Assessment of cytochrome P450 1A in dab as biomarker of exposure to polychlorinated biphenyls and related compounds

Promotor:	Dr. J.H. Koeman Hoogleraar in de Toxicologie
Co-promotor:	Dr. J.P. Boon Senior onderzoeker Nederlands Instituut voor Onderzoek der Zee

Hedwig Maria Sleiderink

Assessment of cytochrome P450 1A in dab as biomarker of exposure to polychlorinated biphenyls and related compounds

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus,
dr. C.M. Karssen,
in het openbaar te verdedigen
op maandag 23 september 1996
des namiddags te vier uur in de Aula
van de Landbouwuniversiteit te Wageningen

Stellingen

behorende bij het proefschrift *Assessment of cytochrome P450 1A in dab as biomarker of exposure to polychlorinated biphenyls and related compounds* van Hedwig Sleiderink

1. De keuze voor de schar als monitoring species kan achteraf op wetenschappelijke gronden als juist worden beschouwd.
-dit proefschrift

2. De EROD bepaling in schar is een goede graadmeter voor de temperatuur van zeewater.
-dit proefschrift

3. Indien men vervuiling van PCBs en verwante stoffen wenst te monitoren door middel van CYP1A metingen in Noordzee schar, verdient het aanbeveling deze metingen aan het eind van de herfst uit te voeren.
-dit proefschrift

4. In tegenstelling tot wat Roose *et al.* aannemen, zijn er wel degelijk verschillen tussen de geslachten waar te nemen bij de CYP1A metingen in de schar.
-Roose, P., K. Cooreman en W. Vyncke (1993). ICES E:15
-dit proefschrift

5. De CYP1A meting in schar kan de chemische analyse van PCBs en verwante stoffen niet geheel vervangen.

6. Aangezien wordt aangenomen dat lipofiele contaminanten in biota proportioneel accumuleren met het vetgehalte, is het beter concentraties van lipofiele contaminanten te kwantificeren op basis van massa en vetgehalte dan op basis van massa alleen.

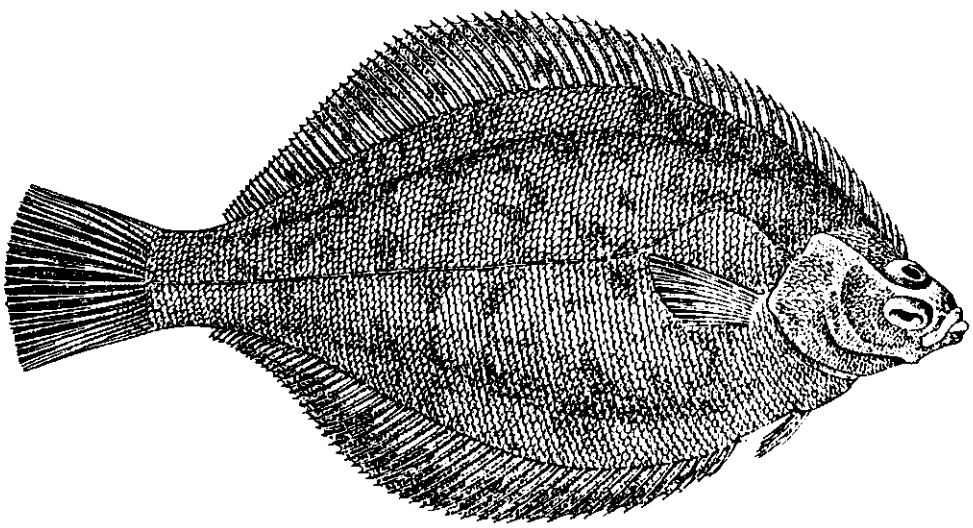
7. Het is onbegrijpelijk dat het gebruik van evertebraten in dierproeven niet geregeld is in de Wet op de dierproeven.

8. De Van Dale zou zijn gezag niet moeten laten gelden op het gebied van de taxonomie en de schar, net als biologen, *Limanda limanda* moeten noemen.

9. Gebrek aan toekomstperspectief in de academische wereld is de reden dat veel jonge onderzoekers de wetenschap de rug toe keren. Er is hier sprake van een verloren generatie.

10. Vanuit een bewustzijn dat wetenschap niet waardevrij doch veeleer waarheidsvrij is zouden wetenschappers zich kunnen en moeten inzetten voor doordacht en geschraagd beleid.

Contents



The research reported in this thesis has been carried out in the framework of the researchgoals of the group Organic Micropollutants of the Department Chemical Oceanography and Marine Pollution and the Applied Science Department (BeWON) of the Netherlands Institute for Sea Research. The objective of the group is to study the behaviour and toxic effects of organic micropollutants in the marine environment.

Chapter 1

Introduction

General background

The marine environment is contaminated with a range of chemicals released by industries and urban communities. In particular, estuarine and coastal areas are exposed to relatively high levels of contamination compared to shelf seas or oceans. Gradually more and more information is gathered about the impact of these environmental chemicals on aquatic life.

