

Due to intrinsic individual differences between the natural eiders in our experiment, correlation of the measured parameters with the individual internal dose is essential in studying effects. This is even more important for field studies where exposure occurs via the diet and thus the individual food choice of the animals is an extra source of variation.

Acknowledgements

We would like to thank JSJ van de Sant, E v Arnhem, W Mullié, and members of the Department of Toxicology, Wageningen, for helping us out at crucial moments.

CHAPTER 3

EFFECTS OF POLYHALOGENATED AROMATIC HYDROCARBONS (PHAHS) ON BIOCHEMICAL PARAMETERS IN CHICKS OF THE COMMON TERN (*STERNA HIRUNDO*)*

ABSTRACT

Common tern (*Sterna hirundo*) eggs were collected from eight breeding colonies that differed in degree of polyhalogenated aromatic hydrocarbon (PHAH) pollution. The eggs were artificially incubated and the chicks sacrificed within 12 hr after hatching. Plasma thyroid hormones, hepatic T4-UDP-glucuronyltransferase (T4-UGT) activity, plasma retinol and yolksac retinoid levels were measured and compared with yolksac PHAH residues (PCBs, PCDFs and PCDDs) and hepatic EROD and PROD activities. No significant differences were observed between colony average levels of plasma thyroid hormones (TT4, FT4 and TT3) nor plasma retinol levels and T4-UGT activities that could be related to colony average PHAH levels. However, average colony yolksac retinyl ester levels did show significant differences. When correlated for individual terns, significant correlations between all these parameters and PHAH levels or EROD or PROD activities were found. The correlation between hepatic T4-UGT and EROD and PROD activities was very strong, suggesting a concomitant induction of these drug metabolizing enzymes. The PHAH levels were expressed as TEQs based on TEFs as proposed by Safe (1990, 1994). In addition to plasma retinol, another retinoid was found which resembles 3,4-didehydro-retinol (vitamin A2) in chromatografic and spectroscopic behaviour. This putative plasma vitamin A2 was significantly positive correlated with the yolksac TEQs and with the hepatic EROD activity. Esters of both retinol and putative vitamin A2 were detected in the yolksac. The average yolksac vitamin A2 levels of the cleanest Zeewolde colony were significantly greater than the average levels in the other colonies. It was not possible to correlate the yolksac retinoids with residue levels or TEQs for individual terns, as these had not been analyzed in the same yolksacs. Common tern chicks that required a longer period of incubation before hatching had significantly smaller concentrations of yolksac retinyl palmitate and greater PHAH levels. The ratio plasma retinol over yolksac retinyl palmitate was significantly increased.

**Based on: Murk AJ, Bosveld ATC, Van den Berg M and Brouwer A (1994): Effects of polyhalogenated aromatic hydrocarbons (PHAHs) on biochemical parameters in chicks of the common tern (Sterna hirundo). Aquat. Toxicol. 30: 91-115*

INTRODUCTION

Polyhalogenated aromatic hydrocarbons (PHAHs) such as polychlorinated biphenyls (PCBs), polychlorinated dibenzofurans (PCDFs) and polychlorinated dibenzo-*p*-dioxins (PCDDs) are ubiquitous environmental toxicants which accumulate especially in aquatic food chains. PHAHs can induce a wide spectrum of adverse health effects in laboratory and wildlife species (Safe, 1986a; Nebert, 1989; Safe, 1990; Brouwer, 1991). In fish-eating birds, exposure to PCBs has been associated with embryonic mortality, growth retardation, hepatomegaly, morphological abnormalities, impaired reproduction, alterations in vitamin A and thyroid hormone metabolism, and behaviourial changes (Gilbertson and Fox, 1977; Hoffman *et al.*, 1987; Gilbertson, 1989; Gilbertson *et al.*, 1991; Kubiak *et al.*, 1989; Murk *et al.*, 1994a). PCDDs, PCDFs and planar PCB congeners initiate their effects mainly through binding to the arylhydrocarbon (Ah) receptor followed by the induction of specific gene products. Most often studied are increased Cytochrome P450IA1 production and related enzyme activities, such as ethoxyresorufin-O-deethylase (EROD), and aryl hydrocarbon hydroxylase (AHH) activities. These gene products may catalyse PHAHs metabolism or disturb the physiology of the animal (Safe, 1986a; Brouwer, 1991). However, for some toxic endpoints such as neurotoxicity, tumour promotion, alterations in vitamin A and thyroid hormone metabolism, there seems to be no absolute requirement for a planar configuration of PHAHs, while also the metabolites of PHAHs have been shown to perform adverse effects (Brouwer, 1991).

The annual load of PCBs to the North Sea is calculated to be 11-17 metric tonnes (Klamer *et al.*, 1991). Inputs of PCBs into the North Sea are contributed by the Atlantic Ocean and atmospheric deposition (together 60-70% of the total input), the Straights of Dover, Baltic Sea, rivers, sewers, (harbour) sludge and industrial calamities. The toxic load that is released by the highly polluted rivers Rhine, Meuse and Scheldt is transported along the Dutch coast towards the Wadden Sea and the German Bight. This has resulted in median PCB-138 levels in the sediment of 117 $\mu g \cdot kg^{-1}$ organic carbon in the Wadden Sea and 75 $\mu g \cdot kg^{-1}$ organic carbon in the German Bight. Not surprisingly PCB levels in eggs of sea birds breeding at the German North Sea coast stayed relatively high or increased between 1981 and 1990 (Becker *et al.*, 1992). In the mesohaline zone of the Westerchelde, Stronkhorst (1993) measured 230 μg PCB-138$\cdot kg^{-1}$ suspended matter (ash-free dry weight) in 1987 till 1989. In common tern carcasses and common tern eggs, average levels of 5930 and 5310 μg PCB-153$\cdot kg^{-1}$ ash-free dry weight were found, respectively. Stronkhorst (1993) suggested that common terns in Saeftinghe (the eastern part of the estuary) could be at risk for reproductive impairment, since PCB-concentrations were found in the same range as reported for Green Bay (Lake Michigan, USA) where

effects such as reduced hatchability, reduced body weight, increased incubation time and congenital abnormalities have been observed by Kubiak *et al.* (1989).

To set quantitative and verifiable ecological objectives for the Dutch management of the North Sea and inland waters, a selection of 60 indicators has been made (Ten Brink *et al.*, 1991). These indicators are selected species, that should represent a cross section of the ecosystem. One of these target species is the common tern (*Sterna hirundo*), a fish eating bird that breeds especially in the Delta region in the south-west part of The Netherlands. Terns have been shown to be susceptible to pollutants such as organochlorine pesticides (Koeman, 1971) and PCBs (Kubiak *et al.*, 1989). Furthermore it is to be expected that terns build up a considerable PCB burden within a few weeks after their arrival from their wintering sites in West-Africa.

This study of the suitability of common terns as biomonitoring species is a follow-up of a comparative study with one-day-old cormorant (*Phalacrocorax carbo*) chicks from two Dutch colonies which differed in degree of pollution. In this cormorant study significant concentration-effect relationships were found for mono-*ortho*-PCBs versus EROD and PROD (pentoxyresorufin-O-deethylase) activity, head length, plasma thyroid hormone and hepatic retinyl palmitate levels (Van den Berg *et al.*, 1994). Despite their toxic responses to PHAH exposure, cormorants were judged not to be suitable as bioindicator species. Firstly because their nests are very hard to reach as in The Netherlands they breed high in trees. Secondly because large intercolony variations in residue concentrations and EROD and PROD activities were found, possibly due to the very large foraging areas.

Common terns are also specialized predators of the aquatic food-chain but their nests are much more accessible and they forage mostly within 10 km from their breeding place (Stienen and Brenninkmeier, 1992; Rossaert *et al.*, 1993). Terns may be sensitive for effects of PHAH contamination as well. Correlations have been shown between levels of non- and mono-*ortho*-PCBs and decreased hatchability, increased incubation period and decreased parental attentiveness in Forster's terns (*Sterna forsteri*) Green Bay, Lake Michigan (Kubiak *et al.*, 1989). Hoffman *et al.*, (1987) observed a positive correlation between hepatic AHH activity and weight loss and abnormal functioning of the thyroid gland in Forster's tern chicks. Becker (1991) found a reduced hatching success for common terns that coincided with increased PCB levels. This reduction was, however, not statistically significant.

At lesser levels of contamination more sensitive parameters than obvious adverse effects are needed to determine effects of chemicals such as PHAHs. It is known that PCBs, PCDDs and PCDFs may alter thyroid hormone and vitamin A metabolism in laboratory species such as rat, mouse (Spear *et al.*, 1988; Brouwer, 1991; Chen *et al.*,

1992) and birds such as herring gull (*Larus argentatus*), ring dove (*Streptopelia risoria*) and eider duck (*Somateria mollissima*) (Spear *et al.*, 1990; Murk *et al.*, 1994a). Both thyroid hormone and vitamin A play essential roles in embryonic development and physiological functions such as energy metabolism and reproduction (Falconer, 1984; Kar and Chandola, 1985; Dawson 1989a,b; Thompson, 1970). Therefore vitamin A and thyroid hormone may function as indicators for both exposure to PCBs and for the animals homeostasis.

In this experiment common tern eggs were collected from seven colonies in The Netherlands and one colony in Belgium, which differed in degree of pollution. The eggs were artificially incubated in the laboratory. Within 12 hr after hatching the common tern chicks were sacrificed. Several tissues were collected for further investigations. Growth parameters of the chicks, PCB/PCDD/PCDF concentrations in the yolksac and hepatic EROD and PROD activities were measured and are reported elsewhere (Bosveld *et al.*, 1994). This paper describes the possible adverse effects of *in ovo* PHAH exposure on vitamin A and thyroid hormone status in blood plasma and yolksac of one-day-old common tern chicks from different breeding colonies. In the same colonies an ecological survey was performed on the reproductive success of the common terns (Rossaert *et al.*, 1993). Correlations of the ecological results with physiological and chemical parameters are described elsewhere (Murk *et al.*, 1996a).

Materials and Methods

Animals and treatment

Fifteen common tern eggs (the second egg of nests of three eggs) were collected from seven breeding colonies in The Netherlands and one in Belgium during the period May and June 1991. The colonies were chosen to represent control and contaminated sites, and were situated at Zeewolde in Flevoland (Zeew); the isle of Griend in the Wadden Sea (Grnd); Slijkplaat in the Haringvliet (Slpl); the Prinsesseplaat in the Oosterschelde (Prpl); the Land van Saeftinghe in the Westerschelde (Saef); Terneuzen (Tern); the Westplaat at the North Sea coast near Oostvoorne (Wpl) and at the North Sea coast near Zeebrugge in Belgium (Zeeb) (Figure 3.1). The eggs were artificially incubated in a laboratory incubator at a temp of 37.5 °C with a relative humidity of 50-60%. Within 12 hr after hatching the young terns were weighed and sacrificed under ether anaesthesia. Livers and yolksacs were weighed immediately, snap frozen in liquid nitrogen and stored at -70°C. Blood was centrifuged and the plasma was stored at - 20°C.

PCDD, PCDF and PCB residues were analyzed in yolksacs by GC-MS and GC-

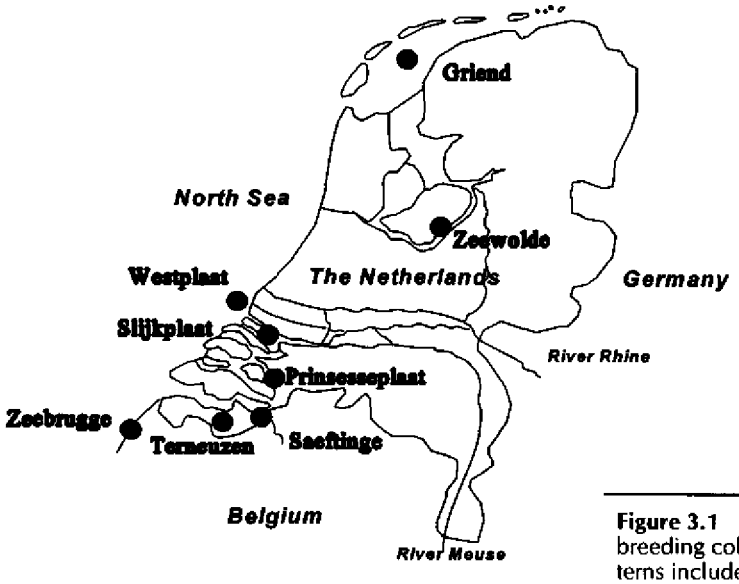


Figure 3.1 Locations of breeding colonies of common terns included in this study, in the Netherlands and Belgium.

ECD , respectively (Bosveld *et al.*, 1993). The individual PHAH levels were transformed into Toxic Equivalents (TEQs), using Toxic Equivalency Factors (TEFs) as proposed by Safe (1990, 1994). Di-*ortho*-PCBs were not used in the TEQ calculations.

Liver microsomes were prepared as described in Bosveld *et al.* (1994). Protein content was analyzed according to the method described by Bradford (1976). 7-ethoxyresorufin-O-deethylase (EROD) and 7-pentoxyresorufin-O-depenthylase (PROD) activities were analyzed fluorimetrically according to the method of Rutten *et al.* (1987).

Plasma thyroid hormone assays

Total thyroxin (TT4), total triiodothyronine (TT3) and free thyroxin (FT4) levels were determined in, respectively, 10, 25, and 25 μl aliquots of plasma, by chemiluminescence immunoassay, using commercially available kits (Amerlite assay kits, Amersham Internat. plc., Amersham, UK). Thyroid hormone levels were calculated from the luminescence data with the Securia computer program of Amersham.

Plasma and yolksac retinoid analysis

Because of the small amounts of plasma available, aliquots of 25 μl were diluted with 25 μl of demineralized water. They were subsequently spiked with retinyl acetate as an

internal standard and extracted with 50 μl methanol and 100 μl diisopropylether. The ether phase was filtered over a 0.45 μm Millipore filter and evaporated under nitrogen gas. The residue was dissolved in 50 μl methanol. Aliquots of 20 μl extract were analyzed on a reverse phase silica RP C18 column (Perkin Elmer, Pecosphere 3*3) with methanol/water (85:15) as eluent according to the method described by Morse and Brouwer (1995). To determine yolksac retinoids, the yolksacs were first weighed, homogenized in 1 ml of Tris-buffer pH 7.4 and extracted in aliquots of 50 μl as described for plasma retinoids. The dried residues were dissolved in methanol:ethyl acetate 1:1 instead of 3:1 because of the relatively large fat content compared to liver extracts. Sampling and running conditions were performed as described for hepatic retinoids by Morse and Brouwer (1995) except that the samples were not cooled during HPLC-analysis to prevent blockage of the column by clotting fat. All handlings were performed at 4°C in the absence of direct daylight. Identification of retinoids was based on co-chromatography with standard retinoids measured at 326 nm. Quantification was performed using standard curves of retinol or retinyl palmitate. HPLC retinoid data were integrated and calculated using Merck D-6000 HPLC manager software.

Further identification of some plasma and yolksac retinoids was performed by dr. A Barua, a member of the Vitamin A Research group of Prof. J.A. Olson. He used reversed phase HPLC (Column Waters Assoc. "Resolve" 5 μ x 3.9 mm x 30 cm) coupled to a Photo-Diode Array detector (Waters, model 991), and as solvent acetonitrile/dichloroethane/methanol (85:10:5) with 0.05% ammonium acetate. The flow rate was set at 1.5 ml·min^{-1}.

Thyroxin (T4) UDP-glucuronyltransferase assay

Thyroxin UDP-glucuronyltransferase (T4-UGT) activity was measured basically as described by Beetstra *et al.* (1991). Hepatic microsomes were incubated with ^{125}I-T4 and the co-factor Uridine Diphosphate Glucuronaat (UDPGA). Because of the small amounts of microsomes available, the microsomal protein in the reaction mixture was diluted to 1 mg·ml^{-1} instead of 2 mg·ml^{-1} with buffer containing 10 mM MgCl2. The reaction mixture consisted of 50 μl substrate solution (100.000 cpm ^{125}I-T4, 4 μM T4), 50 μl cofactor solution (20 mM UDPGA and 30 mM $MgCl_2$) and 100 μl microsomal preparation (containing 1 mg protein·ml^{-1}). The mixtures were incubated in duplicate at 37°C. Blanks were incubated in the absence of UDPGA. The reaction was stopped after 120 min by adding 200 μl ice cold methanol. After 10 min on ice, the samples were centrifuged for 10 min at 3000 rpm. 200 μl of the supernatant was brought to 1 ml with 0.1 M HCl, and ^{125}I-T4 and ^{125}I-T4-glucuronate (^{125}I-T4-G) were separated by Sephadex LH-20 columns chromatography. Finally the separated fractions were analyzed for radioactivity in a

gamma counter. T4-UGT Production is expressed as nmol T4-UGT formed per min per gram microsomal protein.

Statistical methods

Differences between colony averages were tested with ANOVA. Testing of dose effect relationships was done by unweighted least squares linear regression analysis and *t*-test. The acceptance level was $P<0.05$.

RESULTS

The differences in yolksac PHAH levels, either expressed as ng TEQs·g^{-1} lipid or on a mass basis (μg mono-*ortho*-PCB·g^{-1} lipid) do not differ very much between the different breeding colonies (Table 3.1, Figure 3.9 for mono-*ortho*-PCBs). The pattern shown for the mono-*ortho*-PCBs is the same for the yolksac TEQ levels. The average yolksac PHAH level is significantly less than the other colonies in the Zeewolde colony, and significantly greater in the Slijkplaat colony.

Plasma thyroid hormones

The greatest total thyroxine (TT4) levels were found in the Zeebrugge colony (6.85 ± 3.1 nmol·l^{-1}). The least levels were found in the Terneuzen colony (5.34 ± 1.92 nmol·l^{-1}). No significant differences were observed in average plasma TT4 concentration between the different colonies when expressed as colony means (Table 3.2). Greatest mean plasma free thyroxine (FT4) levels were found again in Zeebrugge (6.21 ± 3.42 pmol·l^{-1}) and least in the Terneuzen colony (4.00 ± 1.05 pmol·l^{-1}). Some average FT4 levels differed significantly between colonies (Table 3.2). However, no trend was found which could be related to colony differences in mean yolksac PHAH-concentrations. Plasma triiodothyronine (TT3) levels were on average again greatest in the Zeebrugge colony (3.93 ± 1.75 nmol·l^{-1}). The Terneuzen and Prinsesseplaat colonies had the least TT3 levels (2.52 ± 1.15 and 2.43 ± 0.53 nmol·l^{-1}, respectively). Although differences existed in average TT3 levels between some colonies (Table 3.2), these differences could not clearly be related to differences in mean yolksac PHAH levels (Table 3.1). Some animals showed extremely high levels of TT4, TT3 as well as FT4. When those levels exceeded the colony means plus twice the standard deviation they were not used in the calculations.

The thyroid hormone parameters did not correlate with yolksac PHAH levels (either expressed as mass or TEQs). However, plasma TT4 levels were significantly correlated with EROD activity: ($\log [TT4] = 0.40 + 0.17 \cdot \log$ (EROD activity); $r = 0.31$, $P<0.05$) and plasma FT4 levels were significantly correlated with PROD activity

Table 3.1 Yolksac PHAH levels (average ± std) expressed as TEQs (without di-ortho PCBs) or as mono-ortho PCB levels.

Parameters	Colonies							
	Zeewolde	Princesseplaat	Griend	Zeebrugge	Saeftinge	Terneuzen	Westplaat	Slijkplaat
TEQs(-DO) ng/g lipid	3.72 ± 1.93[a]	7.93 ± 3.93[ab]	11.42 ± 5.96[ab]	10.36 ± 5.27[ab]	11.47 ± 4.41[ab]	11.39 ± 4.13[ab]	11.79 ± 5.29[ab]	17.82 ± 4.90[b]
mo-PCBs µg/g lipid	6.22 ± 3.30[a]	16.40 ± 5.15[ab]	17.9 ± 10.76[ab]	21.14 ± 14.4[ab]	24.04 ± 9.71[ab]	23.94 ± 10.0[ab]	28.07 ± 6.02[b]	39.66 ± 11.2[b]

[a] Significantly lower than Slijkplaat ($P<0.05$).

[b] Significantly higher than Zeewolde ($P<0.05$).

Table 3.2 Plasma thyroid hormone levels (average ± std) and hepatic T4-UGT activity in common tern chicks from different breeding colonies.

Parameters	Colonies							
	Zeewolde	Princesseplaat	Griend	Zeebrugge	Saeftinge	Terneuzen	Westplaat	Slijkplaat
T4 (nmol/l)	5.90 ± 1.37	6.75 ± 2.29	6.46 ± 2.17	6.85 ± 3.10	6.54 ± 1.52	5.34 ± 1.92	6.50 ± 1.01	5.92 ± 1.57
T3 (nmol/l)	3.47 ± 1.90	2.43 ± 0.52[a]	3.09 ± 1.39	3.93 ± 1.75	2.56 ± 0.99	2.51 ± 1.15	3.31 ± 1.52	3.10 ± 1.32
FT4 (pmol/l)	5.39 ± 1.63	4.48 ± 1.83	4.06 ± 1.64[b]	6.21 ± 3.42	5.18 ± 2.15	4.00 ± 1.05[b]	5.13 ± 1.78	4.43 ± 1.78[b]
T4-UGT act. pmol/min.mg	20.4 ± 5.6	22.4 ± 6.6	22.8 ± 6.2	22.9 ± 5.5	24.3 ± 5.7	23.2 ± 6.3	21.8 ± 3.9	25.4 ± 5.2

[a] Significantly lower than Zeewolde andZeebrugge ($P< 0.05$).

[b] Significantly lower than Zeewolde ($P< 0.05$).

(log [FT4] = 0.45 + 0.32·log (PROD-activity); $r = 0.42$, $P < 0.005$, Figure 3.2).
No correlation was observed with EROD activity.

Thyroxine UDP-glucuronyltransferase activity

Although the available amount of hepatic microsomes was very small, our measurements of thyroxine UDP-glucuronyltransferase (T4-UGT) activity proved to be very reproducible. There were no significant differences in average T4-UGT activity amongst breeding colonies. Greatest T4-UGT activities were found in the Slijkplaat colony (30.4 ± 6.3 pmol T4-G·min^{-1}·mg^{-1} microsomal protein) and least in the Zeewolde colony (24.5 ± 6.7 pmol T4-G·min^{-1}·mg^{-1} microsomal protein) (Table 3.2). When calculated on the basis of individual terns, T4-UGT activity was positively correlated with the hepatic EROD (log (T4-UGT-activity) = 9.3 + 1.9·log (EROD-activity); $r = 0.53$, $P < 0.001$, Figure 3.3) and PROD activities (log (T4-UGT-activity) = 3.3 + 1.4·log (PROD-activity); $r = 0.43$, $P < 0.001$).

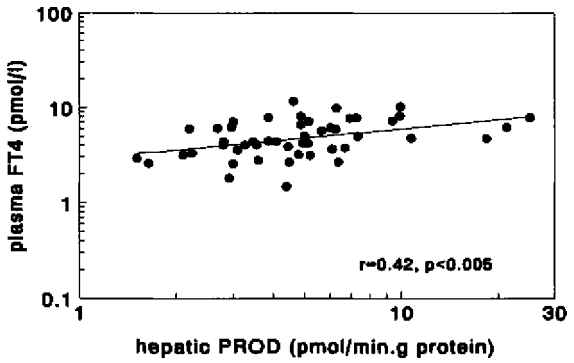


Figure 3.2 Plasma free thyroxin (FT4) levels in one-day-old common tern chicks plotted against hepatic PROD-activity in the same animal.

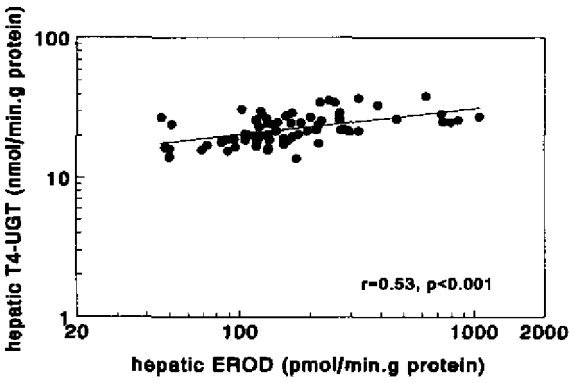


Figure 3.3 Hepatic T4-UGT activity in one-day-old common tern chicks plotted against hepatic EROD-activity in the same animal.

Table 3.3 Plasma and yolksac retinoid levels (average ± std) in common tern chicks from different breeding colonies.

Parameters (ng/ml)	Colonies							
	Zeewolde	Princesseplaat	Griend	Zeebrugge	Saeftinge	Terneuzen	Westplaat	Slijkplaat
Plasma retinol	107.5±49.3	90.3±38.7	61.8±18.6[ab]	57.6±19.6[ab]	72.0±20.6[ab]	85.1±19.9[ab]	96.8±27.8[a]	130.9±47.8
Plasma vit. A2	371.8±118.5	360.3±101.3	380.0±123.3	342.9±58.0	366.6±78.3	390.2±85.9	291.9±67.6	290.9±82.1
Ys ret.palmitate	1.18±1.02	0.82±0.52	1.08±0.17	0.34±0.23[a]	0.44±0.35[a]	0.40±0.15[a]	0.79±0.81	0.93±0.47
Ys. vit. A2 ester	13.24±6.29	7.34±2.53[a]	7.92±2.39[a]	6.23±1.84[a]	5.67±0.92[a]	4.76±1.07[a]	6.18±1.10[a]	7.40±2.16[a]

[a] $P < 0.05$ (Zeewolde)
[b] $P < 0.05$ (Slijkplaat)
Abbreviations: Ys = yolksac; ret. = retinyl; vit. = vitamin.

Table 3.4 Several biochemical parameters (average ± std) in chicks that hatched after 21, 22, 23 or more than 23 days.

Incubation (days)	TT3 (nmol/l)	plasma retinol (A) (ng/ml)	plasma vit.A2 (ng/ml)	Yolksac ret. palmitate (ng/ml)	Yolksac vit. A2 ester (ng/ml)	Ratio Pl. retinol/ Ys ret. palmitate (×100)	Yolksac weight (g)	Yolksac weight relative to body weight (%)
21	3.34±1.52	98.8±45.0	346.1±112.0	0.89±0.32 [a]	10.21±1.55	83.6±29.5	0.52±0.21	3.84±1.47
22	3.15±1.56	81.9±35.4	352.5±91.1	0.81±0.55[a]	6.89±2.86	145.8±87.8	0.61±0.24	4.28±1.53
23	2.84±1.32	93.5±38.5	357.5±91.4	0.50±0.27	6.14±0.82	173.4±96.8[b]	0.57±0.22	4.03±1.57
>23	2.73±1.06	79.1±24.4	361.4±90.5	0.43±0.14	6.05±2.50	198.4±64.0[b]	–	–

[a] Significantly different from '>23 days' ($P<0.05$)
[b] Significantly different from '21 days' ($P<0.05$)
– No average value available (only one measurement)
Abbreviations: Ys = yolksac; ret. = retinyl; vit. = vitamin; Pl = plasma.

No correlation was observed between T4-UGT activity and PHAH levels, either expressed as mass or TEQs.

Plasma retinol and yolksac retinyl palmitate

The average plasma retinol levels per colony are presented in Table 3.3 and Figure 3.4. In this and the other bar-graphs the colonies are ordered depending on the mean TEQ value found in the yolksacs of the tern chicks from that colony: Zeewolde with the least TEQ at the left, Slijkplaat with the greatest TEQ value at the right. Mean plasma retinol levels were greatest in the common tern chicks from the Slijkplaat colony (130.89 ± 49.30 $ng \cdot ml^{-1}$) and least in chicks from the Zeebrugge (57.55 ± 19.60 $ng \cdot ml^{-1}$) and Griend colony (61.82 ± 18.64) (Table 3.3). Apart from Zeewolde and Princesseplaat a trend can be observed showing increasing plasma retinol levels coinciding with increasing yolksac PHAH concentrations (Figure 3.4). No correlation was found with PHAHs, either expressed as TEQs or as a unity of mass. A significant positive correlation was found between plasma retinol levels and hepatic EROD: (log [plRE] = 1.41 + 0.22·log (EROD-activity); r = 0.36, $P < 0.01$) and PROD acyivities: (log [plRE] = 1.74 + 0.22·log (PROD-activity); r = 0.42, $P < 0.01$).

Average yolksac retinyl palmitate levels from the colonies Saeftinge (0.44 ± 0.35 $ng \cdot ml^{-1}$), Terneuzen (0.40 ± 0.15 $ng \cdot ml^{-1}$) and Zeebrugge (0.34 ± 0.23 $ng \cdot ml^{-1}$) were significantly less compared to the other colonies (58-65% less, Table 3.3). Quantification of the yolksac retinol levels was not possible because the levels were very small, barely exceeding the noise (about 0.04 $ng \cdot ml^{-1}$ yolksac).

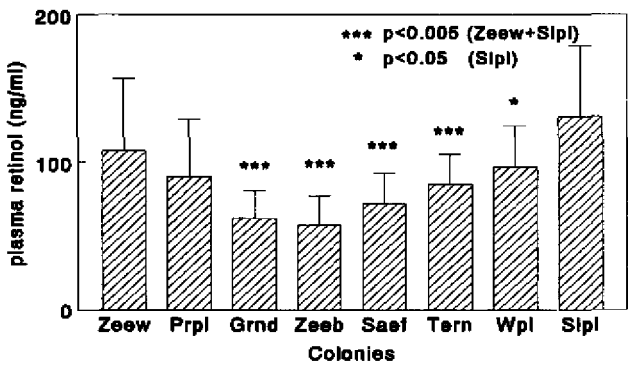


Figure 3.4 Average plasma retinol levels in one-day-old common tern chicks from different breeding colonies (n = 10).

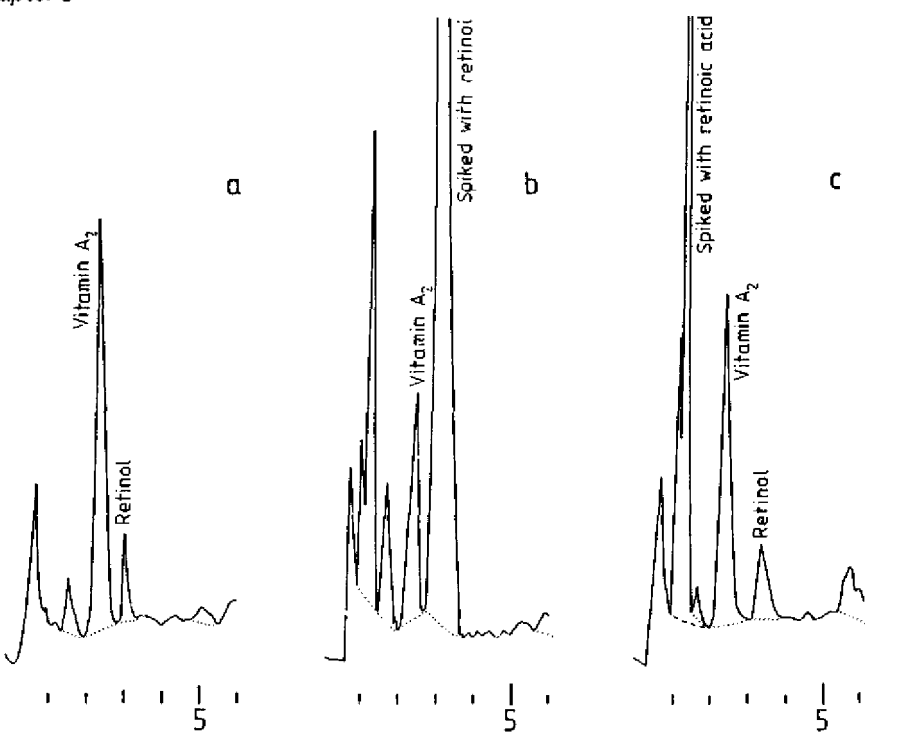


Figure 3.5 HPLC chromatograms (measured at 326 nm) of (a) common tern plasma; (b) common tern plasma spiked with retinol; and (c) common tern plasma spiked with retinoic acid. Marked are retinol (vitamin A) and the putative vitamin A2.

Putative plasma 3,4-didehydroretinol (vitamin A2) and its vitamin A2-ester in yolksac

HPLC chromatograms of plasma extracts from common tern chicks showed a large peak (Rt 2.37 min) eluting just before the retinol peak (Rt 3.06 min) (Figure 3.5a). A similar peak was found in plasma from adult common terns (data not shown). Spiking the plasma demonstrated that the peak represented neither retinol (Figure 3.5b) nor retinoic acid (Figure 3.5c). Given its chromatographic behaviour and UV-absorption at 326 nm, this peak is thought to be a retinoid in nature. Further analysis of the unknown peaks was by Dr Barua (Department of Biochemistry and Biophysics, Iowa State University, USA), using a diode array spectrometer. It was found that the unknown plasma peak had an absorption maximum at 345 nm and a subsidiary peak at 275nm (Figure 3.6). This is compatible with the UV spectrum and chromatographic location of 3,4-didehydro-retinol (vitamin A2). In addition some α-Tocopherol, traces of ß-carotene and a large peak due to ß-cryptoxanthin were found as well. In the yolksacs two peaks were identified (Rt 16.6 and 17.97 min) which both showed absorbance at 345 nm. These peaks are thought to

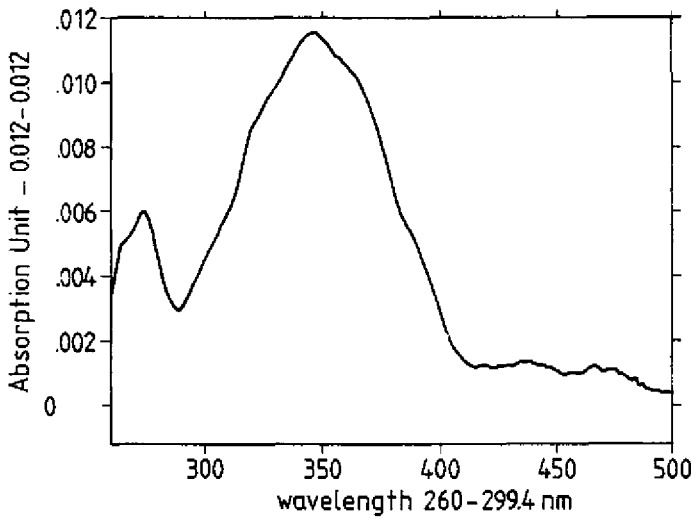


Figure 3.6 Absorbtion spectrum of the putative plasma 3,4-didehydroretinol (vitamin A2).

represent esters of vitamin A2, based on UV absorption spectrum, localization on the chromatogram and shifts to the putative vitamin A2 following hydrolysis (Figures 3.7a and b).

Both plasma and yolksacs of the common tern chicks were bright yellow due to carotenoids. The carotenoid profiles of plasma and yolksac indicated the presence of α- and ß-carotenoids. Quantification of the putative vitamin A2 and vitamin A2 ester was performed by using the standards for retinol and retinyl palmitate, respectively. This gives information on apparent vitamin A2 levels that can be used for comparison on a relative basis.

Putative plasma vitamin A2 levels were on average 4-fold greater than those of plasma retinol in the same animals (Table 3.3). The mean level of plasma vitamin A2 in common tern chicks from the Zeewolde colony (371.8 ± 118.5) was 130% of that of the most polluted colonies Westplaat and Slijkplaat (291.9 ± 67.61 and 291.0 ± 82.1 $ng \cdot ml^{-1}$, respectively) (Table 3.3, Figure 3.8). This difference was, however, not statistically significant. Plasma vitamin A2 levels significantly decreased with increasing hepatic EROD activity and TEQ, respectively: log [vit. A2] = 386-0.14·log(EROD-activity); r = 0.26, $P < 0.05$ and log [vit.A2] = 396-0.10·log(TEQ); r = 0.25, $P < 0.05$.

Assuming that the vitamin A2 ester is a retinoid with a specific optical density similar to retinyl palmitate, the estimated levels were on average 11.8-fold greater than those of retinyl palmitate. The average yolksac levels of the putative vitamin A2-ester of five tern chicks, and the average mono-*ortho*-PCB levels in five other yolksacs from the

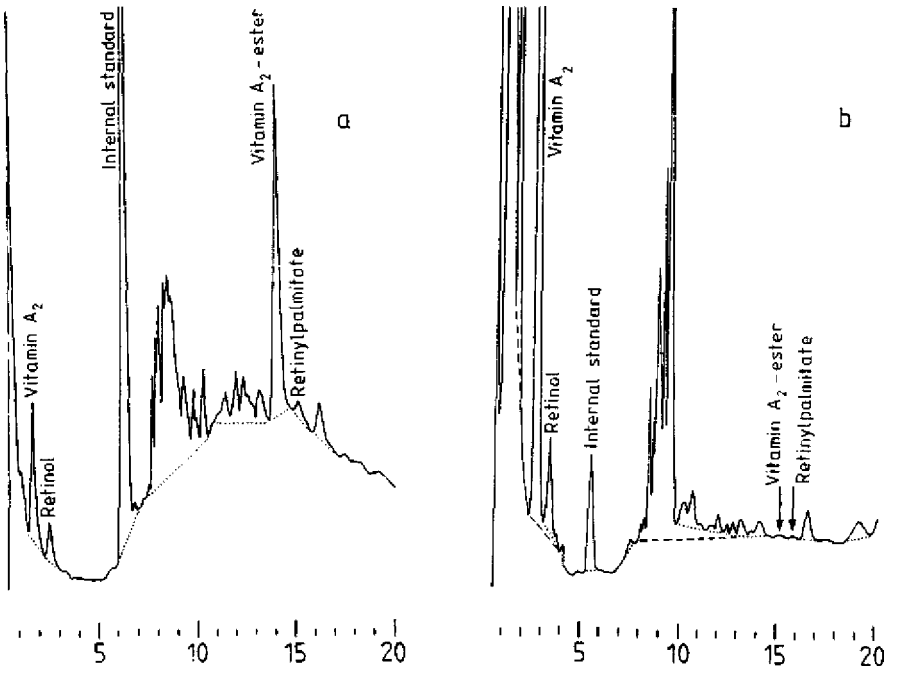


Figure 3.7 HPLC chromatograms (measured at 326 nm) of common tern yolksac retinoids before (a) and after (b) hydrolysis.

same colony are shown in Figure 3.9. The standard deviations can be found in, respectively, Table 3.3 and Table 3.1. The greatest average colony levels of the yolksac vitamin A2-ester were found in terns from Zeewolde (13.24 ± 6.29 ng·ml^{-1} yolksac). This was significantly greater than the average yolksac levels in all other colonies (Table 3.3, Figure 3.9). The Terneuzen colony showed the least yolksac levels of the vitamin A2-ester (4.76 ± 1.07 ng·ml^{-1}). It was not possible to correlate the yolksac retinoids with residue levels or TEQs for individual terns, as these had not been analyzed in the same yolksacs.

