# Molecular and biochemical studies on the Ah receptor pathway in flounder (*Platichthys flesus*)

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# Molecular and biochemical studies on the Ah receptor pathway in flounder (*Platichthys flesus*)

**Harry Besselink**

**Proefschrift**

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# STELLINGEN

1. De rol van PCB's bij het ontstaan van levertumoren bij de bot afkomstig uit Nederlandse kustwateren, is verwaarloosbaar.
*Dit proefschrift*

2. Bij de bot is EROD activiteit geen bruikbare biomarker voor blootstelling aan PHAK's en evenmin voor PAK's wanneer PHAK's aanwezig zijn.
*Dit proefschrift*

3. PCB's hebben bij de bot een overwegend remmende invloed op de activiteit van het cytochroom P4501A, hetgeen tevens leidt tot een verminderde gevoeligheid voor PAK's en PHAK's.
*Dit proefschrift*

4. Palace & Brown gaan er ten onrechte van uit dat transthyretine betrokken is bij het transport van retinol in het bloed van de schar.
*(Palace, V.P. and Brown, S.B., 1994. Environ. Toxicol. Chem., 13:473-476)*

5. Bij het onderzoek naar de carcinogeniteit van complexe mengsels zoal toxafeen, PCB's en dioxines, wordt bij het dierexperimentele onderzoek dikwijls ten onrechte gebruik gemaakt van technische mengsels.

6. Dat er steeds minder vissen met tumoren worden aangetroffen in de zee, moet eerder worden toegeschreven aan overbevissing dan aan een vermindering van de milieuverontreiniging.

7. Magnetische velden kunnen van invloed zijn op de toxische werking van bepaalde chemicaliën.
(*Hurych, J. et al., 1996. Toxicol. Lett., 88:305-311*)

8. Er zijn onvoldoende aanwijzingen dat chemicaliën in het voedsel een bijdrage leveren aan de waargenomen toename van atopische ziektes als astma, rhinitis en eczeem.

9. Een van de belangrijkste dingen in de wetenschap is niet zozeer het verzamelen van nieuwe feiten als wel het ontdekken van nieuwe manieren om erover na te denken.
(*natuurkundige William Lawrence Bragg, 1890-1971*)

10. Strenge milieumaatregelen leiden niet tot stagnatie van de economie.
(Naar: *Ehrlich, P.R. and Ehrlich, A.H., 1996. Betrayal of science and reason.*)

11. De doelstelling van de overheid om het universitair onderzoek meer te richten op maatschappelijke prioriteiten door het overhevelen van gelden uit de rijksbijdrage voor universiteiten naar de tweede geldstroom (NWO), getuigt van weinig vertrouwen in het zelforganiserend vermogen van onderzoekers en universitaire bestuurders.
(*persbericht KNAW, november 1997*)

12. In het kader van het in dit proefschrift beschreven onderzoek, valt het vangen van bot niet te vergelijken met botvangen.

*Stellingen behorende bij het proefschrift:*
*"Molecular and biochemical studies on the Ah receptor pathway in flounder (Platichthys flesus)"*
*Harry Besselink, Wageningen, 16 januari 1998.*

Voor mijn moeder en oma,
aan wie ik veel mooie herinneringen heb.

# Contents

# Chapter 1
# Introduction

Over the past decades, there has been special scientific interest in the occurrence of skin and liver diseases in coastal and marine fish populations. Many scientists have argued that the increased prevalence of some diseases (e.g. fin rot, skin ulcers and neoplasia) in contaminated areas world wide are caused by high concentrations of environmental pollutants, such as polyaromatic hydrocarbons (PAHs) and polyhalogenated aromatic hydrocarbons (PHAHs) (Malins *et al.*, 1988; Metcalfe *et al.*, 1988; Vethaak, 1993). On the other hand, involvement of natural stress factors in the aetiology of diseases could not be ruled out (Vethaak & ap Rheinallt, 1992; Vethaak & Jol, 1996; Vethaak & Wester, 1996). The flounder (*Platichthys flesus*) is a common (flatfish) species on soft substrata in Dutch coastal and brackish waters including the Wadden Sea. Individuals are commonly affected by the above-mentioned diseases (Vethaak & Jol, 1996; Vethaak & Wester, 1996). The possible contribution of anthropogenic contaminants, in particular polychlorinated biphenyls (PCBs), to the aetiology of disease in flounder is the topic of interest in this thesis.

**Environmental pollution: possible causal factors for fish diseases.**

To address the aetiology of fish diseases, epidemiological surveys have been carried out in an attempt to correlate fish diseases with the levels of pollution. The earliest field studies trying to correlate fish diseases, such as skin and liver tumours and fin rot, to pollution were carried out in the United states in the 1960s (Mahoney *et al.*, 1973, Mearns & Sherwood, 1974). Many of the earlier surveys suggested a relationship between pollution and disease, but no causal relationship could be demonstrated. In order to investigate a relationship between prevalences of hepatic neoplasms in bottom-dwelling fish and concentrations of toxic chemicals in the marine environment, Malins and co-workers (1988) conducted field studies over a 5-year period. They demonstrated statistically significant correlations between the occurrence of hepatic neoplasms and levels of PAHs in sediment (Malins *et al.*, 1985) and levels of metabolites of aromatic compounds in the bile (Krahn *et al.*, 1986). Other studies have provided additional evidence for a causal relationship between pollution and the occurrence of fish diseases. Varanasi *et al.* (1986) reported covalent binding of metabolites of benzo(a)pyrene to hepatic DNA in English sole (*Parophrys vetulus*). Covalent binding of aromatic free radicals was only observed in sole from contaminated areas and not in sole from a reference site. Furthermore, PCBs and PAHs were found to be significant risk factors for the occurrence of

neoplastic, preneoplastic, and specific degenerative/necrotic lesions in fish from chemically contaminated urban sites by Myers *et al.* (1987).

The occurrence of certain diseases in fish populations from the North Sea has been related (in partial) to environmental pollutants. The main source of anthropogenic contamination is through riverine outflows, primarily from the Rhine, Scheldt, Meuse, Elbe, Weser, and Thames. As a consequence, coastal areas and estuaries are significantly polluted (Klamer *et al.*, 1991; de Boer, 1995). In the North Sea, studies on fish diseases have focused mainly on dab (*Limanda limanda*) and flounder (Dethlefsen *et al.*, 1987; Vethaak *et al.*, 1992). High prevalences of serious pathological liver conditions, e.g. shrinkage of liver cells and neoplastic liver nodules, in flounder, ruffe (*Gymnocephalus cernua*), and smelt (*Osmerus eperlanus*) from the Lower Elbe were observed by Peters *et al.* (1987) and addressed to high concentrations of pollutants, e.g. PCBs. Using flounder as a local indicator species in Dutch coastal areas and estuaries, a multidisciplinary survey between 1983 and 1987 recorded mainly lymphocystis, skin ulcers and liver neoplasia (Vethaak, 1987). Again, it was suggested that the high prevalences of some of these lesions was related to the presence of high concentrations of environmental pollutant, particularly PAHs and PHAHs. In a further study, the implication of PHAHs or other chemicals in the aetiology of liver neoplasia and possibly lymphocystis was further confirmed in a large-scale mesocosm study using flounder (Vethaak *et al.*, 1996).

**Toxic effects of PCBs in fish**

The majority of toxicological studies with PCBs have been conducted in laboratory animals such as rat and mice. The toxicity of PCBs depends on factors such as: 1) the degree of chlorination; 2) the animal species and strain used for toxicity testing; 3) the sex and age of the animals used; 4) the duration and route of exposure (Safe, 1994). In mammals, PCBs elicit a broad range of adverse health effects (McConnell, 1989; DeVito & Birnbaum, 1994). The most observed effects of PCBs include loss of body weight (Allen *et al.* 1974; Baumann *et al.*, 1983), immunotoxicity (Tryphonas *et al*, 1991), neurotoxicity (Tilson *et al.*, 1990), endocrine effects (Brouwer *et al.*, 1997), reproductive toxicity (Allen *et al.*, 1979), dermal toxicity (McConnell, 1989) as well as carcinogenicity (Kimbrough *et al.*, 1975).

Many of the above mentioned pathological and biochemical changes have also been observed in fish exposed to PCBs and related compounds. Long-term i.p. exposure of carp to the commercial PCB mixture Delor 103 resulted in a lower weight gain as compared to control fish (Svobodová *et al.*, 1994). A number of environmental pollutants are reported to affect reproductivity (Colborn *et al.*, 1993; Peterson *et al.*, 1993). In fish, PCBs have been shown to interfere with steroid metabolism thus modulating reproductive success. Freeman & Idler (1975) demonstrated 11β-hydroxylation of testosterone in brook trout (*Salvelinus fontinalis*) after three weeks of exposure to Aroclor 1254. Alterations in hepatic steroid metabolism upon

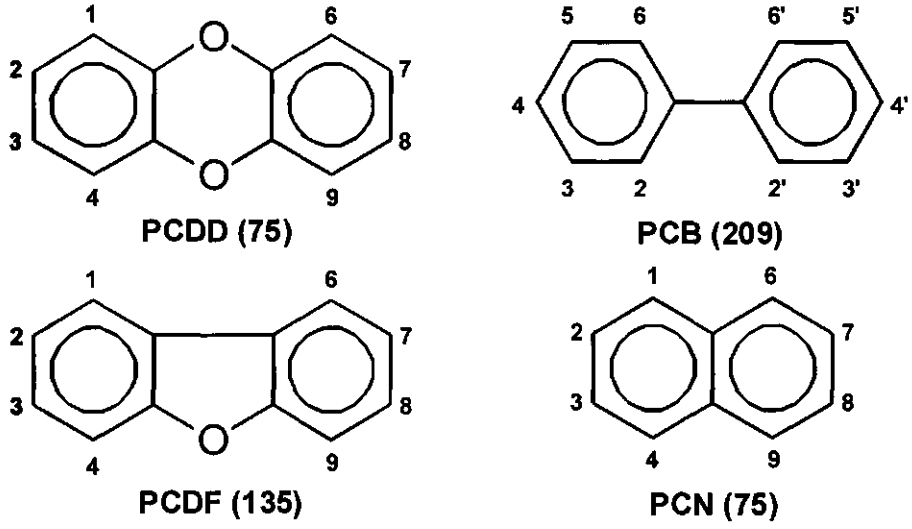


**Figure 1.1** Molecular structure and ring position of PHAHs (polyhalogenated aromatic hydrocarbons): PCDD (polychlorinated dibenzo-*p*-dioxin), PCDF (polychlorinated dibenzofuran), PCB (polychlorinated biphenyl), and PCN (polychlorinated naphthalene). The numbers between brackets indicate the number of possible congeners.

PCB exposure were also observed in carp (*Cyprinus carpio*) by Yano & Matsuyama (1986). Reports on effects of PHAHs on the immune system in fish are ambiguous. Modulation of the immune response was observed in rainbow trout upon exposure towards TCDD (Spitsbergen *et al.*, 1986). Suppression of B-cell mediated immunity in chinook salmon (*Oncorhynchus shawytscha*) was reported by Arkoosh *et al.* (1994) upon exposure to PCBs. In contrast, chronic exposure of Aroclor 1254 did not affect the humoral immune system in rainbow trout (Cleland *et al.*, 1988). Endocrine disruptive effects in fish were reported by Leatherland & Sonstegard (1978). Coho salmon (*Oncorhynchus kisutch*) fed diets containing a PCB mixture of Aroclor 142 and 1254 for 3 months showed reduced plasma levels of triiodothyronine. Furthermore, changes in retinoid homeostasis were observed by Palace & Brown (1994) in Lake char (*Salvelinus Namaycush*) eight weeks after a single oral administration of 3,3',4,4',5-pentachlorobiphenyl. Finally, very little evidence based on experimental research was found for the carcinogenicity of PCBs in fish. Nevertheless, considering the similarity between

toxicological effects in mammals and fish, the presence of a common mechanism of action of PCBs and related compounds is suggested.

**Ah-receptor pathway, a common mechanism?**

The molecular mechanism of action of PHAHs has been studied extensively in mammals (fig. 1.2). It is initiated by binding of the xenobiotic to a soluble cytosolic receptor, named the aryl hydrocarbon (Ah) receptor. The non-occupied Ah receptor is part of a multimeric protein complex which contains two heat shock proteins (Hsp90) and another, not yet fully characterised, protein of about 46 kDa (Hsp50) (Landers & Bunce, 1991; Okey *et al.*, 1994). Upon ligand binding, the AhR is transformed to a DNA- binding form and transported into the nucleus. The transformation includes dissociation of the Ah receptor complex and subsequent association with a protein. This protein, named Arnt (Ah receptor nuclear translocator protein), was cloned by Hoffman *et al.* (1991) and demonstrated to be a component of the DNA-binding form of the ligand-AhR complex. The ligand-AhR-Arnt complex subsequently binds to a specific DNA sequence, termed the dioxin responsive enhancer (DRE), leading to enhanced or inhibited rates of transcription of genes associated with the Ah genetic complex (Okey *et al.*, 1994; Hankinson, 1995). Binding of TCDD to the Ah receptor has been described in mammalian (Denison *et al.*, 1986a,b) as well as some fish species (Lorenzen & Okey, 1990; Hahn *et al.*, 1994). In addition, induction of the cytochrome P4501A enzyme system (the best studied response mediated by the Ah receptor) in fish has been found to be comparable to mammalian induction of CYP1A (Stegeman & Hahn, 1994). These observations underscore the presence of a common mechanism of PHAH induced toxic effects in mammals and fish.

The first, most-studied, and best-understood response shown to be mediated by the Ah receptor, is induction of hepatic cytochrome P450 (Whitlock, 1990). These enzyme-linked cytochromes are involved in the oxidative metabolism of numerous lipophilic exogenous and endogenous substrates, including drugs, PAHs, PHAHs, pesticides, fatty acids, and steroids. In mammals the cytochrome P450 superfamily comprises a group of heme-containing enzymes, which up till now have been classified in 36 subfamilies based on amino acid sequence (Nebert *et al.*, 1991; Nelson *et al.*, 1993). Each subfamily has a distinct, but often overlapping, substrate specificity and are under independent genetic control. The CYP1A subfamily is induced by PAHs, planar PCBs, dioxins and related compounds (3-MC type inducers) (Okey, 1990; Poland & Knutson, 1982; Safe, 1994). Non-planar PCB congeners are inducers of the CYP2B subfamily and are called PB-type inducers. Commercial PCB mixtures exhibit both 3-MC and PB type induction pattern and are often revered to as mixed-type inducers.

In fish, two P450 subfamilies (1A and 2B) have been identified (Stegeman, 1989; Miranda *et al.*, 1990). But unlike corresponding mammalian P450 isoforms, only fish CYP1A is inducible by 3-MC-type inducers whereas the constitutive CYP2B appears to be not induced by

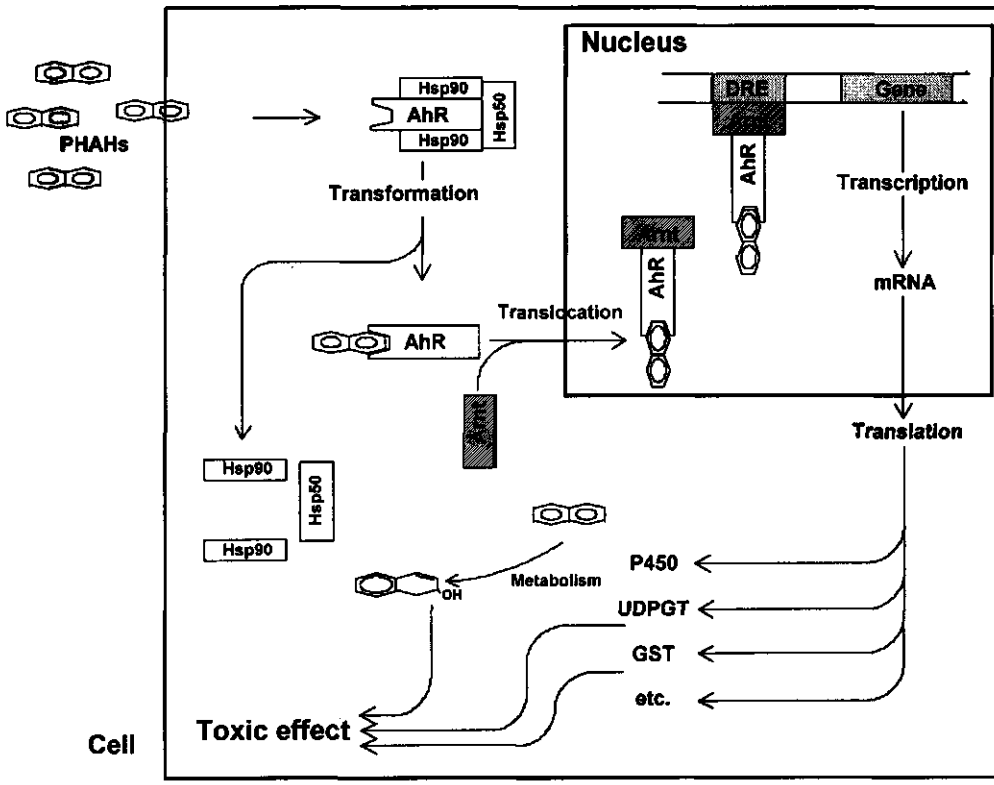


**Figure 1.2** Simplified representation of the Ah receptor mediated mechanism of toxicity of PHAHs. Abbreviations: AhR: arylhydrocarbon receptor; ARNT: Ah receptor nuclear translocator; DRE: dioxin responsive enhancer; GST: glutathione *S*-transferase; Hsp: heat shock protein; P450: cytochrome P450;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

phenobarbital-type inducers (Goksøyr & Förlin, 1992; Stegeman & Hahn, 1994; Kleinow *et al.*, 1990). In fish, induction of CYP1A and associated 7-ethoxyresorufin-*O*-deethylase (EROD) activity have been shown to occur upon exposure to PCBs, PAHs, dioxins and related compounds (Celander & Förlin, 1995; Eggens, 1996; Hahn & Stegeman, 1994; van der Weiden *et al.*, 1994). Since the induction of CYP1A and EROD activity is one of the earliest responses observed in fish upon exposure to contaminants that exert their toxic effect via the Ah receptor pathway, the use of EROD activity as biomarker in fish has been widely advocated (Bucheli & Kent, 1995; Goksøyr,

1995). Payne & Penrose (1975) and Payne (1976) were among the first to use induction of the cytochrome P450 enzyme as a biomarker. Since then, measurement of CYP1A activity has frequently been used to monitor early responses of environmental contamination in fish (Narbonne *et al.*, 1991; Goksøyr *et al.*, 1991; Stegeman *et al.*, 1987).

**Objective and approach of the present study**

Although to date, it is suggested that PHAHs and PAHs are involved in the aetiology of fish diseases, the underlying mechanisms are still largely unknown. To establish a causal relationship between the increased occurrence of fish diseases and an environmental pollutant, an epidemiological approach should be accompanied with experimental work addressing the molecular mechanism of action of the pollutant. Therefore, the research presented in this thesis was undertaken to provide additional information on the mechanism of action of PCBs in the flatfish flounder. The focus was on the mechanistic aspects of the toxic and biochemical effects of PCBs and related compounds, with the aim to provide a sound scientific basis for possible PCB induced effects in flounder, which may or may not correspond to the diseases observed in the wild.

In order to investigate the mechanism of action of PCBs, flounder were caught from the wild and exposed to PCBs and related compounds under controlled laboratory conditions. In **chapter** 2, the procedures for catching, transporting, and disinfecting flounder are given. In addition, the seawater aquarium in use at the Department of Toxicology is described. The sensitivity of flounder towards PCB and TCDD exposure was assessed by measuring hepatic CYP1A induction and possible associated alterations in vitamin A and thyroid hormone homeostasis (**chapter** 3 and 4). In **chapter** 5, the possible interactions between PHAHs and their effect on CYP1A content and activity in flounder was evaluated. Since most, if not all, of the toxic and biochemical effects of PCBs are Ah receptor mediated, a study on the molecular characterisation of the Ah receptor pathway for CYP1A induction, as well as on interference of PCB congeners with the CYP1A catalytic activity in flounder was conducted (**chapter** 6). Finally, the effects on retinoid levels in flounder after long-term exposure to contaminated harbour sludge, are reported in **chapter** 7.

# Chapter 2
# Methodology: Salt Water Recirculation System

In order to investigate the Ah-receptor mediated effects of polyhalogenated aromatic hydrocarbons (PHAHs) in flounder under laboratory conditions, flounder were caught from the wild and transported to the city of Wageningen and held in seawater aquaria at the Department of Toxicology, Agricultural University Wageningen. Here the procedures for catching, transporting, and disinfecting flounder are given. In addition, the seawater aquarium in use at the Department of Toxicology is described.

### Collecting fish

Flounder were caught in the Western Wadden Sea by beam-trawling. Special care was taken to avoid damage to the fish during hauling. Therefore, the ship towed a 5 m beam-trawl for no longer than 15 minutes. After hauling, the net was taken on board and opened. Visibly healthy immature flounder in the length class between 18.0 and 22.0 cm (mainly 2 years of age) were selected and placed in a reservoir filled with seawater. The water was constantly refreshed by pumping in seawater. Flounder remained in these reservoirs until the ship docked and arranges were made to transport the fish by car.

### Transport

Upon arrival in port, flounder were divided over several 75 litres vessels which could be hermetically closed. Crushed ice was placed on the bottom of the vessel after which a large plastic bag was placed on top of the ice. The ice was added to prevent rising water temperatures during transport of the fish. The plastic bag was filled for 1/3 with seawater. Each vessel was loaded with a maximum of 30 fish (Fig. 2.1). To prevent fish dying from suffocation, the water was aerated and the plastic bag filled with technical oxygen. After closing the bag and the vessel airtight, the fish were transported by car.

### Disinfection and acclimatisation

Flounder caught from the wild are potentially infected by parasites, bacteria, and viruses (e.g. Trichodina, Dactylogyrus). To prevent an outbreak of parasites or bacterial and viral diseases, flounder were disinfected. Two methods of disinfection were tried.

**Figure 2.1.** Preparation of flounder for transport by car. A plastic bag was placed into a vessel (75 litres) containing a layer of crushed ice, and filled with seawater ($^1/_3$). A maximum of 30 flounder were placed into the vessel. The seawater was aerated and the bag filled with oxygen, after which the bag and vessel were closed airtight.

Formaldehyde solutions are used as disinfectant because of their antibacterial, antiviral, and antiparasitical properties. The antiparasitical action of formaldehyde is based on its disinfecting properties, which are reducing and cause proteins to precipitate. In addition, formaldehyde treatment results in irritation of the mucous membrane of fish, which stimulates the antiparasitical action (Schäperclaus, 1990). Flounder were placed for $1^1/_2$ - 2 hrs in a well aerated aquarium filled with seawater in which a formaldehyde solution was added to a final concentration of 200 ppm/l. After treatment, flounder were placed in seawater filled aquaria and acclimatised. Although formaldehyde treatment gives good results ultimately, this procedure was not used for disinfecting flounder eventually since the treatment appeared to be very stressful.

The procedure used for disinfecting flounder takes advantage of the fact that flounder is a euryhaline fish species, inhabiting salt as well as fresh waters. Upon arrival, the flounder are placed in fresh water containing aquaria for 24 hrs after which they are transferred to salt water containing aquaria. After 24 hrs flounder are placed again in fresh water aquaria for 1 day. Finally, fish are transferred to salt water aquaria. This procedure is less stressful for the fish. In contrast to flounder, most of the parasites can not cope with fluctuations in salt concentration. Using this method we obtained very good results in disinfecting flounder, making it possible to maintain flounder in our salt water aquaria facilities for more than 4 years without serious health problems.

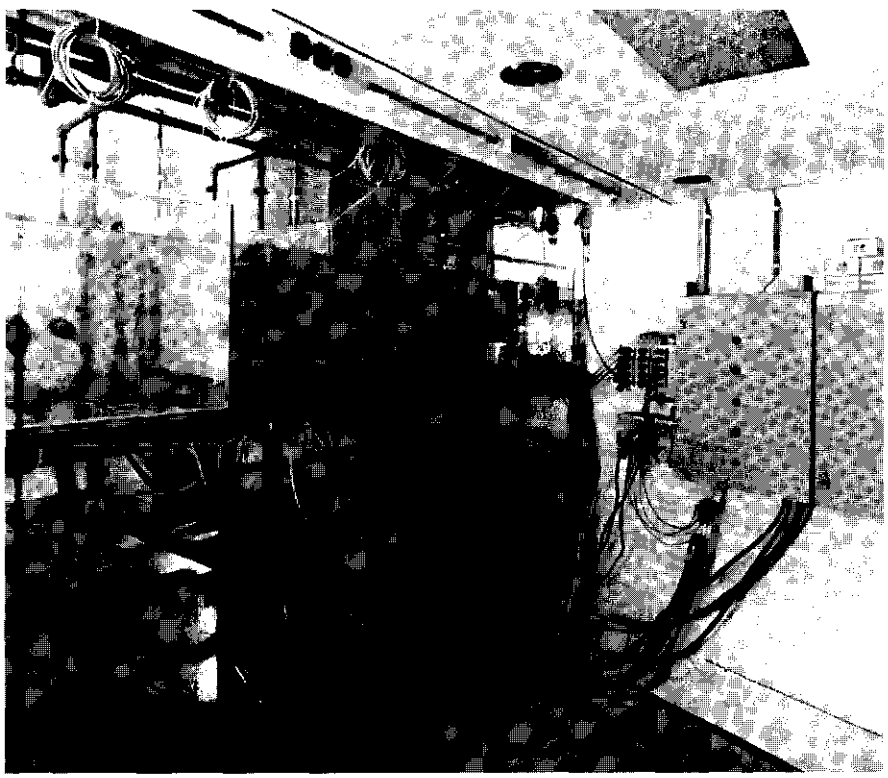

**Figure 2.2.** Semi-synthetic seawater aquarium system as employed at the Department of Toxicology, Agricultural University Wageningen. The aquaria shown (450 $dm^3$) are used for acclimatisation of flounder.

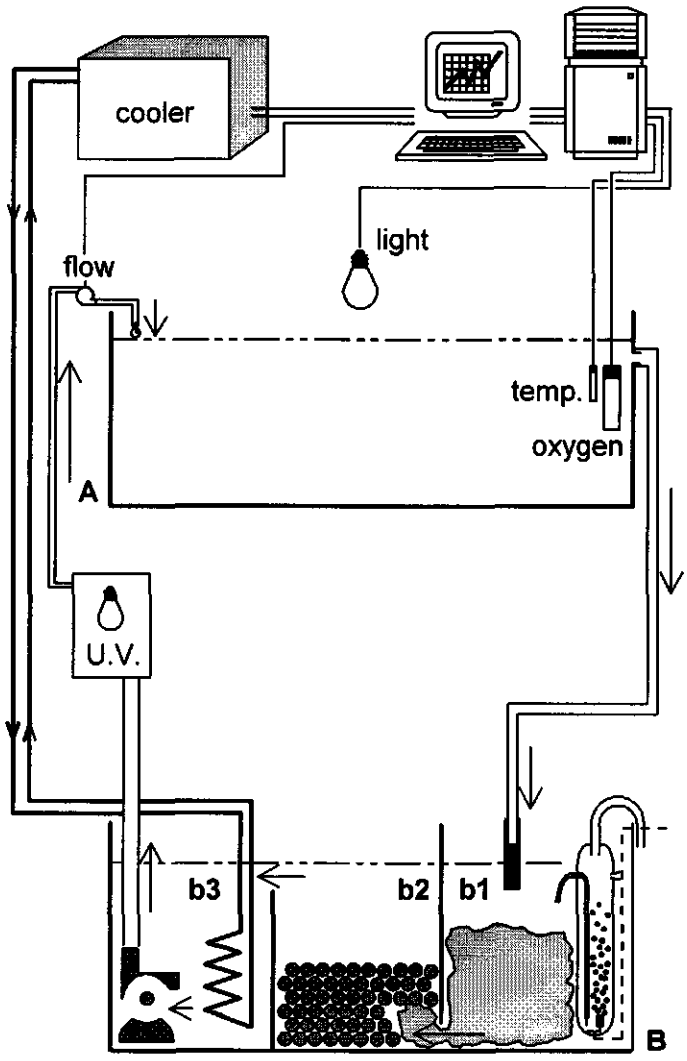


**Figure 2.3.** Schematic representation of the recirculating semi-synthetic seawater aquarium design. The system is composed of: **A** - aquarium (containing fish); **B** - deposition tank. The deposition tank is divided into 3 compartments: **b1**- containing cotton-wool and a protein removal system; **b2** - containing porous hydrobeads used as substrate for bacteria; **b3** - containing a cooling unit and a pump to recirculate the water. Furthermore the aquarium design consists of a UV-light installation and a cooler Using computer software, water temperature, water flow, light regime, water oxygen level and salinity are monitored and regulated continuously.

Prior to *in vivo* experiments, flounder were acclimatised at 13°C for 4 weeks, in 450 $dm^3$ glass tanks with recirculated (biologically filtered) and aerated semi-synthetic seawater under a 12 hr light : 12 hr dark photoperiod regime (Fig. 2.2). Flounder were fed frozen mysis (*Mysis relicta*) and cockles (*Mollusca, Bivalvia, Cerastoderma edule*) alternately at 1% of body weight daily. Following acclimatisation, flounder were transferred to 225 $dm^3$ glass aquaria containing semi-synthetic seawater and a small layer of sediment. Each aquarium was stocked with a maximum of 14 flounder.

**Salt Water Recirculation System**

For laboratory exposure studies with flounder, a recirculating semi-synthetic seawater aquarium design was employed. A recirculation design was used rather than a flow through system with actual seawater because of logistic and financial reasons. Semi-synthetic seawater (HW Marinemix + Bio-Elements, Wimex, Wiegandt, Krefeld, Germany) was prepared in heavily aerated large basins at high salinity (± 60 $^0/_{00}$). Prior to experiments, aquaria were filled with concentrated semi-synthetic seawater and diluted to a salinity of 32 $^0/_{00}$. A disadvantage of a recirculation system is the build up of contaminants in the recirculated water. Therefore, semi-synthetic seawater was filtered and disinfected. Figure 2.3 shows a schematic representation of the semi-synthetic seawater aquarium design used.

The aquarium design was composed of 2 aquaria: one aquarium which contained the fish and a deposition tank. The deposition tank was used to remove contaminants from the recirculated water. The tank was divided into three compartments. The first compartment contained cotton-wool to filter large particles from the water, and a protein removal system. Protein was skimmed by heavy aeration of seawater through a glass-tube. Foam formed was collected outside the deposition tank. The second compartment contained porous hydrobeads which acted as substrate for bacteria. Bacteria were used to biologically filter the semi-synthetic seawater. After filtration, the water entered the third compartment. Here, the water was cooled and pumped through a UV-installation, before returning to the aquarium. UV-irradiation of the semi-synthetic seawater was used for disinfection. During an experiment in which flounder were exposed to PHAHs, the seawater from the aquarium passed a charcoal filter before entering the deposition tank. The charcoal was used to remove traces of PHAHs in the recirculating seawater.

Water quality (ammonium, nitrite, and nitrate levels) was manually monitored daily, whereas light regime, water temperature, water flow, water oxygen levels, and salinity were monitored and regulated continuously using a computer software package (Technische Dient "De Dreyen", Agricultural University Wageningen, The Netherlands).

# Chapter 3
# Low Hepatic 7-Ethoxyresorufin-*O*-Deethylase (EROD) Activity and Minor Alterations in Retinoid and Thyroid Hormone Levels in Flounder (*Platichthys flesus*) Exposed to the Polychlorinated Biphenyl (PCB) Mixture, Clophen A50


**Abstract**

The effect of the polychlorinated biphenyl (PCB) mixture Clophen A50 on hepatic cytochrome P4501A1 dependent EROD (7-ethoxyresorufin-*O*-deethylase) activity, plasma thyroid hormone levels and plasma, kidney, and liver retinoid concentrations of the euryhaline flatfish flounder (*Platichthys flesus*) was determined 2 and 10 days after i.p. (intraperitoneal) injection with 20, 100, and 500 mg Clophen A50/kg body weight. No effect of Clophen A50 on total cytochrome P450 content in flounder liver was observed at both time points. A 6-fold, dose-dependent, significant increase in EROD activity was found at exposure day 10 in flounder receiving 100, or 500 mg Clophen A50/kg body weight. Plasma retinol concentrations were not altered at both time points after Clophen A50 administration, whereas renal retinol levels showed a minor dose-related increase at day 2 and day 10 of exposure. Significant alterations in hepatic retinoid concentrations were observed, which were not dependent on the dose of PCB administered. In addition Clophen A50 administration did not result in a dose-related alteration of total $T_4$ concentrations in plasma. Total $T_3$ concentrations in plasma were only significantly increased at day two after exposure, whereas free $T_4$ concentrations were increased at both time points after Clophen A50 administration. These data indicate that with regard to the parameters investigated and in contrast to other fish species studied, the flounder is not a sensitive species to PCB exposure.



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**Introduction**

In general, many studies have shown that fish from highly contaminated coastal/estuarine waters show increased prevalences of certain skin and liver diseases (including neoplasia) as compared to relatively unpolluted waters (e.g. Peters *et al.*, 1987; McCain *et al.*, 1992; Vethaak & ap Rheinallt, 1992; Vogelbein *et al.*, 1990). There is rapidly growing evidence that polycyclic aromatic hydrocarbons (PAHs) and polyhalogenated aromatic hydrocarbons (PHAHs) may contribute to the etiology of at least some of the diseases, especially liver tumours (Malins *et al.*, 1988; Gilbertson, 1989; Myers *et al.*, 1994; Vethaak, 1994). In the Netherlands, intensive studies have been focused on diseases in the flounder (*Platichthys flesus*) and their possible relationship to chemical contaminants for more than 10 years. The flounder is an euryhaline flatfish species which show relatively high prevalences of lymphocystis (a viral skin disease) and liver tumours in polluted Dutch coastal waters (Vethaak & Jol, 1996; Vethaak & Wester, 1996), whereas locally very high prevalences (up to 50%) of skin ulcers are reported near freshwater drainage sluices (Vethaak, 1992). In addition to chemical contaminants, osmotic stress may play an important contribution to the development of skin ulcers at these specific sites (Vethaak, 1992).

Polychlorinated biphenyls (PCBs) belong to a category of contaminants suggested to be partially responsible for the etiology of at least some of the observed diseases of the flounder. Some of the toxic effects of polychlorinated biphenyls (PCBs) reported in mammals, including skin lesions and carcinogenicity (reviewed in Safe, 1994), show some resemblance with the pathological effects observed in fish from polluted areas.

Most of the toxic effects of PCBs are mediated by the Aryl hydrocarbon (Ah) receptor (Poland & Knutson, 1982; Landers & Bunce, 1991). There is a good structure-dependent correlation between Ah-receptor binding and the potency to induce cytochrome P4501A (CYP1A) (Poland & Glover, 1977; Denomme *et al.*, 1986), the major form of cytochrome P450 induced by PAHs and PHAHs in fish (Stegeman & Hahn, 1994), and associated ethoxyresorufin-O-deethylase (EROD) activity (Stegeman, 1981; Stegeman *et al.*, 1988).