To make an assessment of the impact of chemicals on the marine environment, it is essential to causally relate their presence to changes in biological responses that result from exposure to these environmental chemicals. Consequently, these changes can serve as biological markers (biomarkers) of exposure to specific environmental contaminants. A biomarker is defined as a change in a biological response that can be related to an exposure to, or toxic effect of, an environmental chemical or chemicals (Peakall and Shugart, 1993). These biological responses to chemicals can range from the molecular or biochemical level, to the individual level and ultimately to population or ecosystem level. Responses indicating that exposure to a chemical or class of chemicals has occurred, but that do not provide knowledge of adverse (toxic) effects at the individual level are defined as biomarkers of exposure. Responses indicating both exposure and adverse effects, are defined as biomarkers of effect (Peakall and Shugart, 1993). Biomarkers can be used to assess the joint impact of a whole group of chemicals, since they reflect the cumulative, synergistic or antagonistic effects of these chemicals on biological systems.

The first detectable changes to environmental chemicals take place at biochemical or molecular levels in target organs and tissues. These changes occur before effects at higher levels of biological organisation, such as population or reproduction level, are detected and can therefore provide an early indication of exposure. However, these changes at lower levels of biological organisation do not necessarily imply adverse effects at the higher levels; the toxicological significance of some biomarkers of exposure is yet uncertain.

A number of biochemical systems that can be applied as biomarkers in the aquatic environment have been studied to date. The most studied is the induction of the

biotransformation enzyme cytochrome P450 1A (CYP1A) by polyhalogenated aromatic hydrocarbons (PHAHs). It has been studied experimentally and has been linked to these contaminants in the environment (Goksøyr and Förlin, 1992; Bucheli and Fent, 1995; Kleinow *et al.*, 1987; Payne *et al.*, 1987; Stegeman *et al.*, 1992). For reviews about several physiological, histological and biochemical biomarkers of environmental contamination see Livingstone (1993), McCarthy and Shugart (1990), Huggett *et al.* (1992).

Dab as study organism

Dab (*Limanda limanda*) is the most abundant flatfish species in the North Sea with an estimated biomass of about 2 million tonnes. It shows a large distribution over the entire North Sea with the highest densities in the German Bight (Daan *et al.*, 1990). Both young stages and adults occur in deeper offshore waters as well as in shallow coastal areas (Rijnsdorp *et al.*, 1992; Bolle *et al.*, 1994). Migration is mainly associated with the annual spawning period, peaking between February and April (Rijnsdorp *et al.*, 1992).

Only because of its wide occurrence in the North Sea, dab is already playing an important role in international contamination monitoring programmes, e.g. Joint Assessment and Monitoring Programme of the Oslo and Paris Commissions and the North Sea Task Force Monitoring Plan of the North Sea nations. However, no attempt has been made to investigate the suitability of the organism for monitoring purposes so far; there is very limited information available about the inducibility of the CYP1A enzyme system of dab under experimental conditions or the influence of different biotic and abiotic variables which can influence CYP1A expression in the field.

In this thesis it is investigated whether the CYP1A response in the flatfish species dab from the southern North Sea can be applied as a biomarker of exposure to (planar) polychlorinated biphenyls (PCBs) and related compounds. In addition, it is assessed whether the dab is a suitable organism for monitoring purposes.

PCBs and related compounds in the North Sea

PCBs, polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are members of the group of polyhalogenated aromatic hydrocarbons (PHAHs), that are capable of inducing CYP1A in mammals, birds and fish. Another class of environmental

chemicals, also capable of inducing CYP1A, consists of the nonchlorinated polycyclic aromatic hydrocarbons (PAHs). In this thesis, emphasis has been laid on PCBs, since they are by far the most important class of compounds capable of inducing CYP1A in the southern North Sea. The Dutch coastal zone belongs to one of the heaviest PCB contaminated areas in the world (Lohse, 1991; Klamer *et al.*, 1991; De Boer *et al.*, 1993). In addition, the toxic potential of PCDDs and PCDFs in the North Sea is much lower than for PCBs (De Boer *et al.*, 1993). Moreover, PAHs, PCDDs and PCDFs show a similar distribution pattern as PCBs in the southern North Sea (Ariese, 1993; Boon *et al.*, 1993; Evers *et al.*, 1993).

The annual input of PCBs in the North Sea, calculated to be 11-17 tonnes (Klamer *et al.*, 1991), is contributed by the Atlantic Ocean, Baltic Sea, atmospheric deposition, effluents of rivers, sewers and harbour sludge. The plume of the rivers Rhine, Meuse and Scheldt do not stretch far out into the open sea; contaminants are transported to the northeast along the coastline of Western Europe by the prevailing residual currents.

Since the late 70's the production and sale of PCBs have been ceased in most countries and the use of PCBs has been restricted to certain closed systems. Despite these policy measures, concentrations of PCBs in biota, with the exception of several lower chlorinated congeners, have essentially remained constant in Dutch rivers and the North Sea (De Boer, 1995).