Incubation period

The average incubation period of the eggs artificially bred in the laboratory was 22.2 ± 1.5 days. The measured plasma thyroid hormone and plasma and yolksac retinoid levels were grouped after the incubation period (21, 22, 23 or more than 23 days) of the chicks (Table 3.4). The average yolksac retinyl palmitate levels of chicks that hatched after 21 and 22 days was significantly greater than the average level of chicks that hatched after more than 23 days (Figure 3.10a). The average levels of the putative yolksac vitamin A2 ester

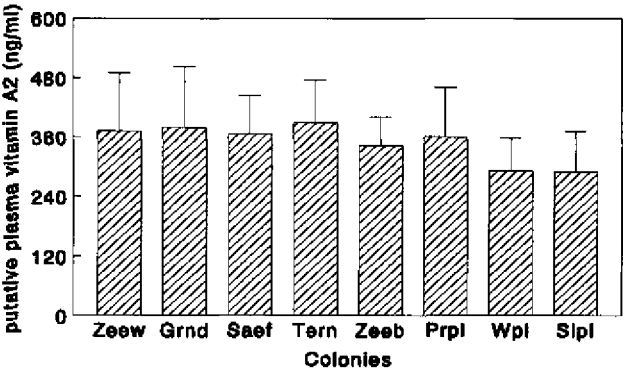


Figure 3.8 Levels of the putative plasma vitamin A2 in one-day-old common tern chicks, quantified using retinol standards.

was also less in terns that hatched after a longer incubation period. This trend was, however, not significant. The ratio plasma retinol and yolksac retinyl palmitate levels was significantly greater in the incubation groups that hatched after 23 days or more compared to the 21-days group (Figure 3.10b), although plasma retinol levels did not differ between the groups (Table 3.4). The average putative plasma vitamin A2 levels were greater with increasing incubation period, but this trend was not statistically significant. The absolute yolksac weights and the relative yolksac weight compared to total body weight did not differ between the incubation groups (Table 3.4). Plasma TT3 levels (the active form thyroid hormone) in the chicks that hatched after more than 23 days of incubation were 82% of the levels in chicks that hatched after 21 days (Figure 3.10c). These levels were, however, not significantly different. No differences were observed for average plasma TT4 and FT4 levels that could be related to yolksac PHAH-levels (data not shown).

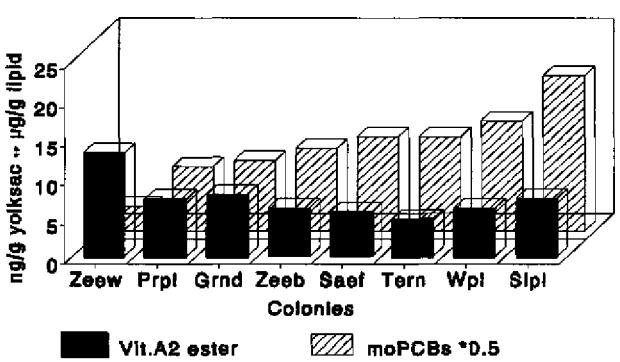


Figure 3.9 Levels of the putative vitamin A2-ester (quantified using retinyl palmitate standards) in 5 yolksacs per colony and of mono-ortho PCBs in 5 other yolksacs of the same colony. Standard deviations can be found in Tables 3.3 and 3.1, respectively.

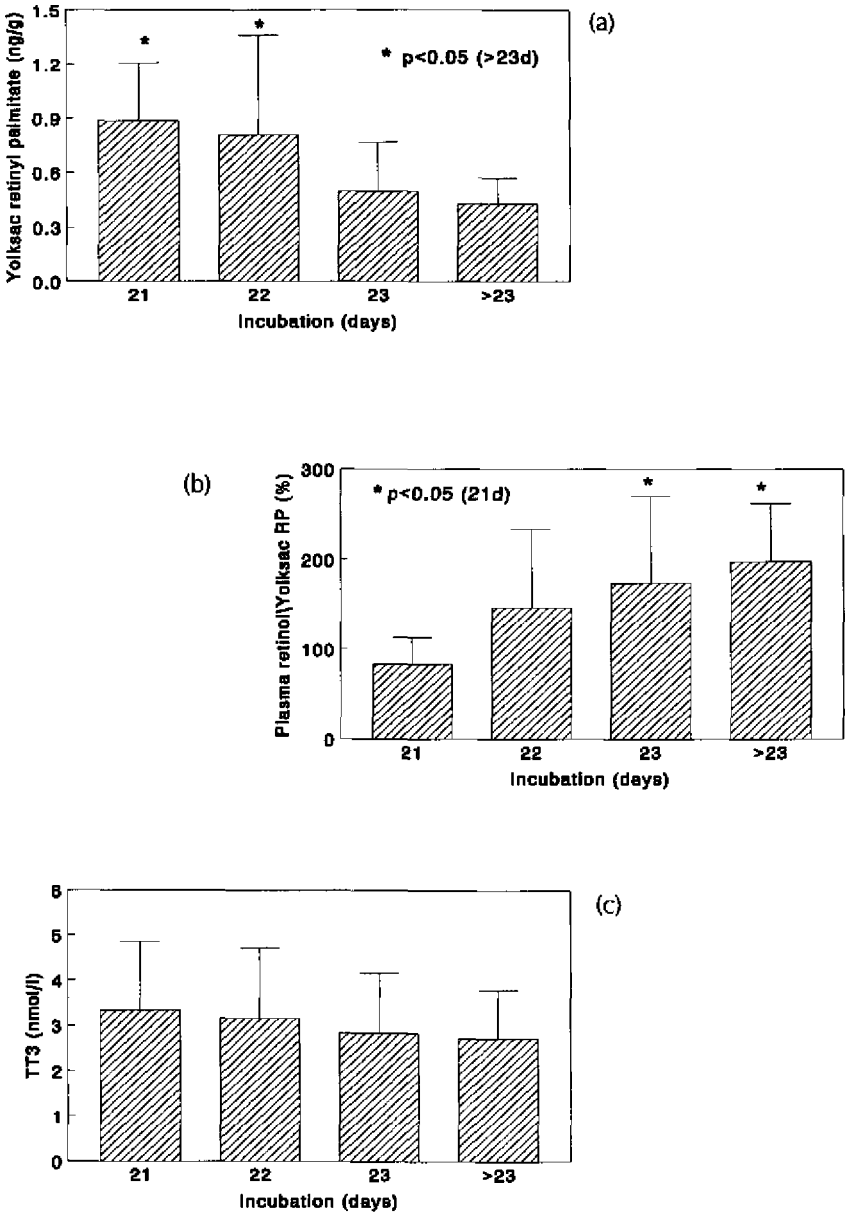


Figure 3.10 Biochemical parameters in chicks that hatched after 21, 22, 23 or more than 23 days of incubation: (a) yolksac retinyl palmitate; (b) ratio plasma retinol over yolksac retinyl palmitate level; (c) plasma TT3 levels.

Discussion

General

The aim of this investigation was to assess the suitability of common terns as indicators for exposure to, and effects of, PHAHs. More specifically this paper describes the possible adverse effects of *in ovo* PHAH exposure on vitamin A and thyroid hormone status in blood plasma and yolksac of one-day-old common tern chicks. Positive correlations were observed between hepatic cytochrome P450 enzyme activity measured as EROD or PROD activity and thyroid hormone parameters TT4 and FT4, T4-UGT activity and plasma retinol and vitamin A2 levels.

The association of thyroid hormone and vitamin A parameters with the level of activity of EROD and PROD suggests that these alterations may be a consequence of exposure to PHAHs. A correlation with yolksac PHAH residues, either expressed as TEQs or as mass, however, was not observed. Until now the reason for this apparent discrepancy is unknown. One argument may be that the levels of PHAHs in the yolksac reflects the body burden of the female parent but does not necessarily reflect the amount absorbed by the one-day old young. The level of hepatic EROD activity on the other hand does reflect the induction status of the cytochrome P450 system within the neonatal terns and thus may better represent internal PHAH levels in the common tern chicks.

Another possible explanation for the existence of correlations with EROD or PROD activity but not with PHAH levels is that also residues that were not measured with chemical analyses may induce EROD or PROD activities and biochemical alterations. Jones and co-workers (1992) observed that the TEQ calculated from PCB, PCDD and PCDF concentrations underestimated the TEQs in an extract of the same avian tissues measured with a H4IIE bioassay by an average of 57%.

Power analysis

Significant relationships were observed only for data that were correlated on the basis of individual terns. Apart from yolksac retinoid ester, no significant effects that could be related tot PHAH-levels were found when group average values were compared between the various breeding colonies. Power analysis demonstrated that due to the large variation in the measured parameters only differences from the controls of at least 35-80% could have been observed (Table 3.5). This calculation was based on a one or two tailed test (depending on the parameter involved) with eight common terns per colony and a probability value of 0.05. Table 3.5 also gives the number of common terns that would have been needed to observe an effect of 25%. The large variation within colonies is probably partially due to the variations in genetic background and the food choice that

Table 3.5 Standard deviations of some of the measured parameters as a percentage of the mean value; the consequences for the minimum statistically significant effect that can be observed using 8 animals per colony; and the minimum number of animals needed to show a significant effect of 25%.

Parameter	Standard deviation (%)	One or two tailed t-test	Min. observable effect (%)	No. animals for 25% effect
Plasma TT4	32	2	52	34
Plasma FT4	39	2	63	51
Plasma TT3	38	2	62	49
T4-UGT-activity	24	1	35	16
Plasma retinol	35	2	57	41
Ys. vit. A2	31	1	45	26
EROD-activity	55	1	80	83

differed between colonies (Murk et al, 1996a). However, even in a semi-field experiment with eider ducks (*Somateria millissima*), fed the same diet and receiving relatively high doses of PCBs (50 or 200 mg Clophen A50·kg^{-1} body weight or 5 or 50 mg PCB-77·kg^{-1} body weight) comparably large variations were observed. Also in such a relatively controlled experiment effects could only be demonstrated when related to internal PCB-levels on the basis of individual birds (Murk *et al.*, 1994a). This suggests that when performing a field study with a natural (bird) species it is important that individual internal doses are measured and/or that the number of animals needed for a chosen minimal observable effect must be calculated in advance.

Thyroid hormones and T4-UGT activity

No significant correlations between plasma TT4, FT4 and TT3 levels and yolksac PHAH levels (either expressed as unity of mass or as TEQ values) were observed. In the Dutch cormorant study (Van den Berg *et al.*, 1994) plasma TT4 and TT3 levels were significantly less in one-day-old chicks from the heavy polluted Biesbosch than from the less polluted Oude Venen breeding colonies. However, the yolksac mono-*ortho*-PCB levels in the cormorant chicks from the Biesbosch colony were on average 4.6-fold greater than in the common tern chicks from the relatively high polluted Slijkplaat colony (184 and 40 μg·g^{-1} lipid, respectively). Mono-*ortho*-PCB levels in cormorants of Oude Venen were on average 9.5-fold greater than the common terns from the relatively clean Zeewolde colony (58 and 6 μg mo-PCB·g^{-1} lipid, respectively). Possibly the PHAH contamination levels of the common terns were not high enough to cause a measurable decrease in plasma TT4/FT4 levels, as thyroid hormones are under strict homeostatic control. In the common tern study only significant positive correlations were found between the plasma TT4 and FT4 levels and hepatic EROD and PROD activity, respectively. Since the EROD

activity represents a PHAH inducible form of cytochrome P450IA1, this correlation suggests a positive association between PHAH contamination and plasma TT4 and FT4.

Although increases in TT4 with PCB-dosage have been reported before for Japanese quail (*Coturnix coturnix japonica*) treated with 200 ppm Aroclor 1254 for 120 days, plasma thyroid hormone levels usually decrease in *in vivo* studies after dosing PCBs. It has been demonstrated that this decrease is at least partly due to competition by PCB-metabolites for the binding site on transthyretine and increased metabolism of thyroid hormone (Brouwer, 1991; Lans *et al.*, 1993; Barter and Klaassen, 1991). Hepatic microsomes of common tern chicks from this experiment, however, hardly produced any PCB-metabolites of 3,4,3',4'-tetrachlorobiphenyl in an *in vitro* experiment, compared to eider duck microsomes (Murk *et al.*, 1994c). This suggests that little competition of PCB-metabolites for the T4 binding site is to be expected at this relatively low level of exposure.

An increase in TT4 and FT4 levels could be the result of decreased 5'-deiodinase activity which converts T4 into T3, the active form of thyroid hormone. It has been demonstrated by Eltom and co-workers (1992) that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin inhibited 5'-deiodinase activity in rats. However, as the 5'-deiodinase activity was not measured in this study, the reason for the apparent positive correlations of TT4 and FT4 with EROD and PROD remains unclear. It can not be excluded that the correlation between EROD and PROD activities and TT4 and FT4 levels reflects an in tandem process of development of inducibility of hepatic metabolizing enzymes (EROD and T4-UGT) and a growth related increase in plasma TT4 and FT4 levels (Harris and Bradshaw, 1984; Darras, 1992).

The T4-UGT activity was not significantly correlated with PHAH levels (expressed as mass or TEQ) but, similar to TT4 and FT4, there was a highly significant correlation with EROD and PROD activities in the same individuals. An increase in T4-UGT activity with increasing PCB levels has been described earlier for laboratory rats (Beetstra *et al.*, 1991; Visser *et al.*, 1993). Morse *et al.* (1993) observed an increase in hepatic T4-UGT activity of 100% in rat fetuses and 40% in neonates after prenatal 3,3',4,4',5,5'-hexachlorobiphenyl and/or 3,3',4,4'-tetrachlorobiphenyl administration. In the fetal hepatic microsomes the EROD activity increased with the dose as well. These data suggest a closely linked relationship between hepatic P450IA1 and T4-UGT activity in terms of Ah-receptor-mediated induction.

Plasma and yolksac retinoids

There was a significantly positive correlation of plasma retinol with hepatic EROD and PROD levels, but not with yolksac PHAH levels. This discrepancy may be due to the fact

that the levels of hepatic EROD/PROD activity may better reflect the internal body burden of PHAHs in the chicks than the levels of PHAHs in the yolksac. The positive correlation of plasma retinol levels are in accordance with the correlation observed in a semi-field experiment with eider ducks (Murk *et al.*, 1994a). Spear and co-workers also found a cytochrome P450 related increase in plasma retinol levels in 3,3',4,4',5,5'-hexabromobiphenyl·kg^{-1} dosed rats (Spear *et al.*, 1988), and in natural populations of herring gulls (*Larus argentatus*) from contaminated colonies compared to relatively clean colonies (Spear *et al.*, 1985, 1990). The increased plasma retinol levels were accompanied by reduced hepatic retinyl palmitate and retinol levels.

Plasma retinol levels can be altered by PCBs and related compounds through at least two pathways. Firstly hydroxy metabolites of PCBs can induce a conformational change in the T4-transport protein transthyretine (TTR) which prevents retinol binding protein (RBP) with retinol from complex formation with TTR (Brouwer *et al.*, 1988; Brouwer 1991). As a result retinol is lost from the circulation by glomerular filtration. However, hepatic microsomes of common terns from this experiment only had a limited capability to produce hydroxy PCB metabolites in an *in vitro* metabolism study (Murk *et al.*, 1994c). Therefore it is unlikely that hydroxy-PCBs may have reduced plasma retinol levels in the common tern chicks. A second mechanism involves interference of PHAHs in the process that regulates storage and mobilization of retinoids in the liver, resulting in an increased mobilisation of retinol to the circulation, decreased retinoid-ester storage, and often increased renal retinoid levels (Brouwer, 1988; Chen *et al.*, 1992). This suggestion is supported by the finding that the levels of yolksac retinoids were greatest in the chicks from Zeewolde (Table 3.3), the cleanest colony (Table 3.1). In the cormorant study hepatic retinyl palmitate levels in chicks from the most polluted Biesbosch area were on average 55% reduced if compared to the Oude Venen area. Due to large interindividual variation this difference was not statistically significant.

No significant correlations were found between yolksac retinoids and hepatic EROD and PROD activity. But again we have to make a distinction between the yolksac, which is a reflection of degree of pollution and availability of vitamin A in the diet of the female tern, and the liver, which is a reflection of the body burden and vitamin A storage of the chick. Therefore it is suggested for future analysis, to compare hepatic retinoid levels with parameters from the chick (EROD, plasma retinol) and to compare yolksac retinoid levels when possible with residue levels in the same yolksac.

In the common tern chicks the putative plasma vitamin A2 (3,4-didehydro-retinol) and yolksac vitamin A2-ester levels were identified. These levels were, respectively, 4- and 12-fold greater than plasma retinol and yolksac retinyl palmitate levels in the same animals, assuming that vitamin A2 and its ester were measurable with a specific optical

density similar to retinyl palmitate. In rats, vitamin A2 is a retinoid with a functional activity of about 40% of that of retinol (Shantz and Brinkman, 1950, ref. in Frolik, 1984). During embryogenesis, however, vitamin A2 plays an important role in tissue positional specification and development (Pijnappel *et al.*, 1993). A significant negative correlation was found between plasma vitamin A2 levels and hepatic EROD activity or TEQ levels. In addition the average yolksac levels of the putative vitamin A2-ester of five tern chicks, show a pattern that is inversely related to that of PHAH levels in the five other yolksacs from the same colony. To our knowledge, vitamin A2 and its ester have not been reported before in laboratory mammals and chicken. However, in flounder (*Platichthys flesus*), rainbow trout (*Salmo gardenerii*) and cormorant we found the putative vitamin A2 in the plasma as well (unpublished results). In the cormorant plasma vitamin A2 levels were less than retinol levels. Perhaps the unknown retinoids are associated with fish and fish eaters as a consequence of specific food sources.

Incubation period

After the data were grouped depending on the incubation period the eggs needed in the incubator untill hatching, significantly greater yolksac retinyl palmitate levels were observed in chicks that needed 21 and 22 days compared to terns needing 23 days or more. Yolksac vitamin A2 showed a similar trend, although not significant. The average ratio of plasma vitamin A over yolksac retinyl palmitate significantly increased with incubation period, till 237% of the animals that needed 21 days. Also the average yolksac PHAH residue levels significantly increased with the incubation period (143% and 172% for, respectively, 22 and 23 days compared to 21 days; Bosveld *et al.*, 1994). An increased mobilisation of vitamin A from the liver to the plasma and decreased retinoid storage are in accordance with findings that are reported earlier (Spear *et al.*, 1985, 1988, 1990; Brouwer, 1988; Chen *et al.*, 1992). As the average yolksac weight and the yolksac weight relative to body weight was the same in all incubation groups (Table 3.4), it is not to be expected that the lesser yolksac retinyl palmitate levels of chicks that hatched later were a result of the longer period that the embryo was feeding on the yolksac. The same goes for the finding that chicks that hatch later have greater yolksac PCB-levels (Bosveld *et al.*, submitted). The suggestion that a longer incubation time is associated with, or is a consequence of, lesser yolksac retinyl palmitate levels is in accordance with studies with chickens, in which it was observed that vitamin A deficiency reduces hatchability. In case of hypovitaminosis A, the large blood vessels that normally appear in the membrane surrounding the embryo after 48 hr of incubation were reduced or did not appear (Thompson, 1970). Based on these findings, it can not be excluded that the incubation period of common tern chicks in the two relatively polluted colonies

Westplaat and Slijkplaat will be prolonged due to in ovo exposure to PHAHs and/or the biochemical effects resulting from that exposure. More experimental work is needed to confirm this hypothesis.

Observations on the incubation period under field conditions in relation to chemical and biochemical parameters will be described elsewhere (Murk *et al.*, 1996a).

Conclusions

It is concluded that, next to hepatic retinoids, yolksac retinoids may be suitable indicators for early effects of PHAHs in the common tern and related fish eating birds.
Due to the large interindividual variations in responses in natural populations, it is important that individual internal doses are measured and/or that the number of animals needed for a chosen minimal observable effect must be calculated in advance.
In general it is speculative to conclude from correlations between effects (such as morphological abberations, EROD activity, vitamin A levels, etc) and PCB levels measured in field experiments that a causal relationship exists. Specific effect parameters based on laboratory experiments with natural species and semi-field studies are needed to prove causal relationships and effects under field conditions.

Acknowledgements

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CHAPTER 4

EFFECTS OF POLYHALOGENATED AROMATIC HYDROCARBONS AND RELATED CONTAMINANTS ON COMMON TERN REPRODUCTION; INTEGRATION OF BIOLOGICAL, BIOCHEMICAL AND CHEMICAL DATA*

ABSTRACT

In eight Dutch or Belgian common tern (*Sterna hirundo*) colonies, breeding biology and food choice were determined, and 15 second eggs were collected from three-egg clutches for artificial incubation, biochemical analysis and analysis of yolksac PHAH levels. Results from these analyses were combined with biological data from the eggs remaining in each clutch. In some breeding colonies severe flooding, rainy and cold weather, and extreme predation caused extensive losses of eggs and chicks. A relationship was found between yolksac mono-*ortho*-(mo-)PCB levels and main food species (fish or insects) of the adult terns before egg-laying. Colony average breeding data differed only slightly, and were difficult to relate to PHAH-levels. When the colonies were grouped after yolksac PHAH-patterns and main food species, significant differences in average egg laying date, incubation period, egg volume and chick weight could be related to differences in yolksac PHAH and retinoid levels, and hepatic EROD activity. The data from all colonies also were combined into one data-set and correlated with the biochemical parameters and PHAH levels. In summary greater yolksac PHAH levels or hepatic EROD-activity correlated with later egg laying, prolonged incubation period and smaller eggs and chicks. Lesser yolksac retinoid- and plasma thyroid hormone levels, and a greater ratio of plasma retinol over yolksac retinoids correlated with later egg laying, prolonged incubation periods and smaller chicks and eggs.

The dynamic environment of the terns had more obvious detrimental effects on breeding success than PHAHs. However, the more subtle effects observed for PHAHs could still be of importance during specific stress circumstances. To monitor site-specific reproduction effects, tree-nesting birds feeding on relatively big and non-migrating fishes would be most suitable. The use of specific biomarkers for exposure and effect is recommended to establish a causal relationship between a certain class of pollutants and an adverse biological effect.

**Based on: A.J. Murk, T.J. Boudewijn, P.L. Meininger, A.T.C. Bosveld, G. Rossaert, T. Ysebaert, P. Meire, S. Dirksen (1996): Effects of Polyhalogenated Aromatic Hydrocarbons and Related Contaminants on Common Tern Reproduction: Integration of Biological, Biochemical and Chemical Data. Arch. Environ. Contam. Toxicol. 31:128-140*

INTRODUCTION

Empirical field studies and toxicological studies suggest that birds can be affected by lipophilic persistent pollutants, such as polyhalogenated aromatic hydrocarbons (PHAHs) and pesticides, that accumulate through the aquatic food chain. In birds PHAHs have been associated with adverse effects such as impaired reproduction, growth retardation, morphological abnormalities, behavioural changes and alterations in vitamin A and thyroid hormone metabolism (Gilbertson and Fox 1977; Koeman *et al.* 1973; Hoffman *et al.* 1987; Gilbertson 1989; Gilbertson *et al.* 1989; Kubiak *et al.* 1989; Spear *et al.* 1990; Walker 1990; Fox *et al.* 1991; Fox 1993; Murk *et al.* 1994a; Van den Berg *et al.* 1995). Although the use and release into the environment of polychlorinated -biphenyls (PCBs), -dibenzofurans (PCDFs) and -dibenzo-*p*-dioxins (PCDDs) is restricted now, levels in depositional zones of the North Sea and in top predators have hardly decreased (Cummins 1988; Klamer *et al.* 1991; Becker *et al.* 1992; Evers *et al.* 1993). Additionally, other PHAHs such as polybrominated diphenyl ethers and polychlorinated terphenyls, have been shown to exert similar effects, probably through the same mechanism (Safe *et al.* 1990 1994; Murk *et al.* 1996b). This means there is still concern for PHAH-effects on top-predators.

To set quantitative and verifiable ecological objectives for the Dutch management of the North Sea, adjacent salt waters and inland waters, 60 indicator species have been selected that are vulnerable to various ecological disturbances and represent a cross section of the ecosystem (Ten Brink *et al.* 1991). The common tern (*Sterna hirundo*) has been recently added, being a specialized top predator of the aquatic food-chain in estuarine areas. Literature data suggest that terns are sensitive for effects of PHAH contamination. For Forster's terns positive correlations have been observed between levels of non- and mono-*ortho*-PCBs and decreased hatchability, increased incubation period and decreased parental attentiveness (Kubiak *et al.* 1989), and between hepatic arylhydrocarbon hydroxylase (AHH) activity (as a measure of PHAH exposure) and weight loss and abnormal functioning of the thyroid gland (Hoffman *et al.* 1987). Becker *et al.* (1991) reported a reduced hatching success for common terns which correlated with increased PCB levels. This reduction was, however, not statistically significant. Common terns offer the advantage of easily accessible nests, as they breed on the ground. Within a few weeks after they arrive from their wintering sites in West and South Africa (Cramp 1985), the terns store reserves for the breeding season. They forage mostly within 10 km from their breeding place (Stienen and Brenninkmeijer 1992; Rossaert *et al.* 1993), therefore their diet is expected to reflect the local degree of contamination. The PCB-118 levels in common tern eggs from the brackish zone of the Dutch Western Scheldt estuary

(0.1-0.7 $\mu g \cdot g^{-1}$ WW, Stronkhorst *et al.* 1993) partially overlap the range reported for Green Bay (0.32-1.56 $\mu g \cdot g^{-1}$ ww) where 75% reproductive impairment was observed (Kubiak *et al.* 1989). Therefore deleterious effects cannot be excluded.

An integrated ecotoxicological study was carried out in 1991 to study the possible effects of PHAHs on the reproduction and development of common tern chicks. Eight colonies were studied, which were expected to cover a gradient in pollution levels. The colonies were (Figure 4.1): Saeftinge, Terneuzen and Zeebrugge in the Western Scheldt area, situated along a gradient towards the North Sea; Prinsesseplaat near the Eastern Scheldt; Slijkplaat and Westplaat in the mouth of the rivers Rhine and Meuse; the isle of Griend in the Wadden Sea and Zeewolde, a relatively clean 'fresh-water colony'. For these colonies the food species, feeding areas and fate of eggs and young were studied. From all colonies the second egg of 15 three-egg-clutches were collected for artificial incubation and chemical and biochemical analysis. More detailed results of the Cytochrome P450IA activity, plasma thyroid hormone levels, T4-glucuronyltransferase (UGT)-activity, plasma and yolk-sac vitamin A and yolk-sac PHAH levels, laboratory incubation period and correlations are described by Murk *et al.* (1994b) and Bosveld *et al.* (1995). This paper focuses on the relationship between the breeding biology in the field, food species before egg laying, and biochemical data and PHAH levels from the (artificially incubated) second egg of the same clutch. More detailed additional observations in the colonies have been reported by Rossaert *et al.* (1993).

MATERIALS AND METHODS

Colonies Studied

Saeftinge is a typical brackish water tidal area in the eastern part of the Western Scheldt estuary (Figure 4.1). There is a high tidal amplitude of 4.5. The birds mainly foraged in the Western Scheldt. The colony of Terneuzen is situated in the middle of a sluice-complex. There is no danger of flooding. The terns in Terneuzen were more aggressive towards intruders than in any of the other colonies. The sluice complex and the Western Scheldt were the main foraging areas. The Zeebrugge colony is situated in the harbour of Zeebrugge and holds over 50% of the Belgian breeding population of the common tern. Most of the common terns foraged in the North Sea, but during early breeding season several terns were also foraging in adjacent fresh water areas. The Prinsesseplaat is a tidal flat which separates the freshwater lake 'Zoommeer' from the Eastern Scheldt. The common terns bred in two (sub-)colonies. Generally the birds foraged in the Eastern Scheldt, but in early May many terns were foraging on emerging Chironomids in the

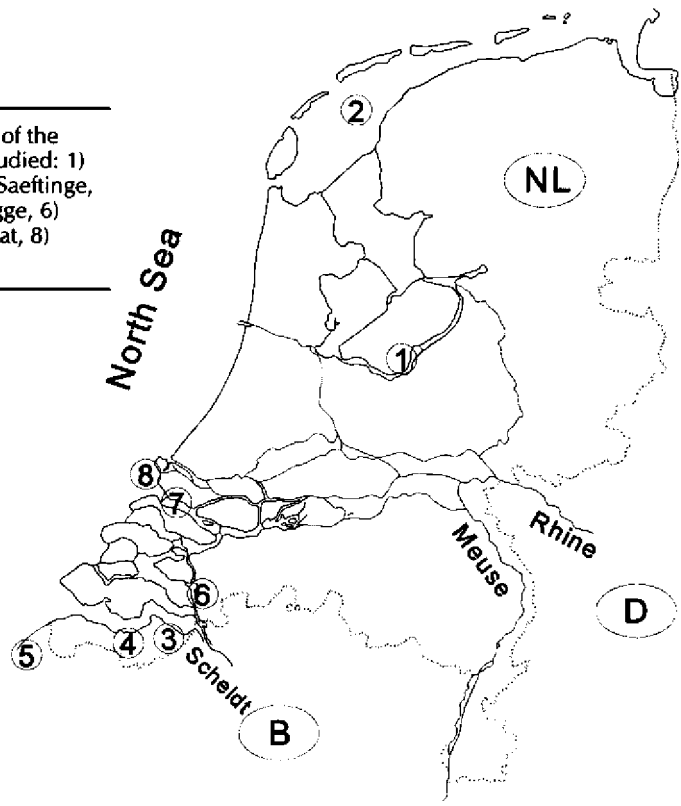

Figure 4.1 Locations of the common tern colonies studied: 1) Zeewolde, 2) Griend, 3) Saeftinge, 4) Terneuzen, 5) Zeebrugge, 6) Prinsesseplaat, 7) Slijkplaat, 8) Westplaat.

Zoommeer. The Slijkplaat is a sandy, shallow flat, situated in the western part of the Haringvliet, a freshwater area with a tidal amplitude of 30-40 cm. The Haringvliet-sluices is the most important foraging area, especially during low tide when (fresh) water of the Haringvliet is discharged into the sea. Westplaat is a man-made bird island along the North Sea. During spring tides the lower parts of the island are flooded. Foraging took place in the surroundings of the Westplaat and near the Haringvliet-sluices when these were discharging into the sea. The Zeewolde colony is situated on the top of a sand depot along a dyke, ca. 2.5 m above surface level. The common terns foraged in the fresh-water lakes Wolderwijd and Veluwemeer around the colony. Shortly before egg-laying, hundreds of common terns were seen foraging on emerging chironomids. Griend is a small island in the western part of the Dutch Wadden Sea. The terns mainly forage within the Wadden Sea in water above tidal flats and in gullies (Klaassen 1992).

Weather Conditions

Data on daily precipitation, temperature and wind speed for the period May 1st-July 31st 1991, were extracted from monthly reports of the Royal Netherlands Meteorological

Institute (KNMI). May 1991 was one of the coldest Mays of this century in The Netherlands, with an average temperature of 10°C. Night frost occurred on a large scale until the end of the month. There were 153 hr of sunshine compared to an average of 205 hr. June 1991 was extremely wet, very cold and exceptionally cloudy. It was the wettest Dutch June of the century with 122 mm of precipitation, against 62 mm normally. There were 119 hr of sun compared to 207 hr normally. Due to this combination it was considered the worst June of this century, resulting in very unfavourable conditions for young birds. July 1991 was warm, sunny and generally dry, although there were several days with heavy showers during the first half of the month.

Field Procedures and Number of Visits

The observations in the colonies started late April-early May 1991 during pair-formation and ended in the second half of July when chicks had fledged. Nests were marked with numbered wooden stakes placed 20-30 cm from the nests. During each visit new eggs were marked, and the number of eggs, damage and/or mortality and number of hatched eggs were recorded. During the peak of egg-laying the nests were checked daily (Saeftinge, Terneuzen and Zeebrugge) or every two days in the other colonies. Before and after this peak the colonies were checked less frequently. The visits were carefully planned in order to avoid disturbance and temperature stress of eggs and chicks as much as possible. During visits in and after the hatching period, young terns were searched and checked for developmental abberations, total weight, wing length and total head length. They were banded, or marked with a little picric acid when they were too small for banding. Wherever possible observations on feeding behaviour and prey selection were made.

Recorded Breeding Data

The date the first egg appeared was used to index the laying date of the clutch. In some cases the laying date was calculated from the exact laying date of another egg using the average laying period for an egg, or from the hatching date of the first hatched egg using the average incubation period per egg. To minimize the chance of underestimating the clutch size due to predation or the fact that clutches might not yet have been completed, the content of all marked nests was recorded during all visits. The egg volume was calculated using the Hoyt (1979) formula: volume (ml) = 0.509 x length (cm) x $(\text{width})^2(\text{cm}^2)$. The length and width were measured with sliding callipers to the nearest 0.1 mm. The incubation period of every individual egg was calculated as the time (days) between completion of the clutch and hatching, provided that the exact laying and hatching dates were known.

Laboratory Incubation and Analyses

In each colony, the second egg of 15 three-egg clutches of which the exact laying date of every egg was known, was collected after at least 7 days of incubation. In the laboratory the eggs were weighed and incubated at 37.5°C and a relative humidity of 50-60%. Eggs were turned automatically every 30 min. Within 12 hr after hatching the young terns were weighed and sacrificed. Livers and yolk-sacs were weighed immediately, snap frozen in liquid nitrogen and stored at -70 °C. Blood was centrifuged and the plasma was stored at - 20°C. Of the hatched eggs the eggshell thickness was measured at the equator of the egg after the shell membranes were removed, using a Mitutoyo micrometer.

Yolk-sac PCB, PCDD and PCDF residues were analyzed in yolk-sacs by GC-MS (Bosveld *et al.* 1995). Total levels of mono-*ortho*-(mo-)PCBs were used, or the individual PHAH levels were transformed into Toxic Equivalents (TEQs), using Toxic Equivalency Factors (TEFs) as proposed by Safe (1990, 1994). Di-*ortho*-PCBs were not used in TEQ calculations. Liver microsomes were prepared as described in Bosveld *et al.* (1995). Protein content was analyzed according to the method described by Bradford (1976). 7-ethoxyresorufin *O*-deethylation (EROD) and 7-pentoxyresorufin *O*-depenthylation (PROD) activities were analyzed fluorimetrically according to the method of Rutten *et al.* (1987).

Plasma thyroid hormones levels, total thyroxin (TT4), total triiodothyronine (TT3) and free thyroxin (FT4), were determined in, respectively, 10, 25, and 25 μl aliquots of plasma, by chemiluminescence immunoassay using commercially available kits (Amerlite assay kits, Amersham Internat. plc., Amersham, UK). Plasma retinol and yolk-sac retinylpalmitate were extracted from 25 μl aliquots with methanol/diisopropylether and analyzed with HPLC as described by Murk *et al.* (1994b). The ratio plasma retinol over retinylpalmitate was calculated as a measure of mobilisation of retinol to the circulation or decreased retinoid ester storage.

Statistical Methods

Differences between clustered data were tested with one-way ANOVA. In addition to comparing colony averages, the data from all colonies were combined into one data set and correlated with the biochemical and chemical parameters from the artificially incubated chicks. The biochemical and chemical data from the artificially incubated chicks were divided into 4 groups based on the data for breeding biology in the field. Attention was paid to an equal distribution of the numbers of biochemical and chemical data over the four groups. The acceptance level was set at $P<0.05$. Clear, but not statistically significant, differences that could be related to differences in PHAH contamination are mentioned as 'trends'.

Results

Comparison of parameters for breeding biology

The colony average breeding biology parameters measured in the field are summarized in Table 4.1. The colonies are arranged in order of increasing colony average yolk-sac mono-*ortho*-(mo-) PCB levels. Although the onset of egg laying was well synchronized among the colonies (13-15 May, only Saeftinge and Zeebrugge somewhat later; data not shown) the average laying date of the first egg showed more variation. Westplaat was the latest of all colonies. The average clutch size was largest in Terneuzen and smallest in Kleine Prinsesseplaat and Westplaat. The average egg volume is only calculated of three-egg clutches, because egg volume partially depends on the number of eggs already layed. The eggs were largest in Terneuzen and smallest in Saeftinge, Westplaat and Prinsesseplaat (Table 4.1). Most colonies showed a decrease of egg volume in clutches which were started later (data not shown, Rossaert *et al.* 1993). The average laying period needed to complete a three-egg clutch varied between 2.59 days (Zeewolde) and 3.50 days (Westplaat) (data not shown). The average incubation period of three-egg clutches was longest on Terneuzen and shortest on Griend. The observed differences in colony averages in breeding biology could not be related to differences in average yolk-sac PHAH-levels.

Large differences in hatching success of three-egg clutches were found between Kleine Prinsesseplaat (0.00) or Saeftinge (0.64) compared to Zeebrugge (2.88) and Terneuzen (2.62) (Table 4.2). However, adverse climatological effects and sometimes extreme predation negatively influenced reproductive success in several colonies (Table 4.2). In the Prinsesseplaat colony heavy showers produced a layer of up to 10 cm of water which sometimes took days to disappear, resulting in massive loss of eggs and chicks. Strong winds in combination with spring tide caused additional losses of clutches in Saeftinge and Griend. Severe predation by oystercatchers (*Haematopus ostralegus*) was observed in the Prinsesseplaat colony. A major problem in the Slijkplaat colony was the rapid growth of thistles. Nests got completely overgrown and were difficult to relocate by the observers and sometimes deserted by the birds. In the Zeebrugge, Terneuzen and Westplaat colonies the adverse factors were not extreme and did not result in abnormal losses. In none of the colonies studied hatching failure was a major cause of egg-loss.

The adverse factors mentioned above make it difficult to relate average breeding biology data to differences in PHAH-contamination. Therefore, from here on calculations are restricted to the three-egg clutches that were not reduced by the factors mentioned

Table 4.1 Breeding biology (average ± SD): colony size, date of laying of the first egg, clutch size, egg volume and incubation period of the first egg of all monitored clutches. The colonies are arranged in order of colony average yolksac mono-ortho (mo-) PCB levels, going from relatively low (6 μg/g lipid in Zeewolde) to high (40 μg/g lipid in Slijkplaat) levels.