Cytochrome P4501A is known to be directly involved in the oxidative biotransformation of non-*ortho*-PCBs (Ishida *et al.*, 1991), resulting in the formation of hydroxylated PCB metabolites. Hydroxylated PCB metabolites have been found to interfere with the plasma transport of both thyroid hormone and retinol, by binding to the plasma carrier protein transthyretin (TTR), thus enhancing the elimination of thyroxine and retinol from the circulation (Brouwer, 1987; Lans *et al.*, 1993). In addition, AhR-mediated induction of enzymes like UDP-glucuronosyltransferase (Morse *et al.*, 1993) and acylCoA:retinol acyl transferase (Jensen *et al.*, 1987) may result in a disturbance of the thyroid hormone and retinoid homeostasis.

PHAHs are known to affect retinoid homeostasis in mammals, as well as in fish (Zile, 1992). A possible role of retinoid metabolism in the toxicity of PHAHs is suggested because of the striking resemblance between the pathological effects observed in mammals exposed to PHAHs

and the symptoms associated with hypovitaminoses A, for example keratinisation of epithelia (Chopra, 1983) and suppression of immune function (Safe, 1994; Mark *et al.*, 1983). In addition, while PHAHs are well known tumour promotors (Silberhorn *et al.*, 1990), retinoids have been shown to suppress the process of carcinogenesis *in vivo* (Sporn & Roberts, 1983) and to modulate PHAH induced carcinogenesis (Flodström *et al.*, 1991).

In teleost, the thyroid hormone is considered to be involved in the process of smoltification (Folmar & Dickhoff, 1980; Prunet *et al.*, 1989), metamorphosis (Inui *et al.*, 1989) and osmoregulation (Leloup & De Luze, 1985). Reported changes in plasma thyroid levels in fish could be attributed to the interference of PHAHs with the thyroid homeostasis (Leatherland & Sonstegard, 1978). Hence osmotic stress in combination with PHAH exposure can disturb various physiological and biochemical processes in fish leading to observed pathological changes.

The present study was undertaken to investigate whether flounder are sensitive to PHAH exposure by measuring hepatic CYP1A induction and possible associated alterations in vitamin A and thyroid hormone homeostasis. Clophen A50, a mixture of PCB congeners, was chosen, rather than an individual congener, to better mimic a more realistic wildlife exposure situation.

**Materials and methods**

*Chemicals.* Clophen A50 was a kind gift from Dr.J.P.Boon (Netherlands Institute for Sea Research, Den Burg, Texel, the Netherlands). The following chemicals were purchased from Merck, Darmstadt, Germany: tris(hydroxymethyl)aminomethane (Tris), diisopropylether, hydrochloric acid, sodium chloride, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, glycerol, sucrose and 2,6-di-*ter*-butyl-4-methylphenol (BHT). Bovine Serum Albumin (BSA) was purchased from Sigma Chemie, Bornem, Belgium. Methanol, HPLC grade, was purchased from Janssen Chimica, Tilburg, the Netherlands. Retinol, retinyl palmitate, and retinyl acetate were purchased from Fluka Chemie, Bornem, Belgium.

*Sample collection.* Flounder were collected by beam-trawling between the 13$^{th}$ and 17$^{th}$ of June 1992 in the Western Wadden Sea. To avoid damage to the fish, the ship towed a 5 m beam-trawl for only 10 minutes. After hauling the net was opened and gonadally immature and overly healthy fish in the size class between 18.0 and 22.0 cm (appr. 2 years of age) were selected. The fish were kept in fresh water for 1 day, followed by 1 day in sea water and again 1 day in fresh water to free the animals from parasites. The fish were acclimatized at 13°C for 4 weeks, in 450 dm$^3$ glass tanks with recirculated (biologically filtered) and aerated semi-synthetic seawater (HW Marinemix + Bio-Elements, Wimex, Wiegandt, Krefeld, Germany) under a 12 hr light : 12 hr dark photoperiod regime. Flounder were fed frozen mysis (*Mysis relicta*) and cockles (*Mollusca, Bivalvia, Cerastoderma edule*) alternately 1% of body weight daily throughout the whole experiment.

*Treatment of fish.* After the 4 week acclimatization period, flounder were divided into groups of 10 animals each and transferred to 225 $dm^3$ glass tanks. Two groups received a single i.p. injection of 5 ml corn oil/kg body weight (day 2 and day 10 control groups). Three groups (day 2 of exposure) were i.p. injected without anaesthesia with respectively 20, 100, or 500 mg of Clophen A50/kg body weight, dissolved in corn oil (5 ml/kg body weight). The remaining three groups (day 10 of exposure) received the same doses of Clophen A50 dissolved in corn oil.

*Dissection of fish.* After two and ten days of exposure, one control group and three Clophen A50 dosed groups were stunned by a blow to the back of the head. Body weight and length were determined and blood samples were taken from the caudal vein with a heparinized syringe. Plasma was collected, after centrifugation of the blood at 500 g in a Eppendorf centrifuge and was stored at -20°C until analysis. After collecting blood, fish were killed by cervical transection. Livers were carefully dissected free from gall bladder, washed in ice-cold 0.9% NaCl, weighed and stored at -80°C until preparation of microsomes. Also kidneys were dissected, washed in ice-cold 0.9% NaCl, weighed and stored at -80°C until preparation of homogenates.

*Preparation of homogenates and microsomes.* Individual livers, and kidneys were homogenized in 1.5 ml 50 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose using 10 strokes with a motor-driven Potter-Elvehjem glass and teflon homogenizer. An aliquot of the homogenate was stored at -20°C for renal and hepatic retinoid analysis. The remaining hepatic homogenates were centrifuged at 9,000 g for 30 min. The resulting supernatants were centrifuged at 100,000 g for 90 minutes. The hepatic microsomal pellets obtained from each fish were resuspended in a 0.1 M potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol and stored at -80°C until analysis. All operations were performed at 0-4°C.

*Protein assays.* Protein was measured by the Bio-Rad assay system, using crystalline bovine serum albumin as standard.

*Cytochrome P450 assays.* Total cytochrome P450 content in the microsomal fraction of flounder liver was determined using the method described by Omura and Sato (1964). Microsomal 7-ethoxyresorufin-O-deethylation (EROD) activity was determined according to the method of Prough *et al.* (1978). The assay was optimized for flounder and executed at 25°C on a Hitachi fluorescence spectrophotometer F-2000.

*Extraction and analysis of retinoids.* Plasma, renal, and hepatic retinoids were analyzed using the method of Brouwer *et al.* (1989a) with some minor modifications. Renal homogenate (100 ml), plasma samples, or hepatic homogenate (50 ml) were vortexed with either 100 ml, or 50 ml

methanol, containing retinyl acetate as internal standard and 0.1 % 2,6-di-*ter*-butyl-4-methylphenol (BHT) (w/v) as an anti-oxidant. Plasma and liver retinoids were extracted overnight at -20°C with 100 ml diisopropylether. Kidney retinoids were extracted with 200 ml diisopropylether. After extraction the ether phase was removed and filtered over a 0.45 mm filter (Millipore, Etten Leur, the Netherlands), evaporated under nitrogen and finally resuspended in either 50 ml (plasma and kidney) or 100 ml methanol:ethylacetate (3:1) (0.1% BHT) (liver). All extractions were carried out in duplicate and under dim light. Twenty ml aliquots of resuspended extracts in methanol (0.1% BHT) were analyzed on a HPLC system employing a $C_{18}$ analytical column (Pecosphere, 3.3 cm length and 4.6 mm internal diameter, 3 mm particle size, Perkin Elmer). A Spectra-Physics Analytical HPLC system was used consisting of a P-2000 binary gradient pump, AS-3000 autosampler, UV-1000 UV-VIS detector and a Merck Hitachi D-2500 chromato-integrator. For the detection of retinoids a wavelength of 326 nm was used. Plasma extracts were analyzed using an isocratic system with 15% water and 85% methanol, a flow rate of 1 ml/min and data collection for 7 minutes. Renal and hepatic retinoids were analyzed by 15% water, 85% methanol for 1.5 minutes, followed by a gradient to 100% methanol for 2.5 minutes. Retinyl esters were eluted by running at 100% methanol for 9.5 minutes. Finally the column was re-equilibrated at 15% water and 85% methanol for 1.5 minutes.

*Thyroid hormone analysis.* Plasma total $T_4$ ($TT_4$), total $T_3$ ($TT_3$), and free $T_4$ ($FT_4$) were measured using the Amerlite system (Amersham, UK) and the protocol of the supplier with slight modifications. The $TT_4$ assay reagent was diluted five times with demineralized water; the $TT_4$ standard curve ranged from 0 to 30 nmol $TT_4$/liter; the standard curve for $TT_3$ ranged from 0 to 9 nmol $TT_3$/liter, whereas the standard curve for $FT_4$ ranged from 0 to 100 pmol $FT_4$/liter.

*Statistics.* Statistical analysis was performed by using the software package SPSS/PC+™ (SPSS Inc., Chicago, IL). Data were tested for normality and homogeneity of variance using the Chi-square test and the Bartletts test, after which data were analyzed statistically by a 2-way ANOVA with dose and time as independent variables. To find significant differences between the treatment groups, data were analyzed by one-way analysis of variance, followed by a least significant difference test. Data that did not pass the Chi-square test, or Bartletts test were analyzed for difference to control using the Kruskall-Wallis test. The data are expressed as the means ± standard error of the mean (SEM).

**Results**

*Body weights, condition factors and relative liver- and kidney- weights.* Overall no significant dose-, or time-dependent effects on body weights were observed following Clophen A50 treatment of flounder. Significantly reduced body weight was only observed in the 20 mg Clophen A50/kg

body weight group at 10 days after treatment compared to their initial body weight at day 0 (Table 3.1). In addition, a treatment-unrelated significant increase in body weight compared to the control group was only observed 2 days after i.p. injection of 20 mg Clophen A50/kg body weight.

No Clophen A50 dose-, or time-related effects were observed on the condition factor of flounder after 2 and 10 days of exposure (Table 3.1). The relative liver weights 10 days after i.p. injection of 20, or 500 mg Clophen A50/kg body weight were slightly, though significantly higher compared to the liver weight on day 2 in the same treatment groups. However, there was no clear dose-dependent increase in liver weights compared to controls at either time point of exposure to Clophen A50. Significantly lower liver weights compared to control values were only observed in flounder receiving 20 mg Clophen A50/kg body weight, at two days after exposure. The only significant difference in relative kidney weights was observed on day 10 of exposure compared to day 2 of the same exposure group (500 mg Clophen A50/kg body weight). However, again there was no dose-dependent effect of Clophen A50 on kidney weights.

*Monooxygenase induction.* Total cytochrome P450 content in liver microsomes of flounder was not altered after 2, or 10 days, following exposure to the PCB mixture Clophen A50 (Figure 3.1).

**Table 3.1.** Body weight, condition factor, relative liver and kidney weight of flounder following i.p. exposure to Clophen A50.

| | | Dose of Clophen A50 (mg/kg) | | | |
|---|---|---|---|---|---|
| | (n) | 0 | 20 | 100 | 500 |
| Body weight | day 0 (20) | 104.8 ± 6.6 | 111.5 ± 7.9 | 119.3 ± 7.3 | 121.5 ± 5.7 |
| (g) | day 2 (10) | 99.4 ± 10.0 | 131.4 ± 12.4[a] | 121.4 ± 9.1 | 118.4 ± 8.7 |
| | day 10 (10) | 108.3 ± 8.4 | 92.3 ± 7.7[b] | 114.7 ± 11.5 | 125.3 ± 7.6 |
| Condition factor | day 0 (20 | 1.54 ± 0.04 | 1.51 ± 0.03 | 1.53 ± 0.03 | 1.54 ± 0.02 |
| ($g/cm^3$)[d] | day 2 (10) | 1.57 ± 0.08 | 1.55 ± 0.04 | 1.58 ± 0.03 | 1.56 ± 0.03 |
| | day 10 (10) | 1.52 ± 0.04 | 1.46 ± 0.03 | 1.50 ± 0.04 | 1.53 ± 0.03 |
| Liver | day 2 (10) | 1.20 ± 0.08 | 0.95 ± 0.08[a] | 1.02 ± 0.06 | 1.00 ± 0.05 |
| (% body weight) | day 10 (10) | 1.18 ± 0.07 | 1.32 ± 0.10[b] | 1.11 ± 0.09 | 1.37 ± 0.10[b,c] |
| Kidney | day 2 (10) | 0.21 ± 0.03 | 0.23 ± 0.04 | 0.22 ± 0.02 | 0.20 ± 0.01 |
| (% body weight) | day 10 (10) | 0.24 ± 0.02 | 0.30 ± 0.05 | 0.27 ± 0.04 | 0.33 ± 0.04[b] |

Flounder were i.p. dosed with Clophen A50 on day 0. Number of animals in parentheses. Data are expressed as mean ± S.E.M..

[a]Significant different from control, $p<0.05$.

[b]Significant different from day 2 treatment group, $p<0.05$.

[c]Significant different from 100 mg Clophen A50/kg b.w. treatment group and day 2 treatment group, $p<0.05$.

[d]Condition factor: ((body weight)/(body lenght)$^3$)x100.

Even a dose as high as 500 mg Clophen A50/kg body weight did not show any effect on the total hepatic cytochrome P450 concentration. In contrast, a 6 fold dose-related increase (F=6.604 (df=3), p=0.001) in EROD activity was found in flounder receiving 100, or 500 mg Clophen A50/kg body weight at 10 days after exposure (Figure 3.2a). At two days after exposure, a slight but not significant induction of EROD activity was observed in flounder receiving 20, or 100 mg Clophen A50/kg body weight. Although not time-related, a dose by time interaction was found (F=3.281 (df=3), p=0.026). A similar pattern of changes was found when EROD activity was expressed as the specific activity, e.g. the amount of resorufin formed per minute per nmol cytochrome P450 (figure 3.2b). Again, a dose-dependent induction (F=2.938 (df=3), p=0.04) of the specific activity was observed 10 days after exposure only whereas at day 2 after exposure a slight but not significant increase was found in the low dose group only. In addition, a 2-way ANOVA interaction (dose*time) was observed (F=6.711 (df=3), p=0.001).

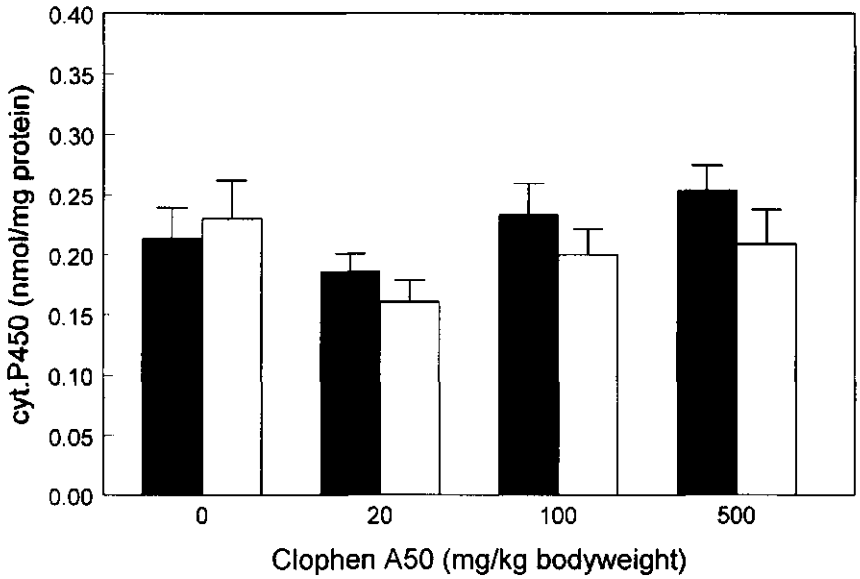


**Figure 3.1.** Effect of Clophen A50 on total cytochrome P450 concentration in flounder hepatic microsomes. Flounder were sacrificed after 2 (represented by black bars) and 10 (represented by white bars) days. Data (n=9) are expressed as means ± S.E.M..

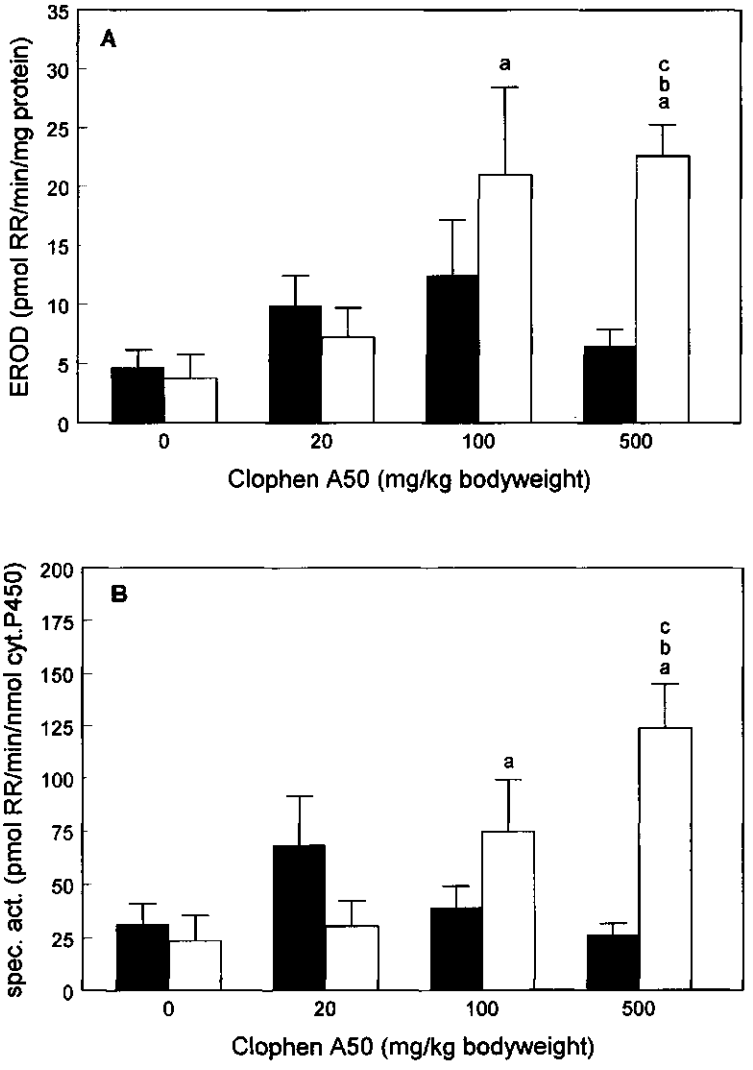


**Figure 3.2.** Hepatic microsomal 7-ethoxyresorufin-O-deethylase (EROD) activity (pmol RR/min/mg protein) (A) and EROD specific activity (pmol RR/min/nmol cat.P450) (B) in flounder, following i.p. exposure to Clophen A50. Flounder were sacrificed at 2 (represented by black bars) and 10 (represented by white bars) days after exposure. (a) indicates a significant increase ($p<0.01$) from cornoil treated groups, (b) indicates a significant difference ($p<0.01$) between the dose group of 500 mg Clophen A50/ kg body weight and the group that received 20 mg Clophen A50/kg body weight, (c) indicates an significant increase ($p<0.01$) compared to the same treatment group at day 2. RR=resorufin formed. Data (n=9) are expressed as means ± S.E.M..

*Plasma, renal, and hepatic retinoid levels.* The effect of Clophen A50 on the plasma and renal retinol concentrations are given in Table 3.2. The plasma retinol concentrations were not affected by exposure to Clophen A50. Only a slight, though significant time-, but no dose-effect on plasma retinol concentrations was observed by 2-way ANOVA analysis ($F=4.170$ ($df=1$), $p=0.045$). A dose-, but not time-related increase of renal retinol levels was observed in Clophen A50 treated flounder ($F=4.012$ ($df=3$), $p=0.011$). At day 2 of exposure, the retinol concentration in the kidney of flounder dosed with 500 mg Clophen A50/kg body weight was significantly higher from control, and 100 mg Clophen A50/kg body weight dosed groups of flounder. At day 10 of exposure, an increase in renal retinol concentrations in the highest dose group was still observed, though the increase was not significantly different from control values.

Large individual differences were observed between individual hepatic retinol concentrations within the same treatment groups (Table 3.3). A significant reduction in hepatic retinol levels was observed between the corn oil treated groups on day 2 and day 10. When expressed as percentage of control, the hepatic retinol content was either decreased (day 2), or increased (day 10) after dosing in the Clophen A50 treated groups compared to controls. These changes were not statistically significant different from controls ($p>0.05$). The individual variability was also large for the retinyl palmitate concentrations. Flounder treated with either 20, or 100 mg Clophen A50/kg body weight showed significantly lower hepatic retinyl palmitate concentrations from the control values on day 2 of exposure, however no changes were observed

**Table 3.2.** Retinoid content in plasma and kidney of flounder following i.p. exposure to Clophen A50.

| Dose Clophen A50 (mg/kg) | Plasma retinol (ng/ml) | Kidney retinol (ng/g) |
|---|---|---|
| day 2 | | |
| 0 | 223.43 ± 8.92 (8) | 313.85 ± 27.57[b] (8) |
| 20 | 203.86 ± 13.52 (10) | 344.36 ± 36.98 (10) |
| 100 | 202.14 ± 13.48 (9) | 307.21 ± 30.03[b] (9) |
| 500 | 188.49 ± 14.61 (9) | 472.34 ± 31.64 (8) |
| day 10 | | |
| 0 | 230.03 ± 20.96 (10) | 306.26 ± 43.26 (9) |
| 20 | 206.98 ± 14.54 (10) | 378.85 ± 64.73 (8) |
| 100 | 239.61 ± 23.63 (10) | 305.84 ± 50.13 (8) |
| 500 | 243.39 ± 21.70[a] (10) | 424.71 ± 65.56 (10) |

Flounder were i.p. dosed with Clophen A50 on day 0. Number of animals in parentheses. Data are expressed as mean ± S.E.M..

[a]: significantly different from day 2 treatment group.

[b]: significantly different from flounder exposed to 500 mg Clophen A50/kg b.w., $p<0.05$.

**Table 3.3.** Hepatic retinoid content of flounder following i.p. exposure to Clophen A50.

| Dose Clophen A50 (mg/kg) | retinol (mg/g) | retinyl palmitate (mg/g) | retinol/retinyl palmitate (ratio) |
|---|---|---|---|
| day 2 | | | |
| 0 (n=10) | 47.42 ± 9.88 | 18.67 ± 3.18 | 2.49 ± 0.51 |
| 20 (n=9) | 19.98 ± 3.32 | 14.02 ± 3.04[a] | 1.91 ± 0.39 |
| 100 (n=10) | 48.77 ± 16.70 | 9.26 ± 2.59[a] | 2.07 ± 0.64 |
| 500 (n=9) | 26.74 ± 4.10 | 17.75 ± 3.05 | 2.13 ± 0.49 |
| day 10 | | | |
| 0 (n=9) | 10.54 ± 2.46[b] | 11.23 ± 3.08 | 1.26 ± 0.27[b] |
| 20 (n=10) | 20.48 ± 2.71 | 13.45 ± 2.16 | 1.81 ± 0.28 |
| 100 (n=10) | 18.37 ± 4.72 | 14.20 ± 2.60 | 1.24 ± 0.32 |
| 500 (n=9) | 18.36 ± 3.87 | 15.64 ± 4.12 | 1.32 ± 0.34 |

Flounder were i.p. dosed with Clophen A50 on day 0. Number of animals in parentheses. Data are expressed as mean ± S.E.M..
[a]: significant different from control, $p<0.05$.
[b]: significant different from day 2 treatment group, $p<0.05$.

from the 500 mg Clophen A50/kg body weight dose group. A significant difference in the hepatic ratio retinol/retinyl palmitate was observed between the corn oil treated groups on day 2 and day 10. In addition, a time-, but not dose-related decrease in the ratio retinol/retinyl palmitate was observed by 2-way ANOVA analysis ($F=6.286$ (df=1), $p=0.015$).

*Plasma thyroid hormone levels.* The results on plasma thyroid hormone measurements are given in Table 3.4. Two days after exposure to Clophen A50 no significant differences in the total $T_4$ levels were observed. At day 10, a significant lower level of plasma total $T_4$ was observed only in flounder receiving 20 mg Clophen A50/kg body weight compared to the control and 100 mg Clophen A50/kg body weight treated groups. Significantly higher total $T_3$ levels in plasma as compared to control values were only observed in fish dosed with 100 mg Clophen A50/kg body weight at day 2 of exposure ($p<0.05$). An overall dose-related effect was indicated in plasma total $T_3$ levels by 2-way ANOVA analysis ($F=5.578$ (df=3), $p=0.002$). Plasma free $T_4$ levels were increased in flounder exposed to 500 mg Clophen A50/kg body weight as compared to the other dosed groups at day 2 of dissection. At day 10, flounder receiving 20 mg Clophen A50/kg body weight had decrease free $T_4$ levels as compared to the control, 100, and 500 mg Clophen A50/kg body weight dose groups. A 2-way interaction (dose*time) was found in plasma free $T_4$ levels by ANOVA analysis ($F=3.694$ (df=3), $p=0.016$).

**Table 3.4.** Thyroid hormone concentrations in plasma of flounder following i.p. exposure to Clophen A50.

| Dose Clophen A50 (mg/kg) | plasma total $T_4$ (nmol/l) | plasma total $T_3$ (nmol/l) | plasma free $T_4$ (pmol/l) |
|---|---|---|---|
| day 2 | | | |
| 0 | 4.95 ± 0.89 (10) | 1.95 ± 0.36 (8) | 6.58 ± 1.08 (9) |
| 20 | 4.49 ± 0.60 (10) | 2.32 ± 0.66 (9) | 4.99 ± 0.75 (9) |
| 100 | 5.61 ± 0.77 (9) | 5.34 ± 0.90[b] (10) | 6.67 ± 1.54 (9) |
| 500 | 5.76 ± 0.87 (10) | 2.89 ± 0.43 (10) | 11.43 ± 1.56[b] (10) |
| day 10 | | | |
| 0 | 5.21 ± 0.54 (10) | 2.58 ± 0.53 (8) | 7.00 ± 0.57 (8) |
| 20 | 2.62 ± 0.33[a] (9) | 1.88 ± 0.55 (10) | 3.68 ± 0.40[c] (9) |
| 100 | 5.77 ± 0.57 (9) | 3.19 ± 0.29 (9) | 10.94 ± 1.72 (9) |
| 500 | 4.03 ± 0.52 (10) | 3.16 ± 0.41 (9) | 9.01 ± 0.85 (10) |

Flounder were i.p. dosed with Clophen A50 on day 0. Number of animals in parentheses. Data are expressed as mean ± S.E.M..
[a]: significant different from control and 100 mg Clophen A50/kg b.w. treatment group, $p<0.05$.
[b]: significant different from all other groups at day 2 of dissection, $p<0.05$.
[c]: significant different from all other groups at day 10 of dissection, $p<0.05$.

## Discussion

The results presented in this paper show no effects on total cytochrome P450 concentrations and only a relatively slight induction in EROD activity, indicating that flounder (*Platichthys flesus*) is not very sensitive to exposure to the technical PCB mixture Clophen A50. In addition, minor effects on either plasma, renal, and hepatic retinoid levels and plasma thyroid levels were found, which in most cases was independent of the dose of Clophen A50.

These results are different from results reported on sensitivity of other fish species towards PCB and TCDD treatment. Andersson *et al.* (1985) observed an almost 50-fold increase in EROD activity in rainbow trout *(Salmo gairdneri)* 3 days after a single i.p. injection of 500 mg Clophen A50/kg body weight. In rainbow trout (*Oncorhynchus mykiss*), Hektoen *et al.* (1994) even found a 140-fold induction of EROD activity upon exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) whereas in carp (*Cyprinus carpio*), a 100 fold increase in EROD activity was observed one week after a single i.p. exposure to 0.27 mg TCDD/kg body weight (van der Weiden *et al.*, 1994). The dose-dependent 6-fold EROD induction found in this study indicates therefore a comparably low responsiveness of flounder (*Platichthys flesus*) towards Clophen A50.

A time-dependent increase of the catalytic activity of cytochrome P4501A was found in rainbow trout *(Salmo gairdneri)* by Andersson *et al.* (1985). The EROD activity reached a

maximum 7 days after i.p. administration of 500 mg Clophen A50/kg body weight, whereas at day 3 of exposure only a slight increase was observed. Our results showed a similar time-dependency. In contrast administration of ß-naphthoflavone, or 2,3,7,8-TCDF resulted in induced EROD activities already after 1 day (Celander *et al.*, 1994; Hahn & Stegeman, 1994; Andersson *et al.*, 1985). This indicates potential differences in toxicokinetics and in responsiveness of the various fish species for PHAH/PAH-induced CYP1A induction.

Although a significant increase in EROD activity was found after 10 days, the 6-fold induction was not as high as could be expected from the dose administered. The Clophen A50 concentrations at which induction was observed (100, and 500 mg Clophen A50/kg body weight) correspond to Dioxine Toxic Equivalences (TEQ) of respectively 0.06 and 0.012 mg TEQ/kg body weight (Schulz *et al.*, 1989; Safe, 1994). Oral exposure of flounder to 10 mg 2,3,7,8-TCDD/kg body weight (0.01 mg TEQ/kg body weight) resulted in a 40-fold increase of EROD activity 10 days after administration (Besselink *et al.*, unpublished results). This may suggest that antagonistic and or substrate inhibition effects may play a role in flounder dosed with Clophen A50, which will be reported on in full detail in another paper.

An alternative explanation of the relatively low responsiveness of flounder with respect to Ah-receptor mediated effects, may be that the Ah-receptor levels in flounder are exceptionally low, or that the Ah-receptor pathway is inefficient. Until now, not much data is available on the presence and amount of the Ah-receptor in teleost. Using photoaffinity labelling Hahn *et al.* (1994) observed specific binding of the photoaffinity label 2 azido-3[$^{125}$I]iodo-7,8-dibromodibenzo-*p*-dioxin in hepatic cytosol of 7 species of teleost, including winter flounder (*Pleuronectes americanus*) and rainbow trout (*Oncorhynchus mykiss*). From a study by Bank *et al.* (1992), it could be concluded that rainbow trout contain only small amounts of Ah-receptor (~5 fmol/mg cytosolic protein). Preliminary studies in our laboratory to determine the presence and the amount of the Ah-receptor in flounder indicate the presence of low concentrations of Ah-receptor in flounder hepatic cytosol (Besselink *et al.*, unpublished results).

It is has been shown that PHAHs can alter retinoid- (Brouwer & Van den Berg, 1984; Brouwer *et al.*, 1989a; Zile, 1992), and thyroid hormone (Brouwer, 1989; Lans *et al.*, 1993; Morse *et al.*, 1993) homeostasis in mammals. The results observed in the present study indicate that interference of PCBs with the flounder retinoid- or thyroid hormone homeostasis if it occurs at all, is affected at best only at a minor scale. However, it could be argued that the large individual variability observed in retinoid- and thyroid hormone levels may have made it difficult to identify PCB-related effects. Alternatively, it could be argued that an exposure time of 10 days is to short to observe more pronounced alterations in both retinoid- thyroid hormone levels in fish. Palace & Brown (1994) reported decreased hepatic retinol, dehydroretinol, and retinyl palmitate concentrations and elevated kidney retinyl palmitate levels in lake char (*Salvelinus namaycush*) eight weeks after a single oral dose of 3,3',4,4',5-pentachlorobiphenyl (CB-126). In a 3-year

mesocosm study flounder were kept under similar conditions either on polluted harbour sludge or unpolluted sediment (Vethaak *et al.*, 1993). In this study we observed decreased levels of plasma retinol, hepatic retinol, and the ratio hepatic retinol/retinyl palmitate in flounder kept on the polluted sludge as compared to flounder kept on unpolluted sediment (Besselink *et al.*, unpublished results).

In conclusion, this study indicates that flounder is not a sensitive species to PCB exposure in terms of Ah-receptor mediated induction of cytochrome P4501A activity, and alterations in retinoid and thyroid homeostasis. No conclusions can be drawn from this short-term exposure study towards the possible involvement of PCBs in the development of skin diseases and liver tumours in flounder from Dutch coastal areas, which involves life-time exposure to these chemicals.

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# Chapter 4
# High Induction of Cytochrome P4501A Activity without Changes in Retinoid and Thyroid Hormone Levels in Flounder (*Platichthys flesus*) Exposed to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)


**Abstract**

Oral doses of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) were administered twice within 7 days (total concentration 0.01, 0.1, 1, 10, or 100 μg TCDD/kg body weight) to flounder (*Platichthys flesus*). After 10 days of exposure, flounder were sacrificed and the effects of TCDD exposure on hepatic microsomal total cytochrome P450 content, 7-ethoxyresorufin-*O*-deethylase (EROD) activity and glutathione-*S*-transferase activity were examined. In addition, plasma and hepatic retinoid and plasma thyroid hormone levels were analysed. Overall a good correlation existed between the dose and liver concentrations of TCDD. However, only 0.75% of the total dose of TCDD was retained in the flounder liver. TCDD caused a dose-related, statistically significant induction of the hepatic microsomal total cytochrome P450 content (4.4 fold; $p<0.001$) and associated EROD activity (27 fold; $p<0.001$), without obvious changes in body weight, liver weight, and condition factor. Total glutathione-*S*-transferase activity was not induced in livers of flounder exposed to TCDD. No TCDD induced changes in liver retinoid, plasma retinol, and plasma thyroid hormone parameters were observed. These results indicate that the flounder is an Ah receptor responsive species with respect to CYP1A induction, but not with respect to other mammalian Ah receptor associated responses.



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## Introduction

In the Netherlands, a number of studies have focused on viral skin diseases and liver tumours in flounder (*Platichthys flesus*) and their possible relationship to chemical contaminants and natural stressors over the last decade (Vethaak & Jol, 1996; Vethaak & Wester, 1996). There is evidence that polycyclic aromatic hydrocarbons (PAHs) and polyhalogenated aromatic hydrocarbons (PHAHs) may contribute to the aetiology of at least some of the diseases, especially neoplasia, observed in fish from highly polluted coastal/estuarine waters as compared to relatively unpolluted waters (Malins *et al.*, 1988; Vethaak & ap Rheinallt, 1992; Vethaak, 1993).

Mammalian studies have indicated that most of the toxic effects of PAHs and PHAHs are mediated by the Aryl hydrocarbon (Ah) receptor (Landers & Bunce, 1991). There is a good structure-dependent correlation between Ah receptor binding and the potency to induce cytochrome P4501A (CYP1A) and associated ethoxyresorufin-O-deethylase (EROD) activity (Poland & Knutson, 1982; Goldstein & Safe, 1989). CYP1A is the major form of cytochrome P450 induced by PHAHs in fish as well (Stegeman & Hahn, 1994). Therefore, as a practical spin-off, the induction of the cytochrome P4501A subfamily is commonly used as a biomarker for exposure of aquatic organisms to PAHs, PCBs, dioxins and related compounds (Bucheli & Kent, 1995; Goksøyr, 1995). In addition to CYP1A, the modulation of other biotransformation enzymes, such as certain forms of glutathione-*S*-transferase (Andersson *et al.*, 1985), UDP-glucuronyltransferase (UGT) (Morse *et al.*, 1993), and mammalian acylCoA:retinol acyl transferase (ARAT) (Jensen *et al.*, 1987) have been found or suggested to be under the control of the Ah receptor signal transduction pathway.