PCBs in fish

Bioaccumulation of PCBs in fish is a distribution process and is the ratio between uptake and elimination. The elimation rate can be enhanced by enzyme-mediated metabolism (biotransformation). Especially lower chlorinated CB congeners are subject to biotransformation (Bruggeman, 1983), which is very likely to be catalyzed by the cytochrome P450 enzyme system. In general, bioaccumulation of organic chemicals increases with increasing hydrophobicity. CB congeners that lack vicinal H-atoms in the *meta, para* positions show a selective tissue retention (Tanabe *et al.*, 1987b; Boon *et al.*, 1989; Bright *et al.*, 1995).

Due to their lipophilic character and their extreme stability to chemical breakdown, PCBs, PCDDs and PCDFs bioaccumulate and biomagnify in higher trophic levels of the food chain, including fish, fish-eating seabirds and marine mammals (Walker, 1990; Rappe *et al.*, 1987; Muir *et al.*, 1988; Evans *et al.*, 1991; Ankley *et al.*, 1992; 1993). The last years it has become evident that the much higher levels of PCBs in biota are potentially more dangerous than PCDDs and PCDFs in the North Sea (De Boer *et al.*, 1993) and other marine areas (Niimi

and Oliver, 1989; Tanabe *et al.*, 1987a; 1989; Kannan *et al.*, 1989; Smith *et al.* 1990; Hong *et al.*, 1992).

In general, the PCB distribution patterns of dab from the North Sea showed higher PCB levels along the coast and lower levels towards offshore areas throughout the 80's (Knickmeyer and Steinhart, 1990), which is in accordance with other PCB patterns in water and biota from the North Sea (Boon, 1992; Boon *et al.*, 1985; Schulz-Bull *et al.*, 1991).

In fish, induction of CYP1A enzymes has been observed after exposure to PCBs (Goksøyr and Förlin, 1992; Kleinow *et al.*, 1987; Payne *et al.*, 1987; Stegeman *et al.*, 1992). In addition, PCB exposure has been linked to reduced reproductive success (Spies and Rice, 1988; Von Westernhagen *et al.*, 1987) and laboratory studies have shown that some species are very sensitive to these compounds in their early life stage (Walker and Peterson, 1991). Finally, effects on steroid metabolism have been reported (Thomas, 1989; Miranda *et al.*, 1992).

The most toxic CB congeners are sterically similar to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, showing chlorine substitution at both *para*-, at least two *meta*-positions and non-*ortho* chlorine substitution (coplanar PCBs), like 3,3',4,4'-tetraCB (CB77), 3,3',4,4',5-pentaCB (CB126) and 3,3',4,4',5,5'-hexaCB (CB169) (Safe, 1990; 1994). Mono-*ortho* chlorine substituted CBs also show this dioxin-type toxicity in mammals, although introduction of one chlorine atom on an *ortho* position results in a decreased coplanarity (Cullen and Kaiser, 1984).

The dioxin-type toxic effects are thought to be mediated by an aryl-hydrocarbon (Ah) receptor mechanism (Safe, 1990; Poland and Knutson, 1982; Okey *et al.*, 1994). Since a cytosolic Ah receptor protein has been detected in several fish species (Lorenzen and Okey, 1990; Heilmann *et al.*, 1988; Hahn *et al.*, 1994), a common mechanism of action in fish analogous to that found in mammalian systems appears to exist.

The cytochrome P450 enzyme system in fish

The cytochrome P450 monooxygenases belong to a superfamily of structurally related heme proteins, catalyzing oxidative biotransformation reactions of a wide variety of endogenous and xenobiotic substrates (Nebert *et al.*, 1991; Nelson *et al.*, 1993). The basic biochemistry and characteristics of the cytochrome P450 enzyme system in fish have been described in several reviews (Andersson and Förlin, 1992; Goksøyr and Förlin, 1992; Bucheli and Fent, 1995; Kleinow *et al.*, 1987; Payne *et al.*, 1987; Stegeman *et al.*, 1992).

One of the hallmark features of P450 systems in fish as well as in mammals, is their inducibility by a large variety of compounds (Kleinow *et al.*, 1987). These compounds can sometimes be suitable substrates for the induced enzymes, but this is not necessarily the case. In mammals, a number of non- and mono-*ortho* chlorine substituted CBs cause induction of at least two iso-forms of the cytochrome P450 1A subfamily of enzymes; i.e. cytochrome P450 1A1 and 1A2 (CYP1A1/2). In fish at least one CYP1A form is induced, although a recent study revealed the presence of two CYP1 genes in rainbow trout (Berndtson and Chen, 1994). Relationships to mammalian CYP1A1 have been established for rainbow trout by cDNA analyses (Heilmann *et al.*, 1988) and for scup based on amino acid sequence (Stegeman, 1989; 1992). CYP1A forms in other fish species will preferably be referred to as CYP1A, since sequence data are yet lacking (Stegeman, 1992). Unlike mammals, fish seem to be refractory to induction by di-*ortho* chlorine substituted CBs (Kleinow *et al.*, 1987; Gooch *et al.*, 1989). Several PCDDs and PCDFs are also capable of inducing CYP1A in fish (Van der Weiden *et al.*, 1992; 1994a; 1994b).