Colony	Colony size (clutches studied)	Laying date first egg[a] (± days) (month-day)	Clutch size[a] (eggs)	Egg volume[b] (ml)	(N)	Incubation period[b] first egg (days)	(N)
Zeewolde (Z)	317 (317)	6-4[c] ± 17.8▲^{T}	2.55 ± 0.66	19.74 ± 1.40	(337)	21.83 ± 1.18	(60)
Prinsesseplaat (P)	181 (181)	6-1[d] ± 12.0	2.44 ± 0.67	19.23 ± 2.03	(29)	22.00 ± 0.00	(2)
Kleine Pr.plaat (K)	64 (64)	5-21 ± 3.5	2.11 ± 0.78▼^{ZPBATS}	19.92 ± 1.32	(55)	–	–
Griend (G)	1500 (30)	5-18 ± 1.2	2.37 ± 0.49	19.69 ± 1.20	(33)	21.11 ± 0.88▼^{ZBATWS}	(19)
Zeebrugge (B)	650 (245)	5-25 ± 4.4	2.49 ± 0.79	19.73 ± 1.40	(462)	21.88 ± 0.94▼^{W}	(186)
Saeftinge (A)	107 (107)	5-25 ± 1.7▼^{ZT}	2.60 ± 0.66	19.33 ± 1.42▼^{ZKBTS}	(221)	22.89 ± 0.93▲^{ZB}	(92)
Terneuzen (T)	145 (145)	5-31 ± 9.8	2.76 ± 0.56▲^{ZPKGBWS}	20.12 ± 1.53▲^{ZPGBAWS}	(321)	23.38 ± 1.31▲^{ZGBWS}	(132)
Westplaat (W)	195 (195)	6-8 ± 19.0▲^{ZATS}	2.14 ± 0.75▼^{ZPBATS}	19.25 ± 1.47▼^{ZKBTS}	(183)	22.39 ± 1.50	(33)
Slijkplaat (S)	420 (420)	5-26 ± 10.3▼^{ZT}	2.54 ± 0.63	19.59 ± 1.43	(278)	22.00 ± 1.67	(44)

[a] All clutches; [b] 3-clutches only; [c] Two distinct peaks of laying; [d] Settled late, from K
▼^{X} Significantly less than colony x
▲^{X} Significantly more than colony x
– No incubation completed

in Table 4.2, and from which the second egg was collected for laboratory incubation. The field data from these clutches can easily be compared with the laboratory data of the collected second egg. Additionally, these three-egg clutches were layed during the peak periods of laying in each colony, which is a limited period of time. This is important as egg volumes, clutch size and laying period were found to be related (Rossaert *et al.* 1993). Of these selected three-egg clutches colony averages in breeding biology data were also calculated (data not shown). The average laying dates of the first egg were significantly earlier for Zeewolde, Griend and Slijkplaat compared to the other colonies, but these differences could not be related to differences in PHAH levels. The average incubation period of the first egg was significantly shortest (21 days) on Griend and longest in the Slijkplaat colony (25 days). The average egg volumes and average eggshell thickness (1.3-1.4) did not differ significantly between the colonies. No morphological abberations were observed in the field nor for the eggs that were artificially incubated.

Comparison of Food Choice between Colonies

Due to circumstances differing between colonies, the quality and quantity of the data on food and foraging is different for each colony. However, the data enable at least a qualitative description for all colonies. In general, common terns from coastal colonies mainly foraged on clupeids (herring (*Clupea harengus*) and sprat (*Sprattus sprattus*)), smelt (*Osmerus eperlandus*), sandeel (*Ammodytes spec.*), goby (*Gobius spec.*), whiting (*Merlangius merlangus*), and to a lesser extent on small flatfish, three-spined stickleback (*Gasterosteus aculeatus*), Ruffe (*Gymnocephalus cernuus*) and crustaceans. Especially in the Zeewolde, but also in the Prinsesseplaat colony, emerging chironomids were an important prey in the beginning of the breeding season. For the Prinsesseplaat colony the fish species were not identified. Figure 4.2 presents the main food choice of adults in each colony in the period before egg laying, combined with the average yolk-sac mo-PCB levels. The average yolk-sac mo-PCB levels were least when adults had foraged on large amounts of insects. Yolk-sac mo-PCB levels were highest in the Westplaat and Slijkplaat (significant) colonies where smelt and herring were the main food items. Clupeids were the main food species in the four colonies with intermediate PCB-levels.

Clustering of Colonies Based on Food Choice and PHAH-Pattern

Based on the PCDD/F patterns in yolk-sacs established by principal component analysis (PCA, Bosveld *et al.* 1995), three groups of colonies can be distinguished coinciding with mainly smelt, clupeids, or insects as food choice before egg laying. This PCA revealed that the Slijkplaat and Westplaat colonies, were clearly distinct from all other colonies, and the Zeewolde colony could be distinguished from the other colonies. The five

Table 4.2 Hatching success (average ± SD) and adverse climatological and ecological factors that influenced the reproductive success.

Colony	Hatching success of 3-clutches	(N)	Specific climatological and ecological factors
Zeewolde (Z)	2.24 ± 1.69	(82)	Many dead young due to cold and rainy wheather, many second clutches
Prinsesseplaat (P)	1.88 ± 1.34▼^{ZS}	(32)	Losses of eggs and young through flooding due to rainfall in June
Kleine Pr.plaat (K)	0.00 ± 0.00▼^{ZPGBATWS}	(11)	Heavy egg predation (mainly by a few oystercatchers)
Griend (G)	2.50 ± 0.58	(4)	Losses of young through flooding by high tide (6-13), predation by gulls
Zeebrugge (B)	2.88 ± 0.33▲^{ZPKAW}	(72)	– (some predation by black-headed and herring gull)
Saeftinge (A)	0.64 ± 1.02▼^{ZPGTWS}	(58)	Massive loss of clutches through flooding by high tide (6-13)
Terneuzen (T)	2.62 ± 0.79▲^{ZPKAW}	(90)	– (disturbance through human activity on sluices)
Westplaat (W)	1.76 ± 1.30▼^{ZS}	(29)	– (some nests were washed away or covered under sand)
Slijkplaat (S)	2.46 ± 0.95	(50)	Rapid growing thistles covered many nests, some were abandoned

– No specific adverse climatological or ecological circumstances that resulted in abnormal losses
▼^{x} Significantly less than colony x
▲^{x} Significantly more than colony x

remaining colonies (Prinsesseplaat, Griend, Zeebrugge, Saeftinge and Terneuzen) exhibited a similar PCA-pattern. The average data were recalculated for three groups of colonies: Slijkplaat/Westplaat, Zeewolde and 'Rest'. In contrast to the colony averages, the group-averages show clear differences in both breeding, biochemical and chemical data (Table 4.3). In the Zeewolde colony eggs are layed significantly earlier and are significantly bigger than in the other two groups. Yolk-sac PHAH levels (expressed either as TEQs or as mo-PCBs) and hepatic EROD activity are significantly least and yolk-sac retinylpalmitate levels and vitamin A2 levels are significantly greatest in the Zeewolde colony. In the Slijkplaat/Westplaat group incubation period is significantly longer, chickweight significantly less, and yolk-sac PHAH and hepatic EROD activity significantly greater than in the other two groups. Plasma FT4 levels are greatest in the Zeewolde group and least in the Slijkplaat/Westplaat group, but these differences were not statistically significant.

Clustering of Data Based on Parameters for Breeding Biology

Laying Date. The biochemical and chemical data grouped after laying date of the first egg are presented in Table 4.4. Later layed eggs contained slightly (38%) more PHAH-residues (expressed either as mo-PCBs or as TEQs) than early layed eggs. Earlier eggs (on average 137 days after January 1st) contained significantly (82%) greater yolk-sac retinyl

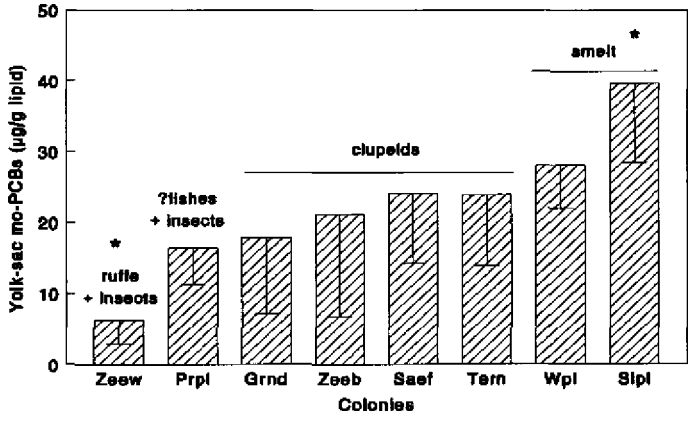

Figure 4.2 Colony average yolksac mo-PCB levels with main food items before egg-laying written above the bars: ruffe (*Gymnocephalus cernuus*); smelt (*Osmerus eperlanus*); clupeids: herring (*Clupea harengus*) and sprat (*Sprattus sprattus*); insects (mainly chironomids) and ?fishes (fish species unknown).
*: mono-ortho (mo-) PCB levels significantly different from the other colonies.

Table 4.3 Group averages (± SD) of breeding data (a) and chemical and biochemical data (b) for colonies grouped after their PHAH-pattern.

A

Colony	Laying date first egg ± SD (days)	Incubation period first egg (days)	Egg volume second egg (ml)	Chick weight second egg (g)
Zeewolde (Z)	127.2 ± 3.6▼^{RS}	21.6 ± 1.2	20.7 ± 1.2▲^{RS}	14.5 ± 1.3
Rest (R)	144.7 ± 4.3	22.0 ± 1.3	19.7 ± 1.4	14.2 ± 1.2
Slijk-/ Westplaat (S)	141.4 ± 3.4	23.1 ± 1.9▲^{ZR}	19.2 ± 1.3	13.4 ± 0.9▼^{ZR}

B

Colony	mo-PCBs µg/g lipid	TEQs ng/g lipid	EROD act. nmol/g.min	Yolks.RP (ng/g)	Yolksac. Vit A2 (ng/g)	Plasma FT4 (pmol/l)
Zeewolde (Z)	6.22 ± 3.30▼^{RS}	3.724 ± 1.763▼^{RS}	138 ± 47	1.18 ± 0.91▲^{R}	13.3 ± 5.6▲^{RS}	5.39 ± 1.54
Rest (R)	20.52 ± 10.20	10.427 ± 4.592	158 ± 78	0.62 ± 0.41	6.4 ± 2.1	4.85 ± 2.29
Slijk-/ Westplaat (S)	32.84 ± 9.80▲^{ZR}	14.112 ± 5.571▲^{ZR}	399 ± 292▲^{ZR}	0.86 ± 0.60	6.8 ± 1.7	4.78 ± 1.70

▼^{X} Significantly less than group X
▲^{X} Significantly more than group X

palmitate levels, 69% greater vitamin A2 levels and an almost 2-fold smaller ratio plasma retinol over yolk-sac retinylpalmitate, than latest layed eggs (on average 150 days). No clear trend was visible for plasma thyroid hormone levels TT4 and FT4. Plasma TT3 levels were slightly greater in earlier layed eggs. The incubation period for the earliest eggs was on average 2 days shorter compared to the latest layed eggs.

Incubation Period. When clustering data based on incubation period in the field, significantly greater yolk-sac mo-PCB or TEQ levels (100%), or hepatic EROD activities (60%) were found in eggs needing 24 days or more for incubation compared to eggs needing on average 20 days (Table 4.5). Because of the small number of eggs for which both incubation period and yolk-sac retinoids were measured, these data were divided over only two groups. Yolk-sac retinylpalmitate and vitamin A2 levels were (not statistically significant) greater in the shortest incubation group. The ratio plasma retinol over yolk-sac retinylpalmitate, however, was significantly greater (3.2-fold) in the longest incubation group. Plasma thyroid hormone (TT3, TT4, FT4) levels were all significantly (50-70%) less in the two longest incubation groups compared to the two shortest incubation groups. The eggs from the shortest incubation group were layed significantly earlier (on average 4 days) than the eggs from the longest incubation group. The chicks (Table 4.5) and eggs (data not shown) from the 2 longer incubation groups were slightly smaller compared to the 2 other groups.

Figure 4.3 shows the ratio of the incubation period of the first egg of a clutch in the field over the incubation period of the second egg in the laboratory incubator. This ratio increases significantly with the incubation period in the field.

Egg Volume and Chick Weight. As average egg volume (whole clutch) and chick weight (second egg) are closely related, these parameters are presented in combination. Yolk-sac PHAH-levels (expressed either as mo-PCBs or as TEQs) were slightly greater (36%) in the smallest eggs and chicks compared to the bigger eggs (Table 4.6a) and chicks (data not shown). Slight differences in average EROD activity were only observed for different chick weight groups (Table 4.6b) but not for different egg volume groups (data not shown). Yolk-sac retinoid levels were slightly greater and the ratio plasma retinol over yolk-sac retinylpalmitate levels less in the biggest eggs. Not enough data were available to make a grouping for yolk-sac retinoid levels based on chick weight. Differences in plasma thyroid hormone (TT4, TT3, FT4) levels that could be related to PHAH contamination, were only observed between groups clustered after chick weight. These differences were only significant for FT4. Egg volume, chick weight and liver weight were significantly correlated with each other. The ratio liver weight over chick weight was on average 2.66-2.73 %, and did not differ between any of the groups.

Table 4.4 Chemical and biochemical data (average ± SD) grouped after egg laying dates (days after January 1st).

Parameter	Group 1 (early) [137.2-137.6][a]		Group 2 [140.1-141.5][a]		Group 3 [143.2-147.3][a]	Group 4 (late) [145.6-150.3][a]	N
mo-PCBs (μg/g)	18.96 ± 10.77		23.07 ± 17.84		23.94 ± 8.19	25.55 ± 11.49	12/13
TEQs (pg/g)	8508 ± 4851		11229 ± 7259		12217 ± 3702	11640 ± 4603	9/10
EROD act. (nmol/g.min)	162 ± 144		294 ± 260		185 ± 77	186 ± 88	18/19
ys ret.palmitate (ng/g)	0.89 ± 0.36	▲[3,4]	1.06 ± 0.47	▲[3,4]	0.42 ± 0.23	0.49 ± 0.42	9/10
ys vit A2 (ng/g)	9.90 ± 2.43	▲[2,3,4]	6.32 ± 1.34		6.13 ± 1.36	5.86 ± 2.06	9/10
plasma retinol (ng/ml)	96.9 ± 53.2		98.1 ± 34.7	▲[3,4]	79.7 ± 22.7	76.9 ± 29.1	23/24
ratio pl.re./ys.r.palm	118 ± 98	▼[4]	146 ± 133		202 ± 98	230 ± 82	9/10
plasma TT3 (nmol/l)	3.32 ± 1.49		3.22 ± 1.51		2.88 ± 1.48	2.78 ± 0.97	22/23
incubation period (days)	21.6 ± 1.84	▼[4]	21.6 ± 1.02	▼[4]	22.3 ± 0.82	23.4 ± 1.49	12/13

[a] The average group-laying dates are not the same for all parameters, as the grouping is performed per parameter, and the total number of data available per parameter varies.

▼[x] Significantly smaller than group x

▲[x] Significantly larger than group x

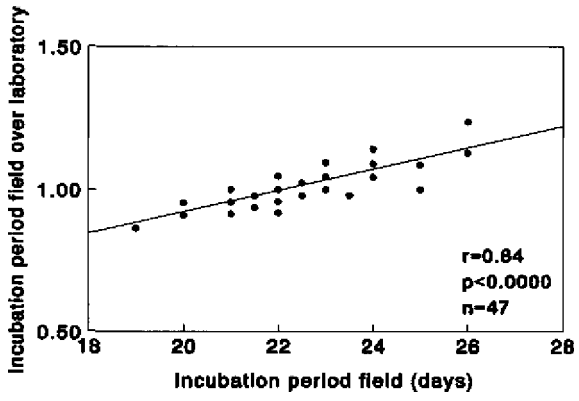

Figure 4.3 The ratio of the egg incubation period in the field over the incubation period in the laboratory incubator, plotted against the incubation period in the field (n = 47).

Discussion

Due to their way of nesting on bare ground close to the shore, common terns are relatively vulnerable to climatological influences and predation if compared to, for example, tree-nesting cormorants. Massive flooding, severe predation and extreme rainy and cold weather had strong negative effects on clutch size, and on hatching and fledging success, and masked possible adverse effects of PHAH-exposure on these parameters. Because of these strong natural influences, only information on egg laying dates, egg volumes and incubation periods could be used for estimating contaminant effects.

Although colony average breeding data could not clearly be related to PHAH-levels, colonies clustered after PCDD/F patterns and main food choice, showed significant differences in both breeding, biochemical and chemical data. Using the data from all colonies combined into one data-set, the breeding parameters could be correlated with yolk-sac PHAH-levels, EROD-activity, yolk-sac retinoid-, and plasma retinol and thyroid hormone levels. These correlations were either statistically significant or clear trends.

Food Choice

On the analogy of observations in North America (Nisbet 1973) female terns could build up a considerable PHAH burden during courtship feeding, when returning from their wintering grounds in Africa to breed in The Netherlands. This period shortly before egg laying is probably the most important period for accumulating PHAH-levels in the eggs.

Table 4.5 Chemical and biochemical data (average ± SD) grouped after incubation period in the field (first egg)

Parameter	Group 1 (short) [20.1-20.9][a]		Group 2 [21.5-21.7][a]		Group 3 [22.5-22.7][a]		Group 4 (long) [24.0-24.7][a]	N
mo-PCBs (μg/g)	12.82 ± 9.12	▼[4]	21.83 ± 11.88		18.44 ± 11.01		26.34 ± 8.72	6/7
TEQs (pg/g)	4951 ± 4080	▼[4]	10440 ± 4099		9016 ± 4851	▼[4]	12467 ± 4291	6
EROD act. (nmol/g.min)	126 ± 42	▼[4]	193 ± 114		171 ± 83		202 ± 86	8/9
ys ret.palmitate (ng/g)	1.07 ± 0.51		0.61 ± 0.34		–		–	7
ys vit A2 (ng/g)	9.31 ± 3.46		7.02 ± 1.26		–		–	7
plasma retinol (ng/ml)	83.1 ± 32.5		78.2 ± 37.2		99.0 ± 35.7		101.0 ± 44.9	10/11
ratio pl.re./ys.r.palm	63 ± 51	▼[2]	204 ± 127		–		–	7
plasma TT4 (nmol/l)	7.02 ± 1.59	▲[3,4]	8.32 ± 2.21	▲[3,4]	5.16 ± 1.58		4.67 ± 1.70	9/10
plasma FT4 (pmol/l)	6.60 ± 2.27	▲[3,4]	7.18 ± 2.10	▲[3,4]	3.67 ± 1.09	▲[4]	3.72 ± 1.04	7
plasma TT3 (nmol/l)	3.95 ± 1.55	▲[4]	4.01 ± 1.84	▲[3,4]	2.55 ± 0.77		2.51 ± 1.16	10/11
chickweight (g)	14.5 ± 1.4		14.4 ± 0.9		14.0 ± 0.8		13.8 ± 1.3	7/8
laying date (days)	140.0 ± 4.1	▼[4]	142.4 ± 4.0		142.6 ± 3.2		144.0 ± 4.6	12

[a] The average incubation periods for the four groups is not the same for all parameters, as the total number of data available per parameter varies.

▼[x] Significantly less than group x

▲[x] Significantly more than group x

High mo-PCB levels were expected based on a Dutch field study with cormorant chicks (Van den Berg *et al.* 1995) where yolk-sac levels of 184 and 58 μg mo-PCBs·g^{-1} lipid were observed in, respectively, the Biesbosch and Oude Venen colony. The average yolk-sac mo-PCB-levels in common tern chicks, however, were only 40 μg·g^{-1} lipid and 6 μg·g^{-1} lipid in, respectively, the most (Slijkplaat) and least (Zeewolde) contaminated colony. The difference in yolk-sac mo-PCB level between these two colonies was only 34 μg·g^{-1} lipid, compared to 126 μg·g^{-1} lipid for the Cormorants. The other six common tern colonies hardly differed in yolk-sac mo-PCB levels (16-28 μg·g^{-1} lipid, Figure 4.2). The relatively low mo-PCB concentrations and small differences between relatively clean and polluted sites are probably consequences of the food choice of the common terns. The terns from the salt water colonies mainly fed on migrating small clupeids, mainly small herring, that were born in the North Sea a few months earlier. Therefore, this food does not reflect the local degree of contamination, and may mask differences in contamination between locations. Small herring contain significantly less PCBs than fishes that overwinter locally, such as flounders (*Platichthys flesus*), 1.3 and 5.2 μg·g^{-1} lipid, respectively, (Stronkhorst *et al.* 1993). Cormorants mainly feed on bigger, often carnivorous, fish that mostly stay in an area with the same degree of pollution. Birds that remain in an estuary all year round, such as oystercatchers, will build up greater levels and better reflect the local degree of contamination, than species such as terns that migrate to less contaminated sites. Stronkhorst *et al.* (1993) observed for PCB-153 a biomagnification factor of 19 for the oystercatcher (egg:cockle) and for common tern only 5.5 (egg:clupeid). After hatching, the chicks are being fed with fishes that are only 1-1.5 times the bill size of the adult tern. These fishes are again much smaller and thus probably less contaminated, than the fishes fed to cormorant chicks.

Considering the relatively small mo-PCB concentrations and the small differences between relatively clean and polluted sites, it is not surprising that hardly any differences in colony average breeding data were found. However, when the colonies were clustered based on yolk-sac PCDD/F patterns, significant differences in both breeding data and biochemical and chemical data were observed. Differences in yolk-sac PCDD/F patterns may be related to the area where the breeding colonies were situated, but they could also be related to the environment the food species originated from. For example, Slijkplaat and Westplaat are the only colonies situated in the sedimentation area of the rivers Rhine and Meuse, but they are also the colonies where the terns (partially) fed on smelt. Additionally, it cannot be excluded that other factors than PHAHs, such as food availability, nutritional quality or the presence of other, unmeasured, contaminants could contribute to the observed differences in group average breeding and biochemical data.

Table 4.6 *Chemical and biochemical data (average ± SD) grouped after (a) average egg volume (all eggs) and (b) chick weight (second egg)*

A

Parameter	Group 1 (big) [21.3-21.6][a]		Group 2 [20.0-20.4][a]		Group 3 [19.2-19.6][a]		Group 4 (small) [17.7-18.1][a]	N	Correlation for chick weight
mo-PCBs (μg/g)	18.64 ± 13.17		20.20 ± 11.31		26.12 ± 10.79		26.84 ± 12.88	12/13	comparable
TEQs (pg/g)	9735 ± 5664		8850 ± 5281		12841 ± 4448		12599 ± 5867	8/9	comparable
ys ret.palmitate (ng/g)	0.88 ± 0.77		0.78 ± 0.41		0.68 ± 0.42		0.69 ± 0.63	9/10	not enough data
ys vit A2 (ng/g)	8.25 ± 5.50		7.33 ± 1.77		7.50 ± 3.19		6.45 ± 1.82	9/10	not enough data
ratio pl.re./ys.r.palm	135 ± 77		158 ± 97		189 ± 123		211 ± 137	9/10	not enough data
chickweight (g)	15.2 ± 0.8	▲[2,3,4]	14.3 ± 0.7	▲[3,4]	13.7 ± 0.5	▲[4]	12.8 ± 0.8	13/14	comparable
liver weight (g)	0.41 ± 0.04	▲[3,4]	0.38 ± 0.05		0.37 ± 0.03		0.35 ± 0.05	13/14	comparable

[a] The average egg volumes for the four groups are not the same for all parameters, as the total number of data available per parameter varies.
▲[x] Significantly larger than group x

B

Parameter	Group 1 (heavy) [15.1-15.5][a]		Group 2 [14.3-14.5][a]	Group 3 [13.6-13.8][a]	Group 4 (light) [12.5-12.6][a]	N
EROD act.(nmol/g.min)	175 ± 72		280 ± 224	216 ± 158	248 ± 205	9/10
plasma retinol (ng/ml)	77.8 ± 23.7		104.3 ± 33.9	99.0 ± 45.2	90.9 ± 44.2	11/12
plasma TT4 (nmol/l)	8.66 ± 6.90		8.18 ± 3.77	6.17 ± 1.82	5.68 ± 1.56	11/12
plasma FT4 (pmol/l)	9.21 ± 1.54	▲[3,4]	6.41 ± 3.28	4.26 ± 1.23	4.71 ± 1.83	8/9
plasma TT3 (nmol/l)	3.24 ± 1.55		3.86 ± 1.46	2.80 ± 1.30	2.71 ± 1.44	11/12

[a] The average chick weights for the four groups are not the same for all parameters, as the total number of data available per parameter varies.
▲[x] Significantly larger than group x

Correlations between Breeding Biology and Biochemical and Chemical Parameters

The biological data of the first egg were compared with the biochemical and chemical data of the second egg from the same clutch. Stronkhorst *et al.* (1993) showed that the variation in contaminant levels is relatively small within the same clutch, especially if compared to the variation between clutches. In the following not only statistically significant results are discussed, but also the clear trends, as these can be a useful indication for contaminant related effects. Due to the relatively large standard deviation using natural instead of inbred laboratory species, only very strong effects will be statistically significant (Murk *et al.* 1994b). An overview of all observed correlations is presented in Figure 4.4. The arrows indicates the direction of change in biochemical and chemical parameters when the parameters for the breeding biology become more unfavourable. A clear trend is indicated with a dotted arrow, a significant effect with a solid arrow. The results for 'egg volume' and 'chick weight' are combined because the parameters are closely related. A more specific discussion is presented below.

Yolk-sac PHAH Levels and EROD-Activity

Yolk-sac PHAH levels and EROD activity are greater in eggs with more unfavourable breeding parameters (Figure 4.4). This increase is significant for a prolonged incubation period, which is similar to the results found for the eggs incubated in the laboratory (Bosveld *et al.* 1995), and in accordance with the results of Kubiak *et al.* (1989, see below). Chicks from later layed eggs contained greater yolk-sac PHAH levels. This correlation could be stronger if the eggs had been collected during the whole laying period instead of only during the peak period of laying. Additionally, (not significantly) greater PHAH levels and EROD activity were observed for smaller eggs and chicks. These correlations suggest that exposure to PHAHs may have a negative influence on breeding parameters, although a correlation with other pollutants, often present in a similar gradient (Gilbertson 1974; Vethaak 1992) cannot be excluded.

In our study no congenital deformities or bill defects were observed. Nevertheless the concentrations of the three most important mo-PCBs (#118, #156 and #105), total PCB-concentrations and total PCDD/F concentrations in common tern eggs from Slijkplaat and Westplaat were similar or slightly greater than those reported for related species (Forsters and common tern) from industrialized sites in North America where birth defects did occur (Smith *et al.* 1990; Ankley *et al.* 1993; Hoffman *et al.* 1993; Bosveld *et al.* 1995). Therefore, it cannot be excluded that those effects are partially caused by other substances, possibly also acting through the Ah-receptor, as is suggested by the results of Tillitt *et al.* (1992). In their study the hatching success of double-crested cormorant (*Phalacrocorax auritus*) eggs correlated poorly with chemically derived TEQs, but strongly

with TEQs measured in an *in vitro* H4IIE rat hepatoma EROD assay, measuring the biological potency of the total mixture of compounds acting through the Ah-receptor. The composition of mixtures of pollutants could be quite different in the north-American sites compared to those in The Netherlands, resulting in different effects of what seem to be the same levels of a certain pollutant. The hepatic EROD-activity measured in the tern chicks in our study cannot easily be compared with the EROD activity in the H4IIE rat hepatoma EROD assay performed by Tillitt *et al.* (1992), because large differences have been observed in species-specific antagonism after exposure to mixtures of PCBs (Aarts *et al.* 1995). H4IIE's only show slight antagonism, whereas nothing is known yet about occurence of antagonism in common terns.

Yolk-sac and Plasma Retinoid Levels

Later layed eggs come from clutches with significantly lesser yolk-sac retinlylpalmitate levels and significantly greater ratios of plasma retinol over yolk-sac retinylpalmitate in the second egg (Figure 4.4). Eggs that needed a prolonged incubation period came from clutches with matching chicks with a significantly greater ratio of retinol over retinylpalmitate and (not significantly) lesser yolk-sac retinyl ester levels, which is similar to the results for the eggs incubated in the laboratory (Murk *et al.* 1994b). An increased ratio plasma retinol over hepatic retinylpalmitate levels indicates an increased mobilisation of retinol to the circulation and a decreased retinoid-ester storage (Brouwer *et al.* 1988; Chen et *al.* 1992). In mammals as well as in birds disturbances in vitamin A homeostasis have been associated with PHAH-exposure. PHAH-exposure can influence vitamin A homeostasis via at least two mechanisms. PHAH hydroxy metabolites can interfere with the plasma transport of thyroid hormone bound to transthyretine (TTR) combined with retinol transport bound to retinol binding protein, resulting in increased loss of both retinol and thyroid hormone from the circulation. PHAHs can also directly increase the release of retinol from the hepatic store, resulting in decreased hepatic retinylester and increased plasma retinol levels (Spear *et al.* 1985, 1988, 1990; Brouwer *et al.* 1988; Murk *et al.* 1994a). Apart from PHAH influences, dietary intake of vitamin A is very important for vitamin A homeostasis, as are adequate stores of retinyl esters and the finely regulated release into the blood (Zile 1992). Therefore, it cannot be excluded that the observed correlations are a consequence of a weaker nutritional status of the female tern, instead of being related to PHAH-exposure.

Vitamin A is essential for normal reproduction. In chickens, vitamin A deficiency has been associated with reduced hatchability due to reduced or failing appearance of large blood vessels around the embryo (Thompson 1970). Therefore the observed greater ratio plasma retinol/retinyl palmitate in later layed eggs, eggs needing a longer incubation

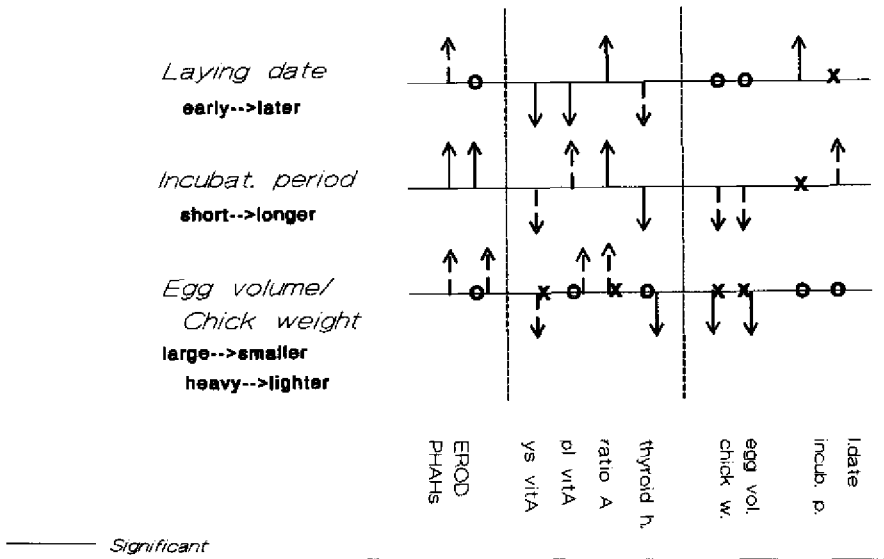

Figure 4.4 Overview of the correlations of the chemical and biochemical data with the biological data of eggs from the same clutches, when clustered on the basis of the biological data. An upwards or downwards directed arrow indicates that the chemical or biochemical parameter mentioned at the bottom increases resp. decreases when the biological parameter becomes less favourable, as mentioned at the left of the figure.

period and smaller eggs are in accordance with this information. Vitamin A also plays an important role in positioning tissues and formation of the skeleton in developing embryos (Pijnappel *et al.* 1993). Apart from development and growth, vitamin A is also essential for the immune function, the integrity of epithelia which are barriers against infections, mucous secretion, and normal vision (Friedman and Sklan 1989; Wobeser and Kost 1992).

The two opposite mechanisms influencing plasma retinol levels upon PHAH exposure, make plasma retinol level alone an unsuitable indicator of PHAH-effects.

Thyroid Hormones

Plasma thyroid hormone levels were significantly less in chicks from eggs needing a prolonged incubation period (TT3, TT4, FT4), in smaller chicks (FT4; TT3 and TT4 a trend), and in chicks from clutches with later layed eggs (TT3 a trend). Also for chicks incubated in the laboratory a negative correlation was found between incubation period and plasma TT3 levels (trend, Murk *et al.* 1994b). Reduction in plasma thyroid hormone levels has often been associated with PHAH-exposure in *in vivo* studies with mammals,

birds and fish, both directly via influencing the metabolism and via hydroxylated PHAH metabolites as mentioned above (Leatherland and Sonstegard 1978; Brouwer *et al.* 1990; Morse *et al.* 1992; Brouwer 1991; Murk *et al.* 1994c). As thyroid hormones play an important role in various physiological processes including energy metabolism, reproduction, development, differentiation and growth (Sharp and Klandorf 1985), the observed effects could be related to reduced thyroid hormone levels.

Breeding Biology

The incubation period is significantly prolonged for eggs from clutches with later laying dates. This is not unusual, as more experienced birds lay their eggs earlier and incubate more efficiently (Hays 1978; Nisbet 1978). Additionally, a greater quality of courtship feeding by the male induces earlier egg-laying and larger eggs and clutches (Nisbet 1973, 1977). However, the observation that eggs from clutches with prolonged incubation periods in both the field and the laboratory have significantly more PHAHs and greater EROD activities suggest that PHAH contamination also plays a role. This could be through influencing vitamin A and thyroid hormone metabolism as was explained earlier. Reduced vitamin A and thyroid hormone levels in a female tern will not only influence egg formation, but also the amount of vitamin A and thyroid hormone passed on to the egg. It would interesting to know whether PHAH exposure also influences the courtship feeding behaviour of the male.

With increasing incubation period of the second egg in the laboratory, the incubation period of the first egg in the field increases even more (Figure 4.3). This suggests that apart from a factor intrinsic to the egg, an additional factor influences the incubation period in the field. This is in accordance with the observation that nest attentiveness and incubation behaviour of herring gulls were negatively correlated with the organochlorine content of the eggs (Fox *et al.* 1978). Also Kubiak *et al.* (1989) demonstrated for Forster's terns that not only factors intrinsic to the egg, but also parental attentiveness impaired reproductive outcome from a PHAH-contaminated site. In their study the differences in mean incubation periods between the contaminated and clean site were, respectively, 4.6 and 8.3 days for laboratory and field incubation. In our study these differences were less extreme, respectively, 2.0 and 4.0 days between the short and long incubation groups in the laboratory (Murk *et al.* 1994b) and the field (Table 4.5). A prolonged incubation period must be considered as adverse, as it imposes a greater risk for the eggs and costs more energy for the adult terns and chicks.

A trend is visible that eggs from clutches needing a prolonged incubation period were smaller and produced smaller chicks. Usually larger eggs need a prolonged incubation period. However, if the reduced egg volume is a consequence of PHAH-

exposure it can be expected that this exposure also increase the incubation period, possibly via decreased vitamin A and thyroid hormone levels.

Consequences for Reproduction

During our study the dynamic environment of the terns had more severe detrimental effects on breeding success than PHAHs, which is in accordance with the results of Becker *et al.* (1991). However, the observed correlations using the whole data set, and the differences in group averages found after clustering colonies after the PHAH-patterns, suggest that in the Dutch situation PHAHs or other contaminants present in the same gradient, have an adverse effect on the measured breeding biology parameters of common terns. It cannot be excluded that the more subtle correlations observed in our study will be more obvious and relatively important under circumstances without the strong adverse ecological and climatological factors. Data on hatching and fledging success are needed, integrating the effects of the quality of the egg, the incubation behaviour of the adult terns, and (with fledging success) direct effects of PHAHs from the food on the chicks. If a parallel can be drawn with mammals, it is to be expected that chicks will be more vulnerable for exposure to PHAHs *in ovo* than post-hatching, as in this period tissues are being formed and hormone levels are being 'fine-tuned'. Even a relatively small maternal dose of the PCB-mixture Aroclor 1254, has been found to result in long-term alterations in retinoid status in the offspring of rats (Morse and Brouwer, 1995) .

Choice of Species for Future Ecotoxicological Field Research

In the effect chain: PHAH-concentration --> biochemical effect in an individual --> effect on population level, the variation in the parameters measured increases continuously. Not only the PHAHs measured, but also other substances are able to act through the same mechanism of action. Biochemical responses are not only influenced by contaminants but also by factors such as physiological condition (age, sex, reproduction, etc), environmental conditions and food quality. The effect at the population level is easily masked by ecological factors such as massive predation, severe flooding, and extreme weather conditions. If the goal of a study is to establish whether effects of substances on populations occur, it is important to choose a suitable species for the specific compounds and effects of interest, but not so vulnerable for other, drastic influences. For reproduction studies, tree-nesting birds are more suitable than common terns which breed on the ground close to the waterline. Additionally, it is important that the exposure of the species studied is site specific. Species that stay in the same area all year around and/or feed on bigger fishes that are present all year around, better reflect

the local degree of pollution than species, such as the common tern, that feed on relatively small and migrating fishes.

Use of Biomarkers for Future Research

A correlation between the concentration of a certain class of chemicals and an observed adverse biological effect is just an indication that a causal relationship may exist. The use of biomarkers for exposure and effect can help to establish a causal relationship between a certain class of pollutants and an observed adverse biological effect. Biomarkers for exposure will indicate the potency of the whole mixture of, often partially unknown, compounds that an organism is exposed to. This presents a more realistic measure of the exposure, including interactions between the compounds, than measurement of some individual compounds and calculating the total toxicity. Examples are measurement of EROD-derived TEQs (Tillitt *et al.* 1992) and of luciferase produced by Ah-receptor active compounds (such as PHAHs and PAHs) using the CALUX-assay (chemical activated luciferase expression). The CALUX-assay has already been developed for small samples of blood plasma, sediment and interstitial water (Murk *et al.*, 1996b,e). Biomarkers of effect should be developed and validated in experimental studies, where hormonal and physiological consequences of different dosages of a pollutant of interest can be assessed, while all other factors are kept constant (Brouwer *et al.* 1990). The biological consequences of hormonal and physiological alterations must then be studied under field conditions. A complicating factor is that endocrine disrupters may only influence physiological functions under specific stress circumstances. Therefore, at the best, it will be possible to indicate critical levels for physiological conditions (such as vitamin A or thyroid hormone) or pollutants. Due to unpredictable and complex ecological circumstances, however, it will be impossible to foretell exact population effects if certain levels are reached.

ACKNOWLEDGEMENTS

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CHAPTER 5

IN VITRO METABOLISM OF 3,3',4,4'-TETRACHLOROBIPHENYL IN RELATION TO ETHOXYRESORUFIN-O-DEETHYLASE ACTIVITY IN LIVER MICROSOMES OF SOME WILDLIFE SPECIES AND RAT*

ABTRACT

A qualitative study was performed of the capacity of hepatic microsomes of several wildlife species to metabolize 3,3',4,4'-tetrachlorobiphenyl (TCB). Hepatic microsomes of species environmentally exposed to polychlorinated biphenyls (PCBs): harbour porpoise (*Phocoena phocoena*), harbour seal (*Phoca vitulina*), common tern (*Sterna hirundo*), and hepatic microsomes from species experimentally exposed to PCBs: eider duck (*Somateria mollissima*), rainbow trout (*Salmo gairdneri*), flounder (*Platichthys flesus*), and Wistar rat were incubated with ^{14}C labeled TCB ([^{14}C]TCB). The mammals and birds were able to metabolize TCB at a rate that was correlated with their ethoxyresorufin-O-deethylase (EROD) activity. No [^{14}C]TCB metabolism was observed in the fish, despite elevated EROD activity in the trout. HPLC analysis of diisopropylether extracts of the microsomal incubations indicated the presence of 4-OH-, 5-OH-, and 6-OH-tetrachlorobiphenyl metabolites and a yet unidentified metabolite. The ratio of the different hydroxy metabolites formed varied for the various species.