Ah-mediated induction of these enzymes can result in depletion of essential components of the intermediating metabolism, e.g., retinoid depletion by ARAT inhibition and thyroid depletion by UGT induction. In addition, the AhR-mediated formation of hydroxy polychlorinated biphenyl (PCB-OH) metabolites, that compete with $T_4$ for plasma protein binding in mammals, adds considerably to the thyroid depleting effects of these compounds (Brouwer, 1989; Lans *et al.*, 1993). In mammals it is hypothesised that the retinol and thyroid disrupting effects of e.g. PCBs may play an essential role in the toxico-pathological effects like tumour promotion and skin disorders (Brouwer, 1987).

In a previous study we observed a low responsiveness of flounder towards the commercial PCB mixture Clophen A50 (Besselink *et al.*, 1996). The aim of the present investigation is to determine the responsiveness of flounder towards 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most potent Ah receptor agonist and most toxic PHAH congener by measuring hepatic CYP1A and other xenobiotic metabolising enzymes. We also investigated whether TCDD exposure would result in endocrine disrupting effects with respect to retinoid and thyroid hormones, similar to those observed in mammals. Flounder were orally dosed to mimic a

more natural exposure. In addition, the liver retention of orally administered TCDD was studied by adding a small amount of $^{3}$H-labelled TCDD to the exposure solution.

**Materials and methods**

*Chemicals.* 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (>99 % pure) was obtained from Promochem, Wesel, Germany. [$^{3}$H]-radiolabeled 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (32 Ci/mmol) was a generous gift of Dr. H. Poiger (Swiss Federal Institute of Technology, Zürich, Switzerland). 1-chloro-2,4-dinitrobenzene (CDNB) was obtained from Aldrich (Aldrich Chemical Compagny, Inc, Milwaukee, USA). Tris, $Na_2EDTA$, diisopropylether, hydrochloric acid, sodium chloride, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, glycerol, sucrose, 2,6-di-*ter*-butyl-4-methylphenol (BHT) and hydrogen peroxide (30% w/w) were obtained from Merck, Darmstadt, Germany. Bovine Serum Albumin (BSA) was purchased from Sigma Chemie, Bornem, Belgium. Glutathione (reduced) and methanol (HPLC grade) were obtained from Janssen Chimica, Tilburg, the Netherlands. Retinol, retinyl palmitate and retinyl acetate were purchased from Fluka Chemie, Bornem, Belgium. $TT_4$, $TT_3$ and $FT_4$ Amerlite kits were obtained from Amersham (Amersham, Aylesbury, Buckinghamshire, United Kingdom).

*Sample collection.* Flounder were collected between day 3 and 6 of May 1994 in the Western Wadden Sea. The ship towed a 5 m beam-trawl for only 10 minutes to avoid damage to the fish, after which the net was hauled and opened. Immature fish in the length class between 18.0 and 22.0 cm (mainly 2 years of age) were selected. To free captured fish from parasites (e.g. Trichodina, Dactylogyrus), the animals were kept in fresh water for 1 day, followed by 1 day in sea water and again 1 day in fresh water. The fish were acclimatised at 13°C for 4 weeks, in 450 $dm^3$ glass tanks containing sediment with recirculated (biologically filtered) and aerated semi-synthetic seawater (HW Marinemix + Bio-Elements, Wimex, Wiegandt, Krefeld, Germany) under a 12 hr light : 12 hr dark photoperiod regime. Flounder were fed previously frozen mysis (*Mysis relicta*) and cockles (*Mollusca, Bivalvia, Cerastoderma edule*) alternately, approximately 1% of body weight daily throughout the whole experiment.

*Treatment of fish.* After the 4 week acclimatisation period, flounder were divided into 6 groups of 8 animals each and transferred to sediment containing 225 $dm^3$ glass tanks. At day 1 and 7 of the experiment gelatin capsules filled with 200 µl of a solution containing either $3.5*10^{-4}$, $3.5*10^{-3}$, $3.5*10^{-2}$, $3.5*10^{-1}$ or 3.5 µg TCDD dissolved in cornoil were orally injected into the stomach of 5 groups of fish. One group was injected with 200 µl corn oil only (control). The final concentrations of TCDD administered were 0, 0.01, 0.1, 1, 10 or 100 µg TCDD/kg body weight. In addition, the TCDD dosed flounder received a small amount of [$^{3}$H] radiolabelled TCDD

(60,000 dpm) by adding the [$^3$H] labelled TCDD to the exposure solution. The gelatin capsules were administered as described by Boon *et al.* (1992).

*Dissection of fish.* Ten days after the first exposure, flounder were captured by netting and stunned by a blow to the back of the head. Body weight and length were determined and blood samples were taken from the caudal vein with a heparinized syringe. Plasma was collected, after centrifugation of the blood at 500 g in an Eppendorf centrifuge and was stored at -20°C until analysis. After collecting blood, fish were killed by cervical transection. Livers were carefully dissected free from the gall bladder, washed in ice-cold 0.9% NaCl, weighed and stored at -80°C until preparation of homogenates and microsomes.

*Preparation of homogenates and microsomes.* Individual livers were homogenised in 2.5 ml 50 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose, using 10 strokes with a motor-driven Potter-Elvehjem glass and teflon homogenizer. An aliquot of the homogenate was stored at -20°C for hepatic retinoid analysis. To determine the amount of [$^3$H]-TCDD present in the liver of flounder, 1.3 ml aliquots of the homogenates were treated with 3.6 ml soluene (Packard, USA) and 200 µl 30% (w/w) hydrogen peroxide to dissolve and decolorize the homogenate, followed by scintillation counting (15 ml Hionic Fluor, Packard, USA) in a 1600 TR Liquid Scintillation Analyzer, Packard. The remaining amount of hepatic homogenates were centrifuged at 9,000 g for 30 min. The resulting supernatants were centrifuged at 100,000 g for 90 minutes. The hepatic microsomal pellets obtained from each fish were resuspended in a 0.1 M potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol and stored at -80°C until analysis. All operations were performed at 0-4°C.

*Protein assays.* Protein was measured by the Bio-Rad assay system, using crystalline bovine serum albumin as standard.

*Cytochrome P450 assays.* Total cytochrome P450 content in the microsomal fraction of flounder liver was determined using the method described by Omura & Sato (1964). Microsomal 7-ethoxyresorufin-O-deethylation (EROD) activity was measured in hepatic microsomes according to the method of Prough *et al.* (1978). The assay was adapted for use in 96 well plates and a fluorospectrophotometric plate reader (Cytofluor 2350, Fluorescence Measurement System, Milipor, USA). The reaction conditions (in a total volume of 200 µl) were 0.1 M Tris-HCl buffer, pH 7.8, 1 mg BSA/ml, 0.1 mM NADPH, 0.6 µM ethoxyresorufin, and 100 µg microsomal protein/ml. The reaction mixture was pre-incubated at 25°C for 5 minutes, the reaction was started by adding NADPH, and the incubation was stopped after 5 minutes at 25°C by the addition of 50 µl 1 M NaOH. The resorufin formed was detected fluorimetrically and compared to a calibration

curve (0-150 nM resorufin). All incubations were in duplicate and corrected for a blank without NADPH.

*Glutathione-S-transferase activity.* Cytosolic glutathione-*S*-transferase activity (GST) activity was measured in hepatic cytosol according to the method of Habig *et al.* (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The assay was adapted for use in 96 well plates and a spectrophotometric plate reader (Thermo-max microplate reader, Molecular Devices). The reaction conditions (in a total volume of 250 µl) were 0.1 M potassiumphosphate buffer, pH 6.5, 1 mM EDTA, 1 mM glutathione (GSH), 1 mM CDNB and 200 µg microsomal protein/ml. After pre-incubating for 2 minutes at 25°C, the reaction was started by adding CDNB. The formation of the glutathione conjugate was measured for 1 minute with 6 second intervals at 340 nm.

*Extraction and analysis of retinoids.* Plasma and hepatic retinoids were analysed as described previously (Besselink *et al.*, 1996). In short, plasma samples or hepatic homogenate (50 µl) were extracted overnight at -20°C with 50 µl methanol, containing retinyl acetate as internal standard and 0.1 % 2,6-di-*ter*-butyl-4-methylphenol (BHT) (w/v) as an anti-oxidant, and 100 µl diisopropylether. After extraction the ether phase was removed, filtered, evaporated under nitrogen and finally resuspended in either 50 µl methanol (0.1% BHT) (plasma) or 100 µl methanol:ethylacetate (3:1) (0.1% BHT) (liver). Twenty µl aliquots of resuspended extracts were analysed on a HPLC system employing a $C_{18}$ analytical column (Pecosphere, 3.3 cm length and 4.6 mm internal diameter, 3 µm particle size, Perkin Elmer). A Spectra-Physics Analytical HPLC system was used consisting of a P-2000 binary gradient pump, AS-3000 autosampler, UV-1000 UV-VIS detector and a Merck Hitachi D-2500 chromato-integrator. For the detection of retinoids a wavelength of 326 nm was used.

*Thyroid hormone analysis.* Plasma total $T_4$ ($TT_4$), total $T_3$ ($TT_3$) and free $T_4$ ($FT_4$) were measured using the Amerlite system (Amersham, UK) and the protocol of the supplier with slight modifications. The $TT_4$ assay reagent was diluted five times with demineralized water; the $TT_4$ standard curve ranged from 0 to 30 nmol $TT_4$/liter; the standard curve for $TT_3$ ranged from 0 to 9 nmol $TT_3$/liter; the standard curve for $FT_4$ ranged from 0 to 100 pmol $FT_4$/liter.

*Data treatment and statistics.* Data were tested for normality and homogeneity of variance using the Chi-square test and the Bartletts test. Once they passed both tests, dose-dependent effects were examined statistically by a 2-way ANOVA with TCDD as independent variable. Significant differences between the treatment groups were analysed by one-way analysis of variance followed by a least significant difference test. Data that did not pass the Chi-square test or Bartletts test were analysed for differences from the control using the Kruskall-Wallis test. Correlation between the

administered dose of TCDD and concentrations of TCDD in hepatic tissue were evaluated using the software package SlideWrite Plus, Version 5.00 (Advanced Graphics Software, Inc., Carlsbad, CA, USA) according to the following equation: $y=a_0+a_1*x$. Sigmoidal dose-response relationships and Calculated No Effect Levels (CNELs) were derived by using either the log total concentration TCDD/kg body weight administered or the log hepatic TCDD concentration. Calculations were carried out using the software package SlideWrite Plus, Version 5.00 (Advanced Graphics Software, Inc., Carlsbad, CA, USA) according to the following equation: $y=a_0+a_1/(1+\exp(-(x-a_2)/a_3))$, where y is the effect at dose x; $a_0$ = average minimum; $a_1$ = (average maximum-$a_0$); $a_2$ = middle of transition (x at inflection point ≡ log($ED_{50}$); $a_3$ = width of transition. Since it is mathematically impossible to make a curve fit with the logarithm of zero, control values were set at log dose -2 ng TCDD/kg b.w. (0.01 ng TCDD/kg b.w. administered) and log hepatic dose -1 pg TCDD/g liver (0.1 pg TCDD/g liver). To derive the CNELs, the average control values plus twice the standard deviation was calculated and projected to the sigmoidal dose-response curve fit. The accompanying x-value corresponds with log(CNEL).

**Results**

*Body weights, condition factors and relative liver weights.* No TCDD-dose related effects were observed on flounder total body weight, liver weight or condition factor throughout the experiment (Table 4.1). In contrast, a minor time-dependent decrease in condition factor was found in fish exposed to corn oil as well as TCDD at day 10 of exposure when compared to their condition factor at day 1 of exposure ($F=4.806$ ($df=1$), $p=0.031$).

*Hepatic TCDD concentrations.* The gelatin capsules used for dosing the fish with TCDD also contained 60,000 dpm of [$^3$H] labelled TCDD at both days of exposure. After correcting the amount of the [$^3$H]-activity in 1.3 ml hepatic homogenate for whole liver weights, the amount of [$^3$H]-TCDD retained in the liver as percentage of the total amount of [$^3$H]-TCDD administered, was calculated. Using this percentage, the actual hepatic TCDD concentration could be calculated. A good positive correlation was observed (Figure 4.1) between the total dose administered and hepatic concentrations of TCDD ($y=-0.26+1.03*x$; $r^2=0.97$; $p<0.01$). However, only a maximum of 0.75% of the total dose of TCDD administered was retained in the liver (data not shown).

*Cytochrome P450 induction.* The total cytochrome P450 content in hepatic microsomes of flounder showed a dose-related induction ($F=23.93$ ($df=5$), $p<0.001$). The lowest dose of TCDD at which a significant elevation of the total cytochrome P450 level was observed was 10 µg TCDD/kg body weight (Figure 4.2). At the highest dose of TCDD administered, a 4.4 fold

**Table 4.1.** Body weight, condition factor and relative liver weight of flounder 10 days after initiation of oral exposure to TCDD.

| TCDD dose (μg/kg body weight) | Body Weight (g) | Condition Factor ($g/cm^3$)[a] | Liver (% body weight) |
|---|---|---|---|
| 0 (8) | 59.2 ± 7.6 | 0.81 ± 0.03 | 0.88 ± 0.07 |
| 0.01 (7) | 80.1 ± 10.0 | 0.83 ± 0.03 | 1.06 ± 0.10 |
| 0.1 (7) | 65.0 ± 7.7 | 0.85 ± 0.03 | 1.27 ± 0.16[b] |
| 1 (7) | 67.3 ± 6.3 | 0.81 ± 0.04 | 0.83 ± 0.08 |
| 10 (8) | 69.5 ± 4.2 | 0.84 ± 0.02 | 0.83 ± 0.05 |
| 100 (8) | 70.9 ± 9.3 | 0.81 ± 0.03 | 1.07 ± 0.16 |

Flounder were exposed to TCDD on day 1 and 7. Number of animals in parentheses. Data are expressed as mean ± SE.
[a]: condition factor: ((body weight)/(body length)$^3$)*100.
[b]: signicantly different from flounder exposed to corn oil, 1 or 10 μg TCDD/kg b.w., $p<0.05$.

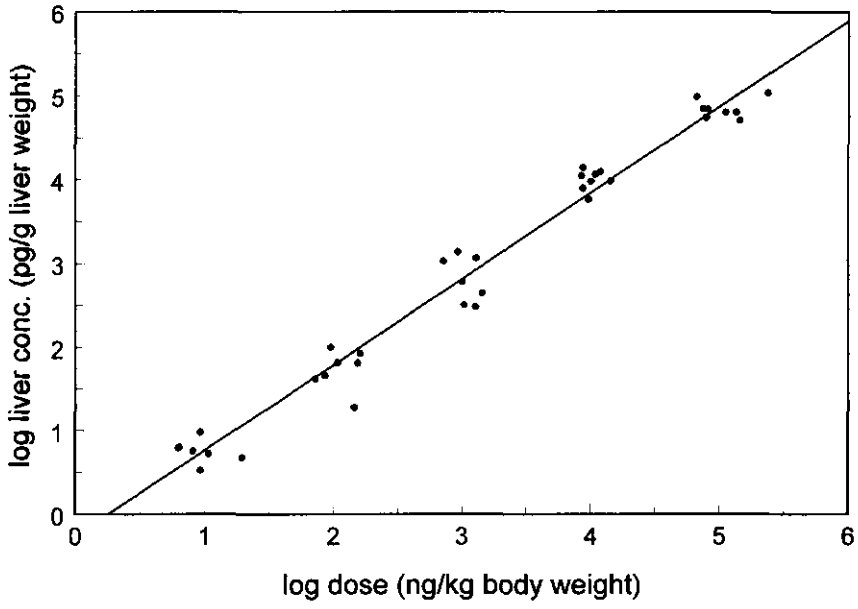


**Figure 4.1.** Relationship between orally administered total TCDD (ng/kg body weight) and hepatic TCDD concentrations (pg/g liver weight) ($y=-0.26+1.03*x$; $r^2=0.97$; $p<0.01$). Hepatic concentrations of TCDD were calculated from radioactivity found in 1.3 ml hepatic homogenate and radioactivity present in the administered dose.

induction of the total cytochrome P450 was observed compared to control levels. In addition, a dose-related induction of the hepatic microsomal EROD activity ($F=18.09$ ($df=5$), $p<0.001$) was observed already at 1 μg TCDD kg body weight (Figure 4.3a). The most pronounced increase in EROD activity (average 27 fold) was observed in flounder exposed to 10 μg TCDD/kg body weight. In contrast to total cytochrome P450 levels, the highest dose of 100 μg TCDD/kg body weight resulted in a slight but not significant decrease in EROD activity as compared to the 10 μg TCDD/kg body weight exposure group. A similar dose-related induction of the EROD specific activity, e.g. the amount of resorufin formed per minute per nmol cytochrome P450, was found ($F=10.49$ ($df=5$), $p<0.001$) (Figure 4.3b). The highest increase in EROD specific activity was also observed in flounder dosed with 10 μg TCDD/kg body weight. Again, the EROD specific activity was lower (not statistically significant) in flounder exposed to 100 μg TCDD/kg body weight as compared to the 10 μg TCDD/kg body weight exposure group.

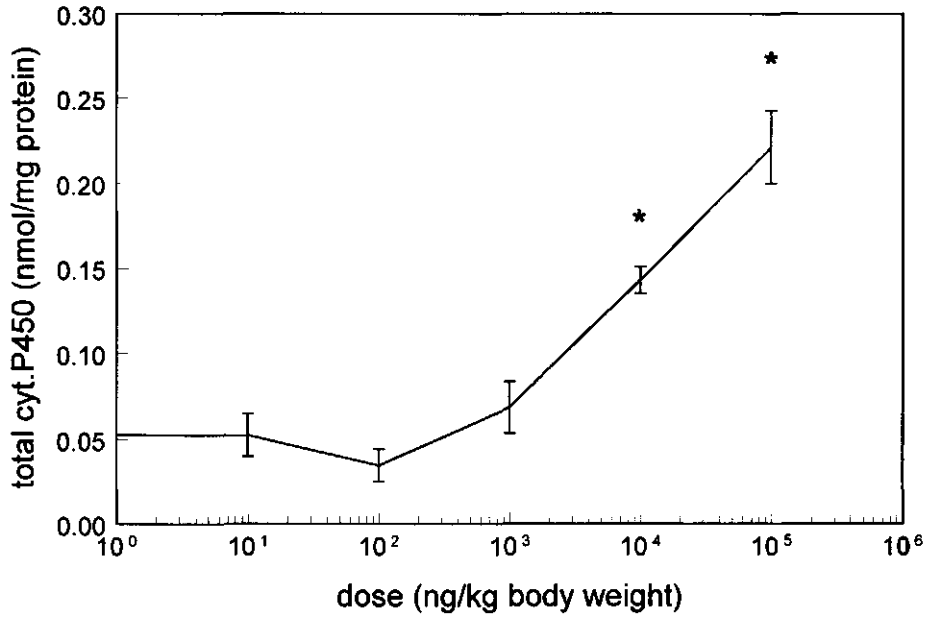


**Figure 4.2.** Total hepatic microsomal cytochrome P450 concentrations in flounder following oral exposure to TCDD. Flounder were sacrificed 10 days after initial exposure. (*) indicates a statistically significant increase ($p<0.001$) from control values. Data ($n=8$) are expressed as means ± SE.

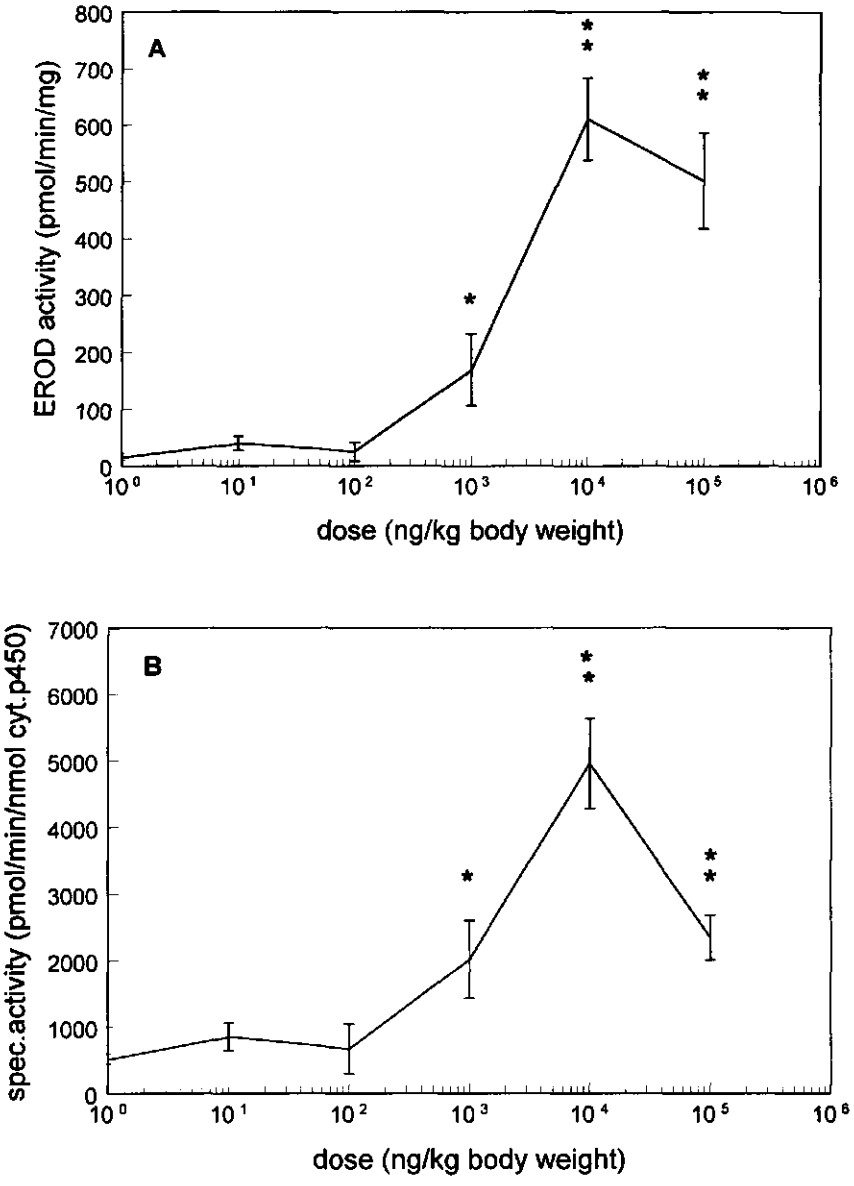


**Figure 4.3.** Hepatic microsomal 7-ethoxyresorufin-O-deethylase (EROD) activity (a) and specific activity (b) in flounder, following oral administration of TCDD. Flounder were sacrificed at day 10 of exposure. (*) indicates a statistically significant increase ($p<0.05$) from control values, (**) indicates a statistically significant difference ($p<0.001$) from control values. Data (n=8) are expressed as means ± SE.

In Figure 4.4a the Calculated No Effect Level (CNEL) is derived from the sigmoidal dose-response relationship of the log total dose and EROD activity after TCDD administration, according to the formula indicated in Material and Methods ($y=32.91+520.10/(1+\exp(-(x-3.21)/0.14))$; $r^2=0.74$; F-statistics=37.02). Using sigmoidal curve fitting, a CNEL of 676.1 ng TCDD/kg body weight was obtained. The $ED_{50}$ for EROD activity induction was calculated to be 1.62 μg TCDD/kg body weight. The same procedure was followed for calculating a CNEL based on the liver TCDD concentration. The dose-response relationship of the log liver TCDD concentration and EROD activity is given in Figure 4.4b ($y=29.50+529.25/(1+\exp(-(x-3.11)/0.21))$; $r^2=0.77$; F-statistics=42.70). Sigmoidal curve fitting resulted in a CNEL of 363.1 pg TCDD/g liver and an $ED_{50}$ of 1.29 ng TCDD/g liver. From Figure 4.4a and 4.4b it is clear that large individual variations of EROD activity were found. In the highest dose group, the EROD activity varied from 109.08 to 938.61 pmol RR/min/mg protein.

*Glutathione-S-transferase activity.* The cytosolic glutathione-*S*-transferase activity was not significantly altered in flounder upon exposure to TCDD. In flounder exposed to cornoil only, the observed GST activity was 0.146 ± 0.03 μmol/min/mg protein. The lowest GST activity was observed in flounder exposed to 0.1 μg TCDD/kg body weight (0.105 ± 0.002 μmol/min/mg protein) whereas the highest dose of 100 μg TCDD/kg body weight resulted in the highest observed GST activity (0.151 ± 0.02 μmol/min/mg protein).

*Plasma and hepatic retinoid levels.* The plasma and hepatic retinoid levels in flounder at day 10 after initiation of exposure are shown in Table 4.2. Plasma retinol levels did not change upon exposure to TCDD. In addition the hepatic retinol and retinyl palmitate concentrations in flounder

**Table 4.2.** Retinoid content in plasma and liver of flounder 10 days after initiation of oral exposure to TCDD.

| Dose TCDD (μg/kg body weight) | plasma retinol (ng/ml plasma) | hepatic retinol (μg/g liver) | hepatic retinyl palmitate (μg/g liver) | hepatic retinol/retinyl palmitate (ratio) |
|---|---|---|---|---|
| 0 (8) | 217.1 ± 23.8 | 24.3 ± 10.6 | 15.3 ± 3.7 | 1.82 ± 0.54 |
| 0.01 (7) | 231.7 ± 24.8 | 18.4 ± 6.0 | 10.0 ± 3.4 | 3.33 ± 1.02 |
| 0.1 (7) | 271.7 ± 37.3 | 18.2 ± 5.4 | 10.2 ± 4.0 | 2.83 ± 0.94 |
| 1 (7) | 202.2 ± 29.8 | 21.5 ± 6.1 | 9.0 ± 3.2 | 2.76 ± 0.59 |
| 10 (8) | 211.9 ± 13.2 | 9.1 ± 2.5 | 15.9 ± 6.1 | 0.96 ± 0.18[a] |
| 100 (8) | 207.9 ± 16.1 | 16.0 ± 5.2 | 19.0 ± 6.2 | 1.46 ± 0.58 |

Flounder were exposed to TCDD on day 1 and 7. Number of animals in parentheses. Data are expressed as mean ± SE.

[a]: significantly different from 0.01 μg TCDD/kg b.w. treatment group, $p<0.05$.

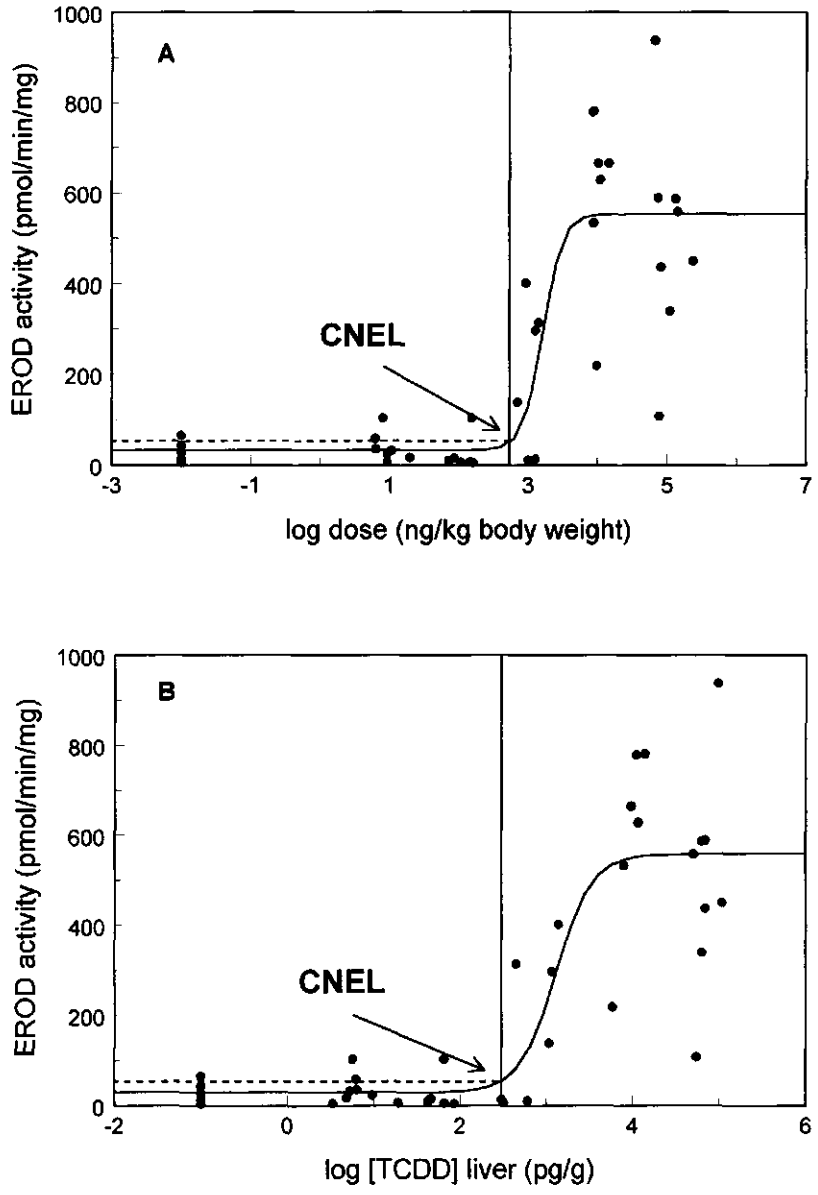


**Figure 4.4.** Sigmoidal dose-response relationship between ethoxyresorufin-*O*-deethylase (EROD) activity and total dose of TCDD/kg body weight administered ($y=32.91+520.10/(1+\exp(-(x-3.21)/0.14))$; $r^2=0.74$; F-statistics=37.02) (a) and hepatic TCDD concentrations ($y=29.50+529.25/(1+\exp(-(x-3.11)/0.21))$; $r^2=0.77$; F-statistics=42.70) (b).

were not altered dose dependently by TCDD, except for a slightly lower but not significant decrease in hepatic retinol concentrations in flounder exposed to 10 μg TCDD/kg body weight. Flounder treated with 10 μg TCDD showed a significant lower hepatic ratio of retinol/retinyl palmitate as compared to flounder treated with 0.01 μg TCDD. However no treatment-related decrease in the hepatic retinol/retinyl palmitate ratio was found.

*Plasma thyroid hormone levels.* Table 4.3 shows the results of the thyroid hormone measurements in plasma of flounder. No dose-dependent changes in either $TT_4$, $TT_3$ or $FT_4$ concentrations were observed. Only total $T_4$ levels in the 100 μg TCDD/kg body weight dosed group decreased significantly ($p<0.05$) compared to the 1 μg TCDD/kg body weight group.

**Discussion**

The major finding of the present study is the observed high inducibility of cytochrome P4501A activity in flounder exposed to TCDD, without changes in retinoid and thyroid hormone levels. The high induction of EROD, the enzyme activity associated with CYP1A, is in agreement with other reports. Fish species such as rainbow trout (*Oncorhynchus mykiss*), mirror carp (*Cyprinus carpio*), or cod (*Gadus morhua*) also have been shown to be sensitive towards TCDD exposure with respect to cytochrome P4501A activity (van der Weiden *et al.*, 1992; van der Weiden *et al.*, 1994; Hektoen *et al.*, 1994).

In the present study a maximum 43 fold (average 27 fold) induction of the hepatic EROD activity was observed in flounder orally exposed to 10 μg TCDD/kg body weight. Other authors report slightly higher cytochrome P4501A activity levels in fish exposed to similar concentrations of TCDD (van der Weiden *et al.*, 1992; Hektoen *et al.*, 1994; Parrot *et*

**Table 4.3.** Thyroid hormone concentrations in plasma of flounder 10 days after initiation of oral exposure to TCDD.

| Dose TCDD (μg/kg body weight) | total $T_4$ (nmol/L) | total $T_3$ (nmol/L) | free $T_4$ (pmol/L) |
|---|---|---|---|
| 0 (8) | 2.59 ± 0.62 | 2.72 ± 0.82 | 5.11 ± 0.55 |
| 0.01 (6) | 2.60 ± 0.68 | 2.90 ± 0.87 | 4.06 ± 0.77 |
| 0.1 (7) | 2.37 ± 0.69 | 1.91 ± 0.99 | 5.33 ± 1.08 |
| 1 (7) | 3.83 ± 0.84 | 1.81 ± 0.70 | 6.85 ± 1.27 |
| 10 (7) | 3.21 ± 0.64 | 1.75 ± 0.45 | 6.00 ± 1.12 |
| 100 (8) | 1.87 ± 0.55[a] | 2.26 ± 0.66 | 4.44 ± 1.01 |

Number of animals in parentheses. Data are expressed as mean ± SE.
[a]: significantly different from 1 μg TCDD/kg b.w. treatment group, $p<0.05$.

*al.*, 1995). In contrast to TCDD exposure, we showed that flounder is not sensitive towards Clophen A50 with respect to CYP1A induction (Besselink *et al.*, 1996). A similar low responsiveness towards PCB exposure was observed in cod (Bernhoft *et al.*, 1994). Preliminary results from our laboratory indicate that *in vitro* the microsomal EROD activity is inhibited by the presence of PCB congeners in the reaction mixture (data not shown). Hence, this could account for the observed low inducibility of CYP1A by PCBs.

The EROD activity and EROD specific activity decreased at the highest dose TCDD administered. Similar observations have been found in a number of species and systems, including fish, mammals, birds and cultured cells at high concentrations of halogenated biphenyls (Hahn *et al.*, 1993; Sawyer *et al.*, 1984; Gooch *et al.*, 1989). This phenomenon was attributed to either cytotoxicity, a decrease in the amount of CYP1A protein, or direct inhibition of CYP1A activity by residual PHAHs present in microsomes (Hahn *et al.*, 1993). At least the first two explanations do not seem likely in our situation since a decrease in EROD activity at the highest concentration of TCDD administered occurred in the presence of high levels of total cytochrome P450.

The relative sensitivity of flounder towards TCDD can be established by comparing the flounder CNEL for EROD activity to Lowest Observed Adverse Effect Levels (LOAELs) from other fish species and rat. From the sigmoidal dose-response relationship as found in our study, the CNEL for flounder towards TCDD was calculated to be 0.68 μg TCDD/kg body weight (0.36 ng TCDD/g liver). In the studies of van der Weiden *et al.* (1992, 1994) LOAELs for rainbow trout and carp of 0.3 and 0.03 μg TCDD/kg body weight respectively, were reported for EROD activity. The LOAEL in rats were observed to be as low as 0.002 μg TCDD/kg body weight for hepatic AHH induction (Goldstein & Safe, 1989). Clearly flounder is sensitive towards TCDD exposure but not as sensitive as rainbow trout, carp, or rat. A similar conclusion can be drawn when the flounder $ED_{50}$ for EROD activity towards TCDD exposure (1.61 μg/kg body weight, 1.29 ng TCDD/g liver) is compared to $ED_{50}$ values for EROD activity reported in other fish species and rat, e.g. 0.91 μg TCDD/kg body weight in rainbow trout (Newsted *et al.*, 1995), 0.048 μg TCDD/kg body weight in carp (van der Weiden *et al.*, 1994), and 0.97 μg TCDD/kg body weight in Wistar rat (Safe, 1994). A more accurate comparison between the relative sensitivity of flounder towards TCDD exposure and the sensitivity of other fish species can be obtained when actual liver concentrations of TCDD are used. Unfortunately, not much data is available on hepatic TCDD concentrations in other fish species.

In the same treatment groups, large individual differences in response towards TCDD exposure were observed in flounder. It could be argued that these large individual differences are a result of individual variations in toxicokinetics. However, we clearly demonstrated that the dosing procedure used, resulted in comparable amounts of TCDD in livers of flounder. In fact, a good correlation between the administered dose and the hepatic TCDD concentration

was found, based on the amount of [$^3$H] radioactivity retained in the liver. An alternative explanation for the observed large individual differences in CYP1A induction, is genetic polymorphism in the flounder population used in this study.