The fact that important classes of inducers of the CYP1A enzyme system in fish, like planar PCBs, PCDDs, PCDFs and PAHs are known aquatic contaminants has stimulated research in this field. CYP1A induction in fish may serve as a biomarker of exposure to especially planar aromatic contaminants (Stegeman and Kloepper-Sams, 1987). A number of studies of the last 20 years reported elevated levels of CYP1A (either catalytic activity, protein or CYP1A mRNA) in fish from contaminated waters (for reviews, see Livingstone, 1993; Haux and Förlin, 1988; Stegeman and Lech, 1991; Goksøyr and Förlin, 1992; Kleinow *et al.*, 1987; Payne *et al.*, 1987). Levels of CYP1A have been related to the content of PCBs, PCDDs and PCDFs in several fish species or their environment, especially in North America (Elskus and Stegeman, 1989; Kloepper-Sams and Benton, 1994; Monosson and Stegeman, 1994; Luxon *et al.*, 1987; Stegeman *et al.*, 1987; 1990; Stein *et al.*, 1992; Munkittrick *et al.*, 1994), but also in Europe (Förlin and Celander, 1993; Galgani *et al.*, 1991; Goksøyr *et al.*, 1991; 1994a; Förlin *et al.*, 1992; Burgeot *et al.*, 1994; Peters *et al.*, 1994) and Australia (Addison *et al.*, 1994; Holdway *et al.*, 1994). Most of these studies were performed in rivers, estuaries and bays; information about offshore areas is limited.

Correlation of CYP1A induction with PAHs, that are also capable to induce the P450-system can be difficult, because they are also very good substrates for the P450-system and therefore rapidly metabolized. Nevertheless, significant correlations between CYP1A induction and PAH levels in sediment have been established (Van Veld *et al.*, 1990; Addison *et al.*, 1994). All these studies confirm the idea that levels of CYP1A can reflect levels of contaminants in the environment and/or in the fish themselves. It must, however, be taken into

account that in many cases the analyzed chemicals do not appear alone, but in a mixture with several other inducing, non-inducing or even induction-inhibiting agents (Goksøyr and Förlin, 1992). Contaminants like the antifouling agent tributyltin (Fent and Stegeman, 1991; 1993) or heavy metals, such as cadmium (George, 1989; Förlin *et al.*, 1986) are known to reduce or degrade CYP1A protein and the related catalytic activity. In experimentally dosed fish, certain PCB congeners inhibited CYP1A activity (Monosson and Stegeman, 1991; Gooch *et al.*, 1989; Boon *et al.*, 1992a). This substrate inhibition was the effect of high PCB concentrations, which are probably not reached in the North Sea.

There are several methods to measure CYP1A induction at different cellular levels, e.g. CYP1A mRNA levels can be measured using specific cDNA probes complementary to the CYP1A mRNA of the fish species studied. Up till now the amount of cDNA probes available is limited. Alternatively, CYP1A protein levels can be determined using specific monoclonal or polyclonal antibodies raised against CYP1A orthologues. Finally, the activity of the CYP1A enzyme can be analyzed using synthetic substrates, e.g. 7-ethoxyresorufin is one of the most commonly used substrates in determining the 7-ethoxyresorufin O-deethylase activity in fish (Förlin *et al.*, 1994).

There is very limited information available about the relation between contaminant levels and CYP1A induction in dab. Relationships between hepatic CYP1A levels (mRNA, protein and catalytic) and sediment levels of particularly PCBs and PAHs have been observed during an ICES/IOC Workshop on Biological Effects Techniques (Bremerhaven Workshop; Renton and Addison, 1992; Eggens *et al.*, 1992; Goksøyr *et al.*, 1992). Here, dab was caught along a gradient from the Elbe estuary in the German Bight to the Dogger Bank during the spawning season in March, which is at the peak of the spawning-related migration period. In the laboratory, induction of CYP1A was found in liver of dab after exposure to sediment containing PAHs and PCBs (Livingstone *et al.*, 1993).

Apart from the influence of inducing or induction-inhibiting agents, the activity and induction of CYP1A in fish can be influenced by a large number of other factors. Influences of endogenous factors like age, sex and reproductive stage (Förlin, 1980; Förlin and Haux, 1989; Elskus *et al.*, 1989; George *et al.*, 1990; Larsen *et al.*, 1992b) have to be minimized to allow for the use of CYP1A induction as a biomarker for environmental contamination by selection of fish according to size, sex, and reproductive stage. Exogenous environmental factors like seasonal variation, water temperature (Egaas and Varanasi, 1982; Andersson and Koivusaari, 1986; Snegaroff and Bach, 1990; Blanck *et al.*, 1989) and diet (George and Henderson, 1992; Lemaire *et al.*, 1992; Goksøyr *et al.*, 1994b) can also influence CYP1A levels.