**Based on: A.J. Murk, D. Morse, J. Boon, A. Brouwer (1994). In vitro metabolism of 3,3',4,4'-tetrachlorobiphenyl in relation to ethoxyresorufin-o-deethylase activity in liver microsomes of some wildlife species and rat. Eur. J. Pharmacol., Environ. Pharmacol. Sect. 270: 253-261.*

INTRODUCTION

Polychlorinated biphenyls (PCBs) are ubiquitous environmental pollutants that accumulate in the environment due to their great lipophilicity and resistance to biodegradation (IPCS, 1993). Due to additional biomagnification, greatest concentrations of PCBs have been found in body lipids of lung breathing top predators of especially the aquatic food chain, such as fish eating birds and mammals. Although internal PCB concentrations increase with the trophic level, the pattern of individual PCB congeners is considerably different in sediments and in species of different trophic levels (Muir *et al.*, 1988; Boon *et al.*, 1989). In general, lesser chlorinated PCB congeners (tetra and less chlorine substitutions) disappear progressively from the congener patterns with increasing trophic level, due to biotransformation. As a general rule, PCB congeners with unsubstituted adjacent *meta* and *para* positions, and congeners with unsubstituted adjacent *ortho*- and *meta*-positions and less than one *ortho*-chlorine are most susceptible to oxidative metabolism, catalysed predominantly by cytochrome P450-dependent monooxygenases (Walker, 1992; Boon *et al.*, 1992).

Cytochrome P450 IA1 (P450IA1) can be induced by several PCB congeners, mediated by the arylhydrocarbon (Ah) receptor. Non-*ortho*-chlorine-substituted PCB-congeners such as TCB (CB-77), 3,4,5,3',4'-pentaCB (CB-126) and 3,4,5,3',4',5'-hexaCB (CB-169), are the most potent PCB congeners with respect to induction of P450IA1 and IA2 and its associated ethoxyresorufin-*O*-deethylase (EROD) activity (Goldstein, 1989; Safe, 1990). P450IA1 is an efficient enzyme involved in the oxidative biotransformation of non-*ortho*-PCBs (Ishida *et al.*, 1991). Non-planar PCBs are mainly metabolized by cytochrome P450 IIB. Several phenolic PCB metabolites and mercapturic acid pathway metabolites, such as methyl sulphonyl PCBs, have been identified in various laboratory species. For example, Darnerud *et al.* (1986) identified a number of hydroxy (OH-) metabolites and a methylsulphone metabolite of TCB in C57/Bl mice and their fetuses. Bergman *et al.* (1992) showed that methylsulphonyl PCBs are relatively persistent metabolites in mink. Yoshimura *et al.* (1987) reported several hydroxylated metabolites of TCB in rats.

Several studies over the past eight years have indicated that phenolic metabolites of PCBs are not just inert intermediate metabolites of PCBs, but do possess metabolite-specific toxic potencies. Stucture requirements for metabolite toxicity are different from those of the parent compounds, planar metabolites are not necessarily more toxic than the non-planar ones (Lans et al., 1993). Hydroxylated PCB metabolites have been found to competitively displace thyroxine from its binding site on the plasma carrier protein transthyretin (TTR), thus enhancing the elimination of thyroxine from the circulation

(Brouwer, 1987; Lans *et al.*, 1993). Hydroxylated PCB metabolites have also been shown to inhibit hepatic thyroxine 5'-deiodinase (Rickenbacher *et al.*, 1989, Adams *et al.*, 1990), interact with oestrogen receptors (Korach *et al.*, 1988), and to uncouple oxidative phosphorylation in mitochondria (Ebner and Braselton, 1987) whereas TCB itself has no such effects (Lans *et al.*, 1990).

The ability of a species to metabolize specific PCB congeners, has mainly been estimated from differences in congener composition of food with that of the predators (Borlakoglu *et al*, 1989; Tanabe *et al.*, 1988; Boon *et al.*, 1992). A limitation of this approach is that it is indirect, not all congeners can easily be separated from each other by gas-chromatography analysis and it is not known what metabolites are being formed. The relatively toxic planar TCB, for example, could not be quantified in most experiments because it co-elutes with the more abundant 2,3,3',4',6-pentaCB (CB-110). No data have been reported on *in vitro* biotransformation of TCB in hepatic microsomes of marine mammals, fish eating birds, and fish. Study of the metabolism of a toxic CB congener in addition to analysing the model substrate EROD, has the additional advantage of being relevant to the possible occurence of the above-mentioned metabolite specific toxic effects in certain species.

Here we report on a qualitative comparative study for *in vitro* metabolism of ^{14}C labeled 3,4,3',4'-tetraCB ([^{14}C]TCB) by hepatic microsomes of several wildlife species. Some of these species, such as harbour seal (*Phoca vitulina*), harbour porpoise (*Phocoena phocoena*) and common tern (*Sterna hirundo*), were environmentally exposed to PCBs and other substances. Also hepatic microsomes from experimentally PCB-exposed eider duck (*Somateria mollissima*), rainbow trout (*Salmo gairdneri*) and flounder (*Platichthys flesus*) were used in this study. The amount and type of OH-metabolites formed were quantified with high performance liquid chromatography (HPLC) and compared with the EROD activities measured in the same microsomes.

Materials and Methods

Origin of microsomes

The liver microsomes used in this experiment originated from environmentally and experimentally exposed species.

Environmentally exposed species

Harbour seal and harbour porpoise were washed ashore alive at the beach of the island of Texel, The Netherlands. The adult male harbour seal was extremely lean, with the

blubber layer completely depleted. The liver contained 130 mg CB-153·mg^{-1} lipid, the blubber 128 and the blood 399 mg CB-153·kg^{-1} lipid. The adult female harbour porpoise had concentrations of 48.9 mg CB-153·kg^{-1} lipid in the liver, 19.2 in the blubber and 87.8 mg·kg^{-1} lipid in the blood. More information about these animals (respectively referred to as HS2 and HP1) is given in Boon *et al.* (1994). The animals died at the mammal rehabilitation centre 'Ecomare', where the livers were removed within 2 hr after death.
Common tern microsomes were obtained from one-day-old chicks, artificially bred from eggs collected in colonies with different degrees of PCB contamination. As only 100 µl of microsomes was avaible per individual for this metabolism study, microsomes were pooled to get 2 portions of 1 mg microsomal protein each. Three pools of microsomes were created, from either 5 animals with a relatively great (around 147 mg CB-153·kg^{-1} yolksac lipid, tern 1), 4 with an intermediate (around 86 mg CB-153·kg^{-1} yolksac lipid, tern 2), and 4 with a lesser mean PCB concentration (around 65 mg CB-153·kg^{-1} lipid, tern 3) (Table 5.1). PCB, PCDF and TCDD concentrations and EROD activities are described in Bosveld *et al.* (1993), the biochemical effects found are described in Murk *et al.* (1994b).

Experimentally exposed species
Eider duck microsomes were prepared from livers of 38-day-old animals killed 10 days after i.p. exposure to 200 mg.kg^{-1} bodyweight (bw) of the commercial PCB mixture Clophen A50 or to 50 mg TCB·kg^{-1} bw (Murk *et al.* 1994a).
Trout microsomes originated from an experiment where trout were orally dosed with 54 or 215 mg Clophen A50·kg^{-1} bw. The animals were killed 10 or 20 days after exposure. Samples of four different animals were used: 215 mg·kg^{-1}, 10 days (trout 1); 54 mg·kg^{-1}, 10 days (trout 2); 215 mg·kg^{-1}, 20 days (trout 3); and 54 mg·kg^{-1}, 20 days (trout 4).
An one-year-old **Flounder** killed 10 days after i.p. exposure to 500 mg Clophen A50·kg^{-1}. Microsomes with the relatively greatest EROD activity were used for this study.
Rat microsomes were obtained from a 16-week-old Wistar rat, that had been given 3 daily i.p. administrations of 30 mg ß-naphtoflavone·kg^{-1} bw, and was killed 24 hr after the last treatment. These microsomes were used as a positive control, since [^{14}C]TCB metabolism has already been characterized in this species (Morse *et al.*, 1995a).

Preparation of microsomes, and measurement of microsomal protein concentrations and EROD activity
Microsomes were prepared according to the method described by Gibson and Skett (1986), and immediately stored at -80°C until further analysis.
Protein concentrations were measured according to the method of Bradford (1976) using

the Bio-Rad protein assay reagent. Measurement of EROD activity was performed as described by Prough *et al.* (1978) at a temperature of 37°C.

In vitro [^{14}C]TCB metabolism and metabolite analysis

[^{14}C]TCB obtained from Sigma Chemical Co., with a specific activity of 37.1 mCi·mmol $^{-1}$ was purified according to the method of Morse *et al.* (1995a). Four hydroxylated metabolites, 2-hydroxy-3,4,3',4'-TCB (2-OH-TCB), 4-hydroxy-3,5,3',4'-tetraCB (4-OH-tetraCB) 5-hydroxy-3,4,3',4'-TCB (5-OH-TCB) and 6-hydroxy-3,4,3',4'-TCB (6-OH-TCB), kindly donated by Dr E Klasson Wehler (Wallenberg Laboratory, Stockholm, Sweden) were used as internal standards for HPLC analysis.

Microsomal incubations and HPLC analysis of TCB metabolites were performed according to the method of Morse *et al.* (1995a). Duplicate portions of 1 mg hepatic microsomal suspension with 10μM [^{14}C]TCB (added in 25 μl acetone) and 50 nM Tris-HCl buffer (pH = 7.5) with a total volume of 900 μl were pre-incubated in glass tubes for 2 min at 37°C. The reaction was started by adding 100 μl of a 10 mM NADPH solution. After 5 min 1 ml ice-cold methanol was added to the incubation mixture. TCB and metabolites were extracted four times with 2 ml of diisopropylether. The ether extract was pooled and dried under nitrogen gas and resuspended in 50 μl of methanol. The samples were stored at -20°C until further analysis. The recovery of mono-hydroxy-TCB (OH-TCB) metabolites using this extraction method was on average 94 $\pm$ 1 % (Morse *et al.*, 1995a).

Aliquots of 20 μl methanol extract enriched with the four standard metabolites were injected on a HPLC on a reversed phase column (Perkin Elmer 30x3 mm coupled to a 83x3 C-18 3 μm column). Separation of metabolites and parent TCB was performed with 78% methanol and 22% water for 15 min followed by 100% methanol for 16 min. Flow rate was 0.8 ml·min^{-1}min and 0.4 min fractions were collected with a Pharmacia fraction collector (LKB.RediFrac). Scintillation fluid (4.5 ml, Ultima-Gold, Packard) was added to the collected fractions and they were counted in a Packard liquid scintillation counter (1600 TR) for a period of 5 min. The pattern of radioactive peaks was compared to the position of the standard metabolites on the UV-absorbance chromatogram (265 nm).

RESULTS

EROD activity

Hepatic microsomal EROD activities differed greatly among the species tested (Table 5.1). EROD activity was greatest in microsomes from the rat (2290 pmol·mg^{-1}·min^{-1}) and the

Table 5.1 Per sample, the EROD activity, total amount of metabolites formed, relative amount of individual metabolites, and ratio amount of metabolites formed/ethoxyresorufin metabolized.

Animal	Exposure (See also M&M)	EROD pmol/mg.m	-▲TCB[a] pmol/mg.mi	4-OH-TCB %[b]	5-OH-TCB %[b]	6-OH-TCB %[b]	unknown %[b]	Ratio (%) ▲TCB/EROD[c]
Porpoise	Environment	471	39.6	20.1	32.9	29.25	17.75	8.4
Seal	Environment	2460	39.7	18	53.8	19.3	8.9	1.6
Tern 1	Environment	130	1.37	10	73.9	14.6	–	1.1
Tern 2	Environment	45	0.94	–	–	–	–	2.1
Tern 3	Environment	11	0.62	–	–	–	–	5.6
Eider 1	CloA50	616	20.7	9.9	76.1	6.9	7.1	3.4
Eider 2	TCB	1050	46.9	9.8	74.4	6.3	9.5	4.5
Trout 1	CloA50(215;10d)[d]	336	0	–	–	–	–	0.0
Trout 2	CloA50(54;10d)[d]	328	0	–	–	–	–	0.0
Trout 3	CloA50(215;20d)[d]	252	0	–	–	–	–	0.0
Trout 4	CloA50(54;20d)[d]	259	0	–	–	–	–	0.0
Flounder	CloA50(500;10d)[d]	2	0	–	–	–	–	0.0
Rat	ß-naphtoflavone	2290	91.4	27.9	23.5	31.3	19.6	4.0

[a] Amount of TCB metabolized
[b] Relative amount of specific hydroxylated metabolites
[c] Ratio between metabolism of TCB over EROD activity
[d] Clophen A50 (dose in mg/kg; number of days of exposure)

environmentally exposed harbour seal (2460 pmol·mg^{-1}·min^{-1}). The EROD activity of the TCB dosed eider was 1050 pmol·mg^{-1}·min^{-1}. Microsomes from the environmentally exposed harbour porpoise and Clophen A50 exposed trout showed intermediate EROD activity (250-450 pmol·mg^{-1}·min^{-1}). Low EROD activities (2-130 pmol·mg^{-1}·min^{-1}) were found in the microsomes of the Clophen A50 exposed flounder and in the environmentally exposed tern chicks.

Total amount of hydroxy-[^{14}C]TCB metabolites formed

The rates of formation of hydroxy-TCB metabolites is given in Table 5.1. Of the species tested, rat microsomes showed the greatest rate of OH-TCB formation, followed by the environmentally exposed porpoise and seal and the experimentally exposed eiders. Although the EROD activity was more than 5-fold greater in the seal than in porpoise microsomes, the rate of metabolite formation was the same for both species. The microsomes of common tern exhibited only limited conversion rates of TCB (between 0.62 and 1.37 pmol·mg^{-1}·min^{-1}). The TCB dosed eider showed the greatest production of metabolites (46.9 pmol·mg^{-1}·min^{-1}), about half of the production of the BNF induced rat microsomes (91.4 pmol TCB·mg^{-1}·min^{-1}). A striking result was the complete absence of metabolite formation in the trout samples, which showed intermediate EROD activity. The EROD activity in the flounder microsomes was very low, and no metabolite formation was observed.

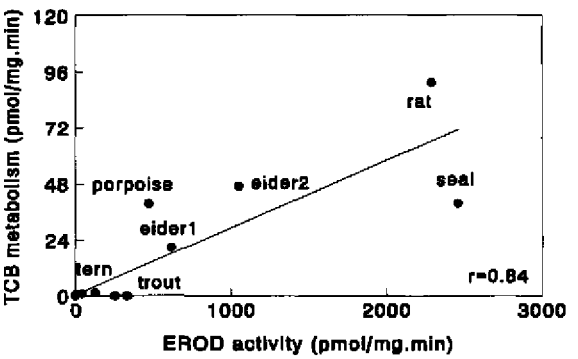
The ratio (x 100) of OH-TCB metabolites formed (pmol·mg^{-1}·min^{-1}) to EROD activity (pmol resorufin·mg^{-1}·min^{-1})(OH-TCB/EROD) of the rat was the same as the mean ratio of both eiders (Table 5.1). The OH-TCB/EROD ratio was greatest in the porpoise (8.4) and least in the tern (1.1 -5.6). The ratio OH-TCB/EROD of the tern microsomes increased with decreasing EROD activity. However, the total amount of metabolites was just at the level of detection in tern 2 and 3. Figure 5.1 shows the correlation of the rate of formation of OH-TCB metabolites with the EROD activity of the microsomes of all species tested.

Relative amount of individual hydroxy-TCB metabolites

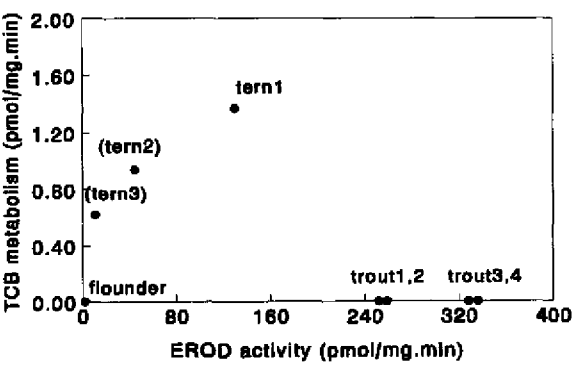
The standard OH-TCB metabolites 2-OH-TCB, 4-OH-tetraCB, 5-OH-TCB and 6-OH-TCB were well separated from each other and the parent compound under the chromatographic conditions used. The sequence of eluted compounds was: 4-OH-tetraCB, 2-OH-TCB, 5-OH-TCB, 6-OH-TCB and finally the parent compound. Figure 5.2 (a-f) gives the OH-tetraCB metabolites extracted from microsomal incubations of different species. No radioactivity was observed at the retention time of 2-OH-TCB (arrows in Figure 5.2). In most chromatograms also a yet unidentified metabolite is visible. Although the total

amount of metabolites could just be detected for terns 2 and 3, this amount was too small to determine the formation of individual metabolites.

The relative contribution of the individual metabolites to the total amount of metabolites is given in Table 5.1. In the microsomal incubations of ^{14}C-TCB in most species five peaks of radioactivity were visible in the HPLC chromatograms, which co-eluted with, respectively, 4-OH-tetraCB, 5-OH-TCB and 6-OH-TCB, an unknown peak and TCB itself. The main OH-TCB metabolite formed is the 5-OH-TCB. This is particularly evident in microsomal incubations of the eider ducks and common tern (± 75%) and the common seal (53.8%). Porpoise and rat microsomes produced roughly equal amounts of 4-OH-tetraCB, 5-OH-TCB, and 6-OH-TCB. Little or no radioactivity was observed other than to TCB in the HPLC chromatograms of trout and flounder microsomal incubations.



(a)



(b)

Figure 5.1 Relationship between ethoxyresorufin-O-deethylase (EROD) activity and amount of metabolites formed by microsomes of various species. (a) all species (b) enlargement of the lower left corner of graph 1a. The TCB metabolism of (tern2) and (tern3) is not very precise.

Discussion

Apart from those of the fish, hepatic microsomes of all animals investigated were able to metabolize TCB *in vitro*. Metabolites were found that co-eluted with 4-OH-tetraCB, 5-OH-TCB and 6-OH-TCB standards, and one unidentified less polar metabolite. No radioactivity was observed at the retention time of 2-OH-TCB. Although the microsomes originated from animals with different conditions and exposures, there was a good correlation between total OH-TCB production and EROD activity in the hepatic microsomes tested, apart from the trout microsomes.

The 5-OH-TCB was the major metabolite formed in the birds (75%) and marine mammals tested (33-54%). Kannan and co-workers (1989a) calculated for PCBs that the carbon position with the greatest frontier (π) electron density is most readily hydroxylated. In a non-chlorinated biphenyl this is the *para* position. If the carbon with the greatest frontier electrons is occupied by chlorine, either a rearrangement occurs (NIH shift) or the carbon with the next greatest electron density is activated for metabolism. For TCB *ortho*-positions have the most favourable electron density, but due to steric hindrance they are least oxidized. The 5,5' positions have the next greatest electron density, which is in accordance with 5-OH-TCB being the principal metabolite found in birds. However, in the rat and porpoise the chlorine shift metabolite 4-OH-tetraCB and the *ortho*-hydroxylated 6-OH-TCB were present in amounts almost equal to those of the 5-OH metabolite. This suggests different substrate specificities for the Cytochrome P450IA-isoenzyme homologues between the species investigated.

Mammals

Most information about TCB metabolite formation by mammals is from studies with laboratory rodents. Ishida *et al.* (1991) showed that 4-OH-tetraCB and 5-OH-TCB were produced at a ratio of 2.2:1 *in vitro* in rat liver microsomes. In our experiment this ratio was 1.2:1. In an *in vivo* experiment, the amount of the 5-OH-TCB in rat faeces was a little larger than that of 4-OH-tetraCB in the first five days after exposure. This discrepancy can be explained by a previous finding that the 5-OH-TCB is excreted much more rapidly than the 4-OH-tetraCB (Yoshimura *et al.*, 1987). Also in mice major metabolites of TCB were 5-OH-TCB and 4-OH-tetraCB. In adipose tissue Klasson Wehler *et al.* (1989) found TCB as well as 4-OH-, 5-OH- and 6-OH-tetraCB.

In our study TCB, which possesses adjacent chlorinated *meta* and *para* positions, is metabolized *in vitro* by both the porpoise and the seal. Tanabe *et al.* (1988) concluded based on a comparison of tissue PCB concentrations, that small cetaceans had no capacity to metabolize PCBs with adjacent non-chlorinated *meta* and *para* positions. This is not

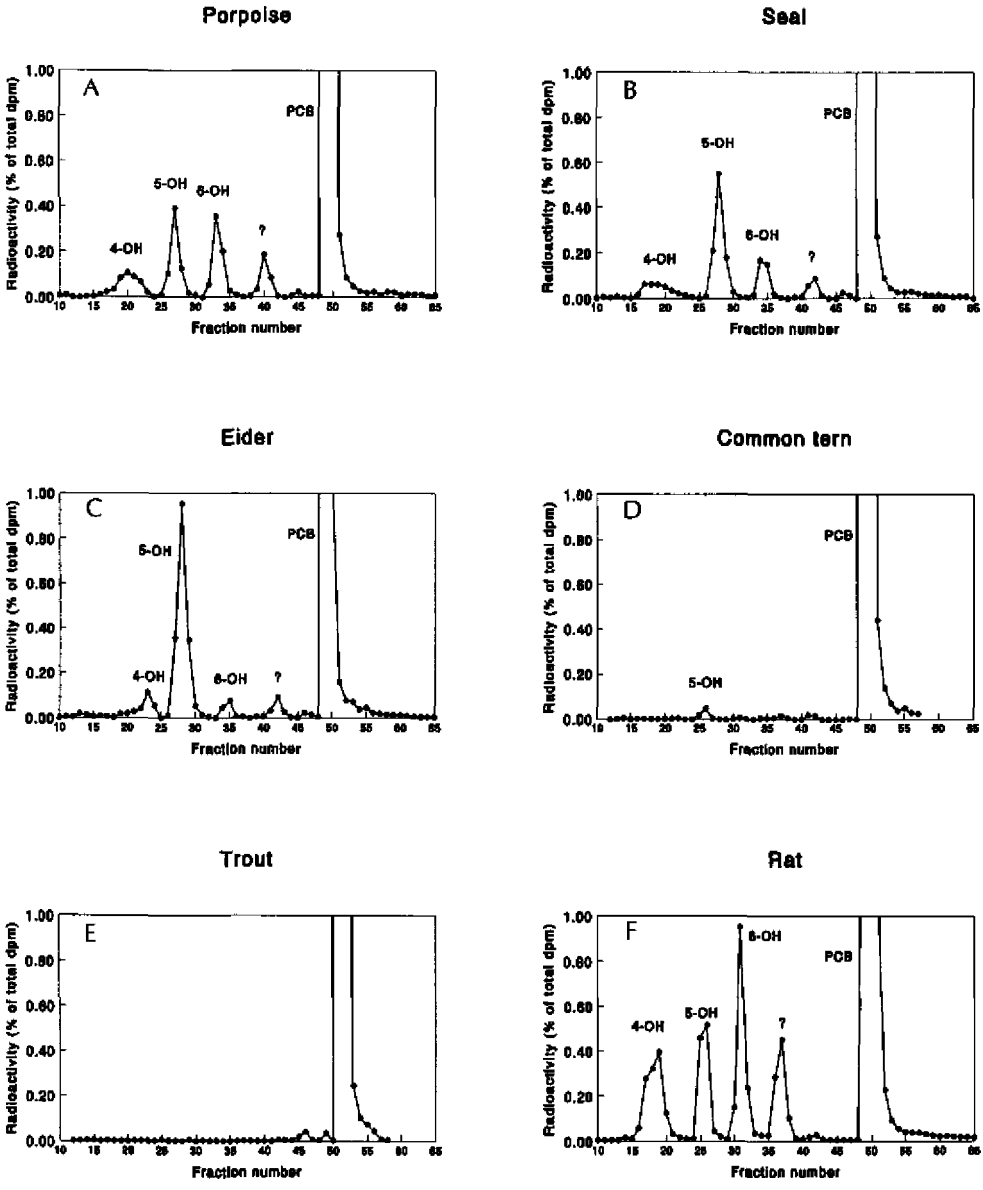


Figure 5.2 The characteristic pattern of TCB and its metabolites per species: porpoise, seal, tern (#1), eider (#2), trout , rat . The codes 4-OH, 5-OH and 6-OH indicate the position of the standard hydroxy-TCB metabolites on the corresponding chromatogram. The arrow indicates the position of the 2-OH-TCB standard.

in contradiction with our data, although no data were available on TCB. The same goes for the conclusion of Boon *et al.* (1992) that seals can metabolize PCBs with a planar configuration when there are adjacent H-atoms present in the *ortho*- and *meta*-positions. Our *in vitro* data suggest that the metabolic capacity of the environmentally exposed porpoise and seal is similar to that of the eiders, and about twice as small as the experimentally induced rat. This is in contradiction with Tanabe *et al.* (1988) who suggested that the capacity of PCB metabolism of small cetaceans (such as porpoises and seals) is extremely small as compared to those of birds and mammals. Kannan *et al.* (1989b) also concluded that terrestrial organisms are better equipped metabolically than marine mammals in degrading xenobiotics. We found that the rat metabolizes about twice as much TCB as the marine mammals. This could be due to a greater exposure to 'animal-plant warfare' (Nebert *et al.*, 1989) of the omnivorous rat compared to the piscivorous marine mammals.

Birds

The only information about TCB metabolism by birds is from chicken embryos and pigeons. Analysis of bile from chicken embryos by Klasson Wehler *et al.* (1990) demonstrated that six days after injection of 0.3 mg [^{14}C]TCB·kg^{-1} egg into the airsac, 4% of the dose was detected in the bile. Three conjugated OH-metabolites were identified: 5-OH-TCB (234 ng per gall bladder), 4-OH-tetraCB (45 ng per gall bladder) and 2-OH-TCB (3 ng per gall bladder). The presence of a dihydroxy-tetrachlorobiphenyl and a dihydroxy-trichlorobiphenyl was also indicated. Borlakoglu *et al.* (1991) also found di-hydroxylated PCB metabolites in Aroclor 1254 treated pigeons and rats. They showed that induction of isoforms of P450 by PCBs results in an enhanced secondary hydroxylation of the mono-hydroxylated metabolites. In our incubations, however, only mono-hydroxylated metabolites were formed. The unknown peak is not polar enough to be di-hydroxylated PCB metabolite. This difference could be due to the differences in incubation time: 60 min against 5 min in our experiment.

On the basis of comparison of PCB congeners in Aroclor and in fish eating sea birds, Boon *et al.* (1989), Borlakoglu *et al.* (1990), Roozemeijer *et al.* (1991) and Walker (1992) concluded that only PCB congeners with unsubstituted adjacent *meta* and *para* positions can be metabolized by birds. Thus, congeners with only unsubstituted adjacent *ortho*- and *meta*-positions appeared to be metabolizable in marine mammals (when no more than one *ortho*-Cl atom is present) but not in birds. These conclusions in regard to birds, however, were only based on measurements of mono-*ortho*-substituted congeners with *ortho-meta* vicinal H-atoms (2,3,3',4,4'-PCB (CB-105), 2,3',4,4',5-PCB (CB-118) and 2,3,3',4,4',5-HCB (CB-156)), because congeners with *ortho-meta* vicinal H-atoms without

ortho-Cl substitution (such as 3,3',4,4'-TCB and 3,3',4,4',5-PCB (CB-126)) were not investigated due to GC separation problems for these compounds, and the relatively small environmental concentrations. The results of the present study show, however, that TCB is rapidly metabolized by the eider hepatic microsomes. Thus, the differences observed in PCB congener patterns *in vivo* may only involve the mono-*ortho*-substituted congeners with *ortho-meta* vicinal H-atoms.

Although predatory birds were expected to have lesser P450 activities than mammals (Walker, 1980), the eider microsomes in this experiment showed great EROD activities and were very well capable of metabolizing TCB. The rate of metabolism was similar to that of the microsomes of the marine mammals tested.

Fish

We observed that exposure of trout to PCBs induced EROD activity. This is in accordance with Melancon and Lech (1983) who found that TCB does induce EROD activity in trout in a dose dependent way. We found, however, that the induced trout were not able to metabolize TCB. Apparently the substrate specificity of P450IA is different between fish and birds and mammals. Two main factors are responsible for substrate specificity towards the cytochromes P450. First the molecular dimensions which determine whether a chemical fits into the binding site. Second the enzyme-substrate complex has to be activated to permit the oxidative metabolism of the substrate (Parke *et al.*, 1990). The last factor involves the position of the frontier molecular orbitals in relation to the shape of the active site of the P450 enzyme. Parke *et al.* (1990) show in a plot of the molecular shape parameter (area/depth^2) against electronic parameter (ΔE) for a number of chemicals metabolized by P450 I, IIB, IIE and IV, that clusters can be observed of chemicals metabolized by certain P450 iso-enzymes. It is conceivable that due to the evolutionary distances the active sites of fish P450IA and mammal/bird P450IA have slightly different requirements for those parameters. As a result ethoxyresorufin could answer the demands of fish P450IA but TCB could not. Additionally it has been demonstrated that, contrary to birds and mammals, fish do not have two iso-enzymes of P450IA (IA1 and IA2) but only one (IA1) that can be induced by planar PCBs (Goksøyr and Förlin, 1992; Stegeman and Kloepper-Sams, 1987). Possibly, ethoxyresorufin is metabolized by IA1 and TCB by IA2. For fish no data on TCB metabolism is available. Of the PCB congener 2,5,2',5'-tetraCB, it was observed that 4-OH-glucuronides were excreted by trout into the bile (Melancon and Lech, 1976). Recent experiments in our laboratory suggest that flounders have little Ah-receptor and cytochrome P4501A activity is vulnerable for substrate inhibition (Besselink *et al.*, 1993, pers. comm.). The EROD-activity in flounder probably

was blocked by the excess of PCBs.

Our findings of the inability of trout and flounders to metabolize TCB is in accordance with the findings of De Boer *et al.* (in press). They analysed the concentrations of some non-*ortho*- and mono-*ortho*-substituted chlorobiphenyls and chlorinated dibenzo-*p*-dioxins and dibenzofurans in 14 marine and freshwater fish species and 4 shellfish species from The Netherlands. In all fish and shellfish but the yellow eel (*Anquilla anquilla*), TCB concentrations were present in two-fold greater relative concentrations than in the technical PCB mixture Aroclor 1254, while the pattern of the other non-*ortho*-substituted PCBs was rather similar to that of Aroclor 1254. This indicates that apart from the eel none of the tested (shell)fish species, including the flounder, can metabolize TCB.

Possible consequences of TCB metabolism

The ability of animals to metabolize PCBs can lead to an increased excretion of these congeners and accordingly to decreased toxicity associated with the parent compound. However, OH-PCB metabolites have been shown to exhibit biochemical effects of their own. With *in vivo* (Brouwer *et al.* 1990a) and *in vitro* experiments (Lans *et al.*, 1994) it has been demonstrated that various hydroxy-polychlorobiphenyls (and -dibenzo-*p*-dioxins and -dibenzofurans) show a competitive interaction with transthyretin (TTR), a plasma transport protein of thyroxine. The greatest competitive binding potency was observed for hydroxylated PCB congeners with the OH-group substituted on *meta* or *para* positions and one or more chlorine atoms substituted adjacent to the hydroxy-group on either aromatic ring or both rings. The metabolites that were formed in our study all had a relative potency greater than that of the physiological ligand thyroxine (Lans *et al.*, in press). All hydroxylated PCBs identified in this study have also been shown to uncouple mitochondrial oxidative phosphorylation (Lans *et al.*, 1990).

Conclusions

From our experiments we conclude that although EROD activity is a measure of the exposure of animals to PCBs and related compounds, it is not a direct measure of the ability of a species to metabolize TCB. The structural requirements for PCB metabolism can be quite different between different animal species. Within the same species (apart from fishes) and when exposed under the same circumstances, there is a relationship between biotransformation of ethoxyresorufin and TCB.

The observed species differences in P450IA1 induction and amount and composition of metabolites formed from TCB may be a consequence of evolutionary

differences in which the divergence of the IA family into the isoforms IA1 and IA2 may play an important role. Also food specialization is expected to be of influence (Nebert *et al.*, 1989). The accompanying differences in detoxification capacity and potential toxicity of metabolites formed pose serious problems for extrapolation of toxicity data from one type of species to another. Identification of the characteristics that make a specific species vulnerable for the toxic actions of PCBs will help to find the species at risk.

ACKNOWLEDGEMENTS

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CHAPTER 6

CHEMICAL-ACTIVATED LUCIFERASE GENE EXPRESSION (*CALUX*): A NOVEL *IN VITRO* BIOASSAY FOR AH RECEPTOR ACTIVE COMPOUNDS IN SEDIMENTS AND PORE WATER*

ABSTRACT

This study demonstrates that the novel *in vitro CALUX* (chemical-activated luciferase expression) assay is a rapid, sensitive assay for assessing the toxic potency of (mixtures of) aryl hydrocarbon receptor (AhR)-active compounds in sediments and pore waters. A rat hepatoma (H4IIE) cell line, stably transfected with a construct containing the dioxin-responsive element (DRE) sequence and the luciferase reporter gene, was used to determine the relative potency or the total activities of AhR-active compounds in sediment and pore water extracts. This novel *CALUX* assay had a detection limit of 0.5 fmol of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The sensitivity and linear working range was slightly better than for the ethoxyresorufin O-deethylase (EROD) assay in H4IIE wild type cells. The primary improvement of the *CALUX* assay compared to the EROD assay however, is that the *CALUX* assay is insensitive to substrate inhibition. The *CALUX* activity induced by organic extracts from 450 mg aliquots of sediment or 250 μl aliquots of pore water, corresponded with the instrumentally analyzed degree of pollution of the sediment. Using pore water, only a simple and rapid extraction procedure was needed, without additional clean-up to prevent cell death. The response from pore water samples in an 8-day early life stage test with zebra fish (*Branchydanio rerio*) corresponded with the *CALUX* induction, although the correlation was sometimes disturbed by heavy metals. Two polychlorinated terphenyl mixtures, the PCB-substitute Ugilec 141, polybrominated diphenylethers, and the PCB-mixture Clophen A50 were tested in the *CALUX* assay, and had induction potencies that were 10^{-4}-10^{-7} compared to TCDD.

*Based on: Murk AJ, Legler J, Denison MS, Giesy JP, Van de Guchte C and Brouwer A (1996): Chemical-activated luciferase expression (CALUX): a novel in vitro bioassay for Ah receptor active compounds in sediments and pore water. Fundam. Appl. Toxicol. **33**, 1:149-160*

Introduction

Sediments at many locations in Dutch waters are heavily polluted with polyhalogenated aromatic hydrocarbons (PHAHs) such as polychlorodibenzo-*p*-dioxins (PCDDs), -dibenzofurans (PCDFs), and -biphenyls (PCBs). Of the large group of PHAHs, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the prototypical and most potent member. PHAHs are persistent and lipophilic and elicit a number of species-specific, toxic responses in vertebrates, including hepatoxicity, body weight loss, thymic atrophy, impairment of immune responses, dermal lesions, reproductive toxicity, alterations in vitamin A and thyroid hormone metabolism, teratogenicity and carcinogenesis (Poland and Knutson, 1982; Goldstein and Safe, 1989; Brouwer, 1991; Giesy et *al.*, 1994; Safe, 1994). Studies with aquatic organisms have concentrated mainly on fish. They have been found to be very sensitive to PHAH-induced toxicity, especially when exposed during early life stages, i.e., egg to larval stadium (Kleeman et *al.*, 1986). Typical toxic responses are malformations, hemorrhages and pericardial edema (Walker et *al.*, 1991, 1992). PHAH-contaminated sediments thus may pose a serious threat to aquatic species and their predators, including fish-eating birds, mammals, and humans. Therefore a rapid, inexpensive assay is needed for monitoring the toxic potency of a great number of samples. We feel that our novel *in vitro* bioassay, with chemical-activated luciferase expression (*CALUX*) based on the mechanism of action of PHAHs, will facilitate rapid assessment of the toxic potency of mixtures of PHAHs in environmental matrices.

Biomarkers for PHAHs

Of the hundreds of existing PHAHs, those with one or no *ortho*-substituted halogen can assume a planar configuration, making them approximate isostereomeres TCDD. The mechanism of action for these relatively toxic, planar PHAHs has been partially elucidated. After binding of the ligand to the cytosolar Aryl hydrocarbon Receptor (AhR), the ligand-receptor complex is activated and translocated to the nucleus, wherein it binds to a dioxin responsive element (DRE) and stimulate transcription of an adjacent gene (Denison and Whitlock, 1995). Examples of induced genes are the phase I and II enzymes cytochrome P450 1A and UDP-glucuronyltransferase (DeVito and Birnbaum, 1994). Although it is not known how cytochrome P450 1A activity contributes to specific PHAH-toxicity, its associated increase in ethoxyresorufin O-deethylase (EROD) activity is often studied *in vivo* and *in vitro* because it is altered in tandem with that of other enzymes and receptor proteins (Brouwer 1991; Tillitt et *al.*, 1991; Jones et *al.*; Eggens et *al.*, 1995). The EROD bioassay, however, poses some disadvantages, such as *in vivo* season-dependent fluctuations in inducibility, low enzyme stability after death of an animal, and

in vitro inhibited activity at greater ligand concentrations (Kennedy *et al.*, 1993; Sawyer *et al.*, 1984; Hahn *et al.*, 1993).

In our laboratories, an additional *in vitro* bioassay for PHAHs has been developed, based on AhR-mediated firefly (*Photinus pyralis*) luciferase gene expression (Aarts *et al.*, 1995; Garrison *et al.*, 1996). A vector containing the luciferase gene under transcriptional control of DREs isolated from the 5-flanking region of the mouse P450 1A1 gene, was stably transfected into a number of hepatoma cell lines, including mouse (Hepa1c1c7) and rat (H4IIE) cell lines. The induction of luciferase activity in the transfected cells, upon exposure to TCDD, is dose-dependent and comparable to that of the natural cytochrome P450 1A activity (Aarts *et al.*, 1995; Garrison *et al.*, 1996, Sanderson *et al.*, 1996).