In mammals it was postulated that the Ah receptor not only regulates the transcription of phase I enzymes but also phase II enzymes (Nebert & Gonzales, 1987; Rushmore & Pickett, 1990). The AhR mediated responses elicited by PHAHs include induction of GST and the alteration of thyroid hormone and retinoid levels (Safe, 1994). In the present study a lack of retinoid and thyroid hormone depletion and an absence of GST induction were observed. This is in contrast to results in other fish species and mammals (Hektoen *et al.*, 1994; Leatherland & Sonstegard, 1978; Palace & Brown, 1994). While Boon *et al.* (1992) observed an increase in GST activity in plaice (*Pleuronectes platessa*), Hektoen *et al.* (1994) reported a significant decrease of GST activity in rainbow trout and no changes in cod. The response of GST activity in fish to xenobiotics thus seems to be ambiguous. In addition a later onset of GST induction as compared to induction of cytochrome P450 enzymes was observed by Andersson *et al.* (1985). Thus the GST activity and cytochrome P4501A activity seems to be regulated differently in fish.

With respect to retinoid and thyroid hormone homeostasis, no alterations were observed in the present study. It is has been shown that PHAHs can alter retinoid (Brouwer *et al.*, 1989a), and thyroid hormone (Morse *et al.*, 1993; Brouwer, 1989) homeostasis in mammals either by modulation of retinoid and thyroid hormone metabolising enzymes (Morse *et al.*, 1993; Jensen *et al.*, 1987) or interference of hydroxy PCB metabolites with the plasma transport of retinol and thyroid hormone (Lans *et al.*, 1993; Brouwer & van den Berg, 1984). The latter mechanism was put forward by Palace & Brown (1994) as a possible explanation for observed retinoid alterations in PCB exposed lake char (*Salvelinus namaycush*). But since transthyretin is not involved in plasma thyroid hormone or retinoid transport in fish (Babin, 1992; Berni *et al.*, 1992), this is not likely to occur in fish. Alternatively, it could be argued that the time of exposure in the present study is too short to observe more pronounced alterations in both retinoid and thyroid hormone levels in fish. In a 3-year mesocosm study flounder were kept under similar conditions either on polluted harbour sludge or unpolluted sediment (Vethaak, 1993). In this study we observed decreased levels of plasma retinol, hepatic retinol, and the ratio hepatic retinol/retinyl palmitate in flounder kept on the polluted sludge as compared to flounder kept on unpolluted sediment (Besselink *et al.*, 1997c).

In conclusion, our study showed that flounder is an Ah responsive species with respect to CYP1A induction but not with respect to modulation of GST activity and alterations in retinoid and thyroid homeostasis. The sensitivity of flounder towards TCDD exposure is slightly lower as compared to other fish species, based on the nominal doses given. In addition, it can be concluded

that oral exposure of flounder using gelatine capsules results in reproducible data as far as the parameters measured in the present study are concerned.

**Acknowledgement**

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# Chapter 5
# Inhibitory Effects of Polychlorinated Biphenyls (PCBs) on 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) Induced Cytochrome P450 1A and Glutathione S-transferase Activities in Flounder (*Platichthys flesus*)

**Abstract**

In the present study flounder (*Platichthys flesus*) were orally treated twice within 7 days with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), Clophen A50, 3,3',4,4',5-pentachlorobiphenyl (CB-126) or combinations of TCDD with either Clophen A50 or CB-126. The total doses administered were equipotent when expressed as TCDD toxic equivalents, using mammalian derived TEF values (0.005 mg TCDD TEQ/kg body weight for TCDD, Clophen A50 and CB-126 and 0.01 mg TCDD TEQ/kg body weight for both combination groups). Ten days after initial exposure, flounder were sacrificed and the effects on hepatic microsomal CYP1A content, 7-ethoxyresorufin-*O*-deethylase (EROD) activity and glutathione-*S*-transferase activity were examined. EROD activity was increased 35.3-, 2.4-, and 13.2-fold in the TCDD, Clophen A50, and CB-126 dosed flounder respectively as compared to cornoil treated flounder. In the combination groups, hepatic EROD activity increased slightly in the TCDD/CB-126 dosed group, whereas a 2.7-fold reduction was found in TCDD/Clophen A50 dosed flounder as compared to TCDD dosed fish. Hepatic CYP1A content was significantly elevated in both combination groups as compared to control levels. In addition, significantly higher CYP1A levels were found in TCDD/Clophen A50 exposed fish as compared to Clophen A50 dosed flounder. The CYP1A levels in the combination groups appeared to be increased in an additive way, as compared to the single compound exposed groups. Total glutathione-*S*-transferase activity was significantly reduced from control levels in both combination groups and the CB-126 treated group. These results show that PCBs inhibits the effect of TCDD on hepatic EROD induction while additive effects are observed for CYP1A protein. In addition, these data indicate a selective inhibition of GST activity by PCBs in the combined exposure situation as it occurs in the wild.

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*Environ. Toxicol. Pharmacol.*, submitted

**Table 5.1.** Exposure groups of flounder, which were dosed orally twice at day 1 and 7 to the indicated doses of PHAHs.

| Dose | Exposure group | | | | | |
|---|---|---|---|---|---|---|
| | control | TCDD | Clophen A50 | CB-126 | TCDD + Clophen A50 | TCDD + CB-126 |
| mg PHAH/kg b.w. | - | 0.005 | 42 | 0.05 | 0.005 + 42 | 0.005 + 0.05 |
| mg TCDD TEQ/kg b.w. | - | 0.005 | 0.005 | 0.005 | 0.01 | 0.01 |

Each exposure group contained 14 flounder at day 1 of the experiment. Composition of Clophen A50 was based on Schultz *et al.* (1989). TEQ values were calculated based on TEF values as reported by Safe (1994).

*Treatment of fish.* After 4 weeks of acclimatisation, flounder were divided into 6 groups of 13 animals each and were transferred to sediment containing 225 $dm^3$ glass tanks. At day 1 and 7 of the experiment gelatine capsules filled with 200 µl of a solution containing either TCDD, CloA50, CB-126, a combination of TCDD and CloA50, or a combination of TCDD and CB-126 dissolved in cornoil were orally injected into the stomach of fish. One group was injected with 200 µl corn oil only (control). The gelatine capsules were administered as described by Boon *et al.* (1992). The concentrations of the different polyhalogenated aromatic hydrocarbons (PHAHs) administered are given in table 1.

*Dissection of fish.* At day 21 of the experiment, fish were captured by netting and stunned by a blow to the back of the head. Body weight and length were determined and after collecting blood from the caudal vein with a heparinized syringe, flounder were killed by cervical transection. Livers were carefully dissected free from the gall bladder, washed in ice-cold 0.9% NaCl, weighed and stored at -80°C until preparation of homogenates and microsomes.

*Preparation of homogenates and microsomes.* Individual livers were homogenised in 2.5 ml 50 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose, using 10 strokes with a motor-driven Potter-Elvehjem glass and teflon homogenizer. The hepatic homogenates were centrifuged at 9,000 g for 30 min. The resulting supernatants were centrifuged at 100,000 g for 90 minutes. The pellets was resuspended in a 0.1 M Tris-HCl buffer (pH 7.6) containing 20% (v/v) glycerol, 1 mM EDTA and 1 mM DTT, and 80°C until analysis. All handlings were performed at 0-4°C.

*Protein assays.* Protein was measured by the Bio-Rad assay system according to the specifications of the supplier, using crystalline bovine serum albumin as standard.

*Cytochrome P450 assays.* Total cytochrome P450 content in the microsomal fraction of flounder liver was determined using a dithionite difference spectrum based on the method described by Omura & Sato (1964). Hepatic CYP1A protein content (3 samples per group) and standards were analysed by denaturing gel electrophoresis on a 6-15% acrylamide gradient gel basically as described earlier (Hahn *et al.*, 1993; Kloepper-Sams, 1987). Scup P450E was used as a reference, which was originally purified from liver of the marine fish scup (*Stenotomus chrysops*) by Klotz *et al.* (1983) and has now been confirmed as a CYP1A by cDNA cloning and sequencing (Morrison *et al.*, 1995). The primary antibody used was MAb 1-12-3 (Park *et al.*, 1986) raised against scup CYP1A. MAb 1-12-3 was kindly provided by J.J. Stegeman (WHOI) and H.V. Gelboin (NCI). After denaturing gel electrophoresis, proteins were transferred onto nylon membranes nitrocellulose and incubated with MAb 1-12-3 (10 µg/ml). As a second antibody alkaline phosphatase conjugated goat anti-mouse IgG (Schleicher and Schuell; 1/5,000 dilution) was used. After visualising flounder CYP1A and standards on Kodak X-AR film using enhanced chemiluminescence (Schleicher and Schuell Rad-Free Chemiluminescence Detection Kit), bands were digitised (Kodak DCS200 digital camera; Adobe Photoshop) and quantified by video imaging densitometry, using NIH Image software. Microsomal 7-ethoxyresorufin-O-deethylation (EROD) activity was measured in hepatic microsomes according to the method of Prough *et al.* (1978). The assay was adapted for use in 96 well plates and a fluorospectrophotometric plate reader (Cytofluor 2350, Fluorescence Measurement System, Millipore, USA) as described by Besselink *et al.* (1997b).

*Glutathione-S-transferase activity.* Cytosolic glutathione-*S*-transferase activity (GST) activity was measured in hepatic cytosol according to the method of Habig *et al.* (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The assay was adapted for use in 96 well plates and a spectrophotometric plate reader (Thermo-max microplate reader, Molecular Devices) and performed as described by Besselink *et al.* (1997b).

*Data treatment and statistics.* Statistical analysis was performed by using the software packages SPSS/PC+$^{TM}$ (SPSS Inc., Chicago, IL, USA) and TOXSTAT 3.2 (University of Wyoming, Laramie, WY, USA). Data were tested for normality and homogeneity of variance using the Chi-square test and the Bartletts test. Data that passed both tests were subjected to one-way analysis of variance followed by a least significant difference test to evaluate significant differences between treatment groups. Data that did not pass the Chi-square test or Bartletts test were analysed for differences from the control using the Kruskall-Wallis test. Data are expressed as the means ± standard error of the mean (S.E.M.).

**Table 5.2.** Body weight, condition factor and relative liver weight of flounder at 21 days after initiation of oral exposure to either corn oil, TCDD, Clophen A50, CB-126, TCDD in combination with Clophen A50 or TCDD in combination with CB-126.

| | | Exposure group | | | | | |
|---|---|---|---|---|---|---|---|
| | (n) | control | TCDD | Clophen A50 | CB-126 | TCDD + Clophen A50 | TCDD + CB-126 |
| Body Weight (g) | day 0 (14) | 66.2 ± 1.6 | 62.6 ± 1.3 | 72.4 ± 3.1 | 72.1 ± 3.5 | 69.0 ± 2.9 | 66.5 ± 2.0 |
| | day 21 (14) | 63.5 ± 1.6 | 59.5 ± 1.5 | 68.7 ± 3.0 | 69.5 ± 3.7 | 66.2 ±2.7 | 64.2 ± 2.1 |
| Condition Factor ($g/cm^3$)[a] | day 0 (14) | 0.97 ± 0.02 | 0.98 ± 0.02 | 1.00 ± 0.02 | 0.97 ± 0.03 | 0.96 ± 0.02 | 0.99 ± 0.02 |
| | day 21 (14) | 0.95 ± 0.02 | 0.95 ±0.02 | 0.96 ± 0.02 | 0.94 ± 0.02 | 0.93 ± 0.02 | 0.96 ±0.01 |
| Liver (% body weight) | day 21 (9) | 0.85 ±0.07 | 0.77 ± 0.04 | 0.84 ± 0.05 | 0.91 ± 0.06 | 0.90 ± 0.04 | 0.88 ± 0.07 |

Flounder were dosed at day 1 and 7. Number of animals in parentheses. Data are expressed as mean ± S.E.M..
[a]: condition factor: ((body weight)/(body length)$^3$)*100.

## Results

*Body weights, condition factors and relative liver weights.* The effects of PHAH exposure on body weight, condition factor and relative liver weight are given in table 2. Overall no effect was found on body weight following oral exposure of flounder to either TCDD, CloA50, CB-126, a combination of TCDD and CloA50 or, TCDD and CB-126 as compared to control values. A slight but not significant decrease in body weight was observed between day 0 and day 21 of exposure for all treatment groups. Condition factors were not altered as well upon exposure to the various PHAHs as compared to control fish. In addition, slightly lower condition factors were observed at day 21 of exposure as compared to day 0 of exposure. Administration of either one of the PHAHs or combinations of PHAHs used in the present experiment did not result in significantly altered relative liver weights in flounder as compared to control levels.

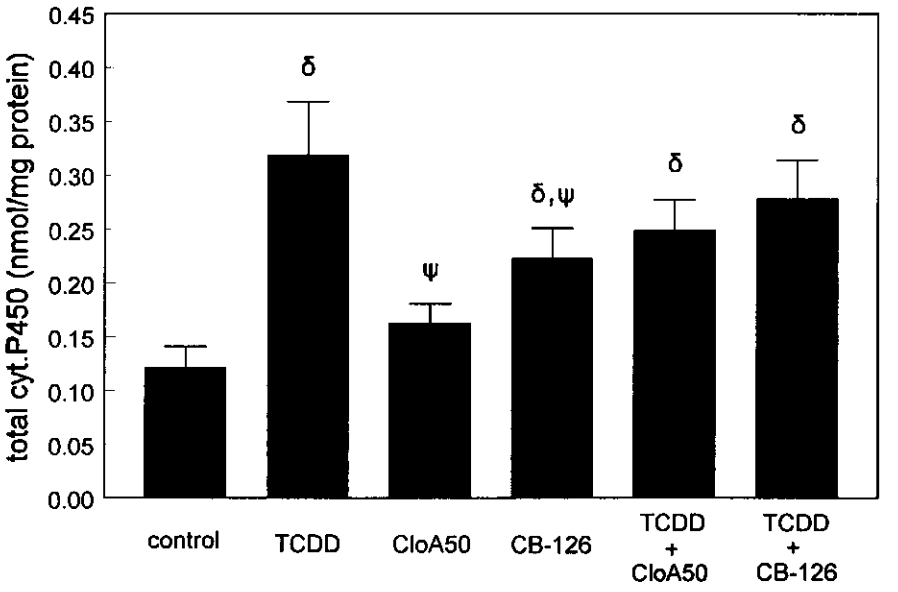


**Figure 5.1.** Total hepatic microsomal cytochrome P450 concentrations (nmol/mg protein) in flounder, following oral exposure to indicated PHAHs. Doses of the various PHAHs are given in Table 1. Flounder were dosed twice at day 1 and 7 and sacrificed at day 21 after initial exposure. (σ) indicates a statistically significant increase ($p<0.05$) from control values, (ψ) indicates a statistically significant difference ($p<0.05$) from the TCDD exposure group. Data (n=13) are expressed as means ± S.E.M..

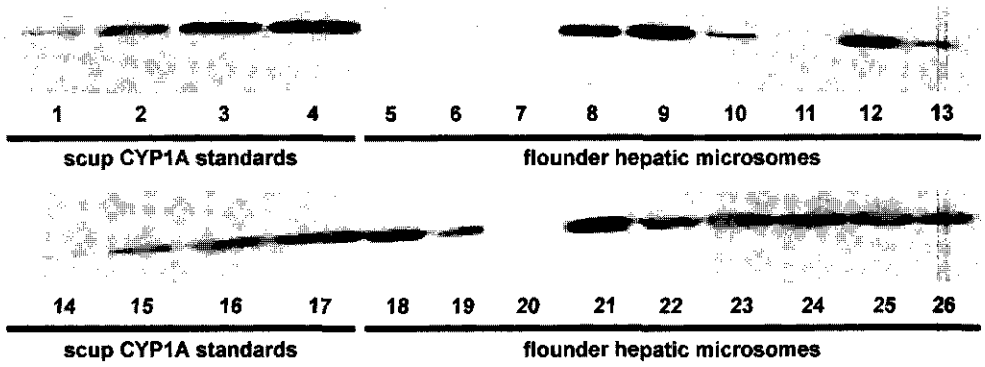


**Figure 5.2a.** Immunoblot of flounder hepatic microsomes using MAb 1-12-3. Hepatic microsomal proteins from individual flounder from the various exposure groups are given in the individual lanes: corn oil (lanes 5-7), TCDD (lanes 8-10), Clophen A50 (lanes11-13), CB-126 (lanes 18-20), TCDD/Clophen A50 (lanes 21-23) or TCDD/CB-126 (lanes 24-26). Four micrograms of microsomal protein per well was loaded on gel. Lanes 1-4 and 14-17 containe purified scup CYP1A1 at 0.1, 0.2, 0.5 and 1.0 pmol per lane.

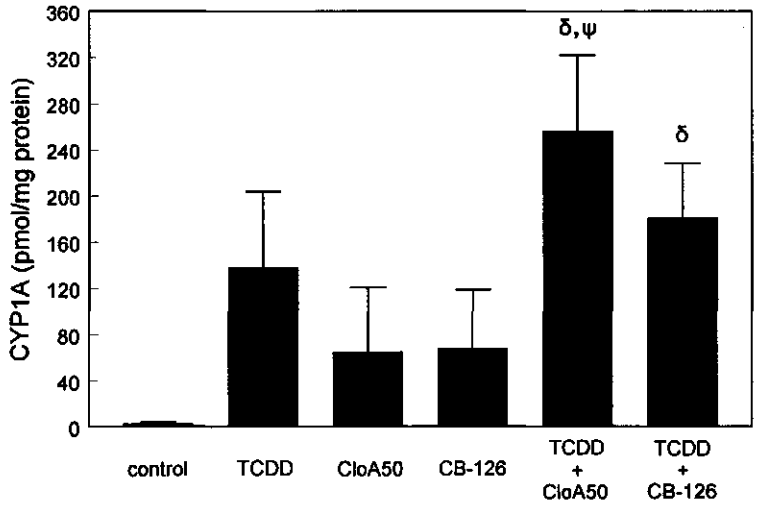


**Figure 5.2b.** Hepatic CYP1A protein concentrations (pmol/mg protein; CYP1A concentrations are expressed as pmol scup CYP1A-equivalents) in flounder, following oral exposure to the indicated PHAHs. Flounder were dosed twice at day 1 and 7 and sacrificed at day 21 after initial exposure. ($\sigma$) indicates a statistically significant increase ($p<0.05$) from control values, ($\psi$) indicates a statistically significant difference ($p<0.05$) from the CloA50 exposure group. Hepatic CYP1A protein concentration are the means ± S.E.M. of the in Fig 2A shown samples (n=3).

*Cytochrome P450 induction.* Total hepatic cytochrome P450 levels were increased significantly in all treatment groups as compared to control levels, except for flounder exposed to CloA50 (fig. 1). Largest increases in total cytochrome P450 levels were observed in flounder treated with TCDD, or TCDD in combination with CB-126 (2.6 and 2.3 fold respectively). Both combination groups(TCDD/CloA50 and TCDD/CB-126) showed slightly lower total hepatic cytochrome P450 levels as compared to the TCDD only dosed group, despite the fact that both combination groups received twice as much TCDD-TEQs. Flounder exposed to CloA50, or CB-126 doses equipotent to TCDD also showed significantly lower cytochrome P450 levels as compared to TCDD treated flounder.

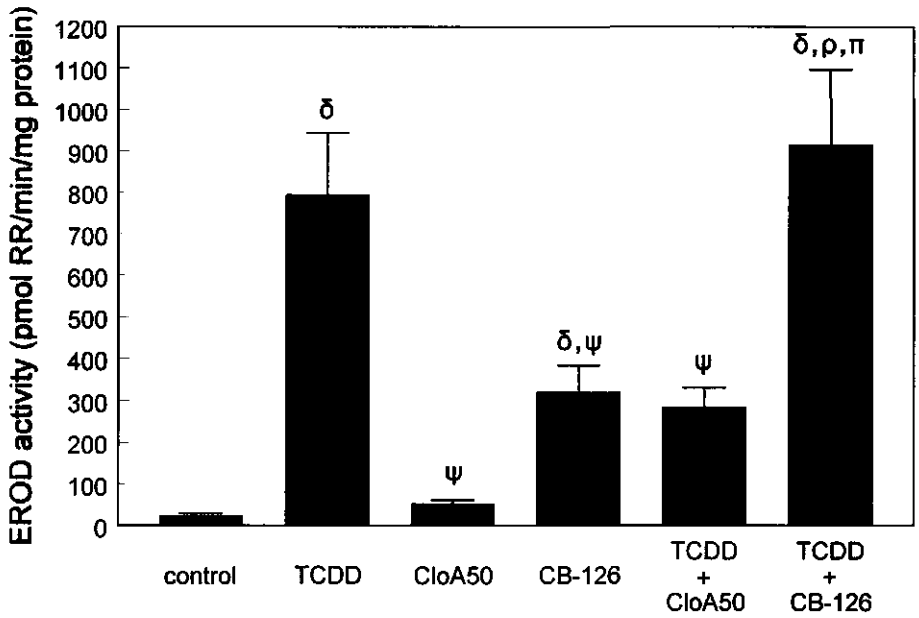


**Figure 5.3.** Hepatic microsomal 7-ethoxyresorufin-O-deethylase (EROD) activity (pmol RR/min/mg protein). in flounder liver, following oral exposure to PHAHs. Doses of the various PHAHs are given in Table 1. Flounder were dosed twice at day 1 and 7 and sacrificed at day 21 after initial exposure. (σ) indicates a statistically significant increase ($p<0.05$) from control values, (ψ) indicates a statistically significant difference ($p<0.01$) from the TCDD exposure group, (ρ) indicates a statistically significant difference ($p<0.01$) from the CB-126 exposure group, and (π) indicates a statistically significant difference ($p<0.01$) from the TCDD/CloA50 exposure group. Data (n=13) are expressed as means ± S.E.M..

**Table 5.3.** EROD activity, EROD activity/total cytochrome P450, and EROD activity/CYP1A in flounder hepatic microsomes 21 days after initiation of oral exposure to either corn oil, TCDD, Clophen A50, CB-126, TCDD in combination with Clophen A50 or TCDD in combination with CB-126.

| | | Exposure group | | | | | |
|---|---|---|---|---|---|---|---|
| | n | control | TCDD | Clophen A50 | CB-126 | TCDD + Clophen A50 | TCDD + CB-126 |
| EROD activity (pmol/min/mg protein) | 13 | 22.5 ± 5.9 | 794.0 ± 150.2$^{\sigma}$ | 53.3 ± 8.2$^{\Psi}$ | 296.1 ± 66.4$^{\sigma,\Psi}$ | 284.1 ± 45.7$^{\Psi}$ | 916.0 ± 181.0$^{\sigma,\rho,\pi}$ |
| EROD/tot. cyt.P450 (pmol/min/pmol cyt.P450) | 13 | 0.2 ± 0.1 | 2.2 ± 0.2$^{\sigma}$ | 0.4 ± 0.1$^{\Psi}$ | 1.5 ± 0.2$^{\sigma,\Psi}$ | 1.0 ± 0.1$^{\sigma,\Psi,\tau}$ | 2.7 ± 0.4$^{\sigma,\rho,\pi}$ |
| EROD/CYP1A (pmol/min/pmol CYP1A) | 3 | 12.7 ± 2.0 | 8.9 ± 2.4 | 9.1 ± 0.5 | 14.0 ± 0.9 | 1.5 ± 0.1$^{\sigma,\Psi,\tau}$ | 10.0 ± 1.2 |

Flounder were dosed at day 1 and 7. n = number of animals. Data are expressed as mean ± SE.M..

$^{\sigma}$: significant different from control group, p<0.05.

$^{\Psi}$: significant different from TCDD exposure group, p<0.05.

$^{\tau}$: significant different from CloA50 exposure group, p<0.05.

$^{\rho}$: significant different from CB-126 exposure group, p<0.05.

$^{\pi}$: significant different from TCDD/CloA50 exposure group, p<0.05.

Liver microsomes of the various treatment groups were loaded on a denaturating gel and separated by electrophoresis. The separated proteins were blotted onto a nitrocellulose filter and incubated with anti-CYP1A antibody (MAb 1-12-3) and a second antibody linked to alkaline phosphatase. Fig. 2A shows the western blots of the various treatment groups. One band was visible which was co-localized to purified scup CYP1A, which was run as a reference in lines 1-4 and 14-17. The staining of the bands was quantified by image analysis and in fig. 2B the relative CYP1A content of the microsomal fractions is given for the various dose groups.

As obvious from the bands, there are large inter-individual differences in the amount of CYP1A protein induced by PHAHs within the treatment groups. For example, TCDD exposed flounder, lanes 8, 9 and 10, showed two (lane 8 and 9) highly induced and one (lane 10) hardly induced individuals. This is also true for CloA50 (lanes 11-13), and CB-126 (lanes 18-20). Surprisingly, in the combined exposure groups there appears to be less individual variation. This

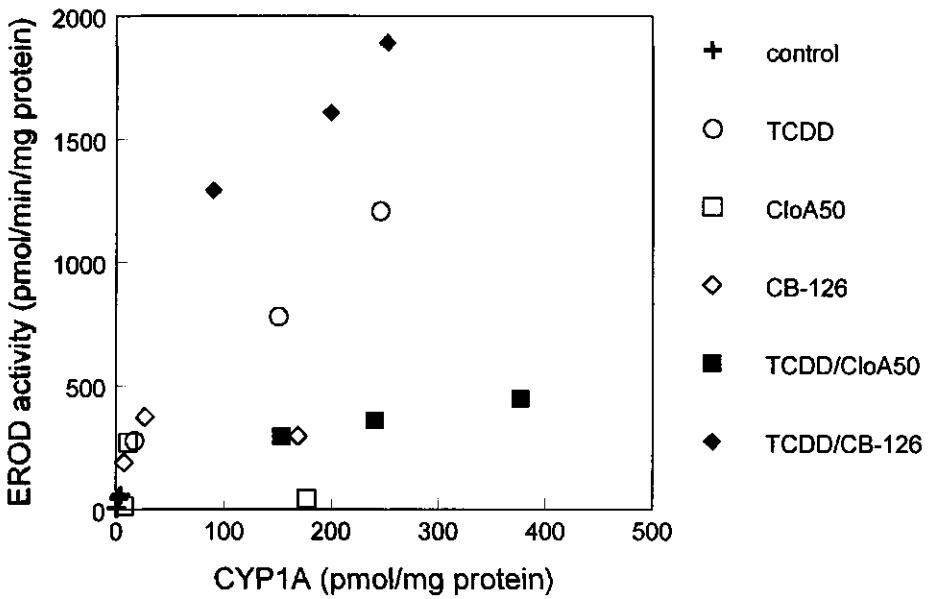


**Figure 5.4.** Relationships between hepatic microsomal relative CYP1A content (pmol/mg protein) and hepatic microsomal EROD activity (pmol/min/mg protein) in flounder following oral exposure to PHAHs. Doses of the various PHAHs are given in Table 1. Flounder were dosed twice at day 1 and 7 and sacrificed at day 21 after initial exposure.

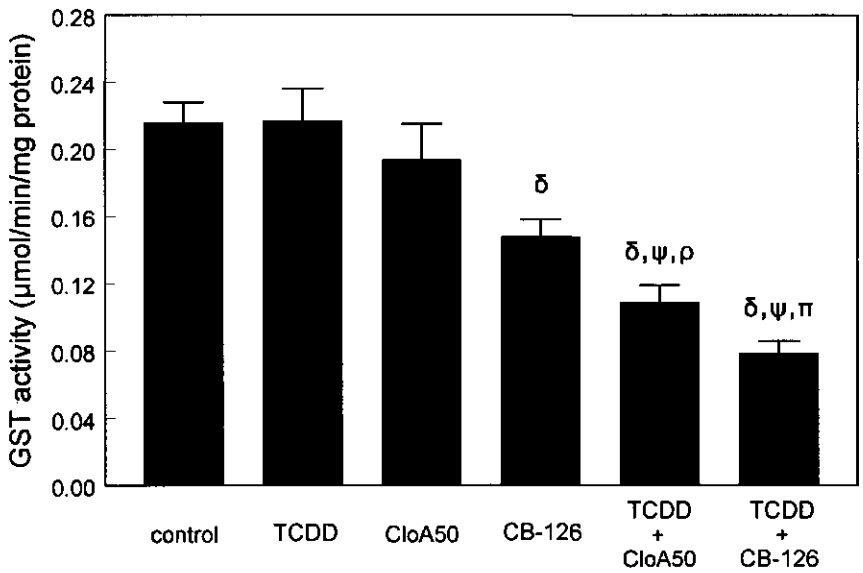


**Figure 5.5.** Hepatic cytosolic glutathione-S-transferase (GST) activity (µmol/min/mg), as measured using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate in flounder following oral exposure to the indicated PHAHs. Doses of the various PHAHs are given in Table 1. Flounder were dosed twice at day 1 and 7 and sacrificed at day 21 after initial exposure. ($\sigma$) indicates a statistically significant decrease ($p<0.01$) from control values, ($\psi$) indicates a statistically significant difference ($p<0.01$) from the TCDD exposure group, ($\rho$) indicates a statistically significant difference ($p<0.01$) from the CloA50 exposure group, and ($\pi$) indicates a statistically significant difference ($p<0.01$) from the CB-126 exposure group. Data ($n=13$) are expressed as means ± S.E.M..

large individual variation within exposure groups is the cause of the low statistical difference between controls and exposure groups, i.e. only in the combined exposure groups the levels of CYP1A protein were significantly different from control levels. Significant differences in CYP1A levels among treatment groups were only observed between TCDD/CloA50 and CloA50 and CB-126 treated fish (Fig 2B). A positive correlation was observed between total cytochrome P450 levels and cytochrome P450 1A concentrations in flounder livers ($y = -84.77 + 746.52*x$; $r^2 = 0.71$; F-statistics = 29.82) (data not shown).

Hepatic EROD activity was significantly increased only in the TCDD, CB-126 and TCDD/CB-126 exposed groups as compared to the control group (Fig. 3). In contrast to total cytochrome P450 (Fig. 1) and CYP1A levels (Fig 2B), the hepatic EROD activity level was not

elevated significantly in the TCDD/CloA50 dosed group as compared to control levels (Fig. 3). In addition, administration of TCDD in combination with CloA50 (total dose: 0.01 mg TCDD-TEQ/kg body weight) brought about a significantly lower EROD activity than administration of TCDD only (0.005 mg TCDD-TEQ/kg body weight). Finally, a significant difference in EROD activity was also observed between TCDD/CloA50 and TCDD/CB-126 treated group, although they both received an equipotent dose of 0.01 mg TCDD-TEQs/kg body weight.

In table 3 the EROD activity (pmol/min/mg protein) as well as the specific activity (EROD activity per pmol cytochrome P450) and turnover number (EROD per pmol CYP1A) are given. With respect to the specific activity, all treatment groups showed higher activity compared to the control group, except for the CloA50 exposure group. The specific activity in both the CloA50 and CB-126 exposure groups was significantly lower than in the TCDD dosed group, whereas only CloA50 dosed flounder showed a significant lower activity in combination with TCDD as compared to TCDD exposed fish, despite the fact that the concentration of TCDD-TEQs in the latter group was half of that administered to the combined group. In contrast, combination of TCDD and CB-126 seemed to be additive. With respect to the turnover number, only the combination group TCDD/CloA50 appeared to be significant decreased as compared to the control, TCDD, and CloA50 exposure groups.

When the relationship between hepatic CYP1A content and hepatic EROD activity was studied (fig. 4), a higher EROD activity with increasing CYP1A content was observed in the TCDD and TCDD/CB-126 exposed groups. Such a relationship was also found in the TCDD/CloA50 exposed group though the slope was much lower when compared to the slopes observed with the TCDD and TCDD/CB-126 exposed groups. In contrast, an initial increase followed by a reduction of EROD activity with increasing CYP1A levels in flounder hepatic microsomes was observed in the CloA50 and CB-126 dosed fish.

*Glutathione-S-transferase activity.* The cytosolic glutathione-*S*-transferase activity appeared to be significantly reduced in flounder exposed to CB-126 and in particular in the combined exposure groups: TCDD/CloA50 and TCDD/CB-126 (fig. 5). Reductions in GST activity up to 29.2%, 47.8%, and 62.2% were found respectively in CB-126, TCDD/CloA50, and TCDD/CB-126 exposed groups as compared to controls and TCDD, or CloA50 treated groups. No reduction in GST activity was observed when flounder were exposed to TCDD, or CloA50 alone.

**Discussion**

The data presented in this paper demonstrate that TCDD, as well as CB-126 and their combinations, are capable of hepatic EROD induction in flounder. However, there may be large differences in outcome of results, when comparing EROD activity and CYP1A specific protein levels in liver microsomes of PHAH administered flounder. The differences are particularly

striking when the results for TCDD and CloA50 alone or the combined TCDD/CloA50 exposure are compared. CloA50 alone hardly shows any EROD induction and significantly inhibited the TCDD induced EROD activity in the combined exposure group. However, when data are expressed as CYP1A protein, no antagonism of TCDD induced CYP1A protein is observed, but instead there is an additive response. These data indicate that EROD activity may be a poor indicator of PHAH exposure in complex exposure situations such as occur in the wild. CYP1A on the other hand, seemed to be a much more reliable indicator of the total exposure to PHAHs.

Induction of hepatic EROD activity as a result of TCDD exposure has been reported in many fish species (Besselink *et al.*, 1997b; van der Weiden *et al.*, 1994; Hektoen *et al.*, 1994). In contrast, induction of EROD activity by PCBs seemed to be dependent on the PCB congener administered and on the fish species studied. A study by Gooch *et al.* (1989) showed that the non-ortho-substituted 3,3',4,4'-tetrachlorobiphenyl was an inducer of hepatic EROD activity in scup (*Stenotomus chrysops*) whereas administration of ortho-substituted PCB congeners failed as inducers of EROD activity in the same fish species. Elevated hepatic EROD activities upon administration of CloA50 were reported in rainbow trout by Andersson *et al.* (1985) and Celander & Förlin (1995). In flounder, we observed only a minor 2-fold induction of hepatic EROD-activity and a maximum 4-fold increase in EROD activity upon administration of 20 and 100 mg CloA50/kg body weight respectively (Besselink *et al.*, 1996). Our present study indicates that part of this discrepancy has to do with inhibitory interactive effects of PCB mixtures with TCDD and related Ah receptor agonists. Also, from mammalian studies it is known that the biochemical response of mixtures of PHAHs can be different from the sum of the individual PHAHs because of interactions among the individual compounds in the mixture. For example, synergistic effects on hepatic monooxygenase activities were observed in mice after co-treatment with 2,2',4,4',5,5'-hexachlorobiphenyl (CB-153) and TCDD (Bannister & Safe, 1987). In contrast, antagonistic effects on aryl hydroxylase activity were found in mice co-treated with Aroclor 1254, or CB-153 and TCDD (Bannister *et al.*, 1987).