The toxicological significance of CYP1A induction towards higher levels of biological organization is yet uncertain, but like in mammals, the induction of CYP1A could be a prerequisite for the formation and presence of reactive PAH metabolites, thus playing a role in the carcinogenesis (Egaas and Varanasi, 1982; Stegeman and Lech, 1991; Varanasi *et al.*, 1987).

Objectives and approach of this thesis

The work reported in this thesis has the following objective: to investigate whether the cytochrome P450 1A response in dab could be used as a biomarker of exposure to PCBs and related compounds in monitoring programmes in the southern North Sea. During the field studies and laboratory experiments, emphasis has been laid on PCBs, because they represent the most important class of inducing compounds in the North Sea; moreover, PCDDs and PCDFs appear to show a similar geographical distribution pattern (Evers *et al.*, 1993).

There is at present no information available to which extent biotic variables, like maturity and sex, or abiotic variables, like water temperature, influence CYP1A expression in dab. For an adequate use of dab as monitoring species this is a prerequisite. Therefore, the first step was to assess the influence of age and sex on the sensitivity of CYP1A induction in order to select the most suitable sex and length class of dab for monitoring purposes. To obtain this information, CYP1A expression in the field in relation to tissue PCB concentrations was studied during winter and autumn (chapter 2). In order to select a suitable annual monitoring period, CYP1A expression was also studied during spring and summer (chapter 3). In addition, this study examined also the effects of water temperature on basal CYP1A levels. When it became evident that water temperature differences had a large influence on CYP1A expression, a field study in late autumn was conducted to check the hypothesis that this would be the most suitable period for annual monitoring (chapter 4).

So far, the induction capacity of CYP1A in dab has received little attention. Therefore, the second part of this thesis describes a number of studies where dab were artifically dosed with a technical PCB mixture (Clophen A40) and several single congeners. Chapter 5 describes CYP1A induction in dab after multiple oral dosing with Clophen A40. As temperature appeared to be an important factor in the field, the influence of temperature on the temporal induction pattern of CYP1A induction for a single non-*ortho* PCB (CB77) was assessed (chapter 6). In addition, the potencies of several other non- and mono-*ortho* CB congeners on CYP1A induction were investigated (chapter 7).

Part I

Field studies

Chapter 2

Sensitivity of cytochrome P450 1A induction in dab (*Limanda limanda*) of different age and sex as a biomarker for environmental contaminants in the southern North Sea *

Abstract

The sensitivity of cytochrome P450 1A (CYP1A) induction as a biomarker for environmental contaminants in the flatfish dab (*Limanda limanda*) was evaluated by studying fish of different age and sex from the southern North Sea. Mature and juvenile dab from both sexes were collected in autumn and winter during two surveys from four different stations with varying levels of polychlorinated biphenyls (PCBs) contamination in the southern North Sea.

All groups of fish exhibited highest muscle PCB concentrations near the Dutch coast. CB153 was always the dominant congener. Since the concentrations of the other congeners measured, covaried to a large degree with CB153, this congener appears to be a good marker for general differences in PCB concentrations.

In summer, bottom water temperature differences of up to 10°C can occur between stratified and vertically mixed areas. This was previously shown to have a strong effect on CYP1A expression. In autumn and winter, stratification has disappeared resulting in almost equal water temperatures between stations of the same survey. CYP1A levels were measured as 7-ethoxyresorufin O-deethylase (EROD) activity and immunoquantitated CYP1A protein concentrations. Highest levels were also found close to the Dutch coast for mature fish from both sexes in October and for juvenile female and mature male fish in February during the spawning season. During this season, gravid female fish had significantly lowered contents of CYP1A protein and EROD activity compared to mature males and juveniles of both sexes.

The sensitivity of CYP1A induction in dab as a biomarker for halogenated aromatic hydrocarbons is highest in mature males when stratification during autumn is lacking.

* Hedwig M. Sleiderink, Ina Oostingh, Anders Goksøyr[1] and Jan P. Boon

[1]Laboratory of Marine Molecular Biology, University of Bergen, Bergen, Norway

Published in *Archives of Environmental Contamination and Toxicology* **28** (1995), 423-430

Introduction

Halogenated aromatic hydrocarbons such as polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) have been released into the environment from human activities, and their residues have been found in almost every component of the world's ecosystem (Safe, 1990). Due to their lipophilic character, these compounds accumulate in fish and other aquatic organisms (Tanabe *et al.*, 1989).

In laboratory studies with fish, the planar compounds cause induction of the isoenzyme cytochrome P450 1A, which will be denoted as CYP1A (Andersson and Förlin, 1992; Goksøyr and Förlin, 1992). Elevated levels of CYP1A have been found in several field studies (Elskus *et al.*, 1989; Goksøyr *et al.*, 1991). Such environmental induction has been associated with increased concentrations of xenobiotic compounds. Therefore, this biochemical parameter may serve as a biomarker of exposure to especially planar aromatic contaminants, although the biological significance of CYP1A induction at higher levels of biological integration is yet uncertain.