For hazard and risk assessment purposes of mixtures of PHAHs, the relative toxic potencies of individual PHAH congeners compared to TCDD have been transformed into toxic equivalency factors (TEFs). The concentrations of the individual congeners measured, multiplied by their respective TEFs, are added up to give the total TCDD toxic equivalency (TEQ) of the mixture (Safe, 1990; Ahlborg *et al.*, 1992). For the PCDD-, PCDF- and PCB-congeners tested so far, the potency to induce *CALUX* activity relative to TCDD (*CALUX*-TEF) has been in accordance with reported TEF values (Denison *et al.*, 1993; Aarts *et al.*, 1995; Garrison *et al.*, 1996; Sanderson *et al.*, 1996).

This study

In this paper we describe the use of the *CALUX* assay for monitoring AhR-active compounds associated with sediments. In addition to PCBs, PCDDs and PCDFs, otherPHAHs, such as polychlorinated terphenyls (PCTs) (Watanabe *et al.*, 1987; De Boer, 1995), tetraclorobenzyltoluenes (the PCB-substitute Ugilec 141) (De Boer, 1995) and polybrominated diphenylethers (PBDEs, widely used as fire retardants) (Watanabe *et al.*, 1987; Andersson and Wartanian, 1992; IPCS, 1994; Pijnenburg *et al.*, 1995) will end up in the organic extracts. The relative potency of these PHAHs in the *CALUX* assay is compared to that of TCDD. Sediment samples from a number of locations in The Netherlands were collected, and extracts of both whole sediment and pore water were tested in the *CALUX* assay. The *CALUX* response of pore water extracts is compared to the biological response in an early life stage (ELS) test with the zebra fish (*Branchydanio rerio*).

Materials and Methods

Chemicals

All chemicals used were of pesticide analysis (pa) or HPLC grade. TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) was purchased from Schmidt B.V. (Amsterdam, The Netherlands). The technical brominated diphenyl ether mixture Bromkal 70-5-DE, and three pure congeners 2,2',4,4'5,5'-hexabromodiphenylether (HBDE), 2,2',4,4',5-pentabromodiphenylether (PBDE), and 2,2',4,4'-tetrabromodiphelylether (TBDE) were provided by Dr. Åke Bergman (Environmental Chemistry, Stockholm University, Sweden). The technical PCB mixture Clophen A50, the PCT mixtures Aroclor 5442 and 5460, and the technical tetrachlorobenzyltoluene mixture Ugilec 141 were provided by Dr. De Boer (Netherlands Institute for Fisheries Research, RIVO, IJmuiden). GC-MS analysis to assure the purity and concentration of the TCDD stock solutions was performed by the Dutch State Institute for Quality Control of Agricultural Products (RIKILT-DLO). For exposure of cells, all chemicals were dissolved in ultra clean dimethylsulfoxide (DMSO; Janssen, Belgium).

CALUX assay

For experiments with individual congeners, rat (H4IIE) or mouse (Hepa1c1c7) hepatoma cell lines, stably transfected with the luciferase (Luc) reporter gene plasmid pGudLuc1.1 were used (further called H4IIE.Luc and Hepa.Luc cells respectively). These cell lines were prepared as previously described in Aarts *et al.* (1995) and Garrison et *al.* (1996) (also called H4L1.1c4 and H1L1.1c7). The cells were grown in 24-well cell culture plates (Costar) in 0.5 ml minimal essential medium (α-MEM, Gibco) with 10% heat-inactivated (h.i.) fetal calf serum (FCS, Gibco) or in 6-well cell culture plates (Costar) in 3 ml α-MEM with 10% h.i. FCS. The cell layer reached 90-100% confluence 24 hr after seeding, and growth medium was replaced with fresh culture medium containing test compounds or extracts in a maximum of 0.5% DMSO. Exposure was in triplicate, and for each assay a TCDD standard series from 1 to 1000 pM was included. After 20-24 hr of exposure the medium was removed and cells washed twice with phosphate-buffered saline (PBS), and the cells were harvested in 250 μl (6-well plates) or 75 μl (24-well plates) cell lysis reagent (Promega), centrifuged for 3 min (6-well plates) or 90 sec (24 wells plates) at 13000*g* and the supernatant frozen at -80°C For measurement of luciferase activity the samples were thawed on ice, 20 μl supernatant was pipetted into a 96-well microtiter plate and 100 μl luciferin assay mix (Promega) at room temperature was added and the plate was mixed for approximately 90 sec on a plate mixer. The light production was measured in an Amerlite Luminometer (Amersham). For each sample the protein content was measured in a microtiter plate at 595 nm, according to Bradford (1976) using protein

assay dye reagent (BioRad) and bovine serum albumin (BSA) as protein standard.

EROD assay

The EROD activity was measured using 96-well microtiter plates, mainly based on the method described by Sanderson et *al.* (1996). Briefly, H4IIE or Hepa1c1c7 wild type cells were seeded in 96-well plates in 100 μl α-MEM. After 24 hr incubation at 37°C the cell layer was 80-90% confluent, and 100 μl of fresh α-MEM containing the test compound in maximal 0.5% DMSO was added to each well. Samples were tested in four or six-fold replicates. After an additional 48-hr incubation, the medium was removed and the wells were rinsed twice with 50% diluted PBS. To each well 20 μl of nanopure water was added to swell the cells and after 15 min incubation at room temperature, the plates were placed at -80°C to lyse the cells. To measure resorufin production, 50 μl of Tris-sucrose buffer (pH 8) with 40 μM dicumerol, followed by 25 μl 10 μM 7-ethoxyresorufin (ER) were added to each well and the plates were pre-incubated for 20 min at 37°C. To start the reaction, 25 μl 1 mM NADPH solution was added per well and the plates were again incubated, 1 hr at 37°C. The resorufin production was measured in a fluorometer (Cytofluor) with an excitation filter at 530 nm and an emission filter at 590 nm. Protein measurement was carried out in the same plates, using the Pierce method (Smith *et al.*, 1985), for which, unlike the Bradford method described above, dilution of samples was not necessary. Samples were left overnight at 37°C to dry until slightly wet. After addition of bicinchoninic acid (Pierce) followed by 30 min incubation at 37°C, the absorbance at 562 nm was measured in a microtiter plate spectrophotometer (Thermomax, Molecular Devices). BSA was used as protein standard.

Substrate Inhibition Test

For the *CALUX* inhibition assay, H4IIE.Luc or Hepa.Luc cells were exposed to 50 pM TCDD in 24-well plates for 24 hr. Upon harvesting and addition of lysis mix, the plates were frozen at -80°C and later thawed on ice prior to luciferase activity measurement. Final concentrations of Clophen A50 ranging from 0.1 to 500 μM and 3,3',4,4-tetrachlorobiphenyl (PCB-77) ranging from 0.01 to 50 μM were reached by adding appropriate concentrations of stock solutions in 4 μl DMSO to the 20 μl of cell lysate in each well. The plates were allowed to stand 5 min at room temperature and then thoroughly mixed for 5 min at 37°C on a plate mixer. Samples of 20 μl were measured for light output as described above. For the EROD assay, H4IIE or Hepa wild type cells were exposed to 50 pM TCDD in 96-well plates for 48 hr. Upon harvesting and addition of nanopure water, plates were frozen at -80°C and later thawed on ice for EROD measurement. Final concentrations of Clophen A50 ranging from 0.1 to 500 μM and PCB 77 ranging from

0.01 to 50 μM were achieved by adding stock solutions in 4 μl DMSO per well shortly after cell lysis at -80°C. The plates were then thoroughly mixed on a plate mixer for 20 min at 37 °C, 50 μl of Tris-sucrose buffer (pH 8) with 40 μM dicumerol, followed by 25 μl 10 μM ER were added to each well, and the plates were pre-incubated for 20 min at 37°C. To start the reaction, 25 μl 1 mM NADPH solution was added per well and resorufin production was measured as described above.

CALUX measurement of sediment and pore water extracts

Sediment samples. Sediment samples collected throughout The Netherlands were obtained from the National Institute for Inland Water Management (RIZA) from the following locations: Ketelmeer (KM1, KM3 and KM13), Oostvaardersplassen (OVP), North Sea Canal (NSC), Drontermeer (DM), Markermeer (MM), Brabantse Biesbosch (BB), Dommel (DOM), Delfland (DL), and Spijkerboor (SB). Sediments were sampled with an Eckman grab and stored in large plastic vials at 5°C. Of the sediment samples used, KM and OVP are routinely assayed in RIZA biomonitoring programs. KM, BB and SB are sedimentation areas of the rivers Rhine and Meuse, and known to be polluted with a wide range of chemicals, including PCBs and dioxins. The OVP is not in direct contact with one of the main rivers and is therefore considered to be a reference site (Maas *et al.*, 1993). Also MM, DM, and DL have low contamination levels in the sediment. NSC and DOM have regional inputs of organic micropollutants and heavy metals, respectively.

Extraction and clean-up of sediment. Sediment samples were homogenated, extraneous overlying water was decanted, and large pieces of debris were removed by sieving the sediment over a coarse sieve (3 mm). Ten grams of sediment was mixed with Na_2SO_4, dried in an oven overnight at 40°C, and extracted with hexane/acetone (1:1) in a soxhlet for 16 hr. After cooling down, the extract was washed with 50 ml of deionized water and 5 ml of saturated NaCl solution, and the hexane fraction was concentrated to 3-4 ml by evaporation. Sulphur was removed using tetrabutyl ammonium sulfite (TBA) (De Voogt *et al.*, 1990; Verbrugge *et al.*, 1991). Further clean-up was performed using a multilayer acid-base silica column consisting of 0.75 g Na_2SO_4 on top of dried silica with, respectively, 0.75 g of 22% and 0.75 g of 40% hexane washed H_2SO_4 and 1 g 33% NaOH on glass wool. After preeluting with 5 ml of hexane the column was loaded with the sample and eluted with 20 ml of hexane followed by 20 ml of hexane/dichloromethane (1:1). The sample was reduced to less than 1 ml by rotoevaporation at 35°C and transferred to 1 ml vials. Just before the extract was completely dried under a gentle, filtered air flow, 100 μl of DMSO was added and the last hexane was evaporated.

Collection and extraction of pore water. Pore water was collected by the method described by Maas *et al.* (1993). Mixed, decanted, and sieved sediment samples of 200

g were added to 500 ml plastic containers and centrifuged for 30 min at 3000*g* at 5°C. The supernatant was carefully decanted into glass erlenmeyer flasks and stored at 5°C. To avoid major chemical or physical changes in its composition, the pore water was not kept longer than 1 week. Samples of pore water (5 ml) were extracted with 5 ml hexane, vigorously vortexed, and centrifuged at 3000*g* for 5 min. The hexane fraction was collected in a hexane-rinsed, glass test tube. This procedure was repeated twice, and the combined hexane fractions were evaporated under a gentle nitrogen flow until only a very small drop was left. To each sample 200 μl of DMSO was added and, after complete evaporation of the remaining hexane, diluted with 3.3 times more DMSO.

Early life stage test with zebra fish

Early life stage (ELS) tests with zebra fish (*B. rerio*) were performed according to the method described by Van Leeuwen *et al.* (1990). Two to six females and two to four males were placed together overnight in special 'brood chambers. Induced by the morning light, the females normally lay up to 600 eggs which are fertilized by the males. The fertilised eggs fall through a mesh at the bottom of the brood chamber into a separate compartment. After disinfection (in a 0.04% formalin solution for 1 min) 20 blastular eggs were transferred to 60-ml glass vials each containing 20 ml of test solution. Undiluted pore water samples and two dilution steps of 1.8 and 3.1 times in dutch standard water (DSW, pH 7.8, hardness = 210 $mg \cdot l^{-1}$ as $CaCO_3$) were prepared in triplicate, added to the eggs and renewed every 2 to 3 d. DSW was used as a blank. A TCDD standard series was included at the concentrations of 0, 25, 50, 500, 1000 and 2000 pM, added in 80 μl DMSO/20 ml DSW and renewed daily. During solution renewal, eggs or larvae were left in the test vessels in a small amount of fluid, while the water was changed. The embryo-larval stages were exposed continuously for 8 d at 25 ± 1°C, without feeding. Water quality parameters (pH, O_2, ammonium, nitrate and nitrite) were checked throughout the test period and remained within acceptable ranges. Dead larvae were enumerated and removed daily, and malformations were recorded. At the end of the test period surviving fish were checked for malformations under a binocular microscope. The end points, percentage mortality and teratogenicity, were corrected by deducting the percentage dead in the control, multiplying the result by 100, and dividing this by 100 minus the percentage mortality in the control (Tattersfield and Morris, 1924).

Calculations of CALUX-TEFs and CALUX-TEQs

For calculation of the TCDD equivalency factors for compounds in the *CALUX* assay (*CALUX*-TEFs) a complete dose-response curve in triplicate was used. Values between 10% and 90% of the maximum were used for Scatchard analysis. The intercept with the

X-axis yields the theoretical maximum *CALUX* response ($CALUX_{max}$); the intercept with the Y-axis the $CALUX_{max}/EC_{50}$ from which the EC_{50} can be calculated. The calculated EC_{50} was always compared with the visually determined values for confirmation. The *CALUX*-TEF value of each compound was calculated as the ratio EC_{50} (TCDD):EC_{50} (compound).

For calculation of TCDD-equivalents in the *CALUX* assay (*CALUX*-TEQs), a complete standard curve of 2,3,7,8-TCDD was used for each cell line. For each 24-well plate the response of the unknown samples were measured (in triplicate) plus three TCDD calibration standards bracketing the TCDD EC_{50}. Based on these calibration standards the complete standard curve was adjusted for plate to plate variation. The standard curve was fitted (1-site ligand fit, function: $y = a_0 * x(a_1 + x)$) using SlideWrite 5.1, which determines the fitting coefficients by an iterative process minimizing the Chi-squared merit function (least squares criterion). The *CALUX*-TEQ value for the unknown sample was interpolated on this curve. The response of the unknown sample is ideally in the range of 10%-100% of the EC_{50} (for H4IIE.Luc 1-10 pM TCDD), and should be further diluted when out of this range. The detection limit is set at the DMSO response plus three times the standard deviation.

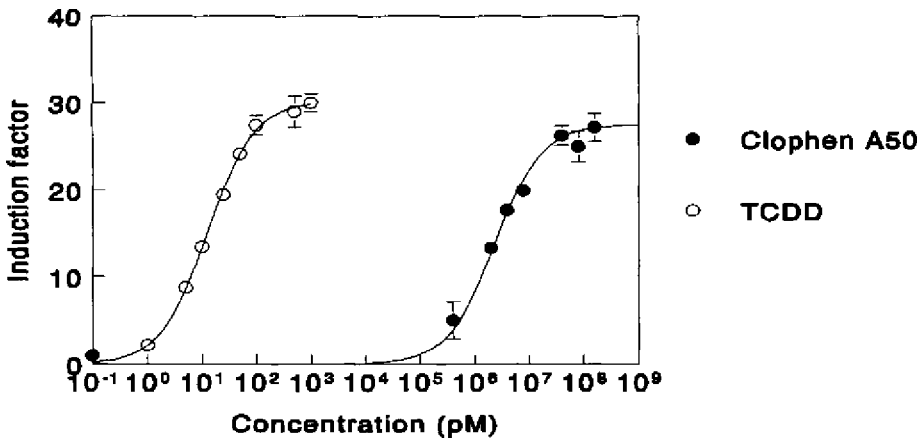

Figure 6.1 Dose-response curves for luciferase induction in H4IIE.Luc cells for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and the technical PCB mixture Clophen A50. Background *CALUX* response was 13.8 relative light units (RLU)/μg protein, induction by 1000 pM TCDD 414.2 RLU/μg protein. Exposure was during 24 hr, for details see M&M.

RESULTS

Induction of CALUX response by PHAHs

The *CALUX* response was sensitive and reproducible for exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxine (TCDD). The detection limit in H4IIE.Luc cells was less than 1 pM, which equals an absolute amount of less than 0.5 fmol/well. The EC_{50} is reached at 10 pM and the curve saturates between 100 pM and 1 nM, with a maximum induction factor of 30 (Figure 6.1). The standard deviation in the *CALUX* assay, performed in triplicate, was generally ≤5%. The dose-response curve for the technical PCB mixture Clophen A50 was comparable to that of TCDD, but the EC_{50} was $5 \cdot 10^5$ times greater and the maximum induction factor was 27, slightly less than for TCDD. Dose-response curves for mixtures of polychlorinated terphenyls (PCT) (Aroclor 5442 and 5460), polybrominated diphenylethers (PBDE) and individual PBDE congeners were similar (data not shown). The EC_{50} values, TEFs, and induction factors at the EC_{50} for *CALUX* activity in H4IIE.Luc cells exposed to these PHAHs are reported in Table 6.1. The *CALUX*-TEF for the technical PCB mixture Clophen A50 and for the PCT mixture Aroclor 5442 were almost identical. Aroclor 5460 was found to be 350 times more potent as a CALUX inducer, and the PBDEs tested were similar to Clophen A50. The *CALUX*-TEF of 2,2',4,4'-TBDE was 10-fold less than for the other PBDEs, comparable to the relatively low induction potency of Ugilec 141. The TEFs for *CALUX* activity determined in H4IIE.Luc or Hepa.Luc cells were almost identical (data not shown).

Table 6.1 Toxic equivalency factors (TEFs) for luciferase induction in H4IIE.Luc cells of several sediment associated compounds, as determined in the *CALUX*-assay. The 50% effect concentration were calculated using Scatchard analysis.

Compound	Molecular weight	EC_{50} (M)	*CALUX* TEF	Induction (fold)[1]
2,3,7,8-TCDD	322	$1.0\ 10^{-11}$	1.0	15.0
Clophen A50	300	$5.0\ 10^{-6}$	$2.0\ 10^{-6}$	13.6
Aroclor 5442	396	$6.3\ 10^{-6}$	$1.6\ 10^{-6}$	14.0
Aroclor 5460	562	$1.8\ 10^{-8}$	$5.6\ 10^{-4}$	8.5
Bromkal 70-5-DE	536	$2.1\ 10^{-6}$	$4.8\ 10^{-6}$	7.3
2,2',4,4'-TBDE	486	$1.4\ 10^{-5}$	$7.1\ 10^{-7}$	7.6
2,2',4,4',5-PBDE	565	$1.7\ 10^{-6}$	$5.9\ 10^{-6}$	4.8
2,2',4,4',5,5'-HBDE	644	$2.3\ 10^{-6}$	$4.3\ 10^{-6}$	9.6
Ugilec 141	320	$1.6\ 10^{-5}$	$6.3\ 10^{-7}$	4.0

[1]The induction factor is determined at the EC_{50}, as the increase in luciferase induction relative to background induction (12.1-13.8 relative light units/μg protein).
Abbreviations: TCDD = tetrachlorodibenzo-*p*-dioxin, TBDE = tetrabromodiphenylether, PBDE = pentabromodiphenylether, HBDE = hexabromodiphenylether

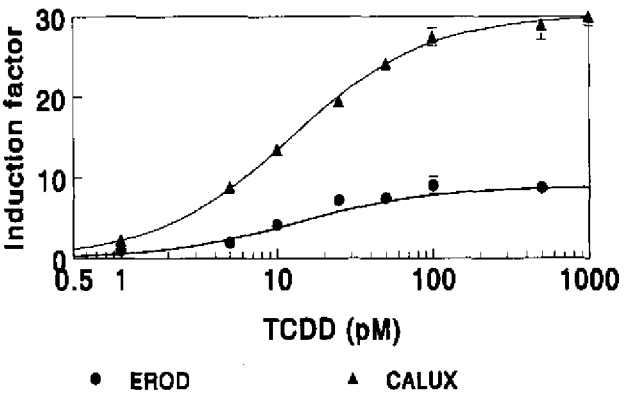

Figure 6.2 Dose-response curves for luciferase induction in H4IIE.Luc cells and ethoxyresorufin O-deethylase (EROD) activity in H4IIE wild type cells for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). For conditions of the luciferase response see Fig. 1. Background EROD activity was 1.3 pmol resorufin (RR)/μg protein.min, induction by 1000 pM TCDD 11.7 pmol RR/μg protein.min. Exposure for EROD assay was during 48 hr, for details see M&M.

The *CALUX* and EROD assay gave similar curves (Figure 6.2), though in the *CALUX* assay the lower detection limit was approximately 2- to 3-fold less and the maximum induction factor was approximately 3-fold higher, the EC_{50}values were somewhat less, and the TEFs slightly higher (both in H4IIE cells). The EC_{50} for TCDD was 16 pM in the EROD assay. The induction factors in the EROD assay for Aroclor 5442 and Aroclor 5460 at the EC_{50} were 4.0 and 3.6, respectively (data not shown), as compared to 14.0 and 8.5, respectively, in the *CALUX* assay. EROD induction by Ugilec was not measurable, and EROD induction by PBDEs was not studied.

Substrate inhibition tests

Inhibition of the responses of the *CALUX* and EROD assays by PCBs were compared in the H4IIE cells (Figure 6.3a). The activity induced by 50 pM of TCDD was measured in the presence of increasing amounts of Clophen A50, added to the lysed cells shortly before the substrates luciferin or ethoxyresorufin respectively, were added. The induction by 50 pM of TCDD for EROD activity was 9.6 pmol resorufin·μg^{-1} protein·min^{-1} while that for *CALUX* activity was 98.9 relative light units (RLU)·μg^{-1} protein. Both responses were set at 100%. The measured EROD activity was reduced to 16% of the original activity after addition of 100 nM Clophen A50. A Clophen A50 concentration of 50 μM completely eliminated EROD activity. No significant inhibition of the *CALUX* activity was

observed at concentrations of Clophen A50 as great as 500 μM. Also 3,4,3',4'-tetrachlorobiphenyl (PCB-77) inhibited EROD activity when added to the lysed cells just before measuring (Figure 6.3b). This inhibition was, however, less pronounced than for Clophen A50, as 40% of the EROD activity was still measurable when 50 μM PCB-77 was added. Again, no significant inhibition was observed for *CALUX* activity (Figure 6.3b). Comparable results were obtained using Hepa cells (data not shown), although the reduction in measurable EROD activity was slightly less than for H4IIE cells. No reduction of the *CALUX* activity was observed in either H4IIE.Luc or Hepa.Luc cells.

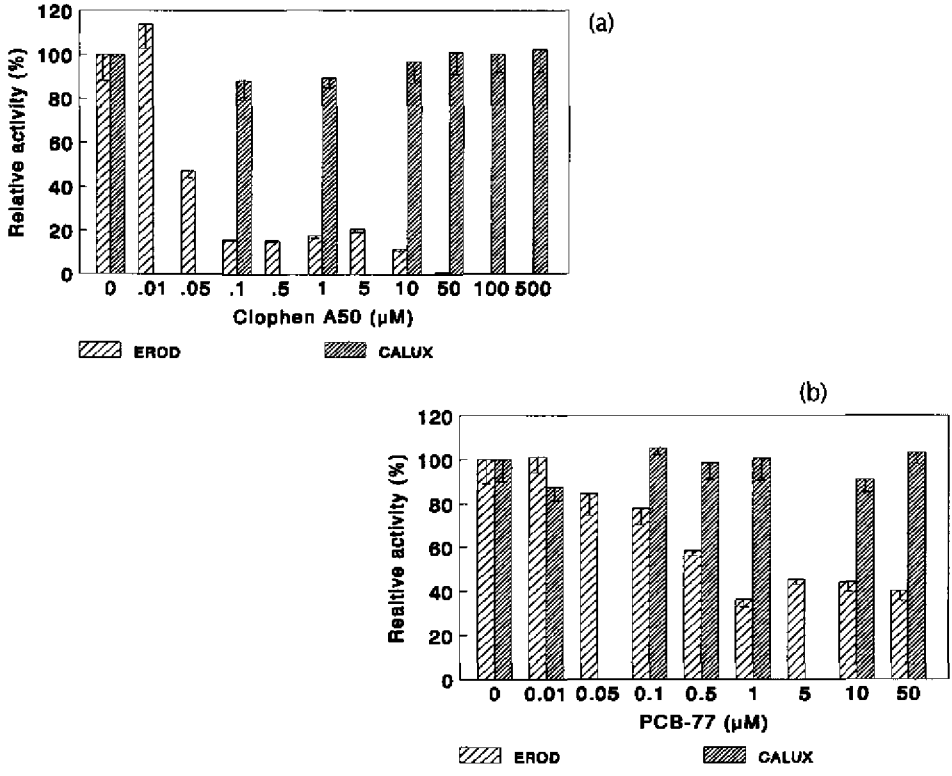

Figure 6.3 Effect of an increasing concentration of (a) Clophen A50 or (b) PCB-77 ,added to lysed H4IIE.Luc or H4IIE.wt cells shortly before measuring, on measurement of respectively the *CALUX* or EROD activity. The *CALUX* and EROD activities had previously been induced by exposure to 50 pM 2,3,7,8-TCDD during 24 hr, and this induction was set at 100%.

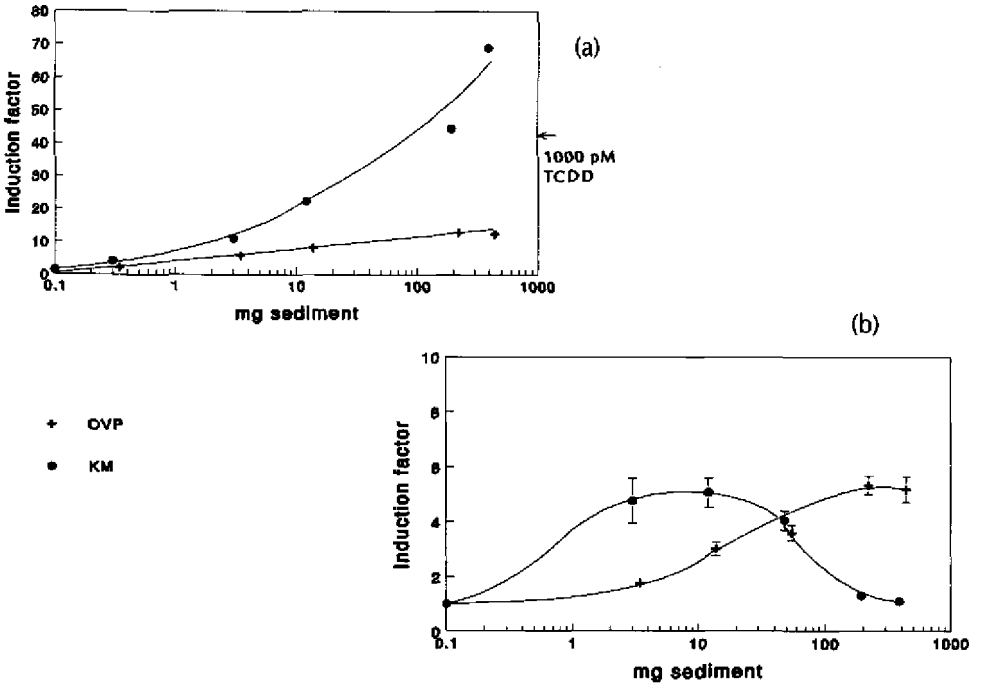

Figure 6.4 a) The *CALUX* response by extracts of Ketelmeer location 1 (KM) and Oostvaardersplassen (OVP) sediment in H4IIE.Luc cells after 20 hr of exposure, expressed as induction factor relative to background luciferase induction (12.1 relative light units/μg protein). b) The same for EROD response in H4IIE wild type cells after 48 hr exposure, background EROD activity 1.1 pmol resorufin/μg protein.min.

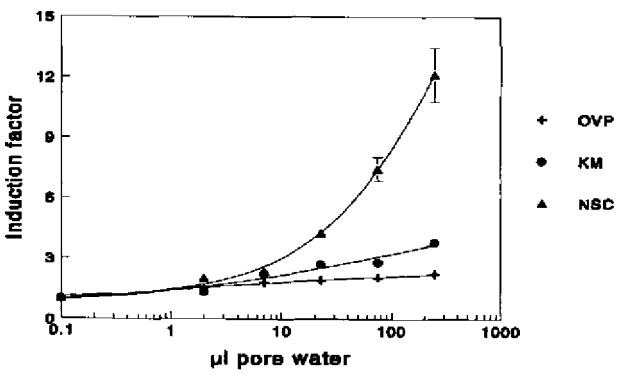

Figure 6.5 The *CALUX* response by pore water extracts in H4IIE.Luc cells after 20 hr exposure, expressed as induction factor relative to background luciferase induction.
OVP = Oostvaardersplassen, NSC = North Sea Canal, KM = Ketelmeer location 13

Induction of CALUX activity by sediment extracts

As an example of the type of responses observed, the dose-response curves for H4IIE cells exposed to sediment extracts from the relatively clean Oostvaardersplassen (OVP) and the polluted Ketelmeer (KM1) are presented for the *CALUX* assay (Figure 6.4a) and the EROD assay (Figure 6.4b). In the *CALUX* assay, the maximum induction factor for TCDD (1000 pM) was 44.6. The extract of 0.3 mg of KM1 sediment resulted in a 4-fold induction of *CALUX*. The extract of 193 mg of KM1 sediment resulted in a *CALUX* response comparable to 1000 pM TCDD. The induction by 387 mg of sediment was approximately 70-fold greater than the DMSO blank, which was greater than the TCDD maximum induction factor. The extract of 441 mg of OVP sediment did not induce *CALUX* activity more than 12.4-fold. The EROD activity was completely inhibited in the presence of KM1 extract at the greater concentrations. Based on the TCDD standards that were used in each *CALUX* assay, the TEQ value of the KM1 sediment was calculated to be 70.0 ± 6.6 fmol·g^{-1} dry sediment and 4.2 ± 0.2 fmol·g^{-1} for the OVP sediment. The recovery of ^{13}C-labeled PCBs and ^{14}C-labeled TCDD in the whole extraction and purification procedure was 85%. Because it was not possible to measure the recovery of each individual sample, the recovery was not used in the calculation of TEQ values.

The sediment extracts not subjected to clean-up, had a dark tar-like appearance (especially the extract of KM1) and caused visible disturbances in the cells, resulting in decreasing protein concentrations and finally cell death. Therefore, unpurified sediment extracts were not tested in further experiments.

CALUX activity by pore water extracts

In contrast to sediment extracts, unpurified pore water extracts did not result in any cell death or reduced protein concentrations. The dose-response curves in H4IIE.Luc cells of pore water extracts from OVP, KM13 and the North Sea Canal (NSC) are presented in Figure 6.5. The extract of 250 μl NSC pore water induced *CALUX* activity 12.1-fold, but did not result in maximum induction. The same volume resulted in 3.8- and 2.2-fold induction for KM13 and OVP respectively. When tested in the EROD assay, the same extracts resulted in 3- to 4-fold less induction (data not shown). Based on the measured TCDD standards, the TEQs calculated for these pore waters were 26.4 fmol·ml^{-1} for KM13, 317.4 fmol·ml^{-1} for NSC and below detection limit for OVP. The detection limit in this *CALUX* assay was 1.9 fmol/well. As 250 μl pore water or less was tested per well, the detection limit was 7.6 fmol·ml^{-1} pore water. The recovery of ^{13}C labeled PCB-101 (2,2',4,5,5-pentachloro biphenyl) and PCB-153 in the extraction procedure was 94%, but the TEQ values were calculated without correction for the average recovery.

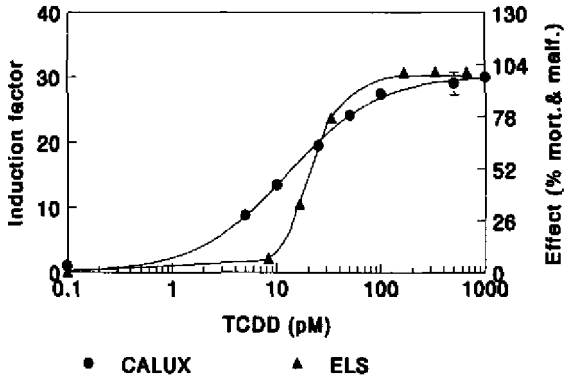

Figure 6.6 Dose-response curves for mortality and malformations after 8 d exposure of eggs of zebrafish (*Branchycardio rerio*) (ELS response) and luciferase production (*CALUX* response) in H4IIE.Luc cells after 24 hr exposure, compared for the same 2,3,7,8-TCDD concentrations. The *CALUX* response is expressed as induction factor relative to background induction. The value at 0.1 pM is in fact the control value (0 pM).

Early life stage test and CALUX assay with pore water

The slope of the dose-response curve for the early life stage (ELS) test with zebra fish in artificial pore water (DSW) spiked with TCDD, was steeper than the slope of the dose-response curve for the *CALUX* assay (Figure 6.6). The EC_{50} (8 d, malformations and mortality) for the ELS assay was 21 ± 2.3 pM TCDD (21 fmol TEQ·ml^{-1}), which was in the same order of magnitude as the EC_{50} of 10 pM TCDD for the *CALUX* assay. At concentrations greater than 35 pM 100% effect was observed, and at concentrations grater than 165 pM all zebrafish larvae were dead within 8 days. Even at the lowest concentration tested (8.3 pM TCDD) 7% malformations were observed.

The results of the *CALUX* assay were compared with those of the ELS test, with pore waters from 10 natural sediments (Table 6.2). The effects varied among pore waters, ranging from 0-100% effect in the ELS assay and from detection limit to 317 fmol TEQ·ml^{-1} pore water in the *CALUX* assay.

Discussion

The results demonstrate that the *CALUX* assay is a rapid, sensitive, and reproducible method of determining the toxic potency of mixtures of lipophilic compounds that bind

to and activate the AhR. Persistent AhR-active compounds that occur in whole sediments or pore water were measured in small aliquots with minimal effort. The *CALUX* assay represents significant improvement relative to the H4IIE.wt assay, which uses endogenous EROD induction as a response. The sensitivity and linear working range of the *CALUX* was slightly better than the EROD assay, but the primary improvement of the *CALUX* assay was that it was insensitive to substrate inhibition, which has been a problem in the EROD assay.

Induction of CALUX response by PHAHs

The TEFs of Clophen A50, Bromkal 70, PBDE and HBDE (Table 6.1), were almost identical, which is in accordance with the results of an *in vivo* experiment with Wistar rats (Von Meyerinck *et al.*, 1990) in which the EROD induction by Bromkal 70 was slightly greater than the induction by Aroclor 1254, a technical PCB mixture comparable to Clophen A50 (Schultz, 1989). The TEF for coplanar and mono-*ortho*-polychlorinated diphenyl ethers (PCDE), based on immunotoxicity and AHH induction in C57BL/6 mice (Safe, 1990), was 10^{-3}. This is about 200-fold greater than the *CALUX*-TEFs that we determined for the di-*ortho*-brominated diphenylethers and the mixture Bromkal 70-5-DE. However, TEFs of di-*ortho*- and mono- or non-*ortho*-PCBs have been reported to differ by 50-200 as well (Safe. 1990). In the *CALUX* assay, the PCT Aroclor 5460 was 20-fold more potent than Aroclor 5442. A comparison of the EROD induction in Spraque-Dawley rats by PCT mixtures Aroclor 5432 and Aroclor 5460 (Toftgard *et al.*, 1986) revealed that Aroclor 5432 was, on a mass base, a more potent inducer than Aroclor 5460. Since Aroclor 5460 is much heavier than Aroclor 5432 or 5442 (Table 6.1) this comparison would be different on a molar base. We did not test Aroclor 5432. The *CALUX*-TEF for Ugilec 141 was 3.2-fold less than that for Clophen A50. This is in accordance with an earlier observation that the EROD activity in C57BL/6 mice dosed with 200 $mg \cdot kg^{-1}$ Ugilec 141 was 3.4-fold less than that of mice dosed with 200 $mg \cdot kg^{-1}$ Aroclor 1254 (Murk *et al.*, 1991). The molar densities of Ugilec 141 and Aroclor 1254 differ less than 10%.

It should be noted that induction values reported for technical mixtures could be partially due to a small percentage of impurities. Additionally, it is important for TEF calculations that the concentrations of the stock solutions used for the assays are validated with GC-MS, as concentrations of carefully prepared stock solutions from different laboratories may vary by a factor of 10. If no validation has been performed, the EC_{50} of the TCDD stock used should be mentioned as well. The maximum *CALUX* induction factors for TCDD may differ slightly between individual assays. This is probably due to slight differences in the condition of the cells, such as the degree of confluency. Three

TCDD calibration concentrations were measured in duplicate on each 24-well plate to allow comparison of TEFs and TEQs determined in different assays.

Substrate inhibition in the EROD assay

The *CALUX* assay offers some advantages when compared to the commonly used EROD assay in H4IIE cells. It is slightly more sensitive and has a 3-fold greater induction factor, but, more importantly, the *CALUX* assay offers the possibility of measuring the presence of less potent inducers such as Clophen A50 and Ugilec 141, as well as low concentrations of compounds in environmental matrices. Since PHAHs are not substrates for luciferase, substrate inhibition by PHAHs will not occur in the *CALUX* assay. The EROD activity is already inhibited at substrate concentrations of 50 nM or more, which are needed for induction by less potent inducers (Figure 6.3a). In contrast to Clophen A50, PCB-77 did not inhibit EROD activity up to 99% at high concentrations (Figure 6.3b). Since PCB-77 is a substrate for cytochrome P4501A1, and readily metabolized in *in vitro* incubation with rat hepatic microsomes (Murk *et al.*, 1994c), PCB-77 could have been metabolized for 40% or more during 60 min incubation in the H4IIE.wt EROD assay. Substrate inhibition has been demonstrated in chicken hepatocytes and rat- and fish hepatoma cell lines (Kennedy *et al.*, 1993; Sawyer *et al.*, 1984; Hahn *et al.*, 1993; Richter *et al.*, 1996). Also in experimentally dosed flounder the EROD activity induced by 5 $\mu g \cdot kg^{-1}$ TCDD was reduced by 66% when dosed simultaneously with 42 $mg \cdot kg^{-1}$ Clophen A50, although the cytochrome P450 protein content increased in an additive manner (Besselink, pers.comm.).

CALUX activity induced by sediment and pore water extracts

Exposure of H4IIE.Luc cells to extracts of KM1 sediment, which is known to contain a number of contaminants, resulted in 17-fold greater *CALUX* activity than did extracts from the relatively clean OVP sediment. Based on chemical analyses, the TEQs of these two locations differ by a factor 20 (unpublished data RIZA). A comparable difference was observed using pore water extracts. The *CALUX* response to 250 μl OVP pore water was less than the detection limit (1.9 fmol/250 μl). The NSC pore water sample contained almost 13-fold more *CALUX*-TEQs than the two sites from the KM. A herbicide producing plant is situated in the NSC, where accidental emissions of PCDD and PCDF have occurred (Turkstra and Pols, 1989). In contrary to whole sediment samples, pore water needed no soxhlet extraction or clean-up steps. This makes the sample preparation much more rapid and the chance of loosing unknown AhR-active compounds with yet unknown optimal recovery conditions much smaller.