To evaluate the potency of the PCB mixture CloA50 and the most potent PCB congener CB-126 to induce cytochrome P450 1A enzyme system with TCDD, flounder were orally exposed to PHAH doses (0.005 mg TCDD TEQ/kg body weight) that should result in equipotent induction of CYP1A activity. The TEF values used to calculate TEQs for CloA50 and CB-126 were based on mammalian TEF values as reported by Safe (1994). CB-126 induced hepatic EROD activity but not to the same extent as TCDD whereas a slight but not significant induction of EROD activity was observed in flounder dosed with CloA50. Furthermore, CYP1A and total cytochrome P450 content in flounder livers were higher in TCDD exposed fish than in equipotently dosed animals with CloA50 or CB-126. Hence, this suggests that using mammalian derived TEF values the toxic response of both the PCB mixture and CB-126 are overestimated in flounder based on hepatic EROD activities and CYP1A protein levels. In an attempt to compare trout derived TEFs

with mammalian derived TEFs, Newsted *et al.* (1995) reported for the PCB congeners CB-77, CB-105, CB-118, CB-126 and CB-169 trout EROD-TEF values (calculated from the ED50s) that were 0.54-, 0.05-, 0.6-, 1.3- and 0.18-fold the mammalian derived TEFs (Safe, 1990) respectively. Therefore, mammalian TEFs for CB-77, CB-105, CB-118 and CB-169 would overestimate the potency of these PCBs in trout, whereas the mammalian derived TEF value for CB-126 would slightly underestimate its potency in trout. In contrast, our study shows that the use of the mammalian derived TEF value for CB-126 overestimates its potency to induce EROD activity in flounder. This is in agreement with Walker & Peterson (1991) and Janz & Metcalfe (1991) who both reported a rainbow trout derived TEF value for CB-126 based on either early-life-stage mortality or AHH activity, that was 0.05-fold lower than the mammalian derived TEF value for CB-126.

Decreased EROD activity have sometimes been attributed to either cytotoxicity or a reduction of CYP1A protein synthesis. In our experiment, both explanations for the lower then expected EROD activity in combined exposure experiments seem unlikely since no indications for cytotoxicity were observed (no effects on condition factor or liver somatic index) and additive concentrations of CYP1A were observed in co-treatment with TCDD/CloA50 compared to CloA50 and TCDD only dosed flounder. A more plausible hypothesis is a direct inhibition of CYP1A catalytic activity by residual PCBs present in the microsomal suspension (Hahn *et al.*, 1993; Gooch *et al.*, 1989). In the present study this hypothesis is supported by the observation that the turnover number is reduced dramatically in the TCDD/CloA50 combination group as compared to the TCDD and CloA50 exposure groups. In addition, we observed that with increasing hepatic CYP1A content, the associated EROD activity, after an initial increase, decreased in both the CloA50 and CB-126 exposed groups. This phenomena is more clear for the CloA50 dosed flounder than the CB-126 exposed fish. *In vitro* inhibition of EROD activity by CB-77 and CB-126 in the presence of increasing CYP1A protein levels were also reported by Hahn *et al.* (1993, 1996) in the fish hepatoma cell line PLHC-1. Direct inhibition of CYP1A catalytic activity by PCBs in flounder hepatic microsomal suspension is under further investigation at our laboratory (Besselink *et al.*, 1997d). In vitro inhibition of hepatic EROD activity in scup microsomes by CB-77 has been reported by Gooch *et al.* (1989).

In contrast to CloA50, co-treatment of flounder with TCDD and CB-126 did not result in a lower CYP1A activity as compared to treatment with TCDD only. In fact, the EROD activity was slightly higher than TCDD only. In addition, CYP1A levels were also slightly higher in the combination group. But both CYP1A activity and CYP1A concentrations in the TCDD/CB-126 treated fish were less than expected on the basis of additivity since the dose administered was 0.01 mg TCDD TEQ/kg body weight. Again, the calculated and administered TEFs overestimated to some extent the observed response. A less-than additive response based on EROD activity was also observed in trout with mixtures of TCDD/CB-126 and TCDD/CB-156 by Newsted *et al.*

(1995). The reasons for this less than additive response are not fully clear but may involve both toxicokinetic as well as saturation of response phenomena in flounder.

Another interesting observation of this study was the strong inhibition of glutathione-S-transferase activity (GST), a phase II enzyme system postulated also to be regulated by the Ah-receptor (Nebert & Gonzales, 1987; Rushmore & Pickett, 1990). The strong inhibition of GST activity was observed only in flounder orally exposed to combinations of TCDD with CloA50 or CB-126, while no effect on GST was observed in the single compound treatment groups except for CB-126 dosed flounder. In a previous study an absence of GST induction was also observed in flounder exposed to a concentration range of TCDD (Besselink *et al.*, 1997b). These results however are in contrast to observation in plaice (*Pleuronectes platessa*) where Clophen A40 caused an increase in GST activity (Boon *et al.*, 1992). Hektoen *et al.* (1994) on the other hand reported a significant decrease of GST activity in rainbow trout and no changes in cod. The reason for the strongly inhibited GST activity in both co-treated groups of flounder is unclear. A possible explanation for these observations might be PCB-metabolite residues present in the cytosol of flounder, thus causing GST substrate inhibition. The presence of PCB-metabolites in flounder have not been established yet. In fact, in vitro metabolism of CB-77 in flounder hepatic microsomes was not detected (Murk *et al.*, 1994), however, it must be mentioned that the microsomes used in that experiment originated from CloA50 dosed flounder, which may have had little induced CYP1A to produce metabolites.

In conclusion, our study indicates that both the CYP1A dependent EROD activity and the total GST activity were strongly reduced in flounder exposed to a combinations of TCDD with the PCB mixture CloA50. The physiological and toxicological consequences of these observations for fish in the wild exposed to complex mixtures of PHAHs are not clear, but may be important with respect to CYP1A and GST dependent conversions of other xenobiotics, such as benzo(a)pyrene and other polycyclic aromatics. Furthermore, since the concentration Clophen A50 to which flounder were orally exposed results in liver concentrations of CB-153 which are in the same order of magnitude as observed in the wild, the use and validity of EROD activity as biomarker for exposure of this species of flounder to environmental pollutants is questionable.

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# Chapter 6
# Low Inducibility of CYP1A Activity by Polychlorinated Biphenyls (PCBs) in Flounder (*Platichthys flesus*): Characterization of the Ah Receptor and the Role of CYP1A Inhibition.


**Abstract**

Several studies have reported a low inducibility of hepatic (CYP1A) activity in European flounder (*Platichthys flesus*) following exposure to mixtures of polychlorinated biphenyls (PCBs). Here we report on mechanistic studies towards understanding this low CYP1A inducibility of flounder, involving molecular characterisation of the Ah receptor (AhR) pathway as well as inhibition of the CYP1A catalytic activity by PCB congeners.

Hepatic cytosolic AhR levels in flounder were determined using hydroxylapatite, protamine sulphate adsorption analysis, or velocity sedimentation on sucrose gradients. AhR levels in flounder (~2-7 fmol/mg protein) were lower than observed generally in rodents (~50-300 fmol/mg protein). Molecular characterisation of the flounder AhR was provided by first-strand cDNA synthesis and amplification of flounder hepatic polyA+ RNA using RT-PCR. A 690 bp product was found, similar in size to a *Fundulus* AhR cDNA. The specificity of the 690 bp band was established by Southern blotting and hybridisation with a degenerate AhR oligonucleotide. The deduced amino acid sequence of the flounder AhR fragment was 59-60% identical to mammalian AhR sequences. Although the AhR is present in flounder cytosol, we were unable to demonstrate detectable amounts of inducible TCDD-AhR-DRE complex in gel-retardation assays.

We further characterised the inhibitory potential of PCB congeners on CYP1A activity in flounder and compared this with inhibitory effects of PCB congeners on rat CYP1A activity. Analysis *in vitro* demonstrated that 3,3',4,4'-tetraCB, 3,3',4,4',5-pentaCB, 2,2',4,4',5,5'-hexaCB, 3,3',4,4',5,5'-hexaCB and the commercial PCB mixture Clophen A50 are potent competitive inhibitors of hepatic microsomal CYP1A catalytic activity in flounder and rat. The $K_m$ for ethoxyresorufin (0.095 μM) in flounder is strikingly close to $K_i$'s found for the tested PCB's. This emphasises the possible involvement of PCB congeners in inhibition of EROD activity in PHAH exposed fish.



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## Introduction

In the aquatic environment, induction of cytochrome P4501A (CYP1A) and associated ethoxyresorufin-O-deethylase (EROD) activity is commonly used as a biomarker for monitoring exposure of fish to environmental pollutants such as polyhalogenated aromatic hydrocarbons (PHAHs) and polyaromatic hydrocarbons (PAHs) (Bucheli and Kent, 1995; Goksøyr, 1995; Stegeman *et al*, 1988; Addison *et al.*, 1994; Monosson and Stegeman, 1994, Eggens *et al.*, 1995). However, fluctuations in CYP1A content and activity have been observed in fish undergoing gonadal recrudescence, suggesting that CYP1A expression may also be regulated by endogenous pathways (Elskus *et al.*, 1992). Moreover, Besselink *et al.* (1996) demonstrated low hepatic CYP1A activity in European flounder (*Platichthys flesus*) upon exposure to the commercial PCB mixture Clophen A50. Decreased responsiveness of CYP1A system in rainbow trout after prolonged exposure to a complex PCB mixture was reported by Celander and Förlin (1995). These observations on low responsiveness and variable results on CYP1A levels and associated EROD activity seriously hamper the further development and use of EROD activity as a reliable biomarker for exposure of fish to PHAHs and related compounds. The reasons for this low responsiveness are not fully understood and may either be a consequence of an "inefficient" Ah receptor pathway for induction of CYP1A activity by PCBs, or may be a consequence of interference of PCB congeners with the catalytic activity of CYP1A in flounder.

The induction of CYP1A is initiated by binding of PAHs, PHAHs, halogenated dioxins or related compounds to the cytosolic aryl hydrocarbon receptor (AhR) (Poland *et al.*, 1976). After ligand binding, two molecules of hsp90 dissociate from the ligand:AhR complex, the receptor translocates into the nucleus where it dimerizes with the Ah receptor nuclear translocator (Arnt) protein and is subsequently converted into its high affinity DNA binding form (Hoffman *et al.*, 1991; Hankinson, 1995). The binding of the ligand:AhR:Arnt complex to its specific DNA recognition site, the dioxin responsive element (DRE), in the promotor region of the CYP1A1 gene leads to enhanced transcription of the CYP1A1 gene (Okey *et al.*, 1994; Hankinson, 1995). Ligand binding to the Ah receptor has been described in mammalian (Denison *et al.*, 1986a,b) as well as some fish species (Lorenzen and Okey, 1990; Hahn *et al.*, 1994). Interactions between ligand-AhR to synthetic DRE have been found in mammals and chicken (Denison *et al.*, 1991; Bank *et al.*, 1992). In fish, Roy *et al.* (1996) showed interactions with DRE in tomcod CYP1A.

In a number of fish species, interference of PCB congeners with the catalytic activity of CYP1A has been suggested. Gooch *et al.* (1989) showed direct inhibition of CYP1A activity by PCB-77 in scup hepatic microsomes. Our own observations showed that Clophen A50 inhibits the effect of TCDD on hepatic EROD activity in flounder with increasing hepatic CYP1A content (Besselink *et al.*, 1997a). These results suggest direct inhibition of hepatic CYP1A catalytic activity by PCBs residues in hepatic microsomal suspensions. Inhibitory or suppressive effects of PCBs on CYP1A activity may therefore interfere with the interpretations

of exposure of fish to environmental contaminants, e.g. PCBs, based on hepatic EROD activity.

In this study we report on the molecular characterisation of the Ah receptor pathway for CYP1A induction, as well as on interference of PCB congeners with the CYP1A catalytic activity in flounder. The occurrence and amount of the Ah receptor in flounder hepatic cytosol was studied. In addition, the ability of the Ah receptor to transform after ligand binding and subsequently bind to its DNA recognition site (DRE) was assessed. We also studied and compared the apparent *in vitro* inhibition of hepatic microsomal CYP1A activity by four PCB congeners and by the commercial PCB mixture Clophen A50 in flounder and, as a comparison, in rat.

## Material and methods

*Chemicals.* Aroclor 1254 was kindly donated by Dr. M. van den Berg of the Research Institute for Toxicology, University of Utrecht, the Netherlands. Clophen A50 was a kind gift from Dr. J. P. Boon (Netherlands Institute for Sea Research, Den Burg, Texel, the Netherlands). [$^3$H]-2,3,7,8-tetrachlorodibenzo-p-dioxin ([$^3$H]-TCDD) (35 Ci/mmol) was obtained from Chemsyn Science Laboratories (Lenexa, Kansas, USA). It was 98% pure as analysed by HPLC (method of Gasiewicz and Neal, 1979). Unlabeled TCDD was a kind gift from Dr. S. Safe. 2,3,7,8-tetrachlorodibenzofuran (TCDF) was purchased from Ultra Scientific (Hope, Rhode Island, USA). Methylated [methyl-$^{14}$C]-ovalbumine was purchased from New England Nuclear Corporation (Boston, Massachusetts, USA). 3,3',4,4'-tetrachlorobiphenyl (PCB-77), 3,3',4,4',5-pentachlorobiphenyl (PCB-126), 3,3',4,4',5,5'-hexachlorobiphenyl (PCB-169) and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB153) (Ballschmitter *et al.*, 1992) were obtained from CN Schmidt B.V. (Amsterdam, The Netherlands). Tween 80 was obtained from Fisher Scientific (Toronto, Ontario). Hydroxylapatite (DNA grade, bio-gel HTP) was purchased from Bio-Rad (Richmond, California, USA). Dimethyl sulfoxide (DMSO) was obtained from Aldrich (Aldrich Chemical Company, Inc, Milwaukee, USA). Tris, HCl, KCl, $Na_2EDTA$, NaCl, NaOH, $NaN_3$, $Na_2MoO_4$, glycerol, and sucrose were obtained from Merck, Darmstadt, Germany. Bovine Serum Albumin (BSA), dithiothreitol (DTT), HEPES, MOPS, aprotinin, leupeptin, pepstatin A, phenylmethylsulfonyl fluoride (PMSF), tosyl-L-lysine chloromethyl ketone (TLCK), and protamine sulphate (Grade X, from salmon) were purchased from Sigma Chemie, Bornem, Belgium. NADPH was obtained from Boehringer Mannheim, Almere, the Netherlands.

*Animals and Cells.* European flounder (*Platichthys flesus*) were caught by beam-trawl in the Western Wadden Sea, the Netherlands (18.0-22.0 cm length class, average age 2 years), disinfected and acclimatised as described earlier (Besselink *et al.* 1997b). Wistar rats (10 weeks old) were obtained from the Laboratory Animal Centre (Wageningen Agricultural University, Wageningen, the Netherlands). Male Hartley guinea pigs (250-300 g) and male Sprague-Dawley

rats (200 g) were obtained from Charles River Breeding Laboratories, Wilmington, DE, USA, and were exposed to 12 h of light and 12 h of dark daily and were allowed free access to food and water. Animals were killed using carbon dioxide and livers were obtained for preparation of cytosol as described below. Killifish (*Fundulus heteroclitus*) were obtained as described in Hahn and Karchner (1995). Mouse hepatoma (Hepalclc7) cells (HEPA-1) were a gift of Dr. T.A. Gasiewicz (University of Rochester, New York, USA).

Flounder (n=12) were orally injected twice at day 1 and 7 with 200 µl gelatin capsules filled with TCDD dissolved in cornoil. The final concentrations of TCDD administered was 10 µg TCDD/kg body weight. Ten days after the first exposure, flounder were killed by cervical transection. Livers were carefully dissected free from the gall bladder, washed in ice-cold 0.9% NaCl, weighed and stored at -80°C until preparation of microsomes. Male Wistar rat (n=3) were dosed i.p. twice at day 1 and 2 with 100 mg Aroclor 1254/kg b.w. in corn oil (0.5 ml). At day 3 of exposure rats were killed. Livers were dissected free from the gall bladder, washed in ice-cold 0.9% NaCl, weighed and stored at -80°C until preparation of microsomes.

*Buffers.* The buffers used for experiments reported in this paper were TS buffer (50 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose), TEGD buffer (0.1 M Tris-HCl buffer (pH 7.6) containing 1 mM EDTA, 1 mM DTT and 20% (v/v) glycerol), HEDG buffer (25 mM HEPES (pH 7.5) containing 1 mM EDTA, 1 mM DTT and 10% (v/v) glycerol), HEDGK buffer (HEDG containing 0.15 M KCl), MEDG buffer (25 mM MOPS (pH 7.5) containing 1 mM EDTA, 5 mM EGTA, 1mM DTT, 0.02% $NaN_3$ and 10% (v/v) glycerol), MEDGM buffer (MEDG containing 20 mM $Na_2MoO_4$), MEDGT buffer (MEDG containing 0.5% (v/v) Tween-80), hydroxylapatite (HAP) suspension (0.1 g HAP per ml MEDGM buffer) and protamine sulphate (PS) suspension (1 mg PS per ml MEDGM buffer).

*Preparation of microsomes.* Individual livers of TCDD treated flounder and Aroclor 1254 treated Wistar rats were homogenised in TS buffer (4 ml/g liver and 2 ml/g liver respectively), using 10 strokes with a motor-driven Potter-Elvehjem glass and teflon homogenizer. The hepatic homogenates were centrifuged at 9,000 g for 30 min. The resulting supernatants were centrifuged at 100,000 g for 90 minutes. After resuspending the pellets in TEDG buffer, the hepatic microsome suspensions of both flounder and rat were stored at -80°C until analysis. All handlings were performed at 0-4°C.

*Preparation of cytosol.* For gel retardation assays, livers of untreated flounder were homogenised in 2.5 ml HEDG buffer using 10 strokes with a motor-driven Potter-Elvehjem glass and teflon homogenizer. After centrifugation of the hepatic homogenate for 30 minutes at 9,000 g, the resulting supernatant was filtered through glass wool to reduce lipid content and

then centrifuged at 100,000 g for 90 minutes. The supernatant (cytosol) was stored at -80$^0$C until use. Sprague-Dawley rat and Guinea pig hepatic cytosol was prepared in HEDG buffer as described by Denison *et al.* (1986a). For velocity sedimentation analysis on sucrose gradient (SG), hydroxylapatite adsorption analysis (HAP) and PS adsorption analysis (PSAA), fresh flounder livers were rinsed in MEDGM buffer and homogenised in 9 volumes of MEDGM buffer plus a mix of proteinase inhibitors (20 μM TLCK, 5 μg/ml leupeptin, 13 μg/ml aprotinin, 7 μg/ml pepstatin A, and 0.1 mM PMSF) (Hahn *et al.*, 1994). Cytosol was prepared and stored as described above. Cytosol from HEPA-1 cells was prepared as described earlier for PLHC-1 cells (Hahn *et al.*, 1993) and stored at -80$^0$C until use.

*RNA isolation.* Flounder hepatic total RNA was isolated using the method described by Chomczynski and Sacchi (1987). Poly A+ RNA was the isolated using the Pharmacia Biotech mRNA Purification kit as described by the supplier (Roosendaal, the Netherlands). *Fundulus* poly A+ RNA was isolated as described earlier (Hahn and Karchner, 1995).

*Ah receptor ligand-binding studies.* Specific binding of [$^3$H]-TCDD to hepatic cytosol was measured using velocity sedimentation analysis (SG) (Tsui and Okey, 1981), hydoxylapatite adsorption analysis (HAP) (Gasiewicz and Neal, 1982) and protamine sulphate adsorption analysis (PSAA) (Denison *et al*, 1984). For SG analysis, aliquots of flounder hepatic cytosol or HEPA-1 cell lysate in MEDGM buffer (5 mg protein/ml and 3.9 mg protein/ml respectively) were incubated with [$^3$H]-TCDD (2 nM) in the presence or absence of TCDF (200 nM as competitor for specific binding) for 2 h at 4$^0$C. Following incubation, 300 μl aliquots were layered onto linear (10-30%) sucrose gradients prepared in MEDGM buffer. Gradients were centrifuged at 372,000g for 2 h at 4$^0$C in a Beckman vertical-tube rotor. After centrifugation, 200 μl fractions were collected and radioactivity was measured in a Beckman LS 5000TD scintillation spectrometer. [$^{14}$C]-ovalbumine (3.6 S) was added onto each sucrose gradient and used as internal sedimentation marker. The HAP (Gasiewicz and Neal, 1982) and PSAA methods (Denison *et al.*, 1984) were modifications of the published methods (Hahn, manuscript in preparation). Aliquots of hepatic cytosol (2 mg protein/ml for flounder cytosol and 1 mg protein/ml for HEPA-1) in MEDGM buffer were incubated with [$^3$H]-TCDD (2 nM) ± TCDF (200 nM) for 2h at 15$^0$C. After incubation aliquots (0.20 ml for HAP assay and 0.25 ml for PS assay) were added to tubes containing 0.25 ml HAP or PS suspension and incubated for 30 minutes with gently vortexing every 10 minutes. One millilitre of MEDGT buffer was added, the pellet resuspended and transferred onto a pre-wetted Whatman GF/A filter in manifold and vacuum was applied. The filter was washed three times with MEDGT buffer after which the filter was placed in a scintillation vial containing 10 ml scintillation fluid. Radioactivity was quantified in a Beckman LS 5000TD scintillation spectrometer.

*Gel retardation analysis.* Specific binding of transformed flounder, Sprague-Dawley rat and guinea pig TCDD-Ah receptor complex to a complementary pair of synthetic oligonucleotides, corresponding to wild type Ah receptor site of mouse DRE3 (Denison *et al.*, 1988a) was determined using gel retardation analysis basically as described by Bank *et al.* (1992). The DRE oligonucleotides were synthesised, purified, annealed and radiolabeled as reported by Denison *et al.* (1988b). Cytosol (2 mg protein/ml for flounder and 16 mg protein/ml for all other species) was incubated with DMSO (20 µl/ml) or 20 nM TCDD in DMSO for 2 h at $20^0$C. Five microlitre of this incubation mixture was added to 16 µl HEDGK buffer containing poly dIdC (100 ng for rat cytosol and 200 ng for guinea pig cytosol; flounder cytosol was incubated with either amounts of poly dIdC), mixed and incubated for 15 minutes at $20^0$C. The incubation was continued for 15 minutes at $20^0$C after addition of $^{32}$P-labeled oligonucleotide (100,000 cpm, ~1.0 ng in 4 µl of HEDG buffer). Analysis of the protein-DNA complexes by polyacrylamide gel electrophoresis and autoradiography was performed as described earlier (Denison *et al.*, 1988a; Denison and Yao, 1991).

*RT-PCR and Southern blotting.* PCR primers AhR-A1 and AhR-B1 were designed and synthesised as described earlier (Hahn and Karchner, 1995; Karchner and Hahn, 1996). Flounder polyA+ RNA purified from 100 µg total RNA was pelleted, washed, and dissolved in 10 µl water. RT-PCR was performed on 3 µl of each sample. *Fundulus* polyA+ RNA (1 µg) was used as positive control for the AhR primers. Conditions were as described in Hahn and Karchner (1995), except annealing was at $50^0$C. Aliquots of the RT-PCR reaction (10 µl) were run on 2% agarose gels, stained with ethidium bromide, and photographed. RT-PCR products were analysed by Southern blotting using oligonucleotide J2u (5'-GGCTAYCAGTTYATYCATGC-3'), targeted to the conserved sequence GYQFIHA (corresponding to amino acids 315-321 of the mouse AhR). The RT-PCR products from four individuals were pooled, cloned into pCNTR (5 prime → 3 prime, Inc.) and sequenced in both directions using SequiTherm and SequiTherm Excel long-read cycle sequencing kits (Epicentre Technologies, Madison, Wisconsin USA) and an automated DNA sequencer (LI-COR, Inc., Lincoln, Nebraska, USA). Four clones were sequenced.

*EROD inhibition studies.* Protein levels in pooled hepatic microsomes were measured by the Bio-Rad assay system according to the specifications of the supplier, using crystalline bovine serum albumin as standard. Microsomal 7-ethoxyresorufin-O-deethylation (EROD) activity was measured in hepatic microsomes according to the method of Prough *et al.* (1978). The assay was adapted for use in 96 well plates and a fluorospectrophotometric plate reader (Cytofluor 2350, Fluorescence Measurement System, Milipor, USA) as described by Besselink *et al.* (1997b). Incubations with flounder tissue contained 37.5 µg microsomal protein/ml and 1 mg BSA/ml,

whereas rat tissue incubations contained 5 µg microsomal protein/ml and 1.03 mg BSA/ml. The extra BSA was added to compensate for differences in total protein concentrations between flounder and rat incubations. EROD inhibition studies with PCB-77, PCB-126, PCB-153, PCB-169 or the commercial PCB mixture Clophen A50 (CloA50) (0-50 µM in DMSO) were performed at different ethoxyresorufin (ER) substrate concentrations (0.1-1.5 µM). Two microlitre of inhibitor solution was added to a mixture containing 0.1 M Tris-HCl buffer (pH 7.8), microsomal suspension, and BSA and shaken for 3 minutes after which 50 µl of ER solution was added. The reaction was started after 5 minutes pre-incubation by adding 50 µl 0.1 mM NADPH and stopped after 5 minutes by the addition of 50 µl 1 M NaOH. The resorufin formed was detected fluorimetrically and compared to a calibration curve (0-150 nM resorufin). All incubations were in duplicate and corrected for a blank without NADPH. Rat hepatic microsomal EROD activity was measured at 37°C whereas for flounder hepatic microsomal EROD activity assays were carried out at 25°C.

*Analysis of inhibition potency.* Curve-fitting of the inhibition curves (EROD activity versus logarithm of the competitor concentration) using non-linear regression was carried out using the software package SlideWrite Plus, Version 5.00 (Advanced Graphics Software, Inc., Carlsbad, CA, USA) at constant ethoxyresorufin concentrations for flounder and rat of 0.6 and 0.4 µM respectively, according to the following equation: $y=a_0+a_1/(1+\exp(-(x-a_2)/a_3))$. The $IC_{50}$ (molar concentration of inhibitor required to reduce EROD activity to 50% of control) value for each competitor was determined by interpolation from the fitted curves. To extract $V_{max}$ and $K_m$ from obtained data, Lineweaver Burk plots were plotted with 1/[ER] in $\mu M^{-1}$ on the x-axis and 1/V ($nmol^{-1}*min*mg$) on the y-axis. In plots of incubations without inhibitor, the y-axis intercept represents $1/V_{max}$ and the intercept with the x-axis represents $-1/K_m$. $K_i$ values were calculated using the formula $K_i = IC_{50}/(1+[ER]/K_m)$, with [ER] concentration for flounder and rat of 0.6 µM and 0.4 µM respectively.

**Results**

*Ah receptor binding studies.* Under the experimental conditions, incubations of HEPA-1 cytosol, used as positive control, resulted in a single specific binding peak for [$^3$H]TCDD (2 nM) as detected by velocity sedimentation on sucrose gradient (Fig. 6.1A). The radioactive [$^3$H]TCDD peak sedimented at fraction 10 (≈9.4 S) and was almost completely abolished in the presence of 200 nM TCDF, indicative of the AhR. Incubations of flounder hepatic cytosol with [$^3$H]TCDD and subsequent velocity sedimentation under the same experimental conditions, resulted in a small but clear peak at fraction 10, which was not detectable in the presence of excess TCDF (Fig. 6.1B). The position of this visualised peak coincided with the sedimentation of the [$^3$H]TCDD-Ah receptor peak from HEPA-1 (≈9.4 S). Ah receptor in HEPA-1 and

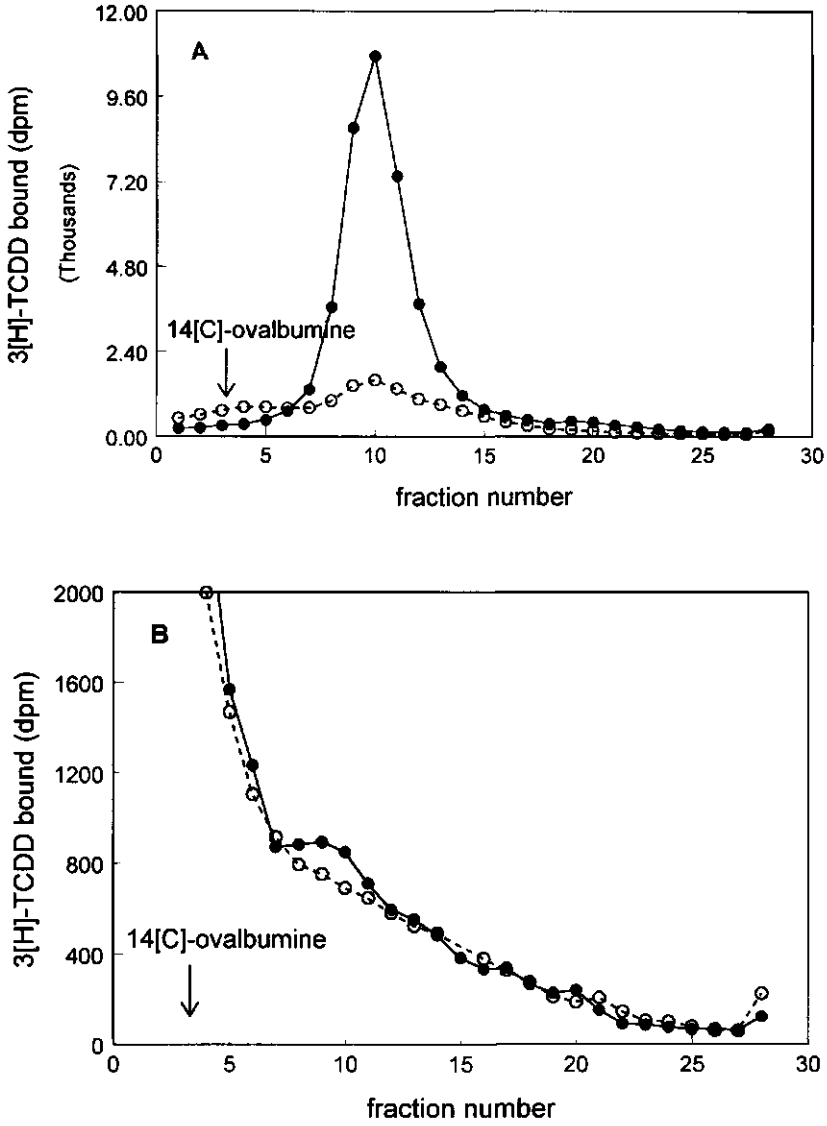


**Figure 6.1.** Typical profiles of [$^3$H]TCDD binding to HEPA-1 (A) and flounder (B) cytosolic Ah receptor analysed by velocity sedimentation on sucrose gradients. Cytosol from HEPA-1 cells (3.9 mg/ml) and flounder (5 mg/ml) was incubated with 2 nM [$^3$H]TCDD in the presence (○) or absence (●) of 200 nM TCDF. Aliquots of 300 µl were analysed by velocity sedimentation on 10-30% sucrose gradients as described under Material and Methods. Arrows indicate the sedimentation position of [$^{14}$C]-ovalbumine (3.6 S).

**Table 6.1.** Concentrations of Ah receptor in hepatic cytosol from various species as determined by velocity sedimentation analysis on sucrose gradient (SG), hydroxylapatite (HAP), and potassium sulphate adsorption analysis (PSAA).

| | Ah-receptor concentration | | |
|---|---|---|---|
| | SG (fmol/mg) | HAP (fmol/mg) | PSAA (fmol/mg) |
| European flounder | 3.83±0.31 (5) | 6.86±1.62 (4) | 1.88±0.35 (3) |
| Winter flounder | 19[f] | 4-8[f] | |
| Rainbow trout | 7.5[c] | 5.1±2.6 (4)[e] | |
| Sprague-Dawley rat | 33 (1)[a] | 97±5 (4)[b] | |
| C57BL/6N mouse | 60±12 (8)[a] | 92+10 (4)[b] | |
| RTH-149 | 29[d] | | |
| HEPA-1 | 361.90 (1) | 72.08 (1) | 31.24 (1) |

Values are expressed as mean ± S.E.M. Number of animals in parentheses. Data obtained from [a]: Okey *et al.* (1979). [b]: Denison *et al.* (1986b). [c]: Heilmann *et al.* (1988). [d]: Lorentzen and Okey (1990). [e]: Bank *et al.* (1992). [f]: Hahn *et al.* (1994).

flounder cytosol were also detected using HAP and PS analysis. The concentrations of Ah receptor in HEPA-1 cells and flounder hepatic cytosol calculated from sucrose gradient, HAP and PS analysis are given in Table 6.1. Flounder Ah receptor levels ranged from 1.88 to 6.86 fmol/mg protein dependent on the assay used. These levels are much lower than Ah receptor concentrations in rodent species and HEPA-1 liver cells. However, also in the rainbow trout and winter flounder, comparably low levels of Ah receptor were observed (Bank *et al.*, 1992; Hahn *et al.*, 1994; Heilmann *et al.*, 1988).

*Gel retardation analysis.* Autoradiograms of interaction of flounder, rat, and guinea pig cytosolic Ah receptor and DRE-containing oligonucleotides in the presence or absence of TCDD are shown in Fig. 6.2. Rat and guinea pig autoradiograms reveal a TCDD-inducible (see arrow) and a constitutive protein-DRE complex. As was previously shown by Denison and Yao (1991), the TCDD-inducible band represents the binding of TCDD-AhR complex to $^{32}$P-labeled DRE-containing oligonucleotides. The constitutive protein-DRE band represents binding of an unknown protein to single-stranded $^{32}$P-labeled oligonucleotides. In contrast to rat and guinea pig, no TCDD-inducible protein-DRE complex was observed in flounder under the same experimental conditions. Additional gel retardation analyses using flounder hepatic cytosol under conditions of high and low ionic strength and different concentrations of poly dIdC also failed to detect TCDD-inducible protein-DRE complexes (data not shown).

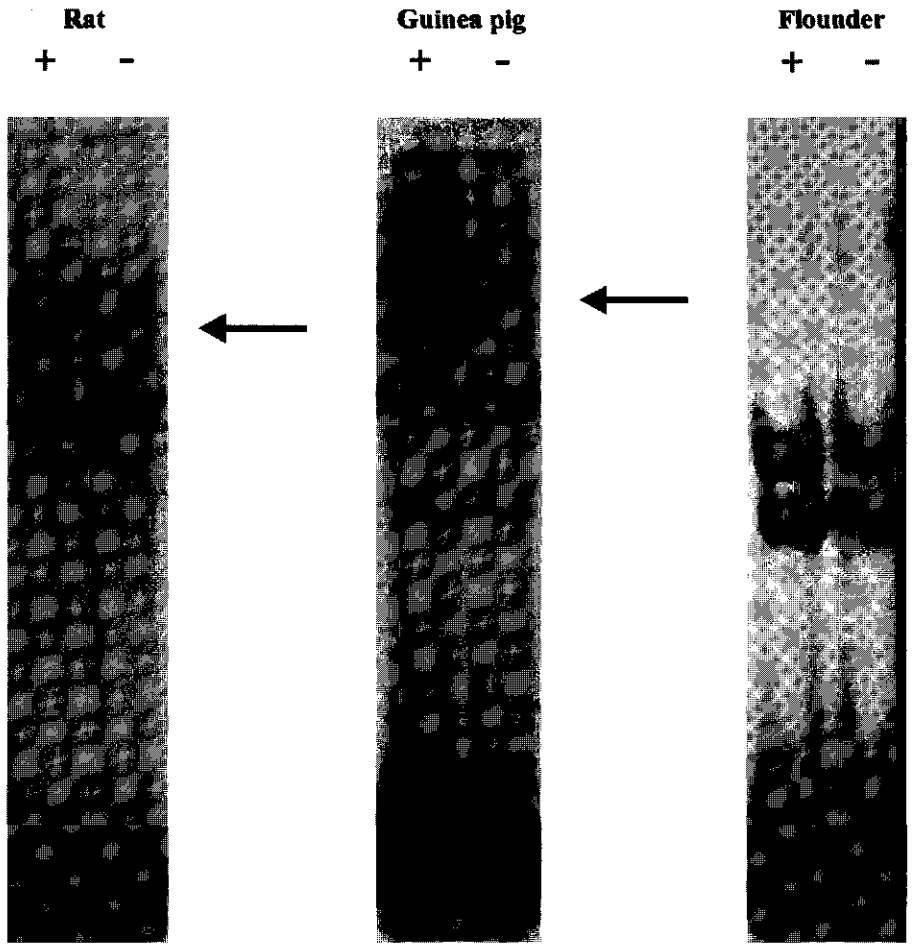


**Figure 6.2.** Autoradiograms of liganded cytosolic Ah receptor and DNA complexes of rat, guinea pig and flounder, analysed by gel retardation analysis. Hepatic cytosol was incubated in the absence (-) or presence (+) of 20 nM TCDD, followed by addition of $^{32}$P-labeled DRE oligonucleotide as described under Material and Methods. Arrows indicate the position of TCDD-inducible protein-DNA complex.