Since the flatfish dab (*Limanda limanda*) is a demersal fish with a large geographic distribution and abundance throughout the North Sea, it is presently being incorporated in pollution monitoring programs in the North Sea (North Sea Task Force, Joint Monitoring Programme). Induction of hepatic CYP1A protein and EROD activity in dab was found after exposure to the technical PCB mixture Clophen A40 (Sleiderink *et al.*, 1995a). The Dutch coastal zone is influenced by the effluents of the rivers Rhine, Meuse, and Scheldt and belongs to one of the heaviest PCB-contaminated areas in the world (Klamer *et al.*, 1991; De Boer *et al.*, 1993). In fish caught in the Dutch aquatic environment, the toxic potential of PCBs, based on toxic equivalency values, is four times more important than that of PCDDs and PCDFs (De Boer *et al.*, 1993).

In the spring and summer, differences in sea water temperature have a strong influence on the induction of CYP1A in dab (Sleiderink *et al.*, 1995c); stratification of the water column can cause differences of bottom water temperatures up to 10°C between different sampling areas in the southern North Sea, making the contaminant response difficult to discern. In late autumn and winter such large temperature differences are not present and these seasons appear more suitable to evaluate the sensitivity of environmentally-induced CYP1A as a biomarker for dioxin-type compounds in dab of different age and sex.

The CYP1A induction response in dab caught from four different stations with varying levels of PCB contamination in the southern North Sea is described and the sensitivity of this

response as a biomarker for halogenated aromatic hydrocarbons in relation to age, sex, and season of capture is discussed.

Materials and Methods

Sampling of fish and microsome preparation

The fish were caught by beam trawling during two surveys with the RV *Aurelia* in the southern North Sea in October 1990 and February 1991 (Fig. 1). The positions and fishing depth (m) were: Site A, 4°2' E, 52°5 N, 25 m; site B, 4°4' E, 53°4' N, 40 m; site C, 4°3' E, 54°6' N, 50 m; site D, 7°0' E, 54°3 N, 38 m. The bottom water temperature in October ranged from 13.5 to 14.6 °C and in February from 3.6 to 4.6°C. No temperature stratification was observed. For each site, specimens of the following four categories were caught: juvenile (size:

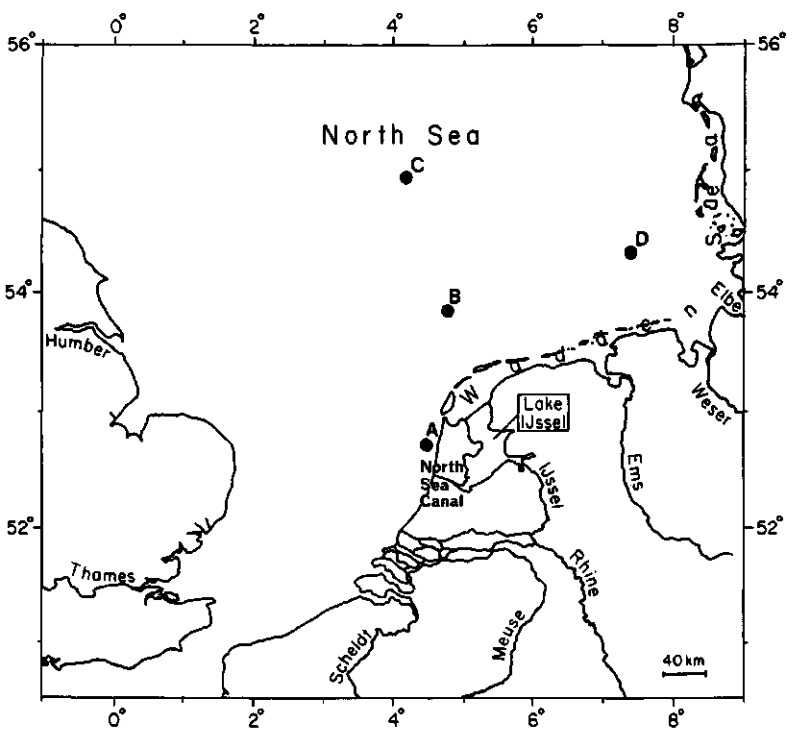


Fig. 1. Location of sampling stations in the southern North Sea.

10-12 cm) and mature (size: 15-20 cm) male and female dab. After capture, the fish were killed by a cephalic blow and cutting of the spinal cord. Due to instability of the research vessel and rough weather, fish and liver weights could not be determined. The livers were excised and washed with an ice cold solution of 1.15% KCl to remove remnants of blood and bile. They were homogenized in 1/2 volume of 99% glycerol. The homogenate was immediately frozen into liquid nitrogen and stored at -80°C for preparation of microsomes in the laboratory. The carcass of the fish was frozen at -25°C for PCB-analysis.

The liver homogenate was further homogenized in approximately 30 ml 1.15% KCl (vol. ratio). The homogenate was centrifuged at 10,000g for 20 min at 4°C. The microsomal fraction was isolated by centrifugation of the supernatant at 100,000g for 1 h at 4°C in a MSE Prepspin 50 ultracentrifuge. The microsomes were resuspended in a 0.1 M Na-K-phosphate buffer pH 7.4, containing 20% (v/v) glycerol, 1 mM EDTA, and 1 mM DTT, frozen and stored in liquid nitrogen.