PHAHs and organochlorine pesticides can be lost during filtration, due to

irreversible adsorption on polyamide filters, and to a lesser degree, on Teflon material (Rood *et al.*, 1995a,b). This may explain why Anderson *et al.* (1995) did not measure any *CALUX* activity using pore water samples from polluted sites, which they filter sterilized over a 0.1-μm membrane. Additionally, Anderson *et al.* (1995) kept collected pore water for approximately 4 months before testing. Whole sediments can be kept at 4°C for more than 112 days. without losing toxicity (Othoudt *et al.*, 1991), however, pore water samples should not be kept longer than 1 week because of chemical changes (Burton, 1991; Hill *et al.*, 1994). When optimizing the pore water assay, we observed that upon filtering ^{3}H-PCB-77-spiked pore water samples over a glass fiber Whatman GF/F 0.7-μm filter, 86-92% of all radioactivity remained on the filter. This was to be expected, since lipophilic molecules such as PHAHs are mainly associated with dissolved and particulate organic carbon, especially in the smallest size fraction (0.22-1 μm) (Muir *et al.*, 1992). PHAHs that are associated with small particles can readily be accumulated by species which filter or ingest organic particles, such as mussels and chironomids (Muir *et al.*, 1992; Ankley *et al.*, 1992) and will be relatively available for uptake through skin or gills after dissociating into the water phase (Swartz *et al.*, 1990; Kjeller *et al.*, 1990; di Toro *et al.*, 1991; Power and Chapman, 1992). Partitioning of organic compounds between the solid phase of sediments and pore water depends on the lipophylicity of the compounds and the presence of dissolved organic matter like humic acids in the pore water (Landrum and Robbins, 1990). Analysis of only the solid phase of sediments does not discriminate between bioavailable and tightly bound contaminants. Therefore, either much additional information is needed for assessment of the bioavailable fraction of sediment associated contaminants or the biologically available fraction could be measured directly using pore water samples.

In vivo validation of the CALUX response

The *CALUX* response has already been chemically validated with pure compounds (Aarts *et al.*, 1995; Garrison *et al.*, 1996) or with mixtures (Postlind *et al.*, 1993). To indicate the hazard of a certain polluted sediment for the ecosystem, the *CALUX* response has to be validated against responses from *in vivo* assays. In this study the *in vivo* response in the ELS assay for a TCDD spiked DSW sample corresponded with the *CALUX* response (Figure 6.6), although the slope of the ELS assay curve was steeper, resulting in a more narrow working range. These results demonstrate that compounds present in pore water samples are directly available for organisms in the water phase, since the fish larvae did not eat during the test period. The response in the 8 day ELS assay with zebra fish has been reported to correlate with the 60-day ELS test for rainbow trout and with a chicken embryotoxicity tests (Van Leeuwen *et al.*, 1990). These species share embryonic develop-

Table 6.2 Concentrations of several contaminants, sediment quality classification, and toxicity of pore water samples. TCDD-equivalents were determined in the *CALUX* assay (*CALUX*-TEQs, fmol/ml), toxicity is expressed as the response in the early life stage (ELS) test with zebrafish (*Branchycardio rerio*) exposed to 10 undiluted pore waters. Concentrations of 3 heavy metals (Cd, Cu, Zn), 10 PAHs and PCB 153 in sediment samples from the same locations (unpublished data RIZA). The sediments were classified relative to sediment quality standards, calculated as standardized sediment (Derde Nota Waterhuishouding, 1993).

Location[1]	Cd mg/kg	Cu mg/kg	Zn mg/kg	Σ10 PAHs mg/kg	PCB-153[2] µg/kg	Classification[5]	*CALUX* TEQ fmol/ml	ELS % effect
KM3	5.97	98.63	873.88	11.43	27.97	4	24.0	42
KM13	12.52	134.44	1333.6	5.0	41.43	4	26.4	100
OVP	1.10	21.64	214.44	0.83	2.08	1	dl[4]	4
BB	5.02	48.59	679.85	10.57	25.78	3	28.4	100
DOM	39.94	311.77	1269.3	6.87	30.36	4	17.6	100
DL	0.73	19.84	126.25	0.36	dl[3]	1	dl[4]	0
SB	7.72	75.40	859.26	7.37	59.32	4	30.8	100
MM	0.75	19.09	137.76	0.81	dl[3]	0	dl[4]	10
DM	0.52	14.20	102.51	5.59	dl[3]	2	dl[4]	0
NSC	2.27	77.32	340.92	25.52	86.21	3	317.4	100

[1] For abbreviations see Materials and Methods.
[2] PCB-153 = 2,2',4,4',5,5-hexachlorobiphenyl.
[3] Below detection limit GC-MS (1 µg/kg).
[4] Below detection limit *CALUX* assay (7.6 fmol/ml in this experimental setup, see also Results: Pore water measurements).
[5] Class 1 meets General Environmental Quality Criteria 2000; Class 2 meets testing value (unpolluted, though further research may be necessary); Class 3 meets signal value (clean-up may be required following further research); Class 4 exceeds signal value (sanitation required) (CCRX, 1990).

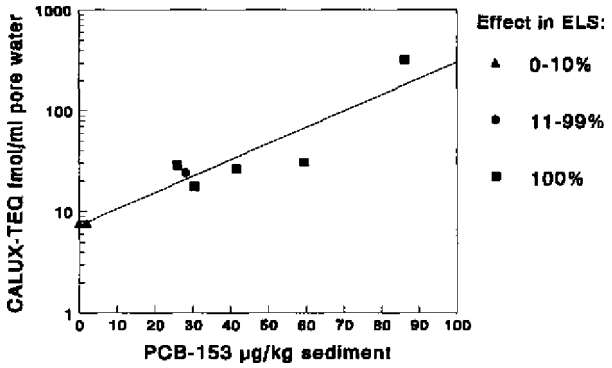

Figure 6.7 Correlation between the 2,3,7,8-TCDD equivalents in pore water determined in the *CALUX* assay (*CALUX* TEQs) and the PCB-153 level in sediment from the same location measured by GCMS (Table 6.2). The different symbols indicate the % mortality and malformations in the early life stage test with zebrafish (*Branchycardio rerio*) performed in the collected pore water (see also Table 6.2).

ment in the absence of maternal metabolism or a placenta barrier. Although the cells used in the *CALUX* assay are mammalian, they are also directly exposed to the toxic compound. The correlation with *in vivo* embryotoxicity in mammals could therefore be less strong.

No clear correlation was observed when comparing the *CALUX* response to extracts of naturally occurring pore waters with the response in the ELS test (Table 6.2). This was to be expected, since the ELS assay will also be influenced by the sometimes great concentrations of heavy metals present in the whole pore water samples (Table 6.2). A better correlation with the *CALUX* response is to be expected with an ELS assay performed with extracts from pore waters. Comparison of the concentrations of PCB-153 in the sediments with the *CALUX* response shows a better correlation (Figure 6.7).

The use of the CALUX assay for hazard assessment of sediments

Given the complexity of the mixtures of PHAHs in sediments and organisms in industrialised countries, chemical analysis can give only a rough impression of the potential risks for the environment. Due to the often small concentrations of individual congeners and the presence of unknown or not routinely measured AhR active substances, such as PBDEs and PCTs that may still contribute to the total TEQs of a sample, there is a risk of

underestimation of the total TEQ. The toxic responses of AhR-active compounds may be additive, as is the case for TCDDs and TCDFs, but for PCBs and non-PCB-like substances both additive and antagonistic interactions have been observed (Safe, 1994; Aarts *et al.*, 1995). These limitations form drawbacks to the TEQ approach. The *CALUX* assay provides a measure of the toxic potency of the whole mixture, including interactions. To be able to correct for differences in quantification due assay to assay variation, three TCDD calibration standards have to be measured with each assay. No changes in EC_{50}-values or *CALUX*-induction relative to TCDD have occurred over the period of 1 year that the cells were in culture.

For assessment of the total biological hazard by contaminants in sediments, a triad approach of combined chemical analyses, bioassays and in situ bottom fauna studies can provide an integrated diagnosis of sediment contamination (Chapman, 1992; Van de Guchte, 1992, 1995). *In vivo* bioassays will give an impression of the total toxic potential and the bioavailability of sediment-associated contaminants. However, they are time-consuming, vulnerable to physical or chemical conditions of the samples, and often provide little information on the cause of toxicity. Additional *in vitro* bioassays like the *CALUX* assay will provide more specific insight into the functional groups of chemicals that are present. In particular the *CALUX* assay using pore water extracts could be a useful tool for rapid and sensitive indication of the toxic potency of biologically available mixtures of AhR-active compounds in sediments. If high *CALUX* responses are observed, chemical analysis is needed to determine the specific compounds responsible for the toxic response.

ACKNOWLEDGEMENTS

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CHAPTER 7

THE *CALUX* (CHEMICAL-ACTIVATED LUCIFERASE EXPRESSION) ASSAY, A SENSITIVE *IN VITRO* BIOASSAY FOR MEASURING TCDD-EQUIVALENTS IN BLOOD PLASMA*

Abstract

Recombinant rat (H4IIE) and mouse (Hepa1c1c7) hepatoma cell lines, containing the firefly (*Photinus pyralis*) luciferase gene under trans-activational control of the aryl hydrocarbon receptor (AhR), were used to determine total AhR-active compounds in blood plasma. Extracts from 250 μl blood plasma aliquots or less, from eider ducks dosed with 3,3',4,4'-tetrachlorobiphenyl (PCB-77) or with the technical PCB-mixture Clophen A50, were used for chemical validation of the *CALUX* (chemical activated luciferase expression) response. For each sample both the fat-containing organic extract and the fat-free, cleaned, extract were tested in the *CALUX* assay. The *CALUX* responses for the extracts were converted into so-called *CALUX*-TEQs (TCDD-equivalents), using a 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) standard curve. The *CALUX*-TEQs in both fatty and cleaned extracts correlated significantly with PCB-77 or PCB-153 levels (depending on the dosage group) determined in blood plasma using GC-MS. For PCB-77 a toxic equivalency factor (TEF) of 1.5 10^{-3} was calculated based on these correlations. In addition, PCB-118 and PCB-156 levels in abdominal fat (assessed with GC-ECD) and hepatic EROD activities correlated well with the *CALUX*-TEQs in both fatty and cleaned blood plasma extracts. Plasma cholesterol and triglyceride levels were determined as a measure of lipid content, in 10 μl aliquots of blood plasma using enzymatic spectrophotometric determination.

In conclusion, we have demonstrated that the *CALUX* assay is a rapid, sensitive assay for assessing the toxic potency of (mixtures of) AhR-active compounds in small samples of blood plasma. The current limit of detection for the *CALUX* assay is less than 0.1 fmol (32 fg) TEQ, which would correspond to roughly 0.3-2 ml blood plasma needed for quantification of TEQs in environmentally exposed species.

**Based on: Murk AJ, Leonards PEG, Bulder AS, Jonas A, Rozemeijer MJC, Denison MS, Koeman JH and Brouwer A (1996): The CALUX (chemical-activated luciferase expression) assay, a sensitive in vitro bioassay for measuring TCDD-equivalents in blood plasma. Submitted to Environmental Toxicology and Chemistry*

INTRODUCTION

It is essential for proper risk assessment of micropollutants occuring in the environment, to be able to measure the internal exposure of wildlife species. Quantification of the toxic potency of the whole mixture of compounds acting via a specific mechanism, instead of only a single or a few representatives, would strengthen the causal relationship between an observed adverse effect and the presence of a (group of) chemical(s).

Polyhalogenated aromatic hydrocarbons (PHAHs) such as polychlorinated biphenyls (PCBs) and polychlorinated terphenyls (PCTs), are ubiquitous pollutants that are especially associated with the aquatic food chain. The highest PHAH concentrations are found in top predators of the aquatic food chain, such as mussel- or fish-eating birds, and fish-eating mammals (Koeman, 1973, Focardi *et al.*, 1988, Duinker *et al.*, 1989; Tanabe *et al.*, 1994). PHAHs elicit a number of species-specific, toxic responses in laboratory and wildlife species, including hepatoxicity, body weight loss, thymic atrophy and impairment of other immune responses, dermal lesions, reproductive toxicity, alterations in vitamin A and thyroid hormone metabolism, teratogenicity and carcinogenesis (Poland and Knutson, 1982; Gilbertson, 1989; Goldstein and Safe, 1989; Brouwer, 1991; Nord, 1992; Safe, 1994; Leonards *et al.*, 1995; Ross *et al.*, 1995).

PHAHs are present as complex mixtures in environmental matrices such as sediments, wildlife and humans. Industrial applications of some PHAHs, such as PCBs and PCTs, have been banned in industrialized countries since the early 1980s. They are, however, still entering the environment for example by leakage from old, so-called closed systems, from recycling of old contaminated materials, leakage from dump sites, and long range atmospheric transport and deposition (Tanabe, 1988). The recent pattern of PCB-contamination suggests their usage still continues in tropical countries (Iwata *et al.*, 1994). Other PHAHs with qualities comparable to PCBs, such as polybrominated- and polychlorinated diphenylethers (PBDEs and PCDEs), are still being produced and used (Pijnenburg *et al.*, 1995). Moreover, environmental input of PHAHs such as polychlorinated-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) is still continuing due to their formation during incomplete combustion in the presence of chlorine (Rappe and Buser, 1989; Rappe, 1991).

As PHAHs are lipophilic, persistent compounds they mainly accumulate in the lipid compartments of organisms. The distribution in the body is dependent on the structure and the physicochemical characteristics of the individual congeners. Excretion depends to a large extend on the metabolism to more polar compounds. The rate of metabolism of PHAHs depends upon halogen content and substitution pattern, the dose and the animal species (Ahlborg *et al.*, 1992). As a result , the absolute and relative

abundance of individual congeners differs greatly between various biological samples (Rappe and Buser, 1989; Norstrom *et al.*, 1990; Duinker *et al.*, 1989).

The mechanism of action for the toxic, planar PHAHs, which are approximate iso-stereomeres of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), has been partially elucidated and involves a receptor mediated proces. After binding of the ligand to the cytosolar aryl hydrocarbon receptor (AhR), the ligand-receptor complex is activated and translocated to the nucleus, wherein it binds to dioxin responsive elements (DREs) followed by stimulation of transcription of adjacent down stream genes (Denison and Whitlock, 1995). Induction of cytochrome P450 1A1 and its associated increase in ethoxyresorufin O-deethylase (EROD) activity is often studied, because its AhR-mediated expression is altered in tandem with that of other enzymes and receptor proteins (Brouwer 1991). The use of the EROD activity for quantifying AhR-active compounds is, however, limited under several circumstances by the phenomenon of substrate inhibition (Murk *et al.*, 1996b, 1996d).

For hazard and risk assessment of mixtures of PHAHs, the relative toxic potencies of individual PHAH congeners compared to TCDD have been transformed into toxic equivalency factors (TEFs). The concentrations of the individual congeners multiplied by their respective TEFs are added up to give the total TCDD toxic equivalency (TEQ) of the mixture (Safe, 1990; Ahlborg et *al.*, 1992). However, the often small concentrations of individual congeners, the presence of unknown or not routinely measured AhR-active substances, the lack of TEF values for several PHAHs, and the possible additive and antagonistic interactions between PHAHs (Safe, 1994; Aarts et *al.*, 1995) are drawbacks to the TEQ approach. In addition, an extensive sample clean-up is needed for chemical analysis of the toxicologically relevant planar PHAHs, in order to separate them from the bulk of other congeners (Nord, 1992). Therefore, a sensitive and quick pre-screening assay is needed for monitoring the toxic potency of whole mixtures of PHAHs in relatively small samples, such as blood plasma.

The CALUX (chemical-activated luciferase expression) assay

A novel *in vitro* bioassay has been developed recently, based on AhR-mediated firefly (*Photinus pyralis*) luciferase gene expression in genetically modified cell lines (Aarts et *al.*, 1995; Garrison *et al.*, 1996). The assay is called the *CALUX* assay (chemical-activated luciferase expression). To produce the *CALUX* cells, a vector containing the luciferase gene under transcriptional control of DREs was stably transfected into mouse (Hepa1c1c7) and rat (H4IIE) hepatoma cell lines, and several other cell lines (Postlind *et al.*, 1993; Aarts *et al.*, 1995; Garrison *et al.*, 1996; Richter *et al.*, 1996). Luciferase induction by TCDD appeared to be dose-dependent, and saturates at ligand concen-

trations greater than 100-1000 nM. For the PCDD-, PCDF- and PCB-congeners tested so far, the relative potency to induce *CALUX* activity correlated well with reported TEF values (Denison *et al.*, 1993; Aarts *et al.*, 1995; Garrison *et al.*, 1996; Sanderson *et al.*, 1996).

Objectives of the present study

The present study was performed to study the possible use of the *CALUX* assay for determining AhR-active compounds present in blood plasma, as a measuring for the internal exposure of animals. *CALUX*-TEQs were determined in crude blood plasma extracts, still containing all lipids, and in sulphuric acid cleaned blood plasma extracts from experimentally dosed eider ducks. The CALUX-TEQs determined in these extracts were compared with chemically determined PCB levels in blood plasma and in abdominal fat, and with the hepatic EROD-activity of the same animal. Plasma cholesterol and triglyceride concentrations were determined and used for normalisation of PHAH levels on a lipid basis.

Materials and Methods

Chemicals

Methanol, diethyl ether, and isooctane were of p.a. grade and purchased from Merck (Germany). Hexane was of HPLC quality (Rathburn, Scotland). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was purchased from Schmidt (The Netherlands) and the purity and concentration of the TCDD stock solution was reconfirmed by GC-MS analysis (Dutch State Institute for Quality Control of Agricultural Products, RIKILT-DLO). Ultra-clean dimethyl sulfoxide (DMSO) was purchased from Janssen (Belgium). Sulphuric acid (z.a., Baker, Holland) was washed with hexane before use in a silica gel 60 (70-230 mesh, ASTM, Merck) column with dried sodium sulphate (p.a., Merck).

Eider ducks

The blood and tissue samples used in this experiment, originated from an earlier performed *in vivo* experiment with eider ducks. The newly hatched eider ducklings were caught on the isle of Vlieland in the Dutch Waddenzee, and kept in large open-air cages as described in Murk *et al.* (1994a). After an acclimation period of 27 days the animals were dosed once intra peritoneally with the vehicle corn oil, 5 or 50 $mg \cdot kg^{-1}$ body weight (bw) 3,3',4,4'-tetrachlorobiphenyl (PCB-77), or with 50 or 200 $mg \cdot kg^{-1}$ bw Clophen A50 (Murk *et al.*, 1994a). Ten days later the animals were killed under ether

anaesthesia, and in addition to tissues, blood was collected and processed to obtain plasma. Blood plasma was stored at -20°C. EROD activities were measured in hepatic microsomes, and a number of mono-*ortho*-and di-*ortho*-PCB congener levels were determined in abdominal fat using GC-ECD, as described in Rozemeijer *et al.* (1995). Lipids were extracted from abdominal fat using pentane and quantified gravimetrically. In this paper only the levels of 2,3,4,4',5-pentachlorobiphenyl (PCB-118) and 2,3,3',4,4',5-hexachloro biphenyl (PCB-156) were used for comparison with the bio-assays, because these congeners contributed most to the total TEQs of the PCB-mixture. For the PCB-77 dosed animals the PCB-77 levels in the abdominal fat were quantified.

Extraction and clean-up of blood plasma

A selection of 25 blood plasma samples was made based on the internal PCB-concentrations measured in abdominal fat, in order to obtain a wide range in concentrations. Blood plasma aliquots of about 1.5 ml in hexane-washed glass centrifuge tubes were denaturated with one volume of methanol. After vigorously mixing, two volumes of hexane were added (about 3 ml) and after mixing again, the tubes were centrifuged for 2 min at 1500g at room temperature. The hexane layer was transferred to another hexane-washed tube, and 1 drop of 6 M HCl per 0.5 ml of plasma was added to the remaining water phase. Subsequently the samples were extracted twice again with hexane as described above. Finally 0.5 ml hexane was added to the water phase, collected again without mixing, and pooled with the rest of the hexane extract. The extraction efficiency of PHAHs from the blood plasma was not determined for each individual sample, as addition of ^{13}C-, or ^{14}C-PCB-standards would influence the *CALUX* measurements. However, the average extraction efficiency was tested using ^{3}H-TCDD spiked eider duck blood samples. The recovery was always 99-101%.

An aliqout of the hexane extract was taken for further clean-up and GC-analysis, the rest was pipetted into a conical screw vial, and evaporated at 30°C under a gentle stream of nitrogen gas. These extracts are further referred to as 'fatty' extracts. Shortly before all hexane was evaporated the desired amount of DMSO was added. After all hexane was evaporated a dilution series of the samples was made in DMSO. For the first dilution step the extracts had to be kept warm (± 30°C) to prevent clotting of the lipids in the pipet. The fatty extracts were tested in 6-well plates using Hepa.Luc cells (see below).

The other part of the hexane fraction of the plasma extract was cleaned over a 20% H_2SO_4 deactivated silica column with 15 ml hexane-diethyl ether (97:3, v/v). This extract is further referred to as 'cleaned' extract. The recovery of PHAHs from the silica-H_2SO_4 column could not be tested for each individual sample for the same reason as

mentioned above. From former experiments however, using ^{13}C-PCB standards under the same standard conditions, we know that this recovery is more than 95%. The cleaned extract was evaporated to 0.5 ml; 1 ml iso octane was added and the extract was quantitatively transferred to a tapered vial and evaporated to approximately 50 μl for GC-MS determination (see below). After GC-analysis, the remaining extract was evaporated, dissolved and diluted in DMSO for *CALUX* measurement in 24-well plates using H4IIE.Luc cells (see below).

CALUX assay

H4IIE.pGudluc1.1 (H4IIE.Luc) or Hepa.pGudluc1.1 (Hepa.Luc) cells, prepared as previously described in Aarts *et al.* (1995) and Garrison *et al.* (1996) (also called, respectively, H4L1.1c4 and H1L1.1c7), were used for experiments performed in, respectively, 24-well or 6-well culture plates (Costar). All cells were grown in minimal essential medium (α-MEM, Gibco) with 10% heat-inactivated (h.i.) fetal calf serum (FCS, Gibco) and 500 μg of G418 (Geneticin, Gibco) per ml of medium, at a temperature of 37°C and 5% CO_2.The *CALUX* experiments with the fatty eider plasma extracts were performed in 6-well cell culture plates in 3 ml α-MEM per well. Due to technical improvements during the course of these studies, the cleaned extracts were tested in 24-well cell culture plates with 0.5 ml α-MEM per well, both without Geneticin. When the cell layer reached 90-100% confluency, 24 hr after seeding, the cells were dosed with the test compounds in a maximum of 0.5% DMSO in growth medium by adding the DMSO to the culture medium (6-well plates) or replacing the medium with fresh culture medium containing the DMSO (24-well plates). Exposure was in triplicate, and on each 24-well plate three TCDD calibration standards, or a TCDD standard series per assay (6-well plates) were included. After 20-24 hr of exposure the medium was removed and cells were washed twice with phosphate-buffered saline (PBS; Oxoid, England). The cells were harvested in 250 μl (6-well plates) or 75 μl (24-well plates) cell lysis reagent (Promega), centrifuged for 3 min (6-well plates) or 90 sec (24 wells plates) at 13000g and the supernatant frozen at -80°C. For measurement of luciferase activity the supernatants were thawed on ice, 20 μl aliquots were pipetted into a 96-well microtiter plate, 100 μl luciferin assay mix (Promega) at room temperature was added and the plate was mixed for approximately 90 sec on a plate mixer (Amersham). The light production was measured in an Amerlite Luminometer (Amersham). The protein content of each supernatant was measured in a microtiter plate at 595 nm (Molecular Devices micro plate reader), according to the method of Bradford (1976) using BioRad protein assay dye reagent and bovine serum albumin (BSA) as protein standard.

GC-MS-method

The cleaned eider blood plasma extracts were analysed for PCB-77 (PCB-77 dosed animals) or PCB-153 levels (rest of the animals), using gas chromatography (GC) coupled to a Saturn II ion trap detector (ITD) (Varian, Walnut Creek, CA). The GC column was a 30 m x 0.2 mm i.d. DB5-MS with a film thickness of 0.2 μm (J&W Scientific, Folsom, CA). A retention gap of 2 m x 0.53 mm i.d. deactivated fused silica (Chrompack, Middelburg, The Netherlands) was used. The helium flow rate was 1.2 ml·min^{-1} at 90°C (2 min) at 14°C·min^{-1} to 240°, next at 15°C·min^{-1} to 270°C with a hold for 15 min at 270°C. For the identification and determination of PCB-77 and PCB-153 a cluster of masses was selected, a single mass was used for the quantification. For further GC and ITD conditions see Leonards *et al.* (1996).

Enzymatic triglyceride and cholesterol assays

Triglyceride concentrations were determined in 10 μl samples of blood plasma by quantitative enzymatic determination using commercially available (Sigma Diagnostics, procedure no. 337) triglyceride reagent (GPO-Trinder) and glycerol standard (250 mg·dl^{-1}). This method is based on enzymatic hydrolysis of triglyceride, resulting in the production of a quinoneimine dye (measurable at 540 nm) directly proportional to glycerol released from triglycerides in the sample.

Cholesterol concentrations were determined in 10 μl samples of blood plasma by quantitative enzymatic determination using commercially available cholesterol reagent (Sigma Diagnostics, procedure no. 352) and standard (200 mg·dl^{-1}). This enzymatic method also yields a quinoneimine dye (measurable at 500 nm) which is directly proportional to the total cholesterol concentration in the sample.

Calculations of CALUX-TEQs and statistics

For calculation of *CALUX*-TEQs a complete standard curve of 2,3,7,8-TCDD was used for each cell line. In each assay, three different TCDD calibration standards, bracketing the TCDD EC_{50}, were measured to correct for assay to assay variation. The standard curve was fitted (1-site ligand fit) using SlideWrite 5.1 and the *CALUX*-TEQ value for an unknown sample was interpolated on this curve. For each sample a dilution series was made, and measured in triplicate. The dilution of the sample that resulted in a *CALUX* response between the EC_2 and EC_{50} of the TCDD response, preferably close to the EC_5, was used for quantification of the sample. This is the most linear part of the dose-response curve, and the quantifications based on this part of the curve are very reproducible.

Statistical analysis of dose-effect relationships was performed by unweighted least-squares linear regression analysis. Differences between group means were tested

Table 7.1 Correlations between *CALUX*-TEQs and PCB-levels in abdominal fat or blood plasma after logaritmic transformation. The three control animals are left out of these correlations because they were below the chemical detection limit for the sample size used.

	Abdominal fat			*Blood*	
	PCB-118	PCB-156	PCB-77	PCB-153	PCB-77
CALUX-TEQ (fatty)	r=0.86 *P*=0.01	r=0.88 *P*=0.02	r=0.94 *P*<0.001	r=0.81 *P*<0.02	r=0.95 *P*<0.001
CALUX-TEQ (cleaned)	r=0.82 *P*<0.01	r=0.82 *P*<0.01	r=0.97 *P*<0.001	r=0.84 *P*<0.01	r=0.96 *P*<0.001

Table 7.2 *CALUX* based TEQs and cholesterol and triglyceride levels in blood plasma, and hepatic EROD activity, in control and PCB-exposed eider ducks.

Parameter	Control[1]	PCB-77		Clophen A50	
		5 mg/kg[2]	50 mg/kg[3]	50 mg/kg[2]	200 mg/kg[3]
CALUX-TEQ (fatty) (fmol/ml)	8.2 ± 6.4	398 ± 401	854 ± 443*	9.2 ± 4.6	37.8 ± 43.2
CALUX-TEQ (fat-free) (fmol/ml)	11.5 ± 8.6	282 ± 112	870 ± 482*	12.4 ± 6.7	53.3 ± 81.1
Hepatic EROD activity (nmol/mg protein.min)	6.7 ± 2.7	59.4 ± 6.4	193.8 ± 126.9*	8.2 ± 5.9	14.4 ± 13.9
Cholesterol (mg/ml)	26.7 ± 1.7	26.7 ± 2.2	26.3 ± 2.9	27.9 ± 2.3	23.9 ± 4.1
Total triglycerides (mg/ml)	6.6 ± 4.5	6.5 ± 4.4	6.4 ± 1.8	6.9 ± 1.4	7.7 ± 1.4
True triglycerides (mg/ml)	4.8 ± 1.5	4.8 ± 0.6	5.0 ± 1.5	5.1 ± 1.7	5.8 ± 1.2

Data are expressed as means ± S.D.

* Significantly different from control with $P<0.05$

[1] n=3; [2] n=5; [3] n=6

using one-way ANOVA. Both calculations were performed using Statistix version 4.0. The acceptance level was set at $P < 0.05$.

Results

The *CALUX* response was found to be very sensitive and reproducible using TCDD as a positive control. The TCDD dose-response curve saturated between 100 pM and 1 nM, the EC_{50} was 10 pM, and the standard deviation was generally ≤5%. The detection limit in H4IIE.Luc cells was less than 1 pM TCDD, which equals an absolute amount of less than 0.5 fmol/well in 24-well plates. A full dose-response curve was presented before (Murk *et al.*, 1996b).

Dilution series of extracts from eider duck's blood plasma induced *CALUX* activities in a dose-related manner, according to a one-site ligand dose-response curve. Figure 7.1 presents dose-response curves for the fatty extracts from plasma of three eiders exposed to either 5 or 50 mg·kg^{-1} bw PCB-77 or 200 mg·kg^{-1} bw Clophen A50, or from a corn oil treated control animal. The *CALUX* activities induced by blood plasma extracts from animals dosed with 50 mg·kg^{-1} bw Clophen A50 (not in the figure) were almost as low as the controls. The standard deviations (STDS) in the measured *CALUX* activities were 5-10%.

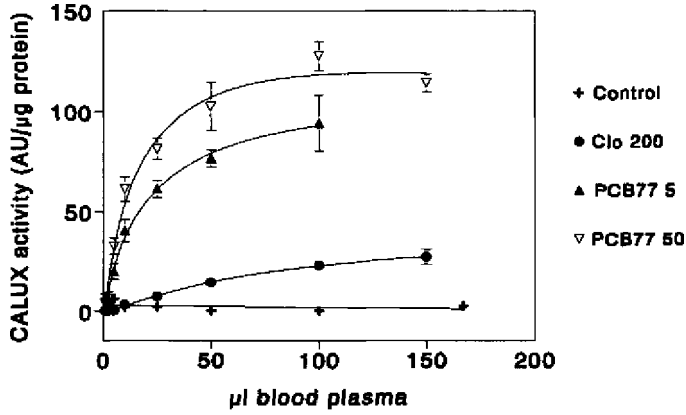

Figure 7.1 *CALUX* induction in Hepa.Luc cells by fatty extracts from a control, 200 mg Clophen A50/kg (CloA50 200), and 5 or 50 mg PCB-77/kg (PCB77 5 or PCB77 50) dosed eider duck.

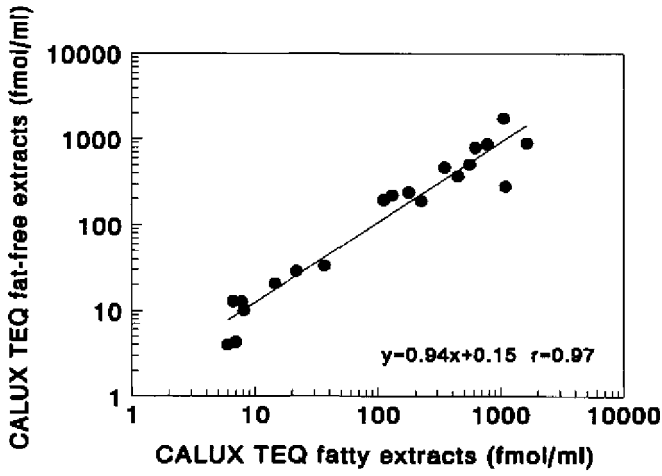

Figure 7.2 Correlation between *CALUX* TEQ determined for uncleaned, fatty blood plasma extracts from experimentally exposed eider ducks, measured in 6-well plates using Hepa.Luc cells, and the same extracts after clean-up, determined in 24-well plates, using H4IIE.Luc cells. For details see Materials and Methods.

The *CALUX*-TEQs determined for the cleaned extracts in 24-well plates, correlated very well with the *CALUX*-TEQs, measured in 6-well plates, for the fatty blood plasma extracts (Figure 7.2; $r = 0.97$).

The PCB-77 (for PCB-77 dosed eiders) or PCB-153 (for Clophen A50 dosed eiders) levels chemically measured in blood plasma correlated significantly with, respectively, PCB-77 levels ($r = 0.98$, $p < 0.001$) or PCB-118 or PCB-156 (respectively $r = 0.96$, $p < 0.001$ and $r = 0.99$, $p < 0.001$) chemically measured in abdominal fat (data not shown). Correlations between *CALUX*-TEQs determined in fatty or cleaned extracts with PCB-levels determined in abdominal fat or blood plasma are presented in Table 7.1. The correlations between PCB-77 (PCB-77 dosed eiders), or PCB-118 and -156 levels (for Clophen dosed eiders) in abdominal fat and the *CALUX*-TEQs determined in the cleaned extracts are presented in Figure 7.3. The three control animals are left out of this figure because the PCB-levels were at, or below, the detection limit. The relationships between the PCB-77 or PCB-153 levels in blood plasma and the *CALUX*-TEQ are comparable (data not shown). From the correlation between the TCDD-equivalents measured with the *CALUX* assay and the chemically determined PCB-77 levels in blood plasma the TEF for PCB-77 in the *CALUX* assay was calculated ([*CALUX*-TEQ]/[PCB-77]). Based on the

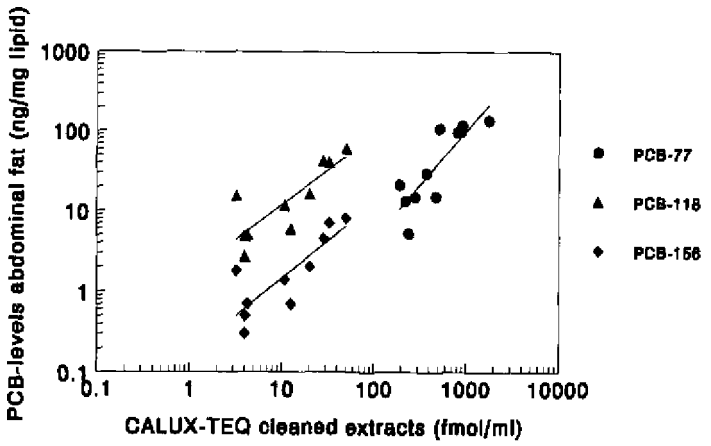

Figure 7.3 Correlation between PCB-118 or PCB-156 levels (for Clophen dosed eiders) or PCB-77 levels (for PCB-77 dosed eiders) determined in abdominal fat using GC-ECD and the *CALUX* TEQ of cleaned blood plasma extracts determined in 24-well plates using H4IIE.Luc cells. For correlations see Table 7.1.

CALUX data for fatty extracts this TEF is 1.6 10^{-3}, based on the cleaned extracts the TEF is 1.5 10^{-3}.

The *CALUX*-TEQs correlated significantly with the hepatic EROD activity, as is shown for the fatty blood plasma extracts in Figure 7.4 ($r = 0.88$, $p < 0.05$). In this figure two different clusters are visible: the control and Clophen A50 dosed animals with low EROD activity and the PCB-77 dosed animals with a relatively high EROD activity. For each exposure group, the average *CALUX*-TEQs, hepatic EROD activity, and cholesterol and triglyceride levels in blood plasma are presented in Table 7.2. Average plasma cholesterol and triglyceride levels were not statistically significant different, although the data suggest a slight increase in triglyceride levels and a decrease in cholesterol levels in the high dose Clophen A50 animals (Table 7.2). All *CALUX*-TEQs mentioned above were expressed on a volume basis, as fmol TEQ·ml^{-1} blood plasma. When expressed on a triglyceride or cholesterol basis, the correlations with chemical data or EROD activity were comparable or slightly less (data not shown).

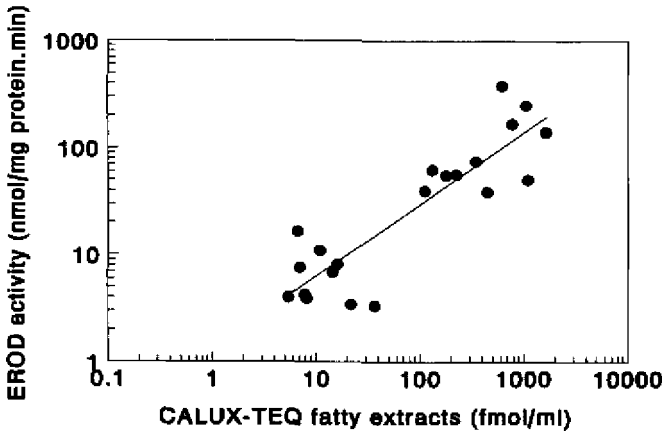

Figure 7.4 Correlation between the hepatic EROD activity and the *CALUX* TEQ for uncleaned, fatty blood plasma extracts, determined in 6-well plates using Hepa.Luc cells (r = 0.88). For details see M&M.

Discussion

The CALUX-TEQ in bloodplasma as a measure of PHAH exposure

The *CALUX* assay proved to be a sensitive, fast and relatively easy method to determine the total toxic potency of mixtures of PHAHs in blood plasma, expressed in TCDD equivalents (TEQs). The *CALUX*-TEQ in blood plasma represents an integrated measure of the internal dose of the AhR-active PCBs in eider ducks, experimentally exposed to either PCB-77 or Clophen A50. The in 6-well and 24-well plates independently performed *CALUX* assays resulted in almost identical *CALUX*-TEQs, confirming the high reproducibility which was already observed in former experiments with pure compounds (Garrison *et al.*, 1996; Murk *et al.*, 1996b; Sanderson *et al.*, 1996). Based on the chemically determined PCB-77 levels and *CALUX*-TEQs in blood plasma of PCB-77 dosed eiders, a TEF of 1.5 10^{-3} was estimated. This value is in the range of the TEFs determined before in H4IIE.Luc cells: 0.7 10^{-3} (Sanderson *et al.*, 1996) and $1.4^{-3}10$ (Murk, unpublished data), and the TEF value of 0.01 proposed by Safe (1994), and 0.5 10^{-3} as proposed by the WHO (Ahlborg *et al.*, 1994).

The results of this study demonstrate that blood plasma extracts can be measured equally well in the *CALUX* assay with and without application of a clean-up procedure.