*RT-PCR and DNA sequencing.* First-strand cDNA synthesis and subsequent amplification of the killifish (*Fundulus heteroclitus)* poly A+ RNA using RT-PCR resulted in a single band of approximately 690 bp (Fig. 6.3 top, lane 3), similar to the size reported by Hahn and Karchner (1995). In addition, reverse transcription and amplification of 4 individual flounder poly A+

RNAs led to a detectable band of approximately 690 bp (Fig. 6.3 top, lanes 4-7) following separation on 2% agarose and staining by ethidium bromide. In all 4 individual samples tested, the flounder AhR was expressed. Southern blotting of the flounder RT-PCR products and hybridisation using degenerate oligonucleotide AhR-J2u revealed a single band of 690 bp for each individual (Fig. 6.3 bottom). The RT-PCR products were cloned and sequenced. The deduced amino acid sequence of the flounder RT-PCR product was 59-60% identical to the PAS domain of mammalian AhRs (Fig. 6.4) and 61% identical to fAhR-1, a *Fundulus* ortholog

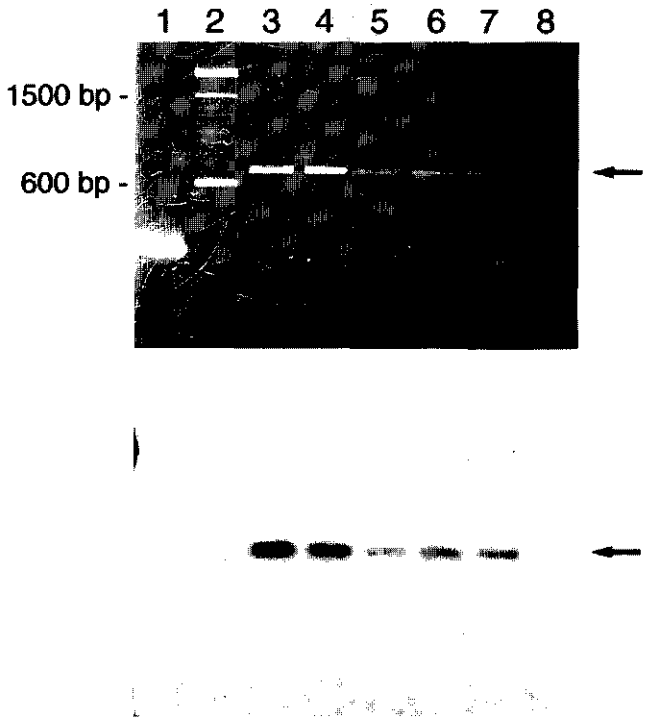


**Figure 6.3.** Ethidium bromide-stained gel (top) and Southern blot (bottom) showing PCR products from amplified *Fundulus* and flounder hepatic poly A+ RNA. For Southern blotting, oligonucleotide J2u was used as probe. Identification of lanes: lane 1, pAW positive control RNA for RT-PCR kit; lane 2, Gibco 100-bp DNA ladder; lane 3, *Fundulus* hepatic polyA+ RNA; lanes 4-7; flounder hepatic polyA+ RNA; lane 8, flounder hepatic polyA+ RNA (minus reverse transcriptase). Arrows indicate hepatic AhR poly A+ RNA PCR products.

**Table 6.2.** Maximum EROD activity ($V_{max}$), Michaelis constant ($K_m$), $IC_{50}$, and inhibition constant ($K_i$) of 4 PCB congeners and Clophen A50 in Wistar rat and flounder hepatic microsomal fraction.

| Species | Compound | $V_{max}$[a] (nmol/min/mg protein) | $K_m$[a] (μM) | $IC_{50}$[b] (μM) | $K_i$[c] (μM) |
|---|---|---|---|---|---|
| Rat | PCB-77 | 3.61 | 0.260 | 0.42 | 0.154 |
| | PCB-126 | 2.90 | 0.236 | 0.24 | 0.088 |
| | PCB-153 | 2.51 | 0.306 | 31.88 | 11.690 |
| | PCB-169 | 2.88 | 0.155 | 0.52 | 0.191 |
| | CloA50 | 2.47 | 0.201 | 4.24 | 1.555 |
| | average ± S.E.M. | 2.87 ± 0.20 | 0.232 ± 0.026 | | |
| Flounder | PCB-77 | 1.11 | 0.100 | 0.61 | 0.084 |
| | PCB-126 | 1.06 | 0.070 | 0.30 | 0.041 |
| | PCB-153 | 1.09 | 0.122 | 19.46 | 2.670 |
| | PCB-169 | 1.02 | 0.092 | 0.62 | 0.085 |
| | CloA50 | 0.93 | 0.093 | 7.39 | 1.014 |
| | average ± S.E.M. | 1.04 ± 0.03 | 0.095 ± 0.008 | | |

a: $V_{max}$ and $K_m$ were derived from Lineweaver Burk plots such as those shown in Fig. 6.7A + B.
b: $IC_{50}$ values (molar concentration of inhibitor resulting in inhibition of EROD activity to 50% of control (no inhibitor)) were calculated by non-linear regression curve fitting of inhibition curves (Fig. 6.5A+B).
c: $K_i$'s were calculated from the non-linear regression curve fits of the inhibition curves shown in Fig. 6.5A+B, using the formula $K_i = IC_{50}/(1+[ER]/K_{m,avg})$, with ethoxyresorufin ([ER]) concentrations of 0.4 μM for rat and 0.6 μM for flounder.

of the mammalian AhRs (Hahn *et al.*, 1997). Interestingly the flounder sequence shared greater amino acid identity (75%) with the *Fundulus* AhR-2 sequence identified recently (Hahn and Karchner, 1995; Hahn *et al.*, 1997).

*EROD inhibition studies.* In order to examine the potency of individual PCB congeners and complex PCB mixtures to interfere with the CYP1A catalytic activity, EROD inhibition studies were performed. In Fig. 6.5A (rat) and 6.5B (flounder), the EROD inhibition curves of the tested PCB congeners are shown at ethoxyresorufin concentrations of 0.4 and 0.6 μM for rat and flounder respectively. From these curves, $IC_{50}$ values were calculated which are given in Table 6.2. The di-ortho substituted PCB-153 was the least potent inhibitor of hepatic microsomal EROD activity in both rat and flounder ($IC_{50}$ values of 31.88 and 19.46 μM respectively). PCB-126 had the greatest inhibitory potency of the PCBs tested in rat and flounder ($IC_{50}$: 0.24 and 0.30 μM respectively). In fact, although the tested PCB congeners showed lower $IC_{50}$ values in rat than in flounder, the order of inhibitory potencies of the PCB congeners was identical in rat and flounder. Calculated $IC_{50}$ values for the commercial PCB mixture were 4.24 and 7.39 μM in rat and flounder respectively, which was lower than

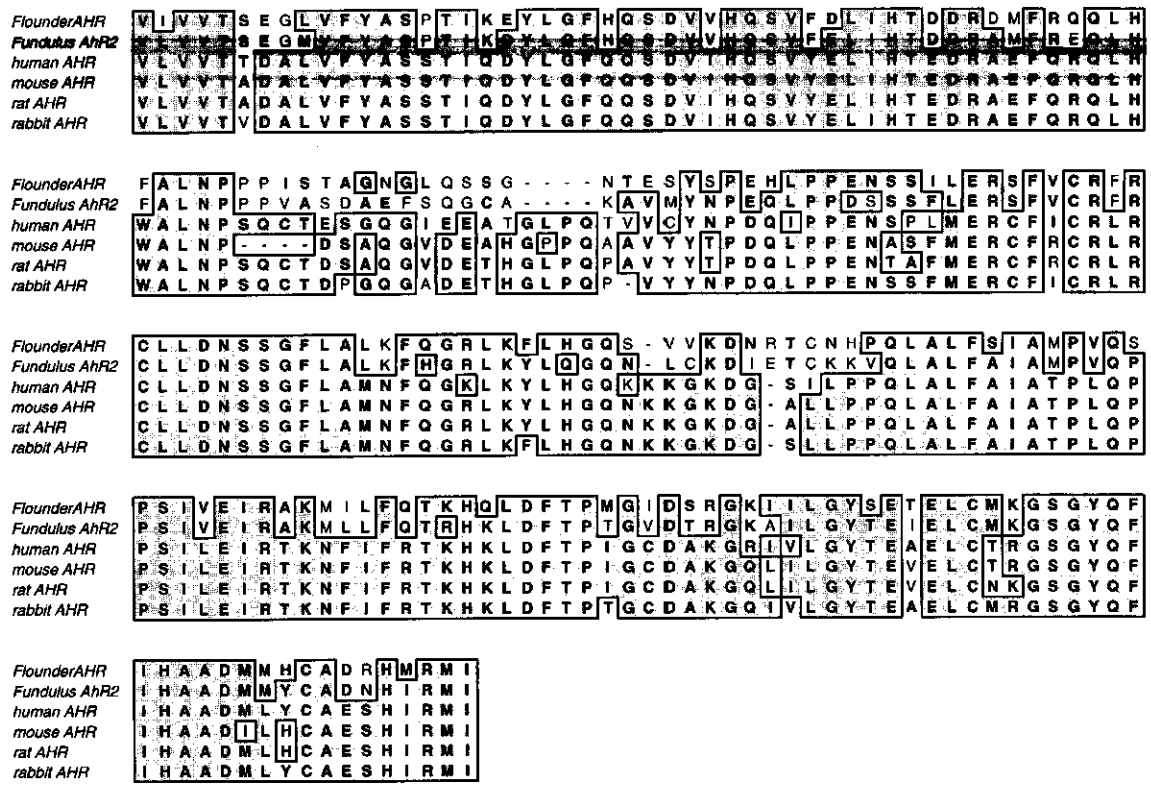


**Figure 6.4.** Deduced amino acid sequence of flounder RT-PCR product aligned with partial amino acid sequences (PAS domain) of other vertebrate Ah receptors. Amino acid sequences were aligned using ClustalW(1.6) (Thompson *et al.*, 1994). GenBank accession numbers for the sequences used are: flounder AhR (XXXXXX); *Fundulus* AhR2 (U29679); human AhR (L19872); mouse AhR (M94623); rat AhR (U09000), rabbit AhR (D38226). Amino acids that are identical in three or more of the sequences are boxed and shaded. Similar amino acids are in bold type.

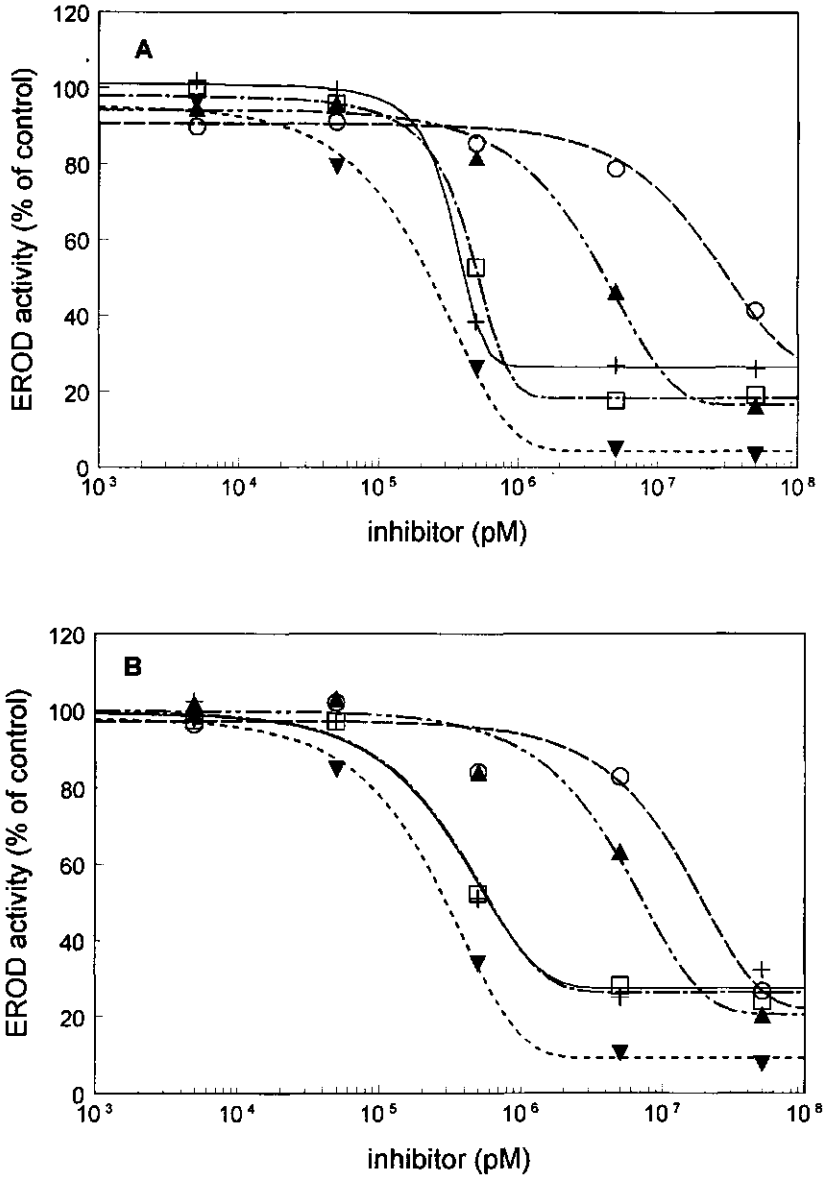


**Figure 6.5.** Inhibition of rat (A) and flounder (B) hepatic microsomal EROD activity by PCB-77 (—+—), PCB-126 (- - -▼- - -), PCB-169 (— - ⊟ - -), PCB-153 (— —O— - ), and CloA50 (— - -▲- · ) with ethoxyresorufin concentration of 0.4 μM for rat and 0.6 μM for flounder. Control EROD activity for rat and flounder are 1.8 and 0.3 nmol/min/mg respectively.

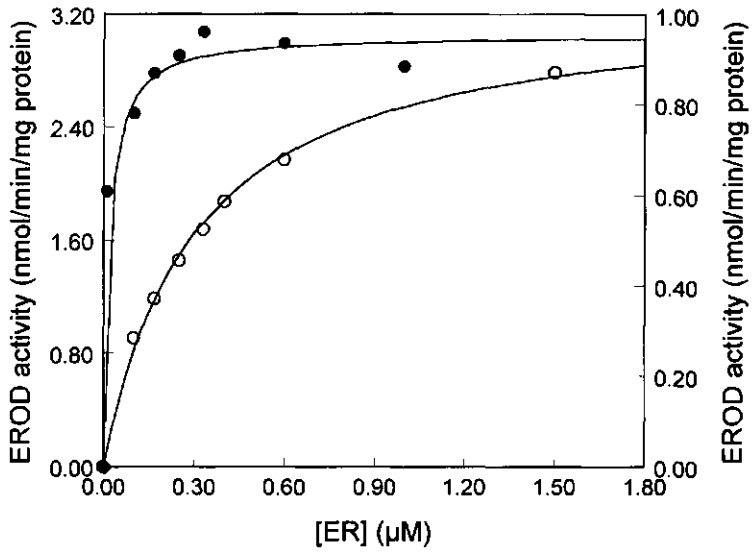


**Figure 6.6.** Substrate saturation curves for CYP1A (EROD) activity of rat (○) (left y-axis) and flounder (●) (right y-axis) hepatic microsomes. Incubations were carried out without inhibitor as described under Material and Methods. Data points are means of duplicates.

PCB-153 but higher than the 3 non-ortho PCBs tested. In Fig. 6.6, substrate saturation curves for the CYP1A (EROD) enzyme of rat (A) and flounder (B) hepatic microsomal fractions are given. Hepatic EROD activity increased with increasing concentration ER. Although the maximum EROD activity in rat is higher than in flounder, substrate saturation of the CYP1A enzyme in flounder microsomes is reached at lower ER concentration than in rats.

To calculate the maximum EROD activity ($V_{max}$) and the Michaelis constant ($K_m$) with ER as a substrate, double reciprocal Lineweaver Burk plots were designed and analysed by linear regression for all inhibitors tested. Fig. 6.7A (rat) and Fig. 6.7B (flounder) show Lineweaver Burk plots with PCB-126 as inhibitor. From the plots it can be concluded that the observed inhibition of hepatic EROD activity by PCB-126 is competitive in nature for both rat and flounder. In fact, all compounds tested were competitive inhibitors of hepatic EROD activity. $V_{max}$ and $K_m$ values, determined for each congener from the Y-axis intercept ($V_{max}^{-1}$) and X-axis intercept ($K_m^{-1}$) are given in Table 6.2. Maximum average CYP1A activity with ER as a substrate was higher in rat (2.87 nmol/min/mg protein) than in flounder (1.04 nmol/min/mg

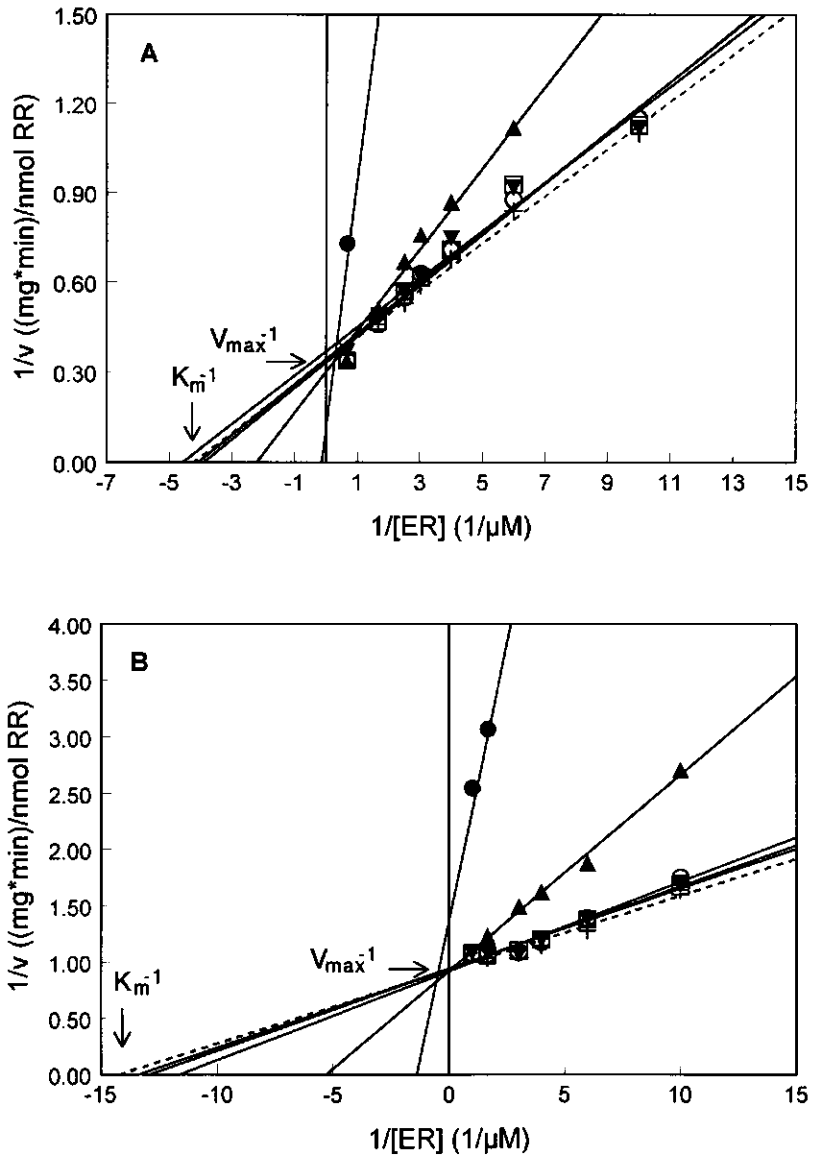


**Figure 6.7.** Lineweaver Burk plots of rat (A) and flounder (B) hepatic microsomal EROD activity with or without addition of inhibitors. The reciprocal values of EROD activity (1/V) are plotted against 1/ER substrate concentrations for incubation without ( — ✚ — ) or with 0.05 nM (—▼— ), 0.5 nM (—□—), 5 nM (—○— ), 50 nM (—▲— ), and 500 nM (—●— ) PCB-126. X-axis intercept for incubation without inhibitor represents $K_m^{-1}$, while y-axis intercept represents $V_{max}^{-1}$.

protein). In contrast, the average Michaelis constant for hepatic EROD activity with ethoxyresorufin as a substrate in rat was higher than in flounder (0.232 μM vs. 0.095 μM). Using average $K_m$ values, inhibition constants ($K_i$) for the tested PCB congeners and CloA50 were calculated (Table 6.2). The inhibition constant $K_i$ was lowest for PCB-126 in rat and flounder (0.088 and 0.041 μM respectively) whereas highest $K_i$'s were observed for the di-ortho PCB-153 (11.690 and 2.670 μM). $K_i$'s for the non-ortho PCB were in the same order of magnitude (rat: 0.088 to 0.191 μM, flounder: 0.041 to 0.085 μM). Intermediate $K_i$'s were calculated for the commercial PCB mixture CloA50 (1.555 and 1.014 μM for rat and flounder respectively).

**Discussion**

In previous studies we reported that flounder exposed to complex PCB mixtures show low hepatic CYP1A activity (Besselink *et al.*, 1996, 1997a). Here, possible molecular mechanisms responsible for the apparent low responsiveness of flounder CYP1A upon exposure to PCBs were investigated. The present studies involved molecular characterisation of the AhR pathway as well as determining the effect of PCB congeners on CYP1A catalytic activity *in vitro*.

This report shows for the first time the positive identification of the AhR in the European flounder (*Platichthys flesus*) by [$^3$H]TCDD binding and by RT-PCR amplification. The hepatic cytosolic Ah receptor is widely distributed in mammalian species and its concentration is commonly found to be 50 to 300 fmol/mg protein (Okey *et al.*, 1979; Denison *et al.*, 1986b). In contrast, limited data are available on the levels of Ah receptor in fish. In trout hepatic cytosol and the trout hepatoma cell line RTH-149, Ah receptor has been demonstrated (Heilmann *et al.*, 1988; Lorenzen and Okey, 1990). More recently, Hahn *et al.* (1994) identified the presence of the Ah receptor in seven species of teleost and elasmobranch fish by photoaffinity labelling with 2-azido-3-[$^{125}$I]iodo-7,8-dibromodibenzo-*p*-dioxin. In the present study we found evidence for the presence of the hepatic cytosolic AhR in flounder using three different AhR binding assays.

Additional evidence for the presence of the cytosolic AhR in flounder liver was provided by amplification of flounder AhR cDNA using RT-PCR (Fig. 6.3 top). The size of the PCR product was 690 bp, identical to recently identified *Fundulus* AhR-2 cDNA (Hahn and Karchner, 1995; Hahn *et al.*, 1997). The specificity of the flounder AhR cDNA was established by Southern blotting and sequencing. All four flounder samples hybridised to an AhR-specific oligonucleotide probe (Fig. 6.3 bottom). Sequencing of the flounder 690 bp product revealed that its deduced amino acid sequence was more related to the *Fundulus* AhR-2 sequence (75 % identical) than to *Fundulus* AhR-1 sequence (61 % identical), which is closer to mammalian AhRs than AhR-2 (Fig. 6.4) (Hahn and Karchner, 1995; Hahn *et al*, 1997). Although the concentrations of Ah receptor detected in flounder (~ 4 fmol/mg protein) using the described

[$^3$H]TCDD binding studies, was in the same order of magnitude as found in other species of fish (Table 6.1), it should be noted that the Ah receptor levels were very low as compared to Ah receptor levels in mammalian hepatic cytosolic tissue (~50-300 fmol/mg protein).

Upon ligand binding and transformation, the cytosolic Ah receptor is transported to the nucleus where it binds to the DRE. Using a complementary pair of synthetic oligonucleotides, corresponding to wild type Ah receptor site of mouse DRE3, Bank *et al.* (1992) demonstrated TCDD-inducible protein-DNA complexes by gel retardation analysis using cytosol from a number of mammalian species, such as rat, guinea pig, mouse cow and sheep, but not in rainbow trout hepatic cytosol. More recently, a TCDD-inducible protein-DRE complex was observed using hepatic cytosol from several fish species (Hahn and Denison, unpublished results). In the present study, we were also able to demonstrate TCDD-inducible protein-DRE binding in hepatic cytosol from rat and guinea pig but failed to do so in flounder hepatic cytosol (Fig. 6.2). As stated by Bank *et al.* (1992), the absence of TCDD-inducible protein-DNA complex in rainbow trout may be due to low levels of Ah receptor in fish or instability of fish Ah receptor. With respect to the levels of hepatic cytosolic Ah receptor, these were low in flounder hepatic cytosol as compared to mammalian levels of cytosolic Ah receptor. Indeed, this might have its influence on the absence of a TCDD-inducible protein-DNA complex, but since no clear correlation was observed between cytosolic Ah receptor levels and the degree of liganded AhR-DNA binding (Bank *et al.*, 1992), other possibilities can not be ruled out.

For instance, prior to interaction with specific regions of DNA (Ah responsive elements), the liganded Ah receptor must undergo transformation into a nuclear form (Okey *et al.*, 1994). Since no DRE binding of TCDD-AhR complex was observed in our study, one or more factors, e.g. Arnt (Hoffman *et al.*, 1991), necessary for transformation might not be present in flounder hepatic cytosol. Furthermore, Bank *et al.* (1992) demonstrated that the nucleotide specific interaction of liganded-AhR complex with DRE is highly conserved between mammals. Single nucleotide substitutions in the critical core consensus motif of the murine DRE resulted in a dramatic decrease in induced AhR-DRE complex formation. Until now, limited data is available on whether the core consensus motif of teleost DRE is similar to the mammalian core motif. In rainbow trout and atlantic tomcod, DRE sequences were demonstrated by Berndtson and Chen (1994) and Roy *et al.* (1996). If differences exist between mammalian and fish nucleotide sequence in the core motif of the DRE, the use of mammalian derived DRE in gel retardation analysis with fish cytosol, might result in undetectable TCDD induced AhR-DRE complexes.

The presented data may suggest that the AhR pathway is only marginally present in flounder with possibly a low responsiveness (no AhR-DRE complexes). This would be in line with reports on low responsiveness of the flounder hepatic microsomal CYP1A enzyme system towards complex PCB mixtures (Besselink *et al.* 1996). However, in previous studies we have

observed high CYP1A inducibility in flounder upon exposure to TCDD, despite low AhR levels and undetectable AhR-DRE complexes. Hence, the flounder hepatic AhR pathway is functional and the apparent low responsiveness of flounder CYP1A upon exposure to PCBs can not solely be attributed to a non-functional AhR pathway. In a previous study we observed inhibitory effects of CloA50 on TCDD induced catalytic activity but in contrast additive effects on CYP1A protein levels (Besselink *et al.*, 1997a) Therefore, we posed interference of PCB congeners with CYP1A catalytic activity as a hypothesis for low responsiveness of CYP1A activity in flounder following exposure to PCB mixtures. Here, the effect of *in vitro* addition of PCB congeners and CloA50 on hepatic microsomal EROD activity in flounder was studied and compared to their effects on rat hepatic microsomal EROD activity.

The results clearly demonstrate the potency of all congeners and CloA50 to inhibit hepatic microsomal CYP1A activity *in vitro* in a competitive way with inhibition constants ($K_i$) close to the Michaelis constant ($K_m$) for ethoxyresorufin. Of the PCB congeners tested, the non-ortho PCB-126 was found to be the most potent inhibitor of the CYP1A activity in both rat and flounder ($IC_{50}$: 0.24 and 0.30 μM respectively), followed by PCB-77, PCB-169 and the di-ortho PCB-153 (Table 6.2). The order with which the tested PCBs were capable of EROD inhibition reflects their potencies to induce CYP1A (Safe, 1994). The commercial PCB mixture CloA50 was intermediate in potency, probably due to the fact that CloA50 is composed of non-ortho- and ortho-substituted PCBs. Overall, the concentration of PCB at which 50% inhibition of CYP1A activity was observed was lower in rat than in flounder, except for the di-ortho-substituted PCB-153.

Surprisingly, inhibition constants ($K_i$'s) calculated for the various tested PCBs were higher in rat than in flounder. This consistently indicates that the cytochrome P4501A system in flounder binds the various PCB congeners with greater affinity than the cytochrome P4501A system in rat. In addition, the present data suggest a higher catabolic efficiency of the flounder CYP1A enzyme system towards ethoxyresorufin as compared to the rat CYP1A system. Fig. 6.6 clearly demonstrates that the maximum EROD activity in flounder hepatic microsomes is reached at lower ethoxyresorufin concentrations than in rat hepatic microsomes. In addition, the flounder Michaelis constant for ethoxyresorufin ($K_m$) is lower than in rat (0.095 and 0.232 μM respectively). The higher catabolic rate of the CYP1A system towards ethoxyresorufin in flounder as compared to rat is also demonstrated when EROD activity is expressed per nmol total hepatic cytochrome P450. In rat, administration of 25 μg TCDD/kg bodyweight results in a specific activity of 3.8 nmol/min/nmol P450 whereas in flounder, a specific activity of 5 nmol/min/nmol P450 is observed after administration of 10 μg TCDD/kg body weight (Lans, 1995; Besselink *et al.*, 1997b).

*In vitro* inhibition of hepatic microsomal EROD activity by PCB congeners was also reported by Gooch *et al.* (1989) in scup (*Stenotomus chrysops*). Inhibition of CYP1A catalytic

activity by PCB-77 was also observed in the teleost hepatoma cell line PLHC-1 (Hahn *et al.*, 1993). Competitive inhibition of CYP1A activity by PCB residues present in hepatic tissue might, at least partly, explain low responsiveness of hepatic CYP1A activity in a number of fish species upon exposure to PCBs (Wirgin *et al*, 1992; Celander and Förlin, 1995). However, other mechanisms can not be ruled out since decreased EROD activity is sometimes accompanied by decreasing levels of CYP1A (Gooch *et al.*, 1989; White *et al.*, 1997). Other mechanisms, such as hepatotoxicity, alterations of heme synthesis and suicide substrate inactivation of CYP1A by reactive metabolites may also be of influence on suppression of hepatic CYP1A catalytic activity (Gooch *et al.*, 1989). In addition, Elskus *et al.* (1992) showed that CYP1A expression and activity in winter flounder (*Pseudopleuronectes americanus*) from PHAHs contaminated sites was regulated endogenously by oestrogens at a pretranslational level. White *et al.* (1997) studied the regulation of scup hepatic cytochrome P4501A1 induction following exposure to PCB-77 and observed a dual effect of transcriptional CYP1A1 mRNA induction and post-transcriptional CYP1A1 protein and activity suppression. Furthermore, species differences in response to environmental PHAH exposure are known to exist (Eggens *et al.*, 1996).

In conclusion, our study clearly demonstrated the presence of the AhR in flounder hepatic cytosol. AhR levels in flounder are clearly lower than in rodents. Although flounder is not as sensitive as rat towards CYP1A induction (Besselink *et al.*, 1997b), the competitive inhibition of the CYP1A catalytic activity by PCBs occurs at similar PCB concentrations in flounder as in rat. In addition, flounder CYP1A seems to be more efficient in metabolising ethoxyresorufin than rat CYP1A. The observed inhibition of EROD activity at PCB levels near the $K_m$ for ethoxyresorufin, underscores the necessity of caution when using CYP1A catalytic activity as dependable biomarker for monitoring exposure of fish to environmental pollutants such as PHAHs.

**Acknowledgement**

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# Chapter 7
# Alterations in Plasma and Hepatic Retinoid Levels in Flounder (*Platichthys* flesus) after Chronic Exposure to Contaminated Harbour Sludge in a Mesocosm Study.


**Abstract**

Flounder (*Platichthys flesus*) were exposed to polluted harbour sludge or to sludge wash-off in mesocosms for three years. At the end of the study, flounder from all mesocosms were sacrificed and plasma and liver were isolated for determination of retinoid levels. In addition, effects of harbour sludge on liver tumour development and skin infections, on spermatogenesis and testicular steroidogenesis, on plasma steroid levels and on induction of the CYP1A enzyme system were determined in flounder. In addition, levels of heavy metals and PHAHs were determined in both flounder and sediment. Here, we present the effects on retinoid levels in flounder. Data on other parameters are presented elsewhere. Retinol concentrations in flounder plasma and liver were significantly reduced in mesocosms containing polluted harbour sludge and sludge wash-off as compared to the control mesocosm. Retinoid stores in the liver, measured as the concentration of retinyl palmitate, were also reduced significantly in the mesocosm containing sludge wash-off as compared to the control mesocosm but not in the directly polluted mesocosm. A negative non-linear association was found between hepatic retinol concentrations and CYP1A protein levels ($r^2$=0.69; $p<0.05$), suggesting the involvement of PHAH and PAH inducible enzymes. The present study clearly indicates that long-term exposure to environmental contaminants in complex matrices decreases retinoid levels in flounder.



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*Aquat. Toxicol.*, in press

## Introduction.

Several studies have indicated the existence of a causal relationship between the occurrence of high incidences of diseases, such as certain skin diseases and liver tumours, in coastal and marine fish populations and the presence of high concentrations of environmental pollutants, such as persistent organohalogens (Peters et al., 1987; Metcalfe et al., 1988; Köhler, 1989; Vethaak. et al., 1996; Vethaak and Jol, 1996; Vethaak and Wester, 1996). The mechanism by which persistent organohalogens may cause these negative health effects in fish is not clear. A possible involvement of the vitamin A metabolism in the toxicity of PHAHs in mammals has been suggested by a number of authors based on the striking resemblance between symptoms associated with a hypovitaminosis A and the toxicopathological effects of PHAH exposure (Kimbrough, 1974; Brouwer, 1987), such as dermatological symptoms (Van Putten et al., 1970) and suppression of immune function (Mark et al., 1983; Safe, 1994). Additional evidence for a role of vitamin A in PHAH toxicity stems from observations in mammals that retinoids suppress the process of carcinogenesis *in vivo* (Sporn and Roberts, 1983) and modulate PHAH induced carcinogenesis (Flodström et al., 1991).