Biochemical analyses

All biochemical analyses were performed on individual livers. 7-ethoxyresorufin O-deethylase (EROD) activity was measured fluorometrically on a Titertek Fluoroskan II plate-reader according to the method of Eggens and Galgani (1992). The concentration of the external standard resorufin (Aldrich Chemie) was assessed, using an extinction coefficient of 73 $mM^{-1}cm^{-1}$ (Klotz *et al.*, 1984). A laboratory reference material was analysed on every plate to obtain an indication for the variance of the results.

The protein assay was performed according to Bradford (1976), with bovine serum albumin as standard.

The total amount of cytochrome P450 ($\sum$P450) was measured spectrophotometrically, using dithionite reduced difference spectra of CO-treated samples according to the method of Omura and Sato (1964), as adapted by Rutten *et al.* (1987), using a Perkin Elmer Lambda 15 UV/VIS spectrophotometer equipped with a scattered-transmission accessory in the scan-mode.

Microsomal CYP1A protein content was quantified immunochemically using an enzyme-linked immunosorbent assay (ELISA) described by Goksøyr (1991), using heterologous rabbit anti-cod CYP1A IgG as primary antibody. The cross-reactivity of this antibody with dab CYP1A protein has been tested previously (Goksøyr *et al.*, 1991).

Polychlorinated biphenyl analyses

The seven chlorobiphenyl congeners usually selected for international monitoring programmes (IUPAC No. 28, 52, 101, 118, 138, 153, 180) were analysed by capillary gas chromatography (GC-ECD). Gas chromatographic determination of PCBs have been described before (Boon, 1985; Boon *et al.*, 1992a; Sleiderink *et al.*, 1995a) and only supplementary information is given here. Determination of PCBs was done on a Hewlett Packard 5880-A gas chromatograph equipped with a narrow bore CP-Sil 8 column (Chrompack). The analytical methods used did not allow the determination of non-*ortho* substituted PCBs (CB77, -126 and -169), but De Boer *et al.* (1993) showed that concentrations of these CB congeners co-varied with concentrations of the congeners determined in this study in fish from the Dutch aquatic environment.

Identification and quantification was made on the basis of a synthetic mixture prepared from standards of the individual congeners. Different dilutions of the same sample were analysed to compensate for the non-linear response of the electron-capture detector (De Boer *et al.*, 1992b). Peaks quantified had a height that differed less than a factor of three from the peak height of that congener in the external standard mixture. At first, CB88 was added as internal standard congener; later this congener was substituted for CB112. Concentrations of the other PCBs were corrected for its recovery. The laboratory currently participates in an ICES intercalibration exercise on PCBs (De Boer *et al.*, 1992b).

Since the entire livers were used for biochemical analyses, PCBs were analysed in muscle of fish (the dorsal part between the backbone and the light skin was used for this purpose). The analyses of PCBs in muscle as a model for the qualitative changes occuring in the entire fish appears legitimate because although absolute concentrations differ between organs and lipids, lipid-based PCB concentrations vary only slightly between different tissues of the same fish (Sleiderink *et al.*, 1995a). Mature male fish from the October survey were analysed individually; other fish were pooled (n=4).

Statistical analyses

The correlation structure between the concentrations of individual CB congeners for each group of fish was examined by performing a principal components analysis on the correlation matrix of the logarithms of the lipid-based concentrations. This data transformation technique facilitates the search for patterns within a set of variables. The figure shows the results in the form of a so-called biplot (Gabriel, 1971). The rationale of this method is explained by De Boer *et al.* (1993).

The different sets of biochemical data were analysed statistically after log transformation by three-way and two-way ANOVA. To examine the correlations between EROD, CYP1A protein, ∑P450 content, and residue PCB concentrations linear regression analyses were performed. EROD, CYP1A protein, and ∑P450 levels were averaged for the same sets of samples that were pooled for the PCB analyses.

Results and discussion

Polychlorinated biphenyl analyses

Tables 1A and 1B list the mean concentrations of the selected CB congeners on a lipid

Table 1A. Percent lipid and levels (ng/g lipid) of polychlorinated biphenyls in muscle of dab from the southern North Sea, caught in October.

Group	Site	% lipid	CB28	CB52	CB101	CB118	CB138	CB153	CB180
Female mature	A	1.2	17	30	88	101	192	224	42
	B	1.0	6	13	39	34	76	88	40
	C	0.7	6	5	11	18	39	54	19
	D	0.6	9	15	43	40	96	112	29
Male mature	A	1.1	24	60	171	162	307	373	93
	B	0.8	8	11	36	45	104	128	51
	C	0.8	7	11	27	37	86	112	45
	D	0.8	15	13	37	52	141	159	84
Female juvenile	A	0.6	24	44	103	97	181	223	44
	B	0.9	6	10	32	29	75	92	50
	C	0.7	8	8	16	15	36	42	16
	D	0.8	11	18	51	55	126	143	30
Male juvenile	A	1.0	31	60	150	127	227	263	73
	B	1.1	8	15	42	39	87	107	45
	C	0.9	6	6	13	13	33	42	15
	D	0.7	12	20	61	58	129	159	34