Skipping the latter step makes the analysis faster, and reduces the probability of unforseen loss of yet unknown compounds of interest on the silica column. However, handling of the fatty extracts, such as in making a dilution range or concentrating the extract, may be more complicated because of the possible precipitation of lipids, unless the extract is carefully kept at about 30°C. In addition, adding a lot of lipid to the growth medium would gradually reduce the availability of PHAHs for the cells. Performing a simple additional Silica-H_2SO_4 clean-up step, circumvents these problems and allows one to concentrate the extract. It is also possible to directly expose *CALUX* cells to untreated blood plasma or serum (data not shown). Although it is tempting to use blood plasma samples as crude as possible for fast pre-screening, recent information suggests that endogenous ligands, or compounds originating from food, may interfere with the response induced by PHAHs (Murk, unpublished results). As these factors may differ depending on food composition, species, sex, age or health status, the results may be misleading.

Measure of lipid content for normalisation

The observed good correlation of the *CALUX*-TEQ in blood plasma with the GC-TEQ in abdominal fat, suggests that PHAHs are relatively evenly distributed over the total lipid phase in a body. Duinker *et al.* (1989) reported that PCBs were present in virtually identical levels in liver, heart, fat tissue, kidney and muscle if expressed on a lipid basis. Only of relatively biodegradable PCBs higher concentrations were present in the liver. Liver deposition appears to be relatively more important for non-*ortho* congeners, but the ratio between liver and adipose tissue concentrations are species dependent (Ahlborg *et al.*, 1992). As blood plays an important role in the transport of PCBs between tissues, it is to be expected that there is a dynamic balance between PCBs levels in blood, liver and other organs. Therefore levels in blood can be used as an overall measure for the internal PHAH dose if normalized on a lipid basis (Aquilar, 1985; Brouwer *et al.*, 1995).

Aquilar (1985) observed that the brain is the only tissue with deviating PCB-levels when expressed on a lipid basis, probably because the basic constituents of the cerebral lipids are phospholipids, in addition to the haem-encephalic barrier which blocks the passage of pollutants to a certain degree. Phospholipids are characterized by a greater polarity than triglycerides and cholesterol, and therefore don't play a great role in the retention of apolar PHAHs. As levels of PHAHs seemed to be essentially linked to triglycerides, standardization should be preferably on the basis of triglyceride weight instead of 'extractable weight', as levels of phospholipids differ among and within tissues. Other studies normalize on the basis of triglycerides and cholesterol (so called 'core fat', Brouwer *et al.*, 1995). Effects on total plasma lipid levels of fasting and feeding are mainly

caused by changes in triglyceride levels (Phillips et *al.*, 1989). However, most laboratories quantify 'extractable lipids' or 'total lipids' gravimetrically. The resulting lipid determinations are very non-specific and differ greatly between laboratories, depending on de extraction conditions and solvents used (QUASIMEME, 1994). One of the problems is that the extracts contain other extractable material as well as lipids, thus measurements are 'extractable weight' rather than only 'extractable lipid'.

The advantage of the kits we used in this study for cholesterol and triglyceride measurement, is that lipids can be specifically quantified in very small aliquots of plasma. In addition, quantification of categories of lipids could give additional information on the physiological condition of organisms. In this study, correlations between *CALUX*-TEQs in blood and PCB-levels in abdominal fat did not improve when expressed on triglyceride or cholesterol basis instead of per ml of plasma. In addition, no differences were observed in triglyceride and cholesterol levels in blood plasma between the exposed eiders and the control group. The eider ducks used for this study, were of the same age, lived under the same circumstances, and were fed the same food. Therefore, their plasma lipid levels were not expected to differ greatly, as was confirmed by the cholesterol and triglyceride measurements. However, when PHAH levels of naturally exposed individuals are to be determined, normalisation on a lipid basis is necessary as for these animals large differences ecological and physiological conditions are to be expected, and lipid composition and lipid levels are influenced by nutritional status, condition, season, maturation, reproductive cycle, sex, species, organ, etc.

Perspectives for CALUX analysis of blood from environmentally exposed species

The experimentally dosed animals used for this study contained relatively high levels of PCBs. The main use for the *CALUX* assay, however, lies in the application for naturally exposed animals including humans. In an integrated ecotoxicological study to establish otter-based quality standards for PHAHs (Hattum et *al.*, 1996), extracts of 1-2 ml aliquots whole blood from naturally exposed otters *(Lutra lutra)* were tested in the *CALUX* assay. The *CALUX*-TEQs ranged from 10-200 fmol·ml^{-1} (3-60 pg TEQ·ml^{-1}) blood. The *CALUX*-TEQs in the livers of these otters and the TEQ based on mono- and non-*ortho*-PCBs correlated very well, and also absolute TEQ levels were comparable (Murk et *al.*, 1996f). With a detection limit of less than 0.5 fmol TEQ/well in 24-well plates, 1 ml of relatively clean otter blood (or 2 ml to prepare 1 ml of blood plasma) is needed to make a small dilution series, to be sure that one of the *CALUX* responses falls within the linear part of the TCDD dose-effect curve. For semi-quantitative information such as 'relatively clean or highly exposed', only one sample needs to be measured (in triplicate), therefore about 0.3-0.5 ml blood plasma is needed. Although the collection of aliquots of 1-2 ml blood

will generally not be a problem for species weighing over 1 kg, it still is too much for smaller species, such as common terns, weighing approximately 100 g. From such species not more than 1 ml of blood can be collected without harming them. If other parameters such as plasma thyroid hormone and retinol levels are to be measured in these samples as well, the amount needed for the *CALUX* assay should be further reduced. At the moment, the *CALUX* assay has been further optimized, and is currently performed in 96-well plates without the need of pipetting cell lysate and with automated injection of substrate in an automated luminometer (Labsystems) (Murk *et al.*, 1996f). Not only does this further reduce the amount of time and material needed for the *CALUX* assay, it also decreases the limit of detection 5 times compared to the method presented here. The limit of detection of the improved *CALUX* assay is less than 0.1 fmol TEQ/well.

Concluding remarks

The results demonstrate that the *CALUX* assay is a rapid and sensitive method to determine the AhR-related toxic potency of compounds present in blood plasma. The usefulness of the *CALUX* assay is especially evident when, due to small sample size or small concentrations of individual congeners, samples would have to be pooled, thus loosing information, or animals have to be killed to get enough material. The *CALUX* assay can also be used for rapid screening of large quantities of samples. Samples inducing a great *CALUX* response could subsequently be chemically analysed to identify the responsible compound(s).

Acknowledgements

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CHAPTER 8

GENERAL DISCUSSION

The research described in this thesis investigates toxic and biochemical effects of PHAHs on juvenile fish-eating birds following experimental or environmental exposure. Based on the toxicological mechanisms of action of PHAHs, biomarkers are developed and validated to identify causal relationships between exposure to PHAHs and adverse effects, even at low levels of exposure. Such tools are needed to address the question whether at present fish eating wildlife species in the Netherlands are exposed to toxic levels of PHAHs.

Overall, the present results confirm the hypothesis that juvenile eider ducks and common terns respond to PHAHs similarly to laboratory species in terms of P450 1A induction and alterations in thyroid hormone and retinoid levels. In addition, the internal PHAH concentrations and biochemical changes can be associated with a number of adverse physiological effects. The observed biochemical and toxic effects are at least partially Ah-receptor (AhR) mediated. However, the observed effects may also partially be a consequence of the toxic action of PHAH metabolites, since hepatic microsomes of PHAH-exposed fish-eating birds and -mammals were found to be able to form hydroxylated (OH) metabolites *in vitro*. An AhR driven reporter gene expression assay has been developed and validated for use as a simple and sensitive biomarker for PHAHs in a number of environmental matrices.

SUMMARY OF THE RESULTS

Juvenile eider ducks and common terns showed AhR responsiveness when exposed respectively dosed with PHAHs in a semi-field experiment (Chapter 2) or exposed environmentally in a field study (Chapters 3 and 4). Internal PHAH levels correlated with hepatic P450 1A induction, measured as EROD-activity, and with hepatic T4-UDP-glucuronyltransferase(GT) activity. For eider ducks PCB-exposure was associated with reduced growth and behavioural activity. In common terns, reduced yolksac retinyl palmitate and plasma thyroid hormone and retinol levels, and a significantly greater ratio of plasma retinol over yolksac retinylpalmitate, coincided with later egg laying and a longer incubation period for the artificially incubated eggs. The incubation period was even more prolonged for the matching, naturally incubated, common tern eggs. Greater yolksac PHAH levels and hepatic EROD-activity were associated with smaller eggs and chicks, later egg laying, and prolonged incubation period (Chapter 4).

The hepatic microsomes of eider duck, common tern, harbour porpoise, harbour seal and rat were found to metabolize the model ^{14}C-labelled PCB-77 (3,3',4,4-tetra-

chlorobiphenyl) at a rate correlating with the EROD activity. The ratio of the 4-, 5-, and 6-OH-metabolites formed varied for the species tested. The birds mainly formed the 5-OH-PCB metabolite. No PCB-77 metabolism was observed in the trout, despite elevated EROD activity (Chapter 5).

The Ah-receptor mediated reporter gene assay, the chemical activated luciferase gene expression (*CALUX*) assay, proved to be a rapid, sensitive and relatively simple bioassay to measure TCDD-equivalents (*CALUX*-TEQs) in extracts of sediment, pore water and small aliquots of blood plasma (Chapters 6 and 7). The assay offers an integrated tool to assess the total AhR-related toxic potency of compounds in a mixture, with a detection limit of less than 0.2 fmol TEQ/well in 96-well plates. Few clean-up and separation step were needed compared to chemical determination of PHAHs, as the assay is not hindered by some lipids (Chapter 7) and the receptor differentiates between relevant or not relevant compounds with regard to the AhR mediated mechanism of toxicity. Also PHAHs such as polyhalogenated diphenyl ethers (PHDEs) and polychlorinated terphenyls (PCTs), which are present in environmental matrices but usually not included in hazard assessment, were found to contribute to the AhR mediated toxicity (Chapter 6).
In the following, these results are discussed in relation to both research questions introduced in Chapter 1 of this thesis.

1 *Do natural fish-eating bird species exhibit toxic effects upon PHAH-exposure, comparable to the effects observed in laboratory species?*

The biochemical changes in vitamin A and thyroid hormone levels observed in eider ducks and common terns, are very much comparable to the effects reported for laboratory rodents and -birds, as was discussed in detail in Chapters 2, 3 and 4. In both bird species, these biochemical changes could at least be partially related to directly AhR-mediated effects or be induced via PHAH metabolites, as is schematically represented in Figure 8.1, and discussed in Chapters 2-5. The observed biochemical changes could also be related to changes in physiological functions, such as moment of egg laying, incubation period and growth. It is probable that the physiological changes are due to causal relationships, as thyroid hormone and vitamin A levels are known to play an important role in growth (McNabb, 1987), embryonic development (Heine *et al.*, 1985; Dersch and Zile, 1993), neurological development (Maden and Holder, 1992), energy metabolism (Hardeveld, 1986), and skeletal development (Thaller and Eichele, 1987; Pijnappel *et al.*, 1993), and fetal PCB-exposure can result in long-term physiological alterations (Morse, 1995). More specifically for birds, vitamin A shortage has been related to reduced formation of the large blood vessels in the membrane surrounding a bird embryo, which may lead to a reduced rate of development or even death of the embryo (Thompson,

1970). In addition, thyroid hormone plays a very important role in normal hatching of especially precocial bird species, which are relatively mature at that moment, such as common terns. Reduced thyroid hormone levels may result in delayed hatching or even death of the embryo in a late stage of development (Whittmann *et al.*, 1993; McNabb, 1987). Large differences have been reported for the physiological role of thyroid hormone shortly before hatching or birth in embryos from precocial species (such as tern, eider and chicken or sheep and monkey) and species which are immature at birth (altricial species such as dove and cormorant or rat and human) (Legrand, 1986; McNabb, 1987). This means that the physiological consequence of reduced thyroid hormone levels may differ between such species as well.

Although PHAHs probably disturb the vitamin A and thyroid hormone homeostasis in fish-eating birds and mammals via comparable mechanisms, an important difference with mammals is that bird embryos develop without physiological interference with the

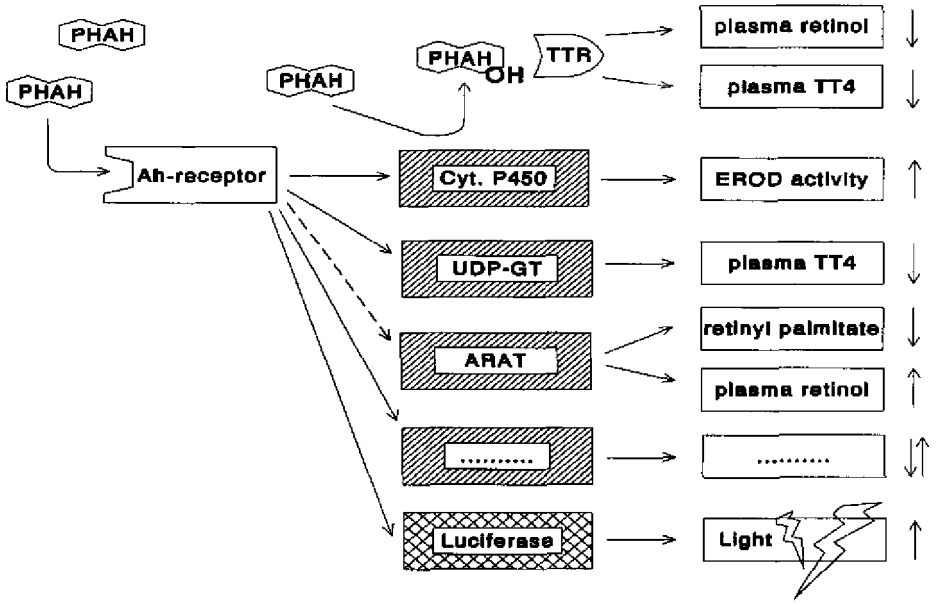

Figure 8.1 Schematic representation of Ah-receptor mediated biochemical effects of PHAHs and effects induced via hydroxylated (OH-) PHAH metabolites. The luciferase gene is present in recombinant cell lines developed for the chemical activated luciferase gene expression (*CALUX*) assay. For further explanation see text.
TTR = transthyretin; TT4 = total thyroxine; EROD = ethoxyresorufin-*O*-deethylase; UDP-GT = uridine-5'-diphosphate glucuronyltransferase; ARAT = acyl-CoA:retinol acyl-transferase; ..... = other (yet unknown) effects.

female bird. The female bird deposits retinol and thyroid hormone from her blood plasma into the egg yolk (Spear *et al.*, 1989; Vieira et *al.*, 1995), and these levels may be influenced as a consequence of PHAH-exposure. In contrast to mammalian embryos, the bird embryo starts to regulate their own vitamin A and thyroid hormone homeostasis during early development (McNabb, 1987; Spear *et al.*, 1989; Dong and Zile, 1995). PHAHs deposited in the egg may cause disturbances in the regulation of this embryonic homeostasis, and OH-PHAHs may play an additional role in these disturbances as well. OH-PHAHs may be deposited into the egg when the female transfers TTR from her blood plasma into the yolk (Vieira *et al.*, 1995). As the tertiary and quartenary structure of chicken and human TTR are supposed to be highly similar (Duan *et al.*, 1991), it is probable that also in birds OH-PHAHs are bound to TTR. Deposition of OH-PHAHs into the egg would be comparable with the selective accumulation of OH-PHAH-metabolites in mammalian embryos from the serum of the mother animal (Morse *et al.*, 1995a; Danerud *et al.*, 1996). In contrast to fetal rats (Morse et *al.*, 1995a), however, chicken embryos have been reported to have PHAH-inducible cytochrome P450 activity (Bosveld *et al.*, 1996), and thus may form additional PHAH-metabolites themselves.

The current knowledge regarding the magnitude of the risk of PHAH-metabolites for wildlife species and man, relative to directly AhR related effects, is still very limited. OH-PCBs have been found to accumulate in blood of, for example, environmentally exposed grey seals (*Halichoerus grypus*) and man (*Homo sapiens*) (Bergman et *al.*, 1994), and in albatross (*Diomedea spec.*) blood in concentrations greater than the parent compounds (Klasson-Wehler and Giesy, pers. comm.). The pattern and amount of metabolites formed after exposure to the same PHAH-mixture may be different between birds and mammals (Chapter 5), resulting in different plasma levels and toxic potencies. Information about the capacity of species to conjugate specific OH-metabolites is needed to assess *in vivo* metabolite levels based on *in vitro* metabolism studies.

These observations discussed above suggest that PHAHs and/or their metabolites may cause biochemical, physiological and developmental effects in birds comparable to those mammals. Specific differences are, however, possible as well, especially in the case of developing embryos.

2 What is the perspective for the use of a receptor-mediated reporter gene assay to assess the total AhR related toxic potency of environmental pollutants?

The AhR-mediated *CALUX* response is induced parallel with other AhR related endpoints, such as P450 1A induction measured as EROD activity and reduction in hepatic retinoid storage (Figure 8.1). Therefore the *CALUX* assay is useful to directly quantify the toxicologically relevant planar PHAHs, as was also suggested by the correlation between the

CALUX response to PHAHs from pore water and the *in vivo* teratogenicity induced in an early life stage test with zebra fish *(Branchydanio rerio;* Chapter 6). Chemical measurement of these PHAH congeners is analytically difficult, time consuming, and will hardly ever be complete (Liem *et al.*, 1991; Leonards *et al.;* 1994a; Bosveld *et al.*, 1995). In addition, a conversion of individual chemical data into TEQs is needed, based on TEF-values which may differ up to a factor of 100 between different sets (Ahlborg *et al.*, 1994). This may resulting in apparently different hazards for the same PHAH mixture. With a receptor mediated assay such as the *CALUX* assay the toxic potency of the mixture is quantified directly, so no conversion is needed. The relatively few clean-up and separation steps needed and the low limit of detection, even allows non-destructive quantification of the internal exposure of relatively small animals (Chapter 7).

The mechanism of *CALUX* induction is comparable to the often used EROD assay, which is based on AhR mediated induction of the natural P450 1A gene. The EROD activity, however, strongly decreases with PHAH concentrations after a maximum induction level has been reached (Hahn *et al.*, 1993; Kennedy *et al.*, 1993; Murk *et al.*, 1996b, 1996d). This may partially be due to a decreased availability of heme, needed for active P450 1A enzyme, caused by porphyrin accumulation (Figure 8.2) (Matteis and Lim, 1994; Kennedy *et al.*, 1995; Tysklind *et al.*, 1995). However, the decrease in EROD activity at higher ligand concentrations is at least partially a result of substrate inhibition (Figure 8.2; Murk *et al.*, 1996b, 1996d). The *CALUX* assay is not influenced by porphyrin accumulation or substrate inhibition, as no heme is needed for production of luciferase and PHAHs are not substrates for luciferase (Figure 8.2).

As some species specific differences in responses to PHAHs, such as antagonism between some di-ortho PCBs and planar PHAHs, were found to occur at the level of the AhR (Figure 8.2; Biegel *et al.*, 1989; Denison and Vella, 1990; Walker and Peterson, 1991; El Fouly *et al.*, 1994; Hahn et al, 1994; Aarts *et al.*, 1995; Richter *et al.*, 1996), it would be interesting to develop *CALUX* cell lines for a number of different wildlife species.

Perspectives for the use of mechanism-based responses as biomarkers for effects of PHAHs under field conditions

It has been demonstrated that thyroid hormone and vitamin A levels can be reduced upon exposure of birds or mammals to PHAHs, and reductions have been associated with disturbances of important physiological functions. Therefore these biochemical parameters are interesting, potentially broad applicable biomarkers for adverse effects of PHAHs under field conditions. An important prerequisite for such use is that the alterations in biochemical parameters are consistent, either increasing or decreasing upon PHAH

exposure. This is evident for parameters such as T4-UDPGT or EROD activity which increase, and hepatic or yolksac retinoid levels which decrease as a consequence of PHAH exposure. Plasma retinol levels, however, can be increased via AhR-related reduced storage capacity of the liver and/or reduced via the action of OH-PHAH metabolites (Chapter 2, Figure 8.1; Jensen *et al.*, 1987; Brouwer *et al.*, 1989; Bank *et al.*, 1989a). Therefore, although knowledge of changes in plasma retinol levels may help to explain mechanisms of toxic action in experimental situations, differences in plasma retinol levels can not be used as an indication for PHAH-exposure and -effect in the field situation. A comparable limitation applies for plasma thyroid hormone levels in free living species. The same As factors such as age, sexe, season, osmotic stress, temperature, ecological stress and food quality will modulate physiologically relevant parameters in a natural way, either good reference values for these background fluctuations are needed, or the animals have to be sampled under comparable conditions.

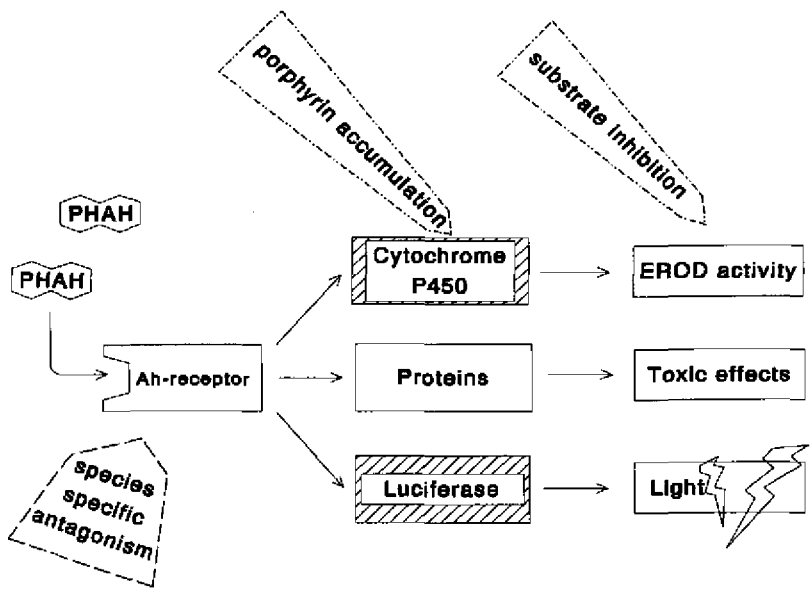

Figure 8.2 Inhibition of EROD activity by porphyrin accumulation and substrate inhibition. The *CALUX* response (measured as light output) is not inhibited by either of these factors. Species specific antagonism at the level of the Ah-receptor influences both cytochrome P450 and luciferase production, and probably also the induction of toxic effects.

It would be very useful to know the critical level of such physiologically relevant parameters, for normal functioning of an animal under realistic field conditions. This critical level, however, can only be roughly indicated as it depends on external as well as internal and temporal conditions. Hypothyroid hatchlings, for example, will be more susceptible to cold stress than hatchlings with normal thyroid hormone levels (Tori and Mayer, 1981), but extra deaths will probably only occur during adverse weather conditions and not with favourable weather. Integrated field studies can yield information about the relationships between exposure, bio-accumulation, biochemical parameters, and physiologically important functions under field conditions. In a recent study, performed to develop otter-based quality objectives for PCBs (DOQOP), a strong negative correlation was found between hepatic vitamin A and TEQ levels in European otters (*Lutra lutra*) (Murk *et al.*, 1996c; 1996f). A greater incidence of infectious diseases was observed in the highly exposed animals (Leonards *et al.*, 1996b), suggesting the biochemical changes partially fall outside of the normal physiological range.

Vulnerable species and life-stages

The biochemical parameters and reporter gene assay reported in this thesis can be useful tools to study species or life-stages with specific (eco)physiological characteristics which may result in extreme exposure or dependence on the PHAH-influenced parameters. Extreme exposure may occur, for example, in female eider ducks when they fast during breeding (Koeman, 1971). Hibernating mammals such as bats and polar bears, which accumulate high levels of PHAHs because of their narrow food choice, are exposed to extra high levels when they use up their fat-reserves during hibernation (Disser and Nagel, 1989; Norstrom *et al.*, 1990; Streit *et al.*, 1995). In addition, thyroid hormones play a specific physiological role when such animals wake up from hibernation (Hulbert, 1985), and depressed thyroid hormone levels could possibly prolong awakening. Also amphibians may be at special risk for exposure to and effects of PHAHs. They depend for their food on the aquatic environment during a large part of their life cycle, have relatively permeable skins, and thyroid hormones play a specific role in amphibian metamorphosis (Shi, 1994) and water-land migration (Grau, 1987; Moriya, 1982). Recent studies in the Department of Toxicology have demonstrated that development of early-life stages and metamorphosis of amphibians are sensitive for the toxic action of PCBs at concentrations occurring in relatively polluted Dutch ponds (Gutleb *et al.*, in prep.).

In general, it is to be expected that species are particularly vulnerable to the toxic actions of PHAHs when the endocrine system has to respond to changing environmental factors such as at the onset of hatching or birth, migration, reproduction, physical stress and illness. Biomarkers for exposure and effect based on the mechanism of toxicity can

Table 8.1 Estimated no-effect and effect concentrations for fish-eating birds and mammals.

All concentrations in ng TEQ/g lipid	Birds		Mammals	
	No effect	Effect	No effect	Effect
Common tern, Forster's tern, cormorant Field data [1]	4, 5, 20			
Common tern, Forster's tern, cormorant Field data [1]		14, 55, 65		
NOEC Bald eagle (range for birds) Literature data [2]	0.2 (0.03-3)			
NOEC hepatic retinoid reduction Field data European otter[4]			2	
NOEC mink reproduction Literature data [3]			1-6	
EC_{90} hepatic retinoid reduction Field data European otter[4]				5
EC_{50} mink reproduction Literature data [5]				5-10
Unaffected seals (repro duction/immune status); (Semi-) field data [6]			0.06	
Affected seals fed Waddensea/Baltic fish (Semi-) field data [6]				0.2

[1] Common tern data based on Murk *et al.* (1994b, 1996a, this thesis)
Forster's tern data calculated from Kubiak *et al.* (1989), assuming an egg contains 4% lipid. Effect: increased incubation period: Cormorant data estimated from Van den Berg *et al.* (1994), assuming the ratio mono ortho-PCB level:TEQs is comparable with common tern; and the Oude Venen colony contains 3.2 times TEQs than the Biesbosch colony. Effect: reduced fledging success.

[2] NOEC for birds based on calculations from Bowerman *et al.*, 1995

[3] NOEC for mink reproduction (litter size and kit survival) based on Leonards *et al.* (1994b), assuming the lipid weight of muscle is 2-3%.

[4] Hepatic retinol and retinylpalmitate reduction, based on the results of the DOQOP project (Murk *et al.*, 1996c; Murk *et al.*, 1996f) The reduction in hepatic retinoid levels correlated with increased prevalence of infectious diseases (Leonards *et al.*, 1996b).

[5] The EC_{50} for mink reproduction (litter size and kit survival) based on Leonards *et al.* (1995), assuming the lipid weight of muscle is 2-3%.

[6] Adverse immunotoxic effects in Harbour seals: 209 pg TEQ·g^{-1} lipid (fed fish from the Baltic Sea), reference seals: 62 pg TEQ·g^{-1} lipid (fed fish from the Atlantic Ocean) (Ross *et al.*, 1995). Adverse reproductive (Reijnders, 1986) and biochemical (Brouwer *et al.*, 1989) effects in harbour seals fed fish from the Dutch Waddensea, containing TEQ levels comparable to Baltic Sea fish (calculated from Boon *et al.*, 1987; De Zwart *et al.*, 1994).

be used to identify and study vulnerable species and life stages, and give an indication of critical PHAH levels.

Possible impact of current PHAH levels on fish-eating wildlife species

In order to compare the sensitivity of the common terns with other fish-eating birds and with fish-eating mammals, and to get an impression whether such species are currently at risk for adverse effects of PHAHs in the Netherlands, some estimated (no) effect concentrations for fish-eating birds and mammals, based on this thesis and some other recent publications are summarized in Table 8.1. Although the comparisons are only rough, as toxic endpoint, determination of lipid content and calculation of TEQs differ between the experiments, these data suggest that the sensitivity of the common terns are in the same order of magnitude as the Forster's terns and cormorants. These birds appear to be about one order of a magnitude less sensitive for PHAHs than the Bald eagle and birds in general based on the NOECs calculated by Bowerman *et al.* (1995) (Table 8.1). The sensitivity of the common terns seems to be slightly less than that of mink and otter. Seals appear to be one magnitude more sensitive than the mink and otter. It can, however, not be excluded that these differences are caused by not yet quantified PHAHs, present in greater quantity in the seals compared to the other species. The *CALUX* assay would be useful in such studies to indicate the total AhR-related toxic potency, including yet not quantified planar PHAHs. Seals could also be more vulnerable for compounds not (directly) acting via the AhR, for example PHAH-metabolites or non-planar PHAHs. Unfortunately, the toxicological potency of not directly AhR-related effects can not yet be quantified.

A NOEC for common tern and otter, based on TEQs only, would be around 2 ng TEQ·g^{-1} lipid. Assuming a bio-magnification factor for TEQs from fish to bird of 20 (Bowerman et *al.*, 1995) the NOEC in fish would be around 0.1 ng·g^{-1} lipid. For otters, with and BCF for TEQs of 95 (Smit et *al.*, 1996), this would be about 0.02 ng·g^{-1} lipid. For seals, assuming a NOEC of 0.1 ng TEQ·g^{-1} lipid and a BCF of 20, the NOEC in fish would be 0.005 ng TEQ·g^{-1} lipid. The levels found in Dutch fresh and salt water fish mostly fall in the range of 0.03 (eel)-1.2 (pike-perch) ng TEQ·g^{-1} fish lipid (calculated from De Boer *et al.*, 1993). These calculations suggest that fish-eating birds and mammals are still at risk for adverse effects of PHAHs in the more polluted parts of The Netherlands.

Future perspectives

Although the use of PCBs and release of PCDD/Fs is strongly reduced, PHAH-release into the Dutch environment still continues as a consequence of leakage from PCB-containing devices that are still in use or dumped; application of PHAH-containing fish oil in

industrial and food products (IPCS, 1993); and increased usage of other persistent PHAHs such as PBDEs (IPCS, 1994; Pijnenburg *et al.*, 1995). PBDEs were found to elicit an AhR related toxic potency comparable to PCBs (Chapter 6). In addition, most of the tested PBDEs, 3,3',4,4'-tetra chlorinated diphenyl ether and the herbicide nitrophen (2,4-dichlorophenyl 4-nitro phenyl ether) were found to compete with T4 for the binding to TTR with a potency comparable to that of the technical PCB-mixture Clophen A50 (unpublished results), after *in vitro* metabolism in P450 1A- or 2B-induced rat hepatic microsomes. It is to expected that several other PHAHs can be metabolized into OH-metabolites with such competitive capacity as well. It is therefore important to continue monitoring accumulation and possible effects of such compounds, especially at places where concentration may be expected, such as sediments, effluents of waste-water treatments, and top predators which reflect the local degree of contamination (see also Chapter 4).

An important question remains whether PHAH concentrations above the NOEC will impair the survival of populations. During the common tern field study, the dynamic environment of the common terns had more detrimental effects on breeding success than the subtle effects which correlated with PHAH levels (Chapter 4). The toxic action of such compounds could impair the recovery from such natural stresses. However, it does not seem likely that the current PHAH levels pose a real threat to the survival of populations of fish-eating birds in The Netherlands, in addition to physical anthropogenic stresses such as habitat destruction, disturbance, traffic and fishing nets. To be able to indicate the implications of exposure to toxic compounds on the fitness of a population, information is needed about the effects on the most important life-cycle stage for the population fitness under realistic conditions (Kammenga, 1995). For the relatively well studied PHAHs this is not even the case for one species. At this moment, therefore, only a rough indication can be presented of the levels of PHAHs above which, or the level of hormones, vitamins or enzyme induction, above or below which adverse population effects may be expected.

MAIN CONCLUSIONS

PHAH exposure of eider ducks and common terns caused alterations in vitamin A and thyroid hormone homeostasis comparable to those described for laboratory rodents. Greater differences are, however, to be expected between developing bird embryos because of differences in physiological contact with the mother animal.

The internal PHAH-concentrations in the eider ducks and common terns could be associated with a number of adverse effects such as reduced growth and behavioural activity, delayed egg laying, a prolonged incubation period and smaller eggs and chicks. Although co-correlation with other pollutants or environmental factors such as food quality can not be excluded, the biochemical alterations correlating with the internal PHAH-concentrations suggest at least a partially causal relationship.

The observed subtle adverse effects suggest that the induced biochemical changes partially fall outside the normal physiological range in which changes can still be compensated.

Hepatic retinoid levels can be used as a physiologically relevant, broad applicable biomarker for adverse effects of PHAHs.

In hepatic microsomes from eider duck, common tern, harbour porpoise, harbour seal and rat, PCB-77 is metabolized at a rate correlating with the EROD activity. The pattern of OH-metabolites formed, however, differs between birds and mammals. Liver microsomes from trout exhibited EROD activity but could not metabolize PCB-77.

A combination of already developed *in vitro* assays can yield information about the capacity of species to form PHAH-metabolites, and the potency of these metabolites to compete with T4 for binding to TTR and possibly other receptors.

The AhR based *CALUX* assay is a rapid, low cost pre-screening alternative for expensive and time consuming chemical analysis of planar PHAHs. This reporter gene assay provides a direct measure for the AhR mediated toxic potency of mixtures of PHAHs.

In parts of The Netherlands, predatory aquatic birds are still at risk for observable adverse effects of PHAHs. It does, however, not seem likely that these PHA-levels are a real threat to the survival of their populations.

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Het onderzoek beschreven in dit proefschrift betreft effecten van polyhalogeen aromatische koolwaterstoffen (PHAKs) op twee predatore aquatische vogelsoorten, en de ontwikkeling van biomarkers voor het vaststellen van blootstelling aan, en effecten van, deze stoffen. Deze biomarkers zijn gebaseerd op het mechanisme van toxiciteit van PHAHs, en daarmee specifiek voor een groep van stoffen met hetzelfde werkingsmechanisme. Op basis van deze biomarkers kan ook inzicht worden verkregen in verschillen in blootstelling, kinetiek en specifieke ecofysiologische eigenschappen van organismen en een indicatie van diersoorten die meer dan anderen kwetsbaar zijn voor de aanwezigheid van PHAKs in het milieu.

Inleiding

Hoewel wetgeving over de toelating en het gebruik van nieuwe en reeds bestaande toxische stoffen tot grote verbeteringen in milieukwaliteit heeft geleid, blijken regelmatig toch nog toxische effecten van stoffen op te kunnen treden. Het gaat hierbij meestal om persistente stoffen die niet acuut toxisch zijn, en toxische effecten kunnen veroorzaken via veelal onverwachte routes. Sublethale effecten vallen vaak minder op, maar kunnen desondanks grote ecotoxicologische gevolgen hebben. Het is daarom van belang om in een zo vroeg mogelijk stadium dergelijke effecten te kunnen detecteren.

PHAKs vormen een grote groep van stoffen, die in mengsels van variërende samenstelling voorkomen in zeer uiteenlopende compartimenten van het milieu. Sommige PHAKs, zoals polychloor dibenzo-*p*-dioxinen (PCDDs) en -dibenzofuranen (PCDFs) zijn ongewenste bijprodukten van incomplete verbranding in de aanwezigheid van chloor en van de produktie van bepaalde stoffen. Andere PHAKs, zoals polychloor bifenylen (PCBs), -terfenylen (PCTs) en -naftalenen (PCNs) zijn vanwege hun gunstige eigenschappen tientallen jaren lang gebruikt in diverse toepassingen. Hoewel de produktie en het gebruik van bepaalde PHAKs, zoals PCBs, door beleidsmaatregelen inmiddels sterk beperkt zijn, verdwijnen veel PHAKs slechts langzaam uit het milieu. Dit is vooral het gevolg van eigenschappen die ze technisch zo bruikbaar maken: persistentie tegen fysische, chemische en biologische afbraak. Van de miljoenen tonnen PHAKs die in de loop der tijd geproduceerd zijn, zal een groot deel pas in de komende tijd in het milieu vrij komen, onder andere via lekkage en verdamping uit gestort afval. Daarbij komt dat bepaalde PHAKs, zoals polybroom bifenylen (PBBs) en vooral polybroom difenyl ethers (PBDEs) nog volop geproduceerd en gebruikt worden als vlamvertragers in tal van toepassingen, zoals meubels en behuizing van electrische apparatuur. Dit betekent dat PHAKs nog lange tijd in het milieu aanwezig zullen zijn en daar problemen kunnen geven.

PHAKs komen in het milieu vooral voor in dierlijke vetten en gebonden aan

organisch materiaal in bijvoorbeeld sedimenten. PHAKs kunnen worden doorgegeven in voedselketens, waarbij de hoogste concentraties in toppredatoren van de aquatische voedselketen worden aangetroffen. Ook kunnen PHAKs worden doorgegeven aan de volgende generatie via eidooier, trans-placentaal transport en melk. De mate van bioaccumulatie van PHAKs verschilt sterk, afhankelijk van de moleculaire structuur. Ook de mate waarin diersoorten PHAKs kunnen afbreken verschilt sterk, afhankelijk van de typen en mate van inductie van biotransformatie enzymen. Over het algemeen zullen meer gehalogeneerde PHAKs sterker bioaccumuleren, hoewel ook het substitutie patroon van de halogeen atomen in het molecuul hierbij een belangrijke factor is. Omzetting van PHAKs tot meer hydrofiele metabolieten heeft niet alleen tot gevolg dat deze sneller uit het lichaam verwijderd worden, maar kan ook weer aanleiding geven tot een nieuw mechanisme van toxiciteit.

Mechanismen van PHAK-toxiciteit

Blootstelling van organismen aan PHAKs kan leiden tot een breed scala aan effecten, waarvan de mate van optreden sterk afhankelijk is van de diersoort. Van PHAKs zijn tenminste twee mechanismen van toxiciteit bekend. De meest belangrijke is via binding aan de cytosolaire 'arylhydrocarbon' receptor (AhR). Vooral PHAKs die een relatief platte structuur kunnen aannemen binden sterk aan de AhR, waarna deze een transformatie ondergaat en naar de celkern wordt getransporteerd. Hier vindt binding van het AhR-PHAK complex aan een specifiek stukje DNA. Deze binding heeft veelal een verhoogde, maar soms ook geremde, transcriptie van nabijgelegen genen tot gevolg, wat kan leiden tot fysiologische verstoringen in het organisme. De via dit mechanisme meest toxische PHAK is 2,3,7,8-tetrachloor dibenzo-*p*-dioxine (TCDD). Het tweede inmiddels bekende mechanisme van toxiciteit start na biotransformatie van een PHAK tot een hydroxy (OH-) metaboliet. Bepaalde OH-PHAKs vertonen een structurele verwantschap met thyroxine (T4), en kunnen dit T4 verdringen van bindingsplaatsen op bijvoorbeeld het transporteiwit in het bloed transthyretine (TTR) of het enzym T4-5'-dejodase. Behalve dat deze competitie kan leiden tot verstoringen in de schildklierhormoon huishouding, leidt het ook tot verlies van retinol (een vorm van vitamine A). Retinol wordt in het bloed normaal aan een retinol bindend eiwit (RBE) in een complex met TTR en T4 getransporteerd. Na binding door een OH-PHAK aan TTR valt dit complex uit elkaar, en kan het kleine retinol-RBE complex door de nieren worden weggefilterd.