From a number of studies it is clear that PHAHs (Hakånsson and Ahlborg, 1985; Brouwer et al., 1989a,b) and PAHs (Leo et al., 1984a) can reduce vitamin A levels in mammalian tissues. The proposed mechanisms by which PHAHs can alter retinoid homeostasis are multiple and may involve modulation of retinoid metabolising enzymes, alterations of hepatic storage capacity (Jensen et al., 1987; Spear et al., 1988) or interference of hydroxy PCB metabolites with the plasma transport of retinol (Brouwer and van den Berg, 1984, 1986). At present, not much data is available on retinoid disrupting effects of organic contaminants in fish (Spear et al., 1992). Palace and Brown (1994) reported altered retinoid concentrations in tissues of lake char (*Salvelinus namaycush*) exposed to 3,3',4,4',5-pentachlorobiphenyl. However, short term (3-10 days) exposure of flounder (*Platichthys flesus*) to either Clophen A50, a commercial mixture of PCBs, or TCDD did not result in altered plasma or hepatic retinoid levels (Besselink et al., 1996, 1997).

In the present experiment, the effects of long-term exposure to environmental pollutants on plasma and hepatic retinoid levels in flounder were investigated. For this purpose, flounder were exposed to chemically contaminated dredge spoil in a mesocosm study design for 3 years. The present study is part of a larger study design, focusing on tumour formation and skin diseases in relation to exposure levels of various environmental pollutants via harbour sludge.

## Material and Methods

*Chemicals.* The following chemicals were purchased from Merck, Darmstadt, Germany: tris(hydroxymethyl)amino methane (Tris), diisopropyl ether, hydrochloric acid, sodium chloride,

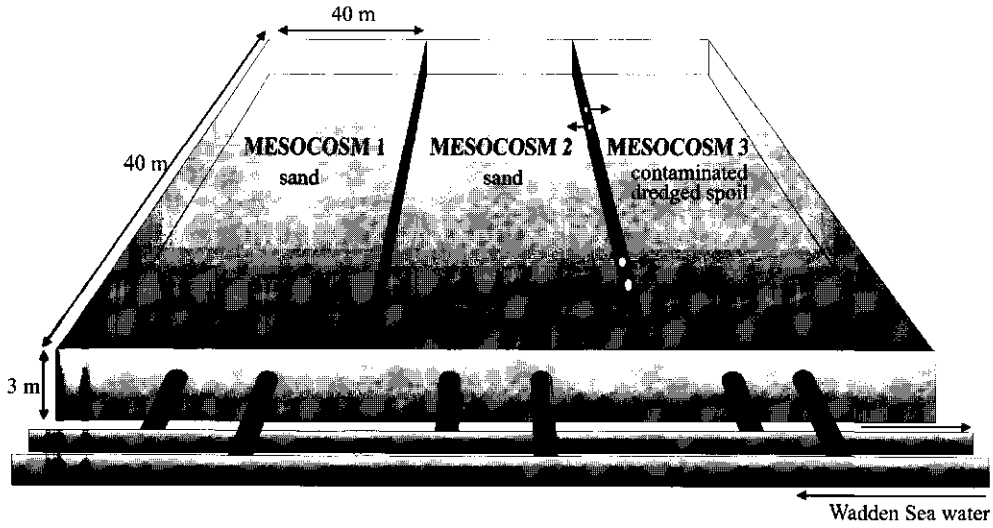


**Figure 7.1.** Schematic view of the three mesocosms. Mesocosm 1 contained relatively clear Wadden Sea sand, with water abstracted from the Dutch Wadden Sea. Mesocosm 2 also contained relatively clean Wadden Sea sand, but the water shared a common circulation with mesocosm 3. The latter was loaded with contaminated dredged spoil from Rotterdam harbour. The water was taken from the Dutch Wadden Sea. Drawing according to Vethaak *et al.* (1996).

sodium citrate, sucrose, 2,6-di-*ter*-butyl-4-methylphenol (BHT) and ethyl acetate (Lichrosolv). Methanol (HPLC grade) was purchased from Janssen Chimica, Tilburg, the Netherlands. Retinol, retinyl palmitate, and retinyl acetate were purchased from Fluka Chemie, Bornem, Belgium.

*Experimental design.* Flounder (*Platichthys flesus*) used in the mesocosm study design were 1-year-old individuals, captured in April 1990 in the Dutch Wadden Sea (Balgzand). They were exposed to chemically contaminated dredge spoil for 3 years at contaminant levels similar to those observed in Dutch coastal waters (Vethaak et al., 1996). Three concrete mesocosm systems of 40x40x3 m were set up as described by Vethaak et al. (1996) (Fig. 1). The reference mesocosm (mesocosm 1) contained relatively clean sandy sediment and water from the Dutch Wadden Sea. Mesocosm 2 contained the same sediment as mesocosm 1 but was indirectly polluted by receiving wash-off from the third polluted mesocosm. The third mesocosm contained contaminated dredge spoil from Rotterdam harbour. The three mesocosms were stocked with 1200, 1200, and 400 fish respectively (Vethaak et al., 1996).

*Sample collection.* Flounder were anaesthetised as described by Janssen et al. (1997). After extracting blood from the caudal vein, 6% sodium citrate in 0.7% sodium chloride was added as anticoagulant (50 µl/ml blood). The blood was centrifuged at 800 g in a Eppendorf centrifuge, the plasma was collected and stored at -20°C until analysis. After collecting blood, flounder were sacrificed by decapitation. Livers were carefully dissected free from gall bladder and a small piece of tissue from each liver was quickly frozen in liquid nitrogen and stored at -80°C until further processing.

*Preparation of liver homogenates.* Individual pieces of liver tissue were homogenised in 1.5 ml of a 50 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose using 10 strokes with a motor-driven Potter-Elvehjem glass and teflon homogenizer. The homogenate was stored at -20°C until hepatic retinoid analysis

*Extraction and analysis of retinoids.* Plasma and hepatic retinoids were analysed using the method of Brouwer et al. (1989a) with some minor modifications. Plasma samples, or hepatic homogenates (50 µl) were vortexed with 50 µl methanol, containing retinyl acetate as internal standard and 0.1 % 2,6-di-*ter*-butyl-4-methylphenol (BHT) (w/v) as an anti-oxidant, and 100 µl diisopropyl ether. Plasma and liver retinoids were extracted overnight at -20°C. After extraction the ether phase was removed and filtered over a 0.45 µm filter (Millipore, Etten Leur, The Netherlands), evaporated under nitrogen and finally resuspended in either 50 µl methanol (0.1% BHT) (plasma) or 100 µl methanol:ethyl acetate (3:1) (0.1% BHT) (liver). All extractions were carried out in duplicate and under dim light. Twenty µl aliquots of resuspended extracts were analysed on a HPLC system employing a $C_{18}$ analytical column (Pecosphere, 3.3 cm length and 4.6 mm internal diameter, 3 µm particle size, Perkin Elmer). A Spectra-Physics Analytical HPLC system was used consisting of a P-2000 binary gradient pump, AS-3000 autosampler, UV-1000 UV-VIS detector and a Merck Hitachi D-2500 chromato-integrator. For the detection of retinoids a wavelength of 326 nm was used. Plasma extracts were analysed using an isocratic system with 15% water and 85% methanol, a flow rate of 1 ml/min and data collection for 10 minutes. Hepatic extracts were analysed by running at 15% water and 85% methanol for 1.5 minutes, followed by a gradient to 100% methanol for 2.5 minutes and 13 minutes at 100% methanol. Finally the column was re-equilibrated at 15% water and 85% methanol for 3 minutes.

*Determination of CYP1A indices.* For the determination of hepatic CYP1A protein content and hepatic EROD activity, pieces of liver were homogenated in a 1.15% KCl solution containing 20% glycerine. After centrifugation of the homogenate in a MSE Europe 24M high-speed centrifuge at 12,500g for 20 min, the supernatant was centrifuged in a Centricon T-1055 ultra-centrifuge at 100,000g for 1h. The remaining microsomal pellet was resuspended in a 0.1 M

phosphate buffer (pH 7.4) (Eggens and Galgani, 1992). Hepatic microsomal EROD activity and CYP1A protein content were then measured as described by Eggens et al. (1996).

*Statistics.* Statistical analysis was performed by using the software package SPSS/PC+™ (SPSS Inc., Chicago, IL). Data were tested for normality and homogeneity of variance using the Chi-square test and the Bartletts test, after which data were analysed statistically by one-way analysis of variance, followed by a least significant difference test. The data are expressed as the means ± standard error of the mean (SEM). Associations between hepatic mixed function oxidase data and plasma and liver retinoid data were evaluated using the software package SlideWrite Plus, Version 5.00 (Advanced Graphics Software, Inc., Carlsbad, CA, USA).

**Results**

In figure 2 typical HPLC elution profiles for retinoid standards, as well as flounder plasma and liver samples are given. The retention times for the various retinoids did not differ between preparations. In flounder plasma only retinol could be detected at 3.5 min elution time (Fig. 2b), whereas in liver samples retinyl palmitate in addition to retinol could be determined at 14.2 and 3.8 min elution time respectively (Fig. 2d). In addition, some minor peaks were observed between 10-13 min elution time, which have not been identified but could represent retinoid esters with a shorter fatty acid tail, or other unsaturated fatty acids.

After 36 months of exposure to polluted harbour sludge, sludge wash-off or reference sand, plasma retinol concentrations were measured in flounder. The results are presented in Table 1. The plasma retinol levels in flounder from the directly, as well as the indirectly polluted mesocosm were significantly reduced (by 30.7 % and 28.1 %, respectively) as compared to plasma retinol levels in flounder from the reference mesocosm. No differences in plasma retinol concentrations were observed between flounder obtained from the two polluted mesocosms.

Hepatic retinol concentrations were drastically reduced (by 81.0 % and 77.0 %, respectively) in flounder exposed directly or indirectly to chemically contaminated dredged spoil for 3 years (Table 1). Large individual differences in retinyl palmitate concentrations in the same treatment groups were observed. Notably, no alterations in retinyl palmitate levels, the retinoid storage form, in flounder from the directly polluted mesocosm were observed compared to the reference mesocosm. However, flounder from the indirectly polluted mesocosm had significantly reduced retinyl palmitate concentrations (43.4 %) as compared to the reference mesocosm. When expressed as hepatic ratio retinol:retinyl palmitate, the ratio in flounder from mesocosm 3 was more decreased then in mesocosm 2 as compared to the reference mesocosm (84.0 % and 63.0 % respectively).

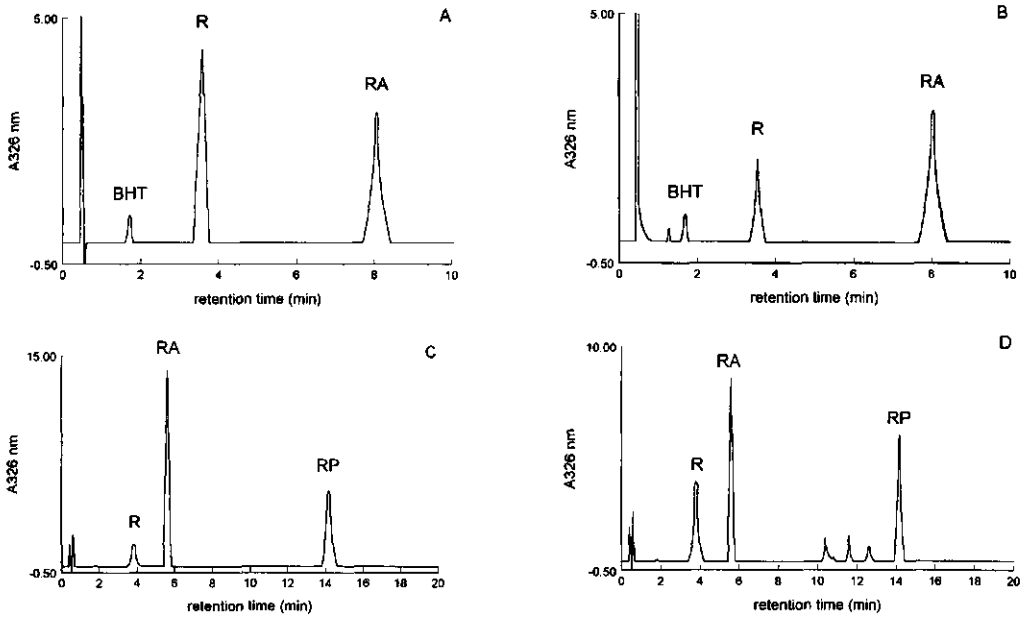


**Figure 7.2.** Typical HPLC chromatograms for (A) a standard solution containing retinol (R) and retinyl acetate (RA) used in plasma retinoid analysis, (B) flounder plasma extract, (C) a standard solution containing retinol (R), retinyl acetate (RA), and retinyl palmitate (RP) used in hepatic retinoid analysis, and (D) flounder liver extract. BHT = 2,6-di-*ter*-butyl-4-methylphenol.

CYP1A protein levels and hepatic EROD activity in flounder from the three mesocosms are given in Table 2. A significant induction in hepatic EROD activity as compared to the reference mesocosm was observed in flounder from the directly contaminated mesocosm 3. In addition, the EROD activity in the flounder from mesocosm 3 was significantly higher than in flounder from the indirect polluted mesocosm 2. Hepatic CYP1A protein levels in flounder from mesocosm 3 were significantly increased as compared to the reference mesocosm 1.

In a number of studies, involvement of the cytochrome P450 enzyme system in the metabolism of retinoids has been shown (Leo et al., 1984; Spear et al., 1988; Roberts et al., 1991; Gilbert et al., 1995). To evaluate the possible involvement of the hepatic cytochrome P450 enzyme system in the reduction of plasma and hepatic retinoid levels in flounder, hepatic

**Table 7.1.** Retinoid content in plasma and liver of flounder following 36 months of exposure to either reference sediment (mesocosm 1), indirectly polluted sediment (mesocosm 2) or contaminated dredged spoil from Rotterdam harbour (mesocosm 3).

| Mesocosm | plasma retinol (ng/ml plasma) | hepatic retinol (μg/g liver) | hepatic retinyl palmitate (μg/g liver) | hepatic ratio retinol/retinyl palmitate (ratio*100) |
|---|---|---|---|---|
| 1 | 68.7 ± 4.4 (27) | 24.8 ± 3.5 (14) | 258.4 ± 22.1 (14) | 10.0 ± 2.3 (14) |
| 2 | 49.4 ± 3.6[b] (37) | 5.7 ± 1.2[b] (19) | 145.6 ± 15.1[a] (19) | 3.7 ± 0.8[b] (19) |
| 3 | 47.6 ± 3.8[b] (21) | 4.7 ± 0.8[b] (14) | 288.9 ± 37.0 (14) | 1.6 ± 0.2[b] (14) |

Number of animals in parentheses. Data are expressed as mean ± S.E..
[a]: significant different from mesocosm 1, $p<0.01$.
[b]: significant different from mesocosm 1, $p<0.001$.

**Table 7.2.** CYP1A indices measured in livers of flounder (*Platichthys flesus*), following 36 months of exposure to either reference sediment (mesocosm 1), indirectly polluted sediment (mesocosm 2) or contaminated dredged spoil from Rotterdam harbour (mesocosm 3).

| Mesocosm | EROD activity (pmol/min/mg protein) | CYP1A protein (abs. 405 nm) |
|---|---|---|
| 1 | 57 ± 10 (15) | 0.057 ± 0.011 (15) |
| 2 | 58 ± 18 (11) | 0.054 ± 0.010 (3) |
| 3 | 166 ± 26[a,b] (14) | 0.105 ± 0.016[a] (15) |

Number of animals in parentheses. Data are expressed as mean ± S.E..
[a]: significant different from mesocosm 1, $p<0.05$.
[b]: significant different from mesocosm 2, $p<0.05$.

mixed function oxidase data and plasma and hepatic retinoid data were correlated. Only data were used from flounder in which both retinoid and mixed function oxidase parameters were analysed. No significant association between EROD activity and plasma or hepatic retinoid levels was found (data not shown). However, a significant negative non-linear association was found between hepatic CYP1A levels and hepatic retinol concentrations (Fig. 3).

In Table 3, data on mean concentrations of organic pollutants and heavy metals in sediment and flounder tissues from each mesocosm are given. A clear gradient of organic pollutants and heavy metals was observed throughout the three mesocosms with lowest levels in the reference mesocosm and highest in the directly polluted mesocosm. This gradient was reflected in CB-153 concentrations in flounder liver, CB-153, CB-77, CB-126, CB-105 and

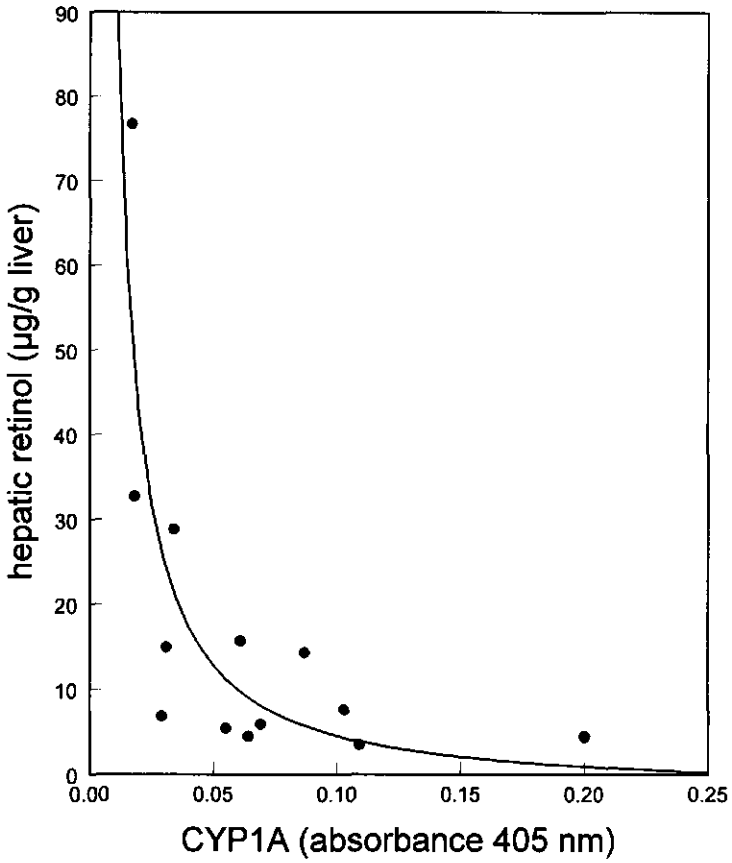


**Figure 7.3.** Negative correlation between hepatic microsomal CYP1A content and hepatic retinol concentrations in flounder (*Platichthys flesus*), chronically exposed for three years to either relatively clean sandy sediment from the Dutch Wadden Sea or contaminated dredged spoil from Rotterdam harbour. Calculations were carried out using the software package SlideWrite Plus, Version 5.00 (Advanced Graphics Software, Inc., Carlsbad, CA, USA) according to the following equation: $y=a_0+a_1 \cdot pow(x, a_2)$, where $a_0 = -1.99$, $a_1 = 0.42$, and $a_2 = -1.19$. F-statistics = 12.16 and $r^2 = 0.69$.

CB-118 concentrations in flounder spawn and 1-OH pyrene concentrations in flounder bile (Table 3). Although a gradient for heavy metals was observed across the mesocosm sediments, no gradient was apparent for Cd, Hg, Pb and Zn in flounder livers.

**Table 7.3.** Concentrations of various pollutants in sediments and flounder liver and spawn, following 36 months of exposure to either reference sediment (mesocosm 1), indirectly polluted sediment (mesocosm 2) or contaminated dredged spoil from Rotterdam harbour (mesocosm 3).

| Pollutant | Units | Mesocosm 1 | | Mesocosm 2 | | Mesocosm 3 | |
|---|---|---|---|---|---|---|---|
| **CB-153** | | | | | | | |
| Sediment < 63 µm | µg.kg$^{-1}$ OC | 58 ± 7 | (7) | 242 ± 76 | (7) | 957 ± 125 | (3) |
| Flounder liver | µg.kg$^{-1}$ lipid | 654 ± 491 | (5) | 1853 ± 1150 | (5) | 3394 ± 1612 | (5) |
| Flounder spawn | µg.kg$^{-1}$ w.w. | 6.1 | (1) | 27 | (1) | 53 | (1) |
| **CB-77** | | | | | | | |
| Flounder Spawn | ng.kg$^{-1}$ lipid | 98 | (1) | 400 | (1) | 745 | (1) |
| **CB-126** | | | | | | | |
| Flounder spawn | ng.kg$^{-1}$ lipid | 15 | (1) | 50 | (1) | 47 | (1) |
| **CB-105** | | | | | | | |
| Flounder spawn | µg.kg$^{-1}$ w.w. | 1.0 | (1) | 3.0 | (1) | 5.6 | (1) |
| **CB-118** | | | | | | | |
| Flounder spawn | µg.kg$^{-1}$ w.w. | 2.4 | (1) | 11 | (1) | 19 | (1) |
| **∑ 3 PAH** | | | | | | | |
| Sediment < 63 µm | mg.kg$^{-1}$ OC | 6318 ± 1583 | (7) | 12553 ± 1596 | (7) | 27665 ± 2452 | (5) |
| **1-OH pyrene** | | | | | | | |
| Flounder bile | ng.ml$^{-1}$ | 128 ± 51 | (52) | 734 ± 227 | (50) | 2400 ± 236 | (36) |
| **Cd** | | | | | | | |
| Sediment < 63 µm | mg.kg$^{-1}$ d.w. | 0.82 ± 0.15 | (3) | 3.20 ± 0.50 | (5) | 8.50 ± 1.50 | (5) |
| Flounder liver | mg.kg$^{-1}$ d.w. | 0.12 ± 0.09 | (3) | 0.15 ± 0.06 | (3) | 0.14 ± 0.10 | (3) |
| **Hg** | | | | | | | |
| Sediment < 63 µm | mg.kg$^{-1}$ d.w. | 0.41 ± 0.06 | (7) | 1.00 ± 0.20 | (7) | 2.40 ± 0.40 | (4) |
| Flounder liver | mg.kg$^{-1}$ d.w. | 0.41 ± 0.29 | (3) | 0.38 ± 0.09 | (4) | 0.26 ± 0.19 | (4) |
| **Pb** | | | | | | | |
| Sediment < 63 µm | mg.kg$^{-1}$ d.w. | 84 ± 10 | (7) | 124 ± 13 | (7) | 175 ± 10.0 | (4) |
| Flounder liver | mg.kg$^{-1}$ d.w. | < 2 | (3) | < 2 | (3) | < 2 | (3) |
| **Zn** | | | | | | | |
| Sediment < 63 µm | mg.kg$^{-1}$ d.w. | 268 ± 31 | (7) | 455 ± 43 | (7) | 680 ± 47 | (4) |
| Flounder liver | mg.kg$^{-1}$ d.w. | 147 ± 48 | (3) | 167 ± 57 | (3) | 118 ± 19 | (3) |

Data from Vethaak et al. (1996) and de Boer et al. (1993). ∑ 3 PAH: Sum of three PAHs benzo[b]fluoranthene, benzo[k]fluoranthene, and benzo[a]pyrene.

## Discussion

The results presented in this paper clearly indicates that plasma and hepatic retinoid levels in flounder exposed for three years to chemically contaminated dredge spoil are dramatically reduced. Across the three mesocosms an upward gradient in sediment PHAH and PAH was observed with increasing level of pollution. The increased pollution level was

reflected in internal levels of PHAHs and PAHs in flounder liver, spawn and bile. A gradient for heavy metals was also apparent in the sediment, however, this did not result in increased flounder liver concentrations (Table 3). These exposure data suggest that PHAHs and PAHs, rather than heavy metals may play a role in the observed reductions in plasma and liver retinoid levels. In the present study a non-linear association between liver retinol concentrations and hepatic cytochrome P4501A levels was observed, which underscores potential involvement of CYP1A inducing compounds, like PHAHs in the reduction of retinoid levels.

In mammals retinoid depleting effects of PHAHs or PAHs are well documented. (Brouwer and van den Berg, 1984; Leo et al., 1984a; Thunberg et al., 1984; Brouwer, 1987; Brouwer et al., 1989a; Hakånsson et al., 1991). In fish, data on retinoid changes as a result of PHAH exposure are limited. Short-time exposure of flounder to either the commercial PCB mixture Clophen A50 or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) had only minor effects on retinoid levels which were not dose-related (Besselink et al., 1996, 1997). In contrast, retinoid reducing effects in tissue of lake char (*Salvelinus namaycush*) were reported by Palace and Brown (1994) eight weeks after a single oral administration of the coplanar 3,3',4,4',5-pentachlorobiphenyl. The duration of the exposure may possibly be of influence in the apparent differences in retinoid responses of fish towards PHAH exposure.

In the present study no alterations in liver retinyl palmitate concentrations were found between heavy polluted and control sediments, except in the indirectly contaminated mesocosm, where a decrease was observed. It should be noted however that in this subset of flounder, there were three individuals with extremely low hepatic retinyl palmitate levels. In addition, it can not be ruled out that differences in food availability and sediment structure among the different mesocosms may have attributed to the observed differences in hepatic retinyl palmitate levels (Vethaak et al., 1996). Most commonly, exposure of mammals to TCDD-like compounds results in a loss of liver retinoid stores (Brouwer et al., 1989a; Mercier et al., 1990). In fish, data on effect of PHAHs on hepatic retinyl palmitate levels are contradictory. While decreased liver retinyl palmitate levels were reported by Palace and Brown (1994) in lake char upon exposure to 3,3',4,4',5-pentachlorobiphenyl and by Spear et al. (1992) in white sucker from a contaminated area, no such changes were found by Gilbert et al. (1995) in rainbow trout after administration of 3,3,',4,4'-tetrachlorobiphenyl. This might suggest that the regulation of retinoid homeostasis and the effect of environmental pollutants like PHAHs on retinoid metabolism in fish and mammals are different.

In mammals, retinoid disturbing effects of PHAHs are reported to be a result of either interference of hydroxy-PCB metabolites with the plasma transport of retinol by transthyretin (Brouwer and van den Berg, 1984; Lans et al., 1993), or modulation of retinoid metabolising enzymes (Zile, 1992). In fish, interference of the plasma retinol transport is not likely to occur since transthyretin is not involved in plasma retinol transport (Shidoji and Muto, 1977; Berni et

al., 1992; Zapponi et al., 1992). With respect to the modulation of retinoid metabolising enzymes, it is known from mammalian studies that in vitro retinoid metabolism is increased by PHAH-inducible cytochrome P450 and UDP-glucuronyl transferase (Leo et al., 1984b; Spear et al., 1988; Bank et al., 1989; Roberts et al., 1991). In rainbow trout, enhanced in vitro 4-hydroxylation of retinoic acid was observed in the presence of NADPH, suggesting cytochrome P450 involvement (Gilbert et al., 1995). Here, a non linear association between hepatic retinol concentrations and hepatic CYP1A protein levels was observed, indicating that enhanced hepatic retinoid depletion is accompanied with increasing CYP1A induction. These observations suggest that the observed alterations in retinoid homeostasis may be a result of induced retinoid metabolism.

More evidence for modulation of retinoid metabolising enzymes by PHAHs rather than interference of hydroxy-PCB metabolites with the plasma transport of retinol as cause for retinoid alterations in flounder, comes from the present observation that the hepatic ratio retinol:retinyl palmitate, a measure for the mobilisation rate of retinol from hepatic retinoid stores, is decreased in flounder caught from the indirect as well as the direct contaminated mesocosm. This indicates reduced mobilisation of hepatic retinoid esters, most probably as a result of increased hepatic retinol metabolism. Nevertheless, modulation of enzymes controlling hepatic retinoid storage (e.g. retinyl ester hydrolase) by PHAHs can not be ruled out. Mercier et al. (1990) and Chen et al. (1992) reported decreases in activity of retinyl ester hydrolase activity upon exposure to PHAHs in mammals.

In conclusion, the present study clearly demonstrates that prolonged exposure of flounder to chemically contaminated sediment results in decreased plasma and liver retinol levels with no overall effect on hepatic retinyl palmitate levels. From mammalian studies it is known that retinoids modulate the cell-mediated immunity (Sidell et al., 1984) and play a major role in the regulation of normal growth and differentiation of epithelia (Chopra, 1983). In addition, retinoids suppress the process of carcinogenesis *in vivo* (Sporn and Roberts, 1983). Modulation of the vitamin A homeostasis in flounder by xenobiotics, e.g. PHAHs, may lead to the development of marginal vitamin A deficiency. This in turn may attribute to the high incidences of skin disorders and liver tumours observed in flounder from contaminated estuaries and coastal areas. Although not conclusive, the observed non-linear association between liver retinol concentrations and hepatic cytochrome P4501A levels may suggest a possible involvement of Ah-receptor mediated PHAH inducible biotransformation enzymes.

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# Chapter 8
# Summary and concluding remarks

The research presented in this thesis focused on the mechanistic aspects of the toxic and biochemical effects of PCBs in flounder (*Platichthys flesus*), with the aim to provide a scientific basis for the suggested involvement of PCBs in the aetiology of diseases observed in flounder. Therefore, the first goal was to study the biochemical effects of PCBs in flounder by investigating the inducibility of cytochrome P4501A and associated EROD activity and glutathione-*S*-transferase activity upon administration to PCBs and TCDD *in vivo*. Secondly, the mechanism of action of PCBs in flounder was investigated by identifying the hepatic microsomal Ah receptor pathway. In addition, the potency of individual PCB congeners and a commercial PCB mixture to specifically inhibit the catalytic CYP1A activity was studied using *in vitro* techniques. Finally, the endocrine disrupting effect of PCBs was studied by analysing the retinoid and thyroid hormone levels in flounder after acute and chronic exposure to either a commercial PCB mixture or contaminated harbour sludge.

**Summary of results**

Short-time exposure of flounder to the technical PCB mixture Clophen A50 showed minor effects on total hepatic cytochrome P450 concentrations and only a relatively slight induction in EROD activity, indicating that flounder is not very sensitive towards i.p. and oral administration of Clophen A50 (**chapter** 3 and 5). The most potent PCB congener (CB-126), in terms of mammalian derived TCDD toxic equivalents (TEQ), was found to be more potent as inducer of CYP1A protein and activity in flounder, but not to the extend as was expected based on TCDD TEQs (**chapter** 5). On the other hand, high induction of the hepatic microsomal CYP1A content and associated EROD activity were observed in flounder orally exposed to TCDD (**chapter** 4). Interestingly, TCDD induced EROD activity in flounder could be inhibited by co-treatment with Clophen A50. In contrast, administration of flounder with a combination of TCDD and CB-126 resulted in an additive effect on EROD activity (**chapter** 5). With respect to induction of hepatic CYP1A protein content, administration of combinations of both Clophen A50 and CB-126 with TCDD resulted in an additive effect compared to exposure of flounder to the individual compounds. These results indicated a direct inhibition of CYP1A catalytic activity by residual PCBs present in the microsomal suspension, rather than inhibition of transcription of the CYP1A gene or translation of CYP1A mRNA. Evidence for direct inhibition of EROD activity by individual PCB congeners and the technical PCB mixture Clophen A50 is presented in **chapter** 6.

The relative sensitivity of flounder towards TCDD exposure was established by comparing the flounder computed no effect level (CNEL) for EROD activity to lowest observed adverse effect levels (LOAELs) from other fish species and the rat (**chapter** 4). The rat was clearly more sensitive towards TCDD exposure, but other fish species, such as carp and rainbow trout, were only slightly more sensitive than flounder.

A number of biotransformation enzymes other then CYP1A are also found or suggested to be modulated by the Ah receptor signal transduction pathway. Among them are certain forms of the phase II enzyme system such as glutathione-*S*-transferase (GST). In contrast to some other fish species and mammals, hepatic cytosolic GST activity in flounder was not altered upon exposure to TCDD (**chapter** 4). Moreover, no effect on GST activity was observed in flounder after Clophen A50 exposure, whereas a slight inhibition was observed upon CB-126 administration (**chapter** 5). In contrast, a strong inhibition of GST activity was observed when flounder were treated with combinations of TCDD and either CB-126 or Clophen A50 (**chapter** 5). An explanation for these observations was not found, but it was suggetsed that GST substrate inhibition by PCB-metabolite residues could have occurred.

To better understand the contradiction in induction pattern of the flounder CYP1A enzyme system towards exposure to either PCBs or TCDD, the hepatic Ah receptor pathway in flounder was characterised (**chapter** 6). In addition, the potency of a number of PCB congeners and Clophen A50 to inhibit the CYP1A catalytic activity in vitro was studied. In **chapter** 6, evidence for the presence of low levels of Ah receptor (1-7 fmol/mg protein) in flounder hepatic cytosol was presented using protamine sulphate and hydroxylapatite analysis and velocity sedimentation on sucrose gradient. The level of Ah receptor in flounder was similar to levels of receptor reported in some other fish species, but much lower than Ah receptor levels reported in mammals. Additional evidence for the presence of the cytosolic Ah receptor in flounder liver was provided by first-strand cDNA synthesis and subsequent amplification of flounder poly A+ RNA using RT-PCR. The specificity of the 690 bp RT-PCR reaction product was established by southern blotting and hybridisation. Subsequent sequencing of the RT-PCR product showed that its deduced amino acid sequence was 75% identical to the killifish species *Fundulus heteroclitus* AhR-2 sequence and 61% identical to the *Fundulus* AhR-1 sequence, which is more similar to mammalian AhRs. Binding of the liganded Ah receptor to the DRE using a complementary pair of synthetic oligonucleotides, corresponding to wild type Ah receptor site of DRE3, could be demonstrated using rat and guinea pig derived cytosol. In contrast, DRE binding of liganded Ah receptor could not be demonstrated using flounder cytosol. These data show that the hepatic AhR pathway is only marginally present in flounder. But since a good induction of CYP1A was observed in flounder exposed to TCDD, the flounder AhR pathway is functional and the apparent low responsiveness of flounder CYP1A towards PCB exposure can not be attributed to a non-functional Ah receptor pathway.

A more plausible explanation for the low CYP1A inducibility upon PCB exposure in flounder is direct inhibition of CYP1A catalytic activity by residual PCBs present in the microsomal suspension. Evidence for substrate inhibition by PCB congeners was provided in **chapter 6.** All of the PCB congeners tested, as well as Clophen A50, were capable of in vitro inhibition of the flounder CYP1A catalytic activity in a competitive way. The competitive inhibition of CYP1A activity by PCBs occurred at similar PCB concentrations in flounder as in rat. CB-126 was found to be the most potent inhibitor of EROD activity whereas CB-153 was least potent. The inhibition constants ($K_i$) of the tested PCBs were close to the Michaelis constant ($K_m$) for ethoxyresorufin. These studies also suggested a higher catabolic efficiency of the flounder CYP1A enzyme system towards ethoxyresorufin as compared to the rat CYP1A system.

Short-term exposure of flounder towards TCDD did not result in either retinoid or thyroid alterations (**chapter** 4). In contrast alterations in retinoid and thyroid hormone levels were observed in flounder exposed to Clophen A50. But such changes were not dependent on the dose of PCB administered (**chapter** 3). These results would suggest that PCBs are less endocrine disrupting in flounder as they are in mammals. However, in a chronic exposure experiment in which flounder were exposed to contaminated harbour sludge for three years, retinol levels in both plasma and liver were reduced (**chapter** 7). In addition, a negative correlation between hepatic retinol concentrations and CYP1A protein levels was observed, indicating involvement of PHAH inducible enzymes.

**Conclusions**

From these studies, the following main conclusions can be drawn:

1. The commercial PCB mixture Clophen A50 only cause minor effects on hepatic microsomal CYP1A protein levels and associated EROD activity in flounder, even at Clophen A50 concentrations as high as 500 mg/kg body weight. This low responsiveness towards Clophen A50 exposure is observed despite a functional hepatic Ah receptor pathway in flounder.
2. The commercial PCB mixture Clophen A50 inhibit the TCDD induced CYP1A activity. This finding can, at least to some extend, be attributed to competitive inhibition of the CYP1A activity by residual PCB congeners present in the reaction mixture.
3. In contrast to rodents, short-term exposure of flounder towards PCBs or TCDD does not induce meaningful retinoid and thyroid hormone disrupting effects.
4. Long-term exposure of flounder towards contaminated harbour sludge causes a marked decline of plasma and hepatic retinoid levels.
5. Exposure up to 3 weeks to PCBs and TCDD does not induce any gross pathological effects in flounder, even at high doses of PHAHs administered.

Comparing the present results with the outcome of studies on mammals and a range of other fish species, the conclusion can be drawn that flounder is relatively insensitive to PCB exposure regarding the parameters studied. Hence this study does not provide evidence for the involvement of PCBs in the development of the lesions observed in flounder from Dutch coastal and estuarine areas.

One may speculate about the nature of other factors involved in the diseases concerned, like pollutants other than PCBs. PAHs for example, are known to bind covalently to DNA after metabolisation, thus possibly initiating carcinogenesis. One may also assume that synergistic actions may occur between certain pollutants. However, the present studies showed that both individual PCB congeners and complex mixtures of PCBs are competitive inhibitors of EROD activity in vitro. If such effects also occur in the wild, PCBs may even antagonise the toxic potential of other pollutants. For instance, inhibition of CYP1A activity by residual PCB congeners might result in reduced formation of reactive PAH metabolites and thus reducing the risk for covalent DNA binding and tumour formation. Hence, this would also decrease the likelihood that PAH initiate carcinogenesis. Furthermore, non-chemical background stressors may also be involved, such as physical disturbance and pathogenic micro-organisms in the case of infectious diseases.

A remark should be made about the usefulness of EROD activity as biomarker for monitoring exposure of fish to environmental pollutants such as PHAHs and PAHs. As a spin-off of the observed competitive inhibition of EROD activity by PCBs, one should reconsider the value of EROD activity as biomarker. On the other hand, induction CYP1A protein levels is not inhibited by PCBs. Therefore, CYP1A protein levels might be a better biomarker than EROD activity for exposure assessment of fish to aromatic compounds.

Finally, in view of the recent developments in science, more emphasis should be put on developing appropriate tools to study the role of environmental pollutants, such as PCBs or related compounds in carcinogenesis. For example, the RAS gene sequence has been identified in flounder (see appendix). Whereas in humans expression of the oncogen is characteristic for a number of tumors, this relation has yet to be elucidated in fish. Nevertheless, the study of alterations in the expression of onco- and suppressor genes might be a useful approach in further studies.

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# Samenvatting

Sinds 1983 wordt door het Rijksinstituut voor Kust en Zee/RIKZ onderzoek gedaan naar biologische indicatoren voor de waterkwaliteit. Het voorkomen van ziekten bij vispopulaties langs de Nederlandse kust wordt gezien als een mogelijke indicator. Een onderdeel van dit onderzoek vormt de studie naar de causale relaties tussen vervuiling van het marine leefmilieu en de frequentie van het voorkomen van visziekten. In 1991 is in samenwerking met de vakgroep Toxicologie van de Landbouwuniversiteit Wageningen een onderzoek gestart naar de mogelijke betrokkenheid van polyhalogeenaromaten bij het ontstaan van de ziekteverschijnselen bij bodemvissen langs de Nederlandse kust (VISEX*LUW project). Centraal in dit samenwerkingsproject staat de mogelijke rol van polychloorbifenylen (PCB's) bij het ontstaan van negatieve gezondheidseffecten bij de bot (*Platichthys flesus*).

In dit proefschrift is het onderzoek beschreven naar de biochemische en toxische effecten van aan PCBs bij de bot. Het belangrijkste doel van dit onderzoek was het verkrijgen van een goede wetenschappelijk onderbouwing van de veronderstelling dat PCB's betrokken zijn bij de ontwikkeling van aandoeningen (o.a. huidaandoeningen en levertumoren) die worden aangetroffen bij de bot langs de Nederlandse kust. De biochemische effecten die onderzocht zijn na *in vivo* blootstelling van de bot aan individuele PCB congeneren, een commercieel mengsel van PCB's (Clophen A50) en dioxine (TCDD), waren de induceerbaarheid van hepatische biotransformatie enzymen (cytochroom P4501A (CYP1A) en glutathion-*S*-transferase). Daarnaast is het toxisch werkingsmechanisme van PCB's in de bot onderzocht door het bestuderen en karakteriseren van de hepatische microsomale Ah-receptor route. Tevens is onderzoek gedaan naar de potentie van PCB's om *in vitro* specifiek de activiteit van het cytochroom P4501A systeem te remmen. Tot slot is gekeken naar mogelijke hormonale verstoringen ten gevolgen van blootstelling van botten aan PCB's, TCDD (acute studies) of vervuild havenslib uit de haven van Rotterdam (chronische studie).

**Samenvatting van de resultaten**

Kort durende blootstelling van botten aan het technisch PCB's mengsel Clophen A50 veroorzaakte geringe veranderingen in de concentratie van het CYP1A eiwit in lever microsomen, terwijl de hieraan geassocieerde enzym activiteit (EROD) slechts weinig toenam. Dit wijst erop dat botten weinig gevoelig zijn voor blootstelling aan Clophen A50 via intra peritoneale of orale weg (**hoofdstuk** 3 en 5). Daarentegen bleek toediening van de meest

toxische PCB congeneer, PCB-126, wel te leiden tot inductie van zowel het CYP1A eiwit als EROD activiteit. Echter, wanneer de toxiciteit van PCB-126 werd uitgedrukt in de van zoogdieren afgeleide TCDD toxische equivalenten (TEQ's), dan bleek het inductieniveau lager te zijn dan als zou mogen verwacht op basis van TCDD TEQ's (**hoofdstuk** 5). Een sterke inductie van het CYP1A eiwit en CYP1A activiteit werd wel waargenomen in botten blootgesteld aan TCDD (**hoofdstuk** 4). Opvallend genoeg bleek de TCDD geïnduceerde EROD activiteit geremd te worden door Clophen A50, terwijl toediening van een combinatie van TCDD met PCB-126 juist een additief effect had op het EROD inductieniveau (**hoofdstuk** 5). Met betrekking tot het CYP1A eiwit werd gevonden dat blootstelling van botten aan combinaties van TCDD met Clophen A50 of PCB-126 leidde tot hogere concentraties CYP1A eiwit in lever microsomen dan blootstelling aan de afzonderlijke chemicaliën. Deze resultaten duiden op een directe inhibitie van de katalytische activiteit van het CYP1A door PCB congeneren nog aanwezig in de lever van Clophen A50 blootgestelde botten. Inderdaad bleken zowel het technische PCB mengsel Clophen A50 als ook een aantal PCB congeneren instaat te zijn de EROD activiteit *in vitro* te remmen (**hoofdstuk** 6). De relatieve gevoeligheid van de bot voor blootstelling aan TCDD werd bepaald door de CNEL (computed no effect level) te vergelijken met de LOAEL's (lowest observed adverse effect level) van andere vissoorten en ratten (**hoofdstuk** 4). Hieruit bleek dat ratten gevoeliger zijn voor blootstelling aan TCDD dan botten, maar dat vissoorten als de karper en regenboog forel in vergelijking de bot slecht weinig gevoeliger zijn.

Er wordt verondersteld dat naast CYP1A nog een aantal biotransformatie enzymen onder controle staan van de Aryl hydrocarbon (Ah) receptor. Hiertoe behoren onder ander een aantal isovormen van glutathion-*S*-transferase (GST). In tegenstelling tot observaties in andere vissoorten en zoogdieren, werd er geen verandering in hepatisch cytosolaire GST activiteit waargenomen in botten blootgesteld aan TCDD (**hoofdstuk** 4). Ook na blootstelling aan Clophen A50 werd er geen effect op de GST activiteit in de bot gevonden, terwijl toediening van PCB-126 een kleine daling in GST activiteit tot gevolg had (**hoofdstuk** 5). Daarentegen werd een sterke remming van de GST activiteit waargenomen in botten blootgesteld aan combinaties van TCDD en Clophen A50 of TCDD en PCB-126 (**hoofdstuk** 5). Als mogelijke verklaring voor deze waarneming kan remming van de GST activiteit door PCB metabolieten aanwezig in het reactiemengsel worden genoemd.

In **hoofdstuk** 6 is de Ah receptor route in de bot gekarakteriseerd, met als doel inzicht te krijgen in het CYP1A inductie patroon na blootstelling aan PCB's en TCDD. Daarnaast is de potentie van Clophen A50 en verschillende PCB congeneren bestudeerd om de CYP1A activiteit *in vitro* te remmen. De aanwezigheid van de cytosolaire Ah receptor (1-7 fmol/mg eiwit) in de lever van de bot is in **hoofdstuk** 6 aangetoond. De concentraties Ah receptor in de bot kwamen overeen met Ah receptor concentraties in andere vissoorten, maar waren

beduidend lager dan de concentraties in zoogdieren. Additioneel bewijs voor de aanwezigheid van de Ah receptor in de bot werd geleverd door first-strand cDNA synthese en amplificatie van poly A+ RNA uit lever van de bot met behulp van RT-PCR. De specificiteit van het 690 bp grote RT-PCR reactie produkt is vastgesteld met behulp van southern blotting en hybridisatie. Sequencing van het reactie produkt toonde aan de aminozuursequentie voor 75% identiek was aan de killifish (*Fundulus heteroclitus*) AhR-2 sequentie en voor 61% aan de killifish AhR-1 sequentie. Hierbij dient te worden opgemerkt dat de killifish Ah-1 een grotere overeenkomst vertoond met AhR's uit zoogdieren. Binding van het TCDD-AhR complex aan de DRE is bestudeerd door gebruik te maken van een complementair paar synthetische oligonucleotiden, corresponderend met wild type Ah receptor (DRE3). Binding van het TCDD-AhR complex aan de DRE werd aangetoond in cytosol afkomstig uit ratten en chinese biggetjes, maar niet in cytosol afkomstig uit botten. Deze resultaten suggereren dat in botten de Ah receptor route niet compleet aanwezig zou zijn. Maar gezien het feit dat een goede CYP1A inductie gevonden werd in botten blootgesteld aan TCDD, kan de lage gevoeligheid van de bot voor PCB's niet alleen verklaard worden door een marginaal aanwezige Ah receptor route.

Een betere verklaring voor de matige induceerbaarheid van de EROD activiteit door PCB's, is directe remming van de CYP1A katalytische activiteit door PCB congeneren nog aanwezig in de microsomale suspensie. In **hoofdstuk** 6 zijn aanwijzingen voor deze substraat remming gevonden. Alle 6 geteste PCB congeneren en Clophen A50 waren in staat de CYP1A activiteit van de bot competitief te remmen. De concentraties waarbij PCB's remming veroorzaakten, waren vergelijkbaar met de PCB concentraties die de rat CYP1A activiteit remden. PCB-126 was de sterkste remmer, terwijl PCB-153 de minst potente remmer van de EROD activiteit was. De inhibitie constanten ($K_i$) van de geteste PCB's bleken in de zelfde orde van grote te liggen als de Michaelis constante ($K_m$) voor ethoxyresorufine. Daarnaast kon uit de resultaten worden geconcludeerd dat het CYP1A enzym systeem van de bot een hogere catabole activiteit ten opzichte van ethoxyresorufine bezat, dan het CYP1A enzym systeem van de rat.

Veranderingen in de vitamine A en schildklierhormoon huishouding van de bot werden niet gevonden na kort durende blootstelling aan TCDD (**hoofdstuk** 4). Daarentegen werden endocriene veranderingen wel aangetroffen in botten blootgesteld aan Clophen A50 (**hoofdstuk** 3). Deze verstoringen in de vitamine A en schildklierhormoon huishouding waren echter gering en bleken niet afhankelijk te zijn van de toegediende dosis PCB. Op basis van deze kort durende blootstellingsstudies zou geconcludeerd kunnen worden dat PCB's geen hormonale verstoringen in de bot veroorzaken. Verlaagde retinol gehalten in zowel plasma als lever van de bot werden wel gemeten in een chronische blootstellingsstudie waarin botten 3 jaar werden blootgesteld aan vervuild havenslib uit de haven van Rotterdam (**hoofdstuk** 7). Tevens

werd een negatieve correlatie gevonden tussen de hepatische retinol concentraties en CYP1A eiwit gehalten. Dit impliceert betrokkenheid van PCB induceerbare enzymen.

**Conclusies**

Uit het beschreven onderzoek kunnen de volgende conclusies worden getrokken:

1. Het commerciële PCB mengsel Clophen A50 veroorzaakt slechts kleine veranderingen in de concentratie van het hepatisch microsomal CYP1A eiwit gehalte en EROD activiteit in de bot, zelfs niet bij zeer hoge concentraties Clophen A50 (500 mg/kg lichaamsgewicht). De lage gevoeligheid van de bot voor Clophen A50 word waargenomen ondanks de aanwezigheid van een functionele Ah receptor route.
2. Het commerciële PCB mengsel Clophen A50 remt de door TCDD geïnduceerde CYP1A activiteit. De remming kan ten dele worden verklaard door competitieve remming van de CYP1A activiteit door PCB congeneren aanwezig in het reactie mengsel.
3. In tegenstelling tot knaagdieren leidt een kort durende blootstelling van botten aan PCB's of TCDD niet tot een belangrijke verstoring van de vitamine A en schildklierhormoon huishouding.
4. Chronische blootstelling van botten aan vervuild havenslib veroorzaakt een drastische verlaging van lever en plasma retinol gehalten.
5. Omvangrijke pathologische effecten als gevolg van een maximaal 3 weken durende blootstelling aan PCB's of TCDD, worden niet aangetroffen in botten, zelfs niet bij zeer hoge concentraties PCB's of TCDD.

Wanneer de resultaten beschreven in dit proefschrift vergeleken worden met de resultaten van blootstelling van andere vissoorten en zoogdieren aan PCB's, dan moet worden geconcludeerd dat de bot relatief ongevoelig is voor blootstelling aan PCB's, met betrekking tot de bestudeerde parameters. Het uitgevoerde onderzoek levert dan ook geen bewijs voor de betrokkenheid van PCB's bij het ontstaan van aandoeningen als huidzweren en lever tumoren bij de bot, afkomstig uit Nederlandse kustwateren.

In plaats van PCB's kunnen andere chemicaliën aanwezig in het marine milieu betrokken zijn bij de ontwikkeling van de beschreven aandoeningen bij de bot. Van PAK's is bijvoorbeeld bekend dat deze groep verbindingen in staat is na metabolisatie covalent te binden aan het DNA en daarmee het carcinogenese proces te initiëren. Van een groot aantal chemische verbindingen is daarnaast bekend dat zij het toxisch effect van andere chemicaliën synergistisch kunnen beïnvloeden. Maar de in dit proefschrift beschreven studies tonen aan dat zowel individuele PCB congeneren als ook Clophen A50 de door TCDD geïnduceerde EROD activiteit *in vitro* competitief remmen. Wanneer deze competitieve remming ook *in vivo* op

treedt, dan bestaat de mogelijkheid dat PCB's een antagonistisch effect hebben op de toxische potentie van andere milieu verontreinigende chemicaliën. Zo kan bijvoorbeeld remming van de katalytische activiteit van het CYP1A eiwit door PCB's resulteren in een afname van de bioactivatie van PAK's met als gevolg een reductie in het aantal gevormde reactieve PAK metabolieten. Een daling in de hoeveelheid reactieve intermediairen verlaagt het risico op covalente DNA binding en daarmee de initiatie van carcinogenese. Naast chemische factoren kunnen ook niet chemische factoren als bijvoorbeeld pathogene micro-organismen en fysische verstoringen, betrokken zijn bij de aetiologie van visziekten.

Een opmerking moet gemaakt worden bij het gebruik van EROD activiteit als biomarker voor de blootstelling van vissen aan anthropogene stoffen. De waarde van EROD activiteit als biomarker moet, gezien het feit dat in dit proefschrift is aangetoond dat aanwezigheid van PCB's in botten leidt tot inhibitie van de EROD activiteit, worden herzien. Inductie van het CYP1A eiwit daarentegen wordt niet beïnvloed door aanwezigheid van PCB's. De bepaling van de concentraties CYP1A eiwit in de lever van vissen is daarom waarschijnlijk een betere parameter voor de mate van blootstelling van vissen aan chemicaliën als PHAK's en PAK's in het marine milieu.

Bij de bestudering van de betrokkenheid van PCB's of aanverwante stoffen bij de ontwikkeling van tumoren in vis moet, gezien de snelle ontwikkelingen in de wetenschap, de nadruk meer komen te liggen op technieken die specifiek de verschillende fasen in het carcinogenese proces belichten. Recentelijk is de sequentie van het ras-oncogen in de bot beschreven (zie appendix). Bij de mens is de expressie van dergelijke oncogenen gekoppeld aan bepaalde vormen van kanker. Een dergelijke relatie is bij vissen nog niet aangetoond. Desondanks kan bij toekomstig onderzoek naar de betrokkenheid van anthropogene stoffen bij de ontwikkeling van tumoren in vissen, bestudering van veranderingen in de expressie van oncogenen of suppressor genen een waardevolle parameter zijn.

# Curriculum vitae

Harry Besselink was born in Arnhem, the Netherlands, on November 9, 1966. Between 1979 and 1985 he was enrolled at Thomas a Kempis College (V.W.O.) in Arnhem. After his graduation in 1985 he moved to Wageningen where he started to study Molecular Science at the Wageningen Agricultural University (WAU). In March 1991 he graduated for his M.Sc. in Molecular Science with specialisation in Biochemistry and Toxicology. During this period, he conducted three 6-months practical research periods at the Department of Biochemistry (WAU) (supervisor: Dr. A.J.W.G.. Visser), the Department of Toxicology (WAU) (supervisor: Dr. Ir. C. den Besten), and the British Industrial and Biological Research Association (BIBRA), Surrey, United Kingdom (supervisors: Dr. B.G. Lake (BIBRA) and Prof. Dr. J.H. Koeman (WAU)). Following his graduation until 1996, he worked as a Ph.D. student at the Department of Toxicology (WAU) on a collaborative study on the biochemical and toxic effects of PCBs in flounder (*Platichthys flesus*), financed in part by grants from the Dutch Ministry of Transport and Public Works and Water Management, National Institute for Coastal and Marine Management/RIKZ, The Netherlands, with supervision of Dr. A. Brouwer (WAU). In September 1997, he was employed as post-doc at the Department of Toxicology on a research program investigating the toxicity of Toxaphene with emphasis on its carcinogenicity, financed by the European Community. The research described in this thesis was conducted between 1991 and 1996.

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# EXPRESSION OF RAS GENE IN FLOUNDER (*PLATICHTYS FLESUS*) AND RED MULLET (*MULLUS BARBATUS*)+

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A member of the *ras* gene family was identified here for the first time in two marine fish, the flounder (*Platichthys flesus*) and the red mullet (*Mullus barbatus*). After RT-PCR the partial sequence of *ras* gene (exons 1 and 2) has been cloned and sequenced from normal liver. At the nucleic acid level, a very high extent of homology with the human *ras* genes is observed (80% to 86%) and a perfect homolgy is observed at the amino acid level. The high conservation of the 5' region of the *ras* gene suggest that the p21 protein has identical function in higher and lower vertebrates. The identification of *ras* gene should be a first step in understanding the molecular basis of carcinogenesis in these species.

Many species of fish which inhabit estuaries and coastal zones are exposed to urban and industrial pollution. Various pollutants including heavy metals, polychlorinated biphenyls (PCBs), pesticides and polycyclic aromatic hydrocarbons (PAHs) have been identified in estuaries and coastal zones. Most of these pollutants are potential carcinogens or mutagens and tend to accumulate in aquatic organisms if they are taken up from water, food and sediment (1-2). A biological monitoring network was set up in 1992 along the French coast to collect new data reflecting biological effects of pollutants such as PAH and PCB on the metabolism of fish. For this purpose, measurement of ethoxyresorufin-O-deethylase (EROD) activity, one of the P450-dependent monooxygenase activity, was used as the exposure index. Highest activity of EROD were observed in liver from marine fish, such as dragonet (*Callionymus lyra*, Linne 1758) and red mullet (*Mullus barbatus*, Linne 1758), living in contaminated sites suggesting a possible role of the liver in the detoxication of environmental pollutants (3-4). Further, field studies by Dunn *et al.* (5) revealed the presence of aromatic DNA adducts in livers of brownbullheads (*Ictalurus nebulosus*) from PAH-polluted areas. These studies revealed that the

+ The nucleotide sequence data reported in this paper appear in the EMBL nucleotide sequence database with the following accession number : X82182 for red mullet sequence and X90910 for flounder sequence.

exposure of fish to environmental pollutants may result to their activation in electrophilic forms subsequently leading to the induction of DNA damage. In a general way, we know that in some cases DNA damage can lead to the fixation of mutations and some of them might play an essential role in the multistage carcinogenic process. Oncogenes activated by point mutation have been identified as genetic targets that may play a key role in carcinogenesis (6).

The most frequently detected oncogenes in human and in rodent malignant neoplasms are members of the *ras* gene family. *Ras* gene family consist of three genes: H-*ras* gene from Harvey sarcoma virus, K-*ras* gene derived from Kirsten sarcoma virus and N-*ras* gene from neuroblastoma (7). They have been identified in mammals, birds, insects, fish, molluscs, plants, fungi and yeasts. These genes code for highly related proteins of 21 kDa, designated as p21, which are composed of 189 amino acids and their high degree of conservation suggest that they play an essential role in the control of cell growth and differentiation. *Ras* genes are converted to active oncogenes by point mutations occuring in either codon 12, 13 or 61. By the use of the NIH3T3 transfection assay, they have been detected in a large variety of human malignancies and in a high percentage of several kinds of rodents tumors. It was demonstrated that *ras* oncogene is activated in high percentage of both spontaneous tumors and tumors induced by genotoxic carcinogens. In addition, studies on several experimental model systems using various chemicals revealed a correlation between the nature of the carcinogen and *ras* mutation (8, 9). Moreover mutations in *ras* genes are thought to be an early event of tumorigenesis (10). In mouse skin epidermis initiated with 7,12-dimethylbenz[a]anthracene (DMBA), Nelson *et al.* (11) demonstrated that activated Ha-*ras* oncogene could be detected one week after the initiation and remained dormant until a tumor promotor induces hyperplasia. For this reason, the use of *ras* oncogene activation as biomarker represent a powerful tool for the molecular epidemiologic evaluation of occupational carcinogenesis.

As for Mammals and rodent, *ras* gene activation is observed in trout hepatocarcinomas induced by carcinogens such as 7,12-dimethylbenz[a]anthracene (12), N-nitrosodiethylamine (13) and Aflatoxin B1 (14). Previous studies have described the incidence of tumors in feral fish population correlated with elevated concentration of environmental chemicals in sediment (15) or tissue (16). Association of such tumors with the presence of environmental chemicals suggests a possible etiology for their induction. Moreover, the genetic events associated with tumorigenesis in feral fish population are not well understood. *Ras* gene activation appears to be a common event of carcinogenesis since Ki-*ras* codon 12 mutations have been observed in *Winter flounder* (17) and in *Tomcod* (18) hepatic tumors. These fish were inhabiting areas contaminated with carcinogenic pollutants such as PCBs and PAHs. The aim to described more precisely the hypothesis that liver tumors in these species are associated with PAH exposure have been succesfully attempt by Fong *et al.* (12) who observed for the first time Ki-*ras* mutation on codon 12 and 61 from hepatic tumors of adult trout after a brief exposure of embryo to PAHs. These results may have important environmental implications because they suggest that a brief exposure to PAHs during sensitive stages of development may affect some fish populations.

In the present study a *ras* gene expressed in the normal liver of two marine fishs is described for the first time. The partial sequence of the first two exons of *ras* gene was obtained from cDNA clones after RT-PCR. We have chosen to work on fish species inhabiting contaminated sites of the French coast and which are currently used in the biological monitoring network such as the flounder

(*Platichthys flesus*) which inhabits the Atlantic Sea, the English Channel and the North Sea and the red mullet (*Mullus barbatus)* which inhabits the Mediterranean Sea. These fish are exposed to mutagenic and carcinogenic pollutants and the identification of *ras* gene is a first step in understanding the molecular basis of carcinogenesis in these species.

## MATERIALS AND METHODS

### *Animals*

Flounder (*Platichthys flesus*) were obtained from the Netherland Institute for Fisheries Research and red mullet (*Mullus barbatus)* were collected by trawl in the Mediterranean sea in April 1994. Portions of the liver were then frozen in liquid nitrogen for RNA analysis.

### *Cloning of cDNA using RT-PCR*

Total RNA were isolated from flounder and red mullet liver according to Chomczynski & Sacchi (1987) (19). One microgram of total RNA was reverse transcribed in a 20-$\mu$l mixture of 25 mM Tris-Hcl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 0.02 M DTT, 500 $\mu$M dNTP in presence of 100 pmoles of antisens primer 5'-ACT TGG TGT TGT TGA TGG CA-3' which correspond to a consensus located in the second exon of various vertebrate *ras* genes (primer RT1) and 200 units of reverse transcriptase RNase H- superscript (Gibco BRL) at 37°C for 1 h. After the incubation the mixture was heated at 95°C for 10 min. For the amplification of the cDNA, the Polymerase Chain Reaction (PCR) (20) was carried out in a 50-$\mu$l PCR mixture containing 10 mM KCl, 10 mM $(NH_4)_2$ $SO_4$, 20 mM Tris-HCl (pH8), 2 mM MgSO4, 200 $\mu$M of each dNTP with the above RT reaction mixture (10 $\mu$l), 1 unit of Vent DNA polymerase (Biolabs, New England) and 30 pmoles of each of the sens primer 5'-ATG ACG GAA TAT AAG CTG GT-3' which correspond to a consensus located at the beginning of the first exon of various vertebrate *ras* gene (primer F1) and the antisens primer RT1. After a heat shock for 5 min at 94°c, the conditions for PCR were at 94°C for 30 s, 60°C for 30 s, 72°C for 20 s (30 cycles) using a thermal cycler (Biometra, Kontron Instrument).

In order to confirm the nature of the PCR products, semi-nested PCR has been carried out: 0.8 $\mu$l of the first PCR was added to the 50 $\mu$l PCR mixture as described previously. The primers used for the semi-nested PCR was the antisens primer 5'-ACA CAG AGG AAG CCC TCT CC-3' (primer RT2) and the F1 primer.

PCR products were isolated and cloned into the Sfr I site of pCR-ScriptTM (SK+) plasmid vector (Stratagene). Three different cDNA clones were sequenced on a 373 A DNA sequencer (Applied Biosystems) according to the dye terminator procedure.

## RESULTS AND DISCUSSION

### *Cloning of Platichthys flesus and Mullus barbatus ras cDNA using RT-PCR*

For cloning *ras* cDNA, RT-PCR was performed using total RNAs from liver tissue of *Platichthys flesus and Mullus barbatus.* Specific oligonucleotide primers derived from a consensus of various vertebrate *ras* gene were used for the Reverse Transcription (RT) and the Polymerase Chain Reaction (PCR) procedure. For each fish a 260 bp PCR product was obtained corresponding to the expected size according to the human sequence. In order to confirm the nature of this PCR product, semi-nested PCR was carried out with the antisens primer RT2 and the F1 primer. A 240 bp fragment was obtained (Fig.1) and this product hybridized with the specific human Ha-*ras* cDNA probe (Oncogene Science) (data not shown).

The cDNA products from *Mullus barbatus* and *Platichthys flesus* were cloned into the pdirect vector (Clontech). The nucleotide sequences of three clones from each fish were determined. Both

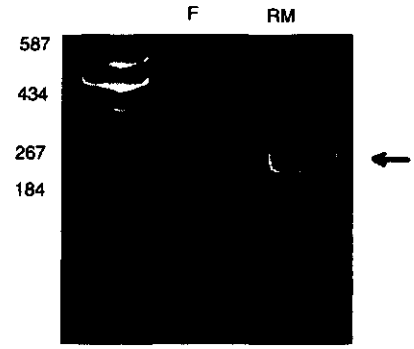


**Fig.1. Electrophoretic patterns of RT-PCR products of total RNAs from liver tissue of Flounder and Red mullet.**
The 240 bp fragment was amplified with the RT2 and F1 primers, separated on 9 % acrylamide gel and stained with ethidium bromide.

nucleotide sequences have been compared with trout, goldfish, dragonet and human *ras* gene (Fig.2). These 240 bp show a very high homology with the first exon and part of the second exon of the different *ras* genes. They present similar homology with the others *ras* genes from fish (from 89% to 93%) and a very high extent of homology with the human *ras* genes is observed (81% to 86%) (Table 1). Higher homology was found between these fish and the human 5' region of Ha-*ras* gene suggesting that we cloned the 5' Ha-*ras* gene of these marine fish. *Ras*-related genes have been identified in normal liver from goldfish (21) and trout (22). In both case, the sequence identified was mostly homologous to human Ki-*ras* gene. It was hypothetize by Nemoto *et al.* (21) that goldfish should possess three kinds of *ras* genes since the autors had isolated various clones which were classified in three groups who belong to the *ras* family. However, the construction of a cDNA library is required to determine whether the 5' region of the *ras*-related gene identified here belongs either to Ha-, K- or N-*ras* .

Base changes who occured among the flounder, red mullet, rainbow trout, goldfish and mammalian *ras* gene are located on the third base of the codon and therefore are neutral for amino acid encoding. Analysis of the predicted amino acid sequence revealed a perfect homology between these fish and other species confirming that the N terminal domain is necessary for the protein function (Fig.3). Recently, we have cloned and sequenced the 5' region of the *ras* cDNA from dragonet (*Callionymus lyra*) (Vincent *et al.*, in preparation). The evolutionary time of the divergence between these three marine fishs (dragonet, red mullet and flounder) and the precedent fish is of about 130 million years and between mammals and fish the evolutionary time is of 350-400 million years (23). It is interesting to examine 5' region of *ras* genes in phylogenetically advanced fish such as flounder (Pleuronectidei family), dragonet (Callionymidei family) and red mullet (Percoidei family). It is well documented that the 5' region of *ras* genes is highly conserved across the evolution. Our results suggest that the N-terminal region of *ras* proteins of marine fish is necessary for the binding of GTP and GDP, as it has been demonstrated in other species (7-24-25).

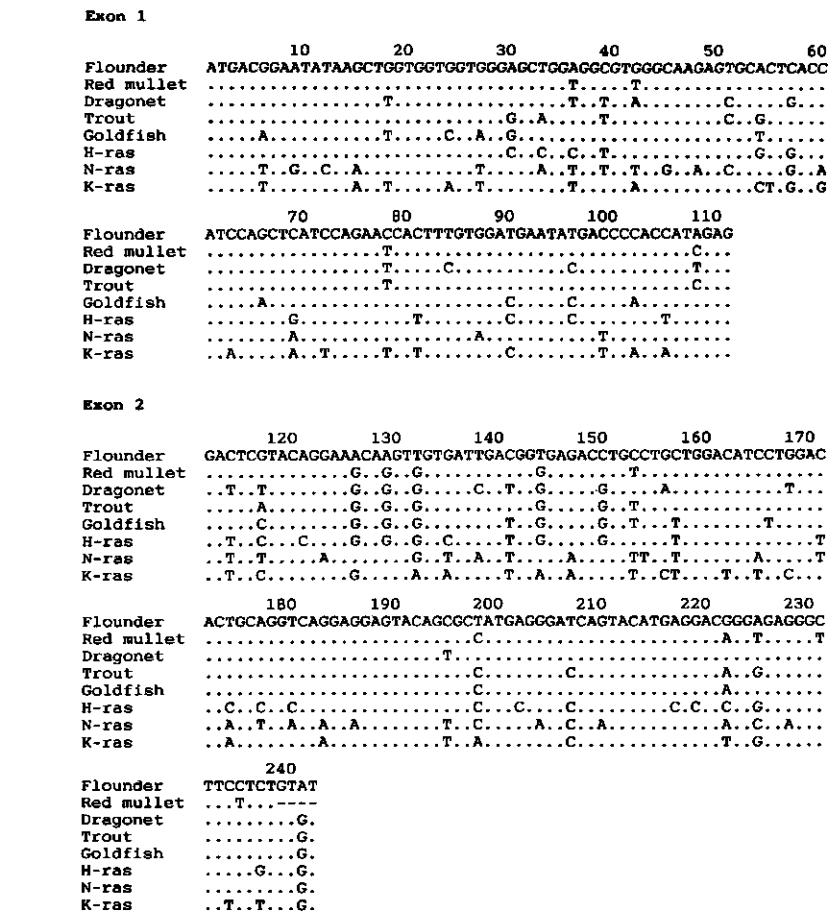


**Figure 2 . Comparison of DNA sequence of RT-PCR products from Flounder and Red mullet with Trout, Goldfish, Dragonet and Human *ras* gene sequences.**

**Table 1 : Comparison of the nucleotide sequences of the Flounder and Red mullet *ras* gene (exon 1 and partial exon 2) with Trout, Goldfish, Dragonet and human *ras* gene sequences**

| | Flounder | Red mullet |
|---|---|---|
| **Ha-ras** | 86% | 84% |
| **N-ras** | 82% | 80% |
| **K-ras** | 82% | 81% |
| **Trout** | 92% | 93% |
| **Goldfish** | 90% | 89% |
| **Dragonet** | 90% | 89% |

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                  10        20        30      ↓ 40        50        60
Flounder    MTEYKLVVVGAGGVGKSALTIQLIQNHFVDEYDPTIEDSYRKQVVIDGETCLLDILDTAG
Red mullet  ............................................................
Dragonet    ............................................................
Trout       ............................................................
Goldfish    ............................................................
H-ras       ............................................................
N-ras       ............................................................
K-ras       ............................................................

                  70        80
Flounder    QEEYSAMRDQYMRTGEGFLC
Red mullet  ....................
Dragonet    ....................
Trout       ....................
Goldfish    ....................
H-ras       ....................
N-ras       ....................
K-ras       ....................
```

**Figure 3 . Homology of the predicted amino acid sequences of Flounder and Red mullet *ras* gene (exon 1 and partial exon 2) with the sequences of the corresponding genes in various species.**The numbers indicate the position from the N-terminal region of the Ha-ras protein. The arrow indicates predicted exon-intron boundaries in Flounder and Red mullet which are the same as those of other species.

The available sequence contains the hot-spot of mutation responsable for the transforming phenotype of *ras* gene. This sequence could be used as a probe to determine whether *ras* gene is overexpressed or mutated in liver tumors of marine fish as we have recently observed in liver hyperplasia of *Callionymus lyra*. A Threonine (ACA) was substituted for alanine (GCA) (Vincent *et al.* in preparation) and should be implicated in initiation of liver tumorigenesis. The census of flounder and red mullet exhibiting various of kinds of neoplasms is currently underway in contaminated sites of the French coast such as the Seine estuary and the golf of Fos sur mer. In the next future, the potential correlation between these neoplasms and the accumulation of contaminants in the fish tissue or between these lesions and the presence of contaminants in the environment will be tested in order to determine whether fish neoplasms could be one of the long term health effects of pollutants.

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