PHAK-effecten in predatore aquatische vogels

Het voorkomen van deze mechanismen van toxiciteit is voornamelijk aangetoond onder laboratorium condities met speciaal hiervoor gefokte dieren. In de praktijk komen hoge

PHAK concentraties echter vooral voor bij aquatische toppredatoren zoals visetende vogels en zoogdieren. Het eerste deel van het onderzoek beschreven in dit proefschrift betreft dan ook de vraag of predatore vogels ook biochemische effecten van PHAKs vertonen, die vergelijkbaar zijn met waargenomen effecten in laboratorium dieren.

Hoofdstuk 2 van dit proefschrift beschrijft een semi-veld experiment waarbij jonge eidereenden zijn blootgesteld aan de 'platte' PCB-77 (3,3',4,4'-tetrachloor bifenyl) of aan het technische PCB-mengsel Clophen A50. De spreiding in de gemeten parameters in deze natuurlijk diersoort was veel groter dan gevonden wordt met speciaal gefokte laboratorium dieren. Groepsgemiddelden waren daardoor dan ook niet significant verschillend. Op basis van correlaties met interne dosis konden echter wel significante effecten van PCB-dosering worden vastgesteld. De resultaten geven aan dat eidereenden gevoelig zijn voor PCB-toxiciteit, en dat de effecten vergelijkbaar zijn met die in laboratoriumdieren. Interne PCB-concentraties correleerden met verminderde plasma T4 en lever vitamine A gehalten, en met een toename in de ratio plasma retinol/lever retinylpalmitaat en met verhoging van de ethoxyresurofine O-deethylase (EROD) activiteit in de lever. Ook werden verminderde groei en loopactiviteit gevonden. In een veldstudie naar mogelijke effecten van PCBs op visdief kuikens, beschreven in de Hoofdstukken 3 en 4, werden biochemische effecten gevonden die vergelijkbaar waren met de in eidereenden gevonden effecten. Van visdief nesten met 3 eieren werd het tweede ei kunstmatig uitgebroed, en na het uitkomen werd het kuiken gebruikt voor het meten van diverse biochemische en chemische parameters. In het veld werd het lot van de twee andere, door de visdieven uitgebroede eieren, geregistreerd. PCB gehaltes konden worden gecorreleerd met verlaagde vitamine A gehalten in de dooierzak, verlaagde plasma vitamine A en schildklierhormoon gehaltes, en met een vergrootte ratio plasma retinol/dooierzak retinylpalmitaat en een geïnduceerde EROD activiteit in de lever. Bovendien konden deze biochemische effecten en de PCB gehalten worden geassocieerd met latere eileg, langere broedduur en kleinere eieren.

De waargenomen effecten passen bij het patroon van AhR-gemedieerd effecten, maar kunnen gedeeltelijk ook veroorzaakt zijn door OH-metabolieten van PHAKs. Uit de in Hoofdstuk 5 beschreven *in vitro* metabolisme studie met lever microsomen van verschillende diersoorten, blijkt dat zowel eidereenden als visdieven in staat zijn om van PCB-77 OH-metabolieten te vormen. Ook in microsomen van een gewone zeehond, bruinvis en rat bleken OH-metabolieten te worden gevormd. De verhouding van de verschillende gevormde OH-metabolieten verschilde tussen de vogels en zoogdieren, maar van door beide groepen gevormde OH-metabolieten is al in eerder onderzoek aangetoond dat deze T4-verdringing kunnen geven.

Hoewel correlaties tussen gehalten aan PHAKs en toxische effecten indicaties

zijn dat effecten van PHAKs ook optreden bij dieren die onder natuurlijke condities leven, hoeven dergelijke correlaties niet te wijzen op een oorzakelijk verband. Wanneer echter ook biochemische of fysiologische veranderingen kunnen worden waargenomen waarvan bekend is dat deze vroege stappen zijn in door PHAKs geïnduceerde mechanismen van toxiciteit, kan meer inzicht worden verkregen in de mogelijke rol van PHAKs in de geconstateerde effecten. In Hoofdstuk 8 wordt nader ingegaan op de mogelijkheden en beperkingen van het gebruik van biomarkers voor effect gebaseerd op kennis van mechanismen van PHAK toxiciteit.

Biomarker voor de toxische potentie van PHAK-mengsels

Dieren worden in het veld meestal blootgesteld aan zeer complexe en deels onbekende mengsels van stoffen. Zelfs van PHAKs zijn al vaak tientallen tot honderden vertegenwoordigers met zeer verschillende toxische potentie aanwezig. Om de rol van PHAKs als mogelijke veroorzaker van bepaalde toxische effecten duidelijker te maken, is het van belang om één maat te hebben voor de toxische potentie van het mengsel, in plaats van de concentratie van een aantal individuele stoffen. De toxische potentie van een PHAK-mengsel wordt nu berekend door de gehaltes van de gemeten stoffen te vermenigvuldigen met hun toxische equivalentie factoren (TEFs), voorzover deze bekend zijn, en vervolgens op te tellen tot de TCDD- of toxische equivalentie (TEQ) waarde van het mengsel. Niet alle relevante stoffen doen echter mee in deze berekening, bijvoorbeeld omdat er geen standaarden voor zijn, men er niet naar op zoek is, of omdat ze onder de detectielimiet liggen. Ook worden mengsel-interacties niet meegenomen in deze bepaling. Om deze beperkingen te vermijden is een AhR gemedieerde reporter gen assay ontwikkeld en gevalideerd, waarmee eenvoudig en gevoelig de toxische potentie van het hele mengsel van PHAKs kan worden gekwantificeerd, inclusief mengselinteracties op het niveau van de AhR (Hoofdstukken 6 en 7). Een duidelijk voordeel van deze receptor gemedieerde assay boven de chemische TEQ bepaling is verder dat de zuivering en scheiding van componenten in milieu extracten minder uitgebreid hoeven te zijn, omdat een beetje vet niet stoort en de receptor onderscheid maakt tussen relevante en niet relevante stoffen. In Hoofdstuk 6 wordt de toepassing van deze zogenoemde *CALUX* ('chemical activated luciferase gene expression') assay beschreven voor sediment- en poriewater monsters. Voor poriewater blijken de *CALUX*-respons en de respons in een teratogeniteitstest met zebravis larven goed overeen te komen. De *CALUX* assay blijkt ook goed toepasbaar om de interne belasting van organismen te bepalen in bloedmonsters (Hoofdstuk 7). Vanwege de lage detectielimiet (< 0.1 fmol TEQ per meting) zijn bloedplasma monsters van minder dan 0,5 ml vaak al voldoende voor kwantificering van de interne dosis van organismen in de vorm van

TEQs. Een belangrijk voordeel van de *CALUX* assay boven het meten van de veel gebruikte EROD activiteit is de afwezigheid van substraatinhibitie (Hoofdstuk 6, Bijlage B). Dit komt doordat luciferase alleen luciferine als substraat heeft, in tegenstelling tot het natuurlijke cytochroom P450 1A enzym, dat naast ethoxyresorufine ook veel PHAKs als substraat heeft.

Belangrijkste Conclusies

PHAKs veroorzaakten in eidereend- en visdiefkuikens veranderingen in de vitamine A en schildklierhormoon homeostase die vergelijkbaar zijn met de effecten beschreven voor laboratoriumdieren. Grotere verschillen zijn te verwachten tussen zich ontwikkelende vogel- en zoogdier-embryo's, vanwege verschillen in fysiologisch contact met het moederdier.

De interne PHAK-concentratie in de eidereenden en visdieven konden worden geassocieerd met een aantal negatieve effecten zoals verminderde groei- en loopactiviteit, latere eileg, langere broedduur, en kleinere eieren en kuikens. Hoewel co-correlatie met andere verontreinigingen of milieufactoren zoals voedselkwaliteit niet kan worden uitgesloten, suggereren de waargenomen biochemische veranderingen in ieder geval een gedeeltelijk causaal verband.

De waargenomen subtiele nadelige effecten suggereren dat de geïnduceerde biochemische veranderingen gedeeltelijk buiten de normale fysiologische range vallen waarbinnen veranderingen nog gecompenseerd kunnen worden.

Vitamine A gehaltes in de lever kunnen worden gebruikt als fysiologisch relevante, breed toepasbare biomarkers voor effecten van PHAKs.

In lever microsomen van eidereend, visdief, bruinvis, gewone zeehond en rat wordt PCB-77 gemetaboliseerd met een snelheid die correleert met de EROD activiteit. De OH-metaboliet patronen verschillen echter tussen de vogels en zoogdieren.
De lever microsomen van forel vertoonden wel EROD-activiteit, maar konden desondanks PCB-77 niet omzetten.

Een combinatie van reeds ontwikkelde *in vitro* assays kan gebruikt worden om de capaciteit te bepalen van diersoorten om OH-PHAKs te vormen, en de potentie van deze OH-PHAKs om T4 te verdringen van TTR.

De AhR gemedieerde *CALUX* assay is een snelle en goedkope assay om TCDD equivalenten te bepalen. Deze reporter gen assay biedt een directe en toxicologisch relevante maat voor de potentie van mengsels van stoffen in milieumatrices tot AhR gemedieerde toxiciteit.

In delen van Nederland lopen predatore aquatische vogels nog steeds het risico op waarneembare nadelige effecten van PHAKs. Het lijkt echter niet waarschijnlijk dat deze PHAK-gehalten op zich, een bedreiging vormen voor de overleving van populaties.

CURRICULUM VITAE

De auteur van dit proefschrift werd op 5 augustus 1959 geboren in Harderwijk. In 1977 behaalde zij het VWO diploma te Katwijk aan Zee. In aansluiting hierop studeerde zij biologie aan de Rijksuniversiteit Leiden. Na het behalen van de kandidaatsdiploma's Milieubiologie en Biochemie, werd het eerste hoofdvak verricht bij de vakgroep Populatiebiologie, het tweede hoofdvak bij de vakgroep Biochemie, en een stage bij Kent State University, Ohio, VS. Na het voltooien van de cursus Milieukunde en de Lerarenopleiding Biologie werd het doctoraal examen behaald in 1985. Hierna trad zij als wetenschappelijk medewerker in in dienst bij het Centrum voor Milieukunde Leiden, gedeeltelijk gedetacheerd bij het Rijksinstituut voor Zuivering van Afvalwater (RIZA) te Lelystad. In 1986 werd zij benoemd als stafmedewerker bij de Gezondheidsraad, met als speciale opdracht het ontwikkelen van de advisering op het gebied van de ecotoxicologie. Sinds augustus 1989 is zij als universitair docent in dienst bij de vakgroep Toxicologie, met als taak het doen van onderzoek en het verzorgen van onderwijs in de ecotoxicologie. Medio 1990 is een begin gemaakt met het onderzoek dat geleid heeft tot het voorliggende proefschrift.

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APPENDIX A

Monitoring exposure and effects of polyhalogenated aromatic hydrocarbons (PHAHs) in European otters (*Lutra lutra*)

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1. Abstract
PHAH levels in otter livers, fish and sediments have been determined using the *in vitro CALUX* (chemical activated luciferase gene expression) bioassay for Ah-receptor active compounds. For fish and otter liver extracts these levels, expressed as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) equivalents (TEQs), correlated well with chemically derived TEQs (GC-TEQs) based on non-, mono-, and di-ortho substituted PCBs. For sediments, however, the so-called *CALUX*-TEQ was much higher than the chemically derived TEQs. In livers from relatively fresh otters, retinol and retinylpalmitate levels were measured, and these vitamin A levels correlated negatively with hepatic GC-TEQ levels. In addition, a strong correlation was observed between PHAH levels in blood and liver on a lipid basis, either expressed as TEQs or individual PHAHs. From these observations it can be concluded that (*CALUX*-)TEQ levels in blood can be used as non-destructive biomarker for PHAH-exposure and accompanying hepatic vitamin A reduction.

2. Introduction
Otter (*lutra lutra*) populations in Europe have declined markedly over the last decades. Polyhalogenated hydrocarbon (PHAH) pollution is considered to be one of the major factors in this decline, in addition to physical threats, such as habitat destruction, traffic accidents and drowning in fishing nets. This assumption was based on toxicological studies with the mink (*Mustela vison*) which is often used as a model for the otter, and on associations between high PCB levels in otters and declining or endangered populations[1,2]. For practical and ethical reasons no toxicological experiments have been conducted with the otter itself. Because the Dutch government aims at the return of the

otter in the Netherlands, insight in the environmentally safe levels of PHAHs permitting survival of viable otter populations is needed[3]. In addition non-destructive biomarkers are needed to monitor exposure and health status of otters, before and after re-introduction in their natural environment.

For hazard and risk assessment of mixtures of PHAHs the concentrations of individual PHAHs multiplied by their respective toxic equivalency factors (TEFs) are added up to give the total TCDD toxic equivalency of the mixture[5]. However, given the complexity of mixtures of PHAHs in environmental matrices, even an extensive chemical analysis can only give an impression of the potency of a mixture[6]. In this study we used a rapid, sensitive *in vitro* assay for assessing the toxic potency of mixtures of PHAHs in livers of accidentally killed otters and in sediment and fish samples collected in the same area. The response in the *CALUX* assay[7,8], using recombinant rat (H4IIE) hepatoma cell lines, is compared with PCB-levels determined using gas chromatography combined with Electron Capture Detection (ECD) or Ion Trap Detection (ITD)[9]. Whenever possible, PHAH levels in the otter livers were compared with PHAH levels in blood samples of the same otters.

One of the mechanisms of toxic action of PHAHs is the reduction of the vitamin A storage in liver, which has been demonstrated in both experimental and field studies[10,11,12]. Vitamin A plays an important role in tissue development in foetuses, reproduction, and resistance against infectious diseases. In relatively fresh otter livers also vitamin A (retinol and retinylpalmitate) levels were determined, and compared with hepatic TEQ-levels. This paper presents the first results of a joint study of which the general outline is presented separately at Dioxin '96[4].

3. Methods

Sample collection and preparation

Dead otters were collected from 1992 to 1993 in Denmark, and the health status was recorded[13]. Relatively fresh liver aliquots of 10-20 g from 12 otters were prepared separately for *CALUX* and GC-ITD or ECD measurement (see below). Samples of about 1 gr were prepared for hepatic retinoid analysis. Blood was collected for chemical and *CALUX* analysis. In five areas in Denmark fish and sediment samples, representative for the otter diet and habitat, were collected in 1995. From each fish species 25 individuals were homogenized using a blender. Fish samples were lyophilized, and sediment samples dried by 60°C.

CALUX-assay

Rat H4IIE.pGudluc1.1 cells prepared as previously described[7,8] were exposed in 24-well cell culture plates and the assay performed as described elsewhere[6]. For calculation of *CALUX*-TEQs a standard curve of TCDD was fitted, and the *CALUX*-TEQ value for the unknown sample was interpolated on this curve.

Chemical analysis

After Soxhlet extraction, lipids were gravimetrically determined in 10% of the liver, blood and fish extract. Another part of the extract (10%) was cleaned over 33% H_2SO_4 deactivated silica. The sediment extract was cleaned over a multi layer column filled with alumina oxide (deactivated with 5% H_2O). This extract was evaporated and partly used for the *CALUX*-assay. The rest was fractionated over a silica gel (5% H_20 deactivated) column and was further separated into three fractions containing non-, mono- and di-ortho substituted PCBs, using a PYE HPLC column[9]. The di- and mono-ortho fractions were measured with GC-ECD, the non-ortho fraction using GC-ITD. The so-called GC-TEQs were caluclated based on TEF-values as described before[5].

Analysis of hepatic retinoids was performed according to Brouwer et al.[10] with aliquots of 50 μl liver homogenate on a reversed phase silica C18 column. Retinoids were detected at 326 nm.

4. Results and discussion

Good correlations were observed between the *CALUX*-TEQs and GC-TEQs for fish and otter liver samples (Figures 1a and 1b). The absolute TEQs were in the same order of magnitude. This was to be expected as TEQs in biota are mainly determinded by non- and mono-ortho PCBs[9,14]. However, for fish sample extracts the TEQ indicated by the *CALUX* -assay was slightly less than the chemically derived TEQs. This could be a consequence of the relatively high levels of di-ortho PCBs in fish compared with the otter[15]. Di-ortho PCBs have been demonstrated to have a slightly antagonistic effect on planar PHAHs in H4IIE cells[7]. The *CALUX*-TEQ and GC-TEQ for sediment extracts also correlated well (Figure 1c). The *CALUX*-TEQs, however, were much (more than a factor of 20) greater than the GC-TEQ. This is probably due to the presence chemically not quantified chemicals, such polyaromatic hydrocarbons (PAHs), which may also be good Ah-receptor agonists, and therefore are determined by the *CALUX* assay. As PAHs hardly bioaccumulate, such differences are not observed in the biotic samples. Relatively little sample preparation and clean-up is needed for the *CALUX* assay [6]. Therefore this assay is a good and fast alternative for more extensive chemical PHAH analysis.

A strong decline of hepatic retinol (Figure 2) and retinylpalmitate (data not shown) levels is observed with a relatively small increase in hepatic GC-TEQ levels. This correlation on TEQ-basis is much better than on the basis of the sum of the 7 standard PCBs. A comparable decrease in hepatic retinol levels was reported for flounder (*Platichthys flesus*) exposed to polluted harbour sediment, although hepatic retinylpalmitate levels did not decrease in these fishes[17]. More otter samples will be analysed to study these relationships further. Good correlations were observed between total PCB-levels in liver and blood on a lipid basis ($y = 0.97x - 0.043$; $r = 0.89$, data not shown). This implies that TEQ-levels in liver can be estimated based on TEQ-levels in blood, which can be collected in a non-destructive manner. There were too litle dead otters available with both a relatively fresh liver and enough blood to measure both hepatic vitamin A and TEQs in blood. However, the correlations observed so far between TEQs in liver and blood, and between TEQ in liver and hepatic vitamin A, suggests that at least a rough estimate of the vitamin A reduction in the liver could be obtained based on TEQ-levels in blood.

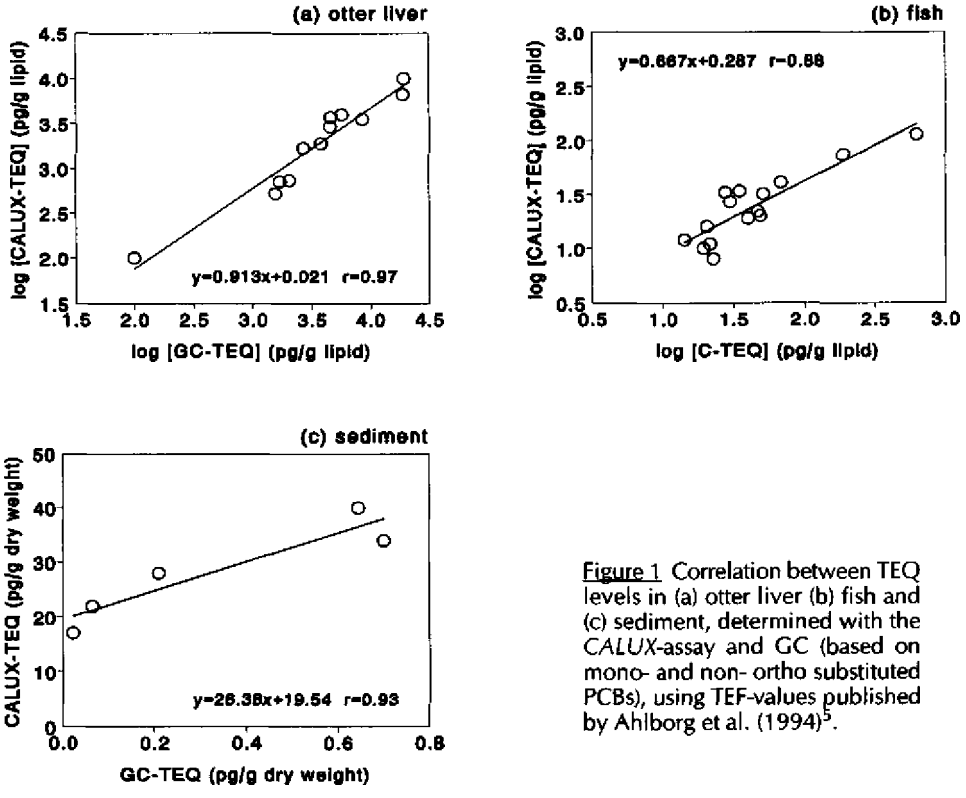

Figure 1 Correlation between TEQ levels in (a) otter liver (b) fish and (c) sediment, determined with the *CALUX*-assay and GC (based on mono- and non- ortho substituted PCBs), using TEF-values published by Ahlborg et al. (1994)[5].

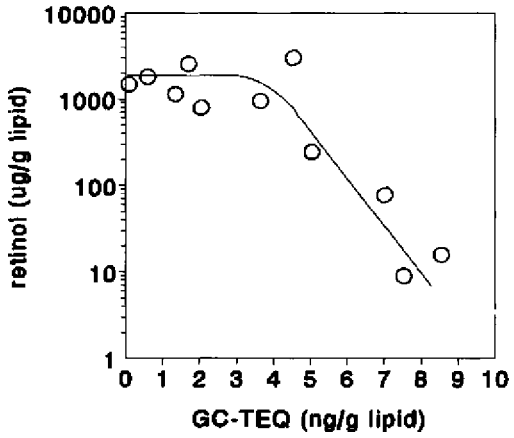

Figure 2 Correlation between TEQ and retinol levels in relatively fresh livers of otters. TEQ-calculations are based on chemically determined PCB-levels using TEF-values published by Ahlborg et al. (1994)[5].

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APPENDIX B

Application of the *CALUX* (chemical activated luciferase gene expression) assay for measuring TCDD-equivalents in sediment, pore water and blood plasma samples

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1. Abstract

The results presented in this paper demonstrate that the *in vitro CALUX* (chemical activated luciferase gene expression) bioassay is a rapid, sensitive assay for assessing the toxic potency of (mixtures of) Ah-receptor active compounds in sediment, pore water and blood plasma. Recombinant rat (H4IIE) and mouse (Hepa1c1c7) hepatoma cell lines, containing arylhydrocarbon receptor (AhR)-mediated luciferase gene expression, were used to determine the *CALUX*-response of sample extracts. Using a 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) standard curve, the *CALUX* responses for the samples were converted into so-called *CALUX*-TEQs (TCDD-equivalents). The detection limit of the *CALUX* assay was 0.5 fmol of TCDD-equivalents. The *CALUX* activity induced by organic extracts from 450 mg aliquots of sediment or 250 μl aliquots of pore-water corresponded well with the instrumentally analysed level of dioxin-like compounds in the sediment. Using pore water, only a simple and rapid extraction procedure was needed, without additional clean-up to prevent cell death. The *CALUX* response both for the fatty, uncleaned organic extract and the cleaned extract of blood plasma from experimentally exposed eider ducks, correlated significantly with the PCB-levels measured in abdominal fat or blood plasma, and with the hepatic EROD activity in the same animals.

An important advantage of the *CALUX*-assay compared to the ethoxyresorufin *O*-deethylase (EROD) assay is that the *CALUX* assay is insensitive to substrate inhibition, which is demonstrated in this paper for 3,3',4,4',5-pentachlorobiphenyl (PCB-126).

2. Introduction

For hazard and risk assessment of mixtures of PHAHs the concentrations of individual PHAHs multiplied by their respective toxic equivalency factors (TEFs) are added up to give the total TCDD toxic equivalency of the mixture[1]. Given the complexity of the mixtures of PHAHs in sediments and organisms, chemical analysis can only give a rough impression of the potential health risks. Due to the often small concentrations of individual congeners and the presence of unknown or not routinely measured AhR active substances, there is a risk of underestimation of the total TEQ. The toxic responses of AhR active compounds may be additive, as is the case for polychlorinated dibenzo -p-dioxins (PCDDs) and -furans (PCDFs), but for PCBs and non-PCB like substances both additive and antagonistic interactions have been observed[2,3]. These limitations are drawbacks to the TEQ-approach. The *CALUX* bioassay provides a measure of the toxic potency of the whole mixture, including interactions.

This paper presents some examples of the use of the *CALUX* assay for monitoring Ah-receptor active compounds in sediment, pore water and blood plasma. Applications of the *CALUX*-assay for human milk and blood samples, butter fat and coconut oil, otter liver and whole blood, and fish samples[4,5,6], and application of the *CALUX* assay for compounds that bind to and activate the estrogen receptor[7], will be presented separately at Dioxin 1996. In pursue of the substrate inhibition tests with Clophen A50 and PCB-77 reported elsewhere[8], possible substrate inhibition of the more potent Ah-receptor agonist PCB-126 is also determined, by comparing the dose-response curves in the EROD and *CALUX* assay.

3. Methods

Sample collection and preparation

Sediment samples differing in degree of pollution were obtained from the National Institute for Inland Water Management (RIZA)were decanted and sieved, ten grams of sediment was mixed with Na_2SO_4, dried in an oven overnight at 40°C, and extracted with hexane:acetone (1:1) in a Soxhlet for 16 hr[8]. Sulphur was removed using tetra butyl ammonium sulphite (TBA). Further clean-up was performed using a multi-layer acid-base silica column consisting of 0.75 g Na_2SO_4, on top of dried silica with 0.75 g of 22% and 0.75 g of 40% hexane washed H_2SO_4, and 1 g of 33% NaOH on glass wool. The column was eluted with 20 ml of hexane followed by 20 ml of hexane:dichloromethane (1:1), dried under a gentle, filtered air-flow, and dissolved in 100 μl of DMSO. Pore water was collected from 200 g decanted and sieved sediment by centrifugation for 30 min at 3000 g at 5°C. The supernatant was carefully decanted into glass erlenmeyer flasks and stored at 5°C. Samples of 5 ml pore water were extracted three times with 5 ml hexane, the

hexane was evaporated under a gentle nitrogen flow, and the extract dissolved in 200 µl of DMSO. Blood plasma was collected from 27 days old eider ducklings, was collected on day 10 after i.p. injection with 5 ml corn oil/kg body weight (bw), 5 or 50 mg PCB-77/kg bw, or with 50 or 200 mg Clophen A50/ kg bw[10]. EROD activities were measured in hepatic microsomes, and PCB levels in abdominal fat (using GC-ECD)[11]. Blood plasma aliquots of about 1.5 ml were denaturated with an equal amount of methanol. The PHAHs were extracted three times with 3 ml of hexane. After the first extraction step, 3 drops of 6 M HCl were added to the water phase. Part of the extract was taken for further clean-up, the rest was evaporated at 30°C under a gentle stream of nitrogen gas, and dissolved in DMSO for use in the *CALUX* assay using Hepa.Luc cells. For the first dilution step these fatty extracts had to be kept warm (± 30°C) to prevent clotting of the lipids in the pipet. The other part of the extract was cleaned over a 20% H_2SO_4 deactivated silica column with hexane-diethyl ether (97:3, v/v). This extract was evaporated, dissolved into isooctane, and analysed by GC. After GC-analysis, the remaining extract was evaporated and dissolved in DMSO for measurement of *CALUX*-activity in H4IIE.Luc cells.

For the ***CALUX-assay***, H4IIE.pGudluc1.1 (H4IIE.Luc) or Hepa1c1c7.pGudluc (Hepa.Luc) cells prepared as previously described[3,9] were exposed to PHAHs in 24-well culture plates[8]. Cells were seeded in 500 µl growth medium and incubated for 24 hr until the cell layer was 80-90% confluent. The medium was replaced by fresh growth medium containing the test compound in maximal 0.5% DMSO. After 24 hours incubation the cells were rinsed twice with 50% diluted PBS (0.5* PBS), 75 µl lysis mix was added, the cells were harvested 15 minutes later and centrifuged for 90 sec. at 13000 g. The supernatant was frozen at -80°C. For luciferase measurement 20 µl supernatant was pipetted in a 96-well microtiter plate, 100 µl luciferin assay mix (Promega) was added and after 90 sec mixing on a plate mixer the light production was measured in an Amerlite Luminometer. For calculation of *CALUX*-TEQs a standard curve of TCDD was fitted, and the *CALUX*-TEQ value for the unknown sample was interpolated on this curve[8]. For the substrate-inhibition test the *CALUX* assay has been performed in 96-well culture plates. Briefly, H4IIE.Luc cells were seeded in 96-well plates in 100 µl growth medium. After 24 hr incubation at 37°C the cell layer was 80-90% confluent, and 100 µl of fresh medium containing the test compound in maximal 0.5% DMSO was added to each well. After an additional 24 hr incubation, the plates were rinsed twice with 0.5*PBS, 20 µl lysis buffer was added, and the plates were placed at -80°C. The plates were measured in a Labsystems Luminometer (Merlin) with automated injection. To be able to correct for differences in quantification due to assay to assay variation, three TCDD calibration standards were measured with each assay.

The ***EROD assay*** was mainly based on the method described before[12]. Briefly, H4IIE wild type cells were seeded in 96-well plates and exposed as described for the *CALUX* assay in 96 well plates, but exposure was during 48 hr instead of 24 hr. Instead of lysis mix, 20 μl of nanopure water was added before the plates were placed at -80°C. To measure resorufin production, the plates were pre-incubated for 20 min. at 37°C, with 50 μl of Tris-sucrose buffer (pH 8) with 40 μM dicumerol, and 25 μl 10 μM 7-ethoxyresorufin (ER) was added to each well. The reaction was started with 25 μl 1 mM NADPH per well, and after 1 hr incubation at 37°C, the resorufin production was measured in a fluorometer (Cytofluor) with excitation at 530 nm and emission at 590 nm.

For the *CALUX* or EROD ***Substrate inhibition assay***, H4IIE.Luc or H4IIE wild type cells were exposed to 50 pM TCDD in 96-well plates for 24 hr, and frozen at -80°C as described above. The plates were thawed on ice prior to luciferase or EROD activity measurement, and final concentrations of PCB-126 ranging from 1 to 10000 nM were reached by adding appropriate concentrations of stock solutions in 4 μl DMSO to each well. The plates were thoroughly mixed on a plate mixer before *CALUX* or EROD activity were measured as described above.

GC-ITD analysis was performed using the isooctane extract of eider duck blood plasma (see above). The gas chromatography (GC) column DB5-MS, 30 m x 0.2 μm (J&W Scientific) was coupled to a Saturn II ion trap detector (ITD; Varian). A retention gap of 2 m x 0.53 mm i.d. deactivated fused silica was used (Chrompack). The GC and ITD conditions are described elsewhere[13].

4. Results and discussion

The *CALUX* response was found to be very sensitive and reproducible using TCDD as a positive control. The detection limit in H4IIE.Luc cells was less than 1 pM, which equals

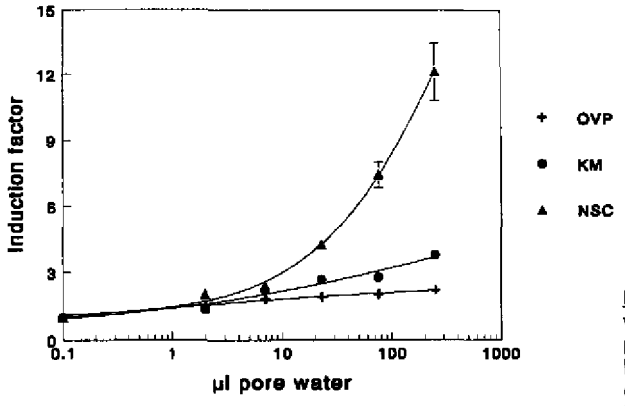

Figure 1 The *CALUX* response by pore water extracts in H4IIE.Luc cells, expressed as induction factor relative to background induction. OVP = Oostvaardersplassen, NSC = North Sea Canal, KM = Ketelmeer

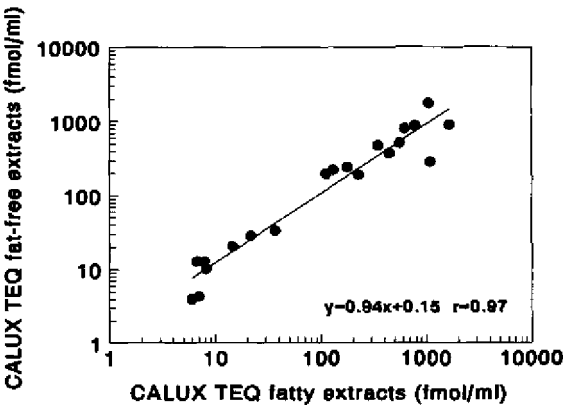

Figure 2 Correlation between *CALUX* TEQ of blood plasma extracts from experimentally exposed eider ducks, with and without clean-up.

an absolute amount of less than 0.5-0.2 fmol/well, depending on method of exposure used[8]. The EC_{50} was 10 pM, the TCDD dose-response curve saturated between 100 pM and 1 nM, and the standard deviation was generally $\leq$ 5%. Exposure of H4IIE.Luc cells to extracts of Ketelmeer (KM) sediment, which is known to be polluted with several PHAHs, resulted in 17 fold greater *CALUX* activity than did extracts from the relatively clean Oostvaardersplassen (OVP) sediment[8]. Based on chemical analyses, the TEQs of these two locations differed by a factor 20. A comparable difference was observed using pore water extracts. Pore water from the North Sea Canal (NSC) contained almost 13 fold more *CALUX*-TEQs than the polluted KM (Figure 1). The high signal in the NSC was probably caused by accidental emissions of PCDD and PCDF from a herbicide producing plant situated in the NSC. Analysis of only the solid phase of sediments does not discriminate between bioavailable and tightly bound contaminants, where pore water samples represent the biologically available fraction of sediment contamination. An advantage of analysis of pore water is that no soxhlet extraction is needed, or clean-up to prevent cell-death. This makes the sample preparation much more rapid and the chance of loosing unknown AhR-active compounds with yet unknown optimal recovery conditions, much smaller.

Extracts of eider duck blood plasma's induced *CALUX* responses in a dose-related manner (Murk et al., submitted). The *CALUX*-TEQs based on the fatty blood plasma extracts correlated significantly with either the PCB-levels in the abdominal fat ($r = 0.80$-0.94, depending on the PCB-congener used), with the hepatic EROD activity of the eider ducks ($r = 0.88$), and with the *CALUX*-TEQs determined for the cleaned extracts (Figure 2; $r = 0.96$). Also the PCB levels in blood plasma extracts correlated well with the *CALUX*-TEQs measured in these cleaned extracts ($r = 0.86$-0.96, depending on the PCB-congener

used) (data not shown; Murk et al., submitted). These results demonstrate that blood plasma extracts can be measured in the *CALUX* assay with and without a clean-up procedure. Performing a simple Silica-H_2SO_4 clean-up, however, offers the possibility to concentrate the extract, and makes it easier to manipulate the extract. The observed good correlation of the *CALUX*-TEQ in blood plasma with the PCB-levels in abdominal fat suggests that the *CALUX*-TEQ in blood plasma provides an integrated measure of toxic potency of the internal dose of the AhR active PHAHs. Although the experimentally dosed eider ducks used for this study contained relatively high levels of PCBs, the *CALUX*-assay has already been applied for measuring TEQs in environmentally exposed species, such as cormorants, otters[6] and humans[4]. About 0.5-1 ml of blood plasma is needed for quantifying *CALUX*-TEQs in blood plasma, depending on the (expected) level of exposure (Murk et al., submitted).

Substrate inhibition

The *CALUX* assay offers some advantages when compared to the commonly used EROD assay in H4IIE cells. It is slightly more sensitive and has a three fold greater induction factor[8,12], but, more importantly, the use of the *CALUX* assay is not limited by substrate inhibition[8]. As is shown in Figure 3, the *CALUX* and EROD induction both increase with increasing PCB-126 concentration. The EROD activity, however, decreases again at concentrations greater than 100 nM, while the *CALUX* activity saturates at the maximum level (Figure 3). For the potent Ah-receptor agonist PCB-126, maximum induction levels are not significantly different from the maximum induction with TCDD. However, the maximum EROD induction declines with decreasing AhR inducing potencies of PHAHs (Murk et al., in prep.). Figure 4 demonstrates the *CALUX* and EROD activity, induced by 50 pM of TCDD, when increasing amounts of PCB-126 are added to the lysed cells shortly before adding the respectively substrates. The measured EROD activity was already reduced to 73% of the original activity by addition of 10 pM of PCB-126, and after addition of 10 nM PCB-126 only 6% of the EROD activity is left. No inhibition of the *CALUX* activity was observed at any of the PCB-126 concentrations tested, which was to be expected, since PHAHs are not substrates for luciferase. These finding are in accordance with previously reported results[8] for H4IIE cell lines, and with results with experimentally dosed flounder, where the EROD activity induced by 5 μg TCDD/kg was strongly reduced when dosed simultaneously with Clophen A50/kg, although the cytochrome P450 protein content increased in an additive manner[14].

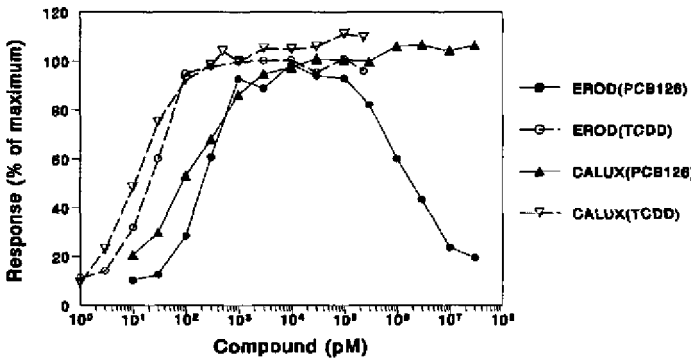

Figure 3 CALUX and EROD induction by PCB-126 in resp. H4IIE.Luc and H4IIE.wt cells. Exposure was during 24 hr in 96-well culture plates. Maximum induction was reached by 1000 pM of TCDD.

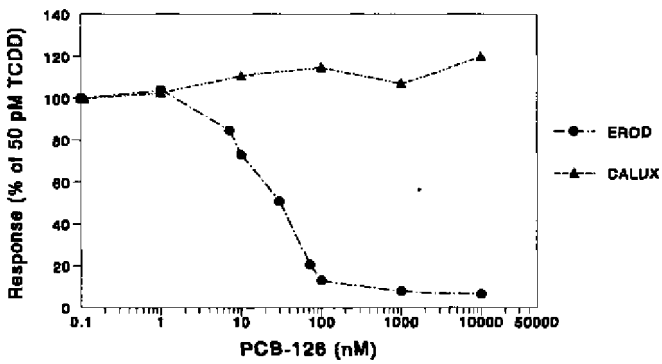

Figure 4 Effect of an increasing concentration of PCB-126 added to lysed H4IIE.Luc or H4IIE.wt cells shortly before measuring the *CALUX* or EROD activities, which were induced by exposure to 50 pM TCDD during 24 hr (this induction was set at 100%).

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