basis in muscle of dab caught during the October and February surveys. An interpretation of the data is given in the biplot of Fig. 2. Apart from plotting the values of the first and second principal component for each group of fish, the figure also shows the correlation between the concentration of each CB congener and the first two principal components by means of a vector. The first principal component represents the absolute value of the individual congeners and the second principal component represents deviations in the CB patterns. The orthogonal projection of each data point on each vector shows its relative position with respect to the data range for the first and second principal component. The length of the vector indicates the reliability of the approximation; if a vector reaches the unit circle, then all variance is explained by the sum of the first and second principal component. The correlation between two congeners is indicated by the product of the cosine of the angle between their vectors and the

Table 1B. Percent lipid and levels (ng/g lipid) of polychlorinated biphenyls in muscle of dab from the southern North Sea, caught in February.

Group	Site	% lipid	CB28	CB52	CB101	CB118	CB138	CB153	CB180
Gravid female	A	0.6	29	57	193	259	531	572	140
	B	0.8	11	17	54	71	145	184	62
	C	0.7	8	7	18	34	73	98	32
	D	0.8	12	28	77	89	71	203	44
Male mature	A	0.8	20	49	155	159	300	338	88
	B	0.7	9	18	52	51	117	144	54
	C	0.7	9	11	28	55	121	159	50
	D	0.8	12	17	64	93	255	293	93
Female juvenile	A	0.7	34	102	303	298	537	630	169
	B	0.7	13	29	74	82	169	207	67
	C	0.7	10	20	38	36	75	87	28
	D	0.7	13	26	66	68	144	160	31
Male juvenile	A	0.6	33	82	204	190	345	404	109
	B	0.7	11	16	39	48	104	123	33
	C	0.7	9	16	29	34	82	97	32
	D	0.9	10	25	56	62	139	151	31

length of the vectors. Orthogonal vectors therefore indicate a zero correlation.

Polychlorinated biphenyl concentrations were always highest at the coastal Site A and lowest at Site C (Oyster Grounds); levels at Site B (Frisian Front) and D (German Bight) were in between; A > B, D > C. Differences in PCB levels between stations were approximately four-fold for mature fish and six-fold for juvenile fish in October and three-fold in February.

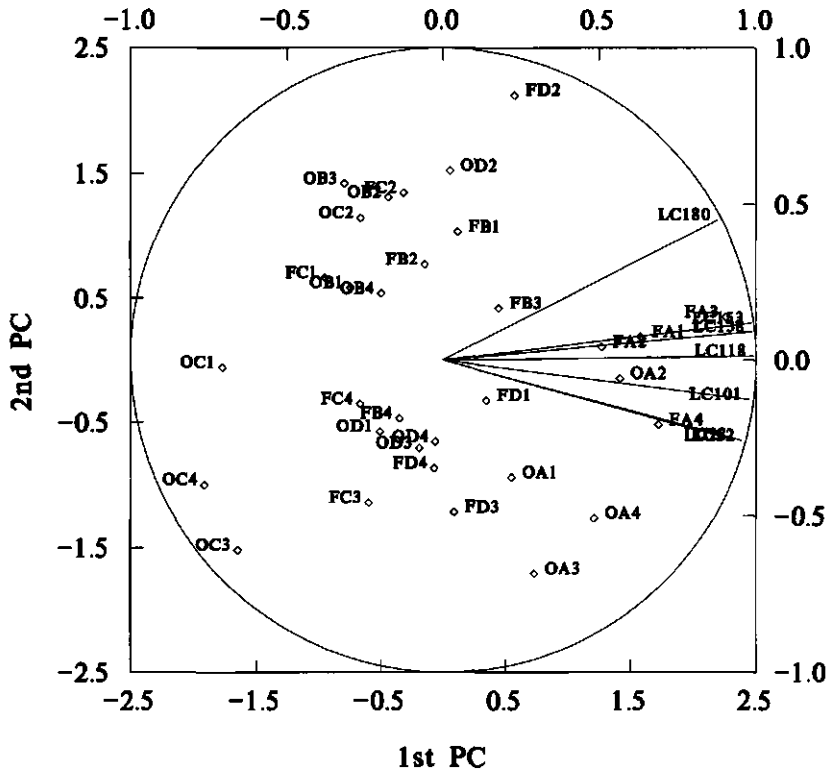


Fig. 2. Bi-plot of principal components analysis of PCB concentrations in samples of mature and juvenile dab from both sexes caught in two different seasons in the southern North Sea. 1st PC=first principal component, 2nd PC=second principal component. The lower *x*-axis and left *y*-axis apply to the individual data points and show the range of values of the 1st and 2nd PC, respectively. The upper *x*-axis and right *y*-axis apply to the vectors and show the variance explained by the 1st and 2nd PC. LCX=ln (CBX) in ng/g lipid; Code ABC: A=season (O=October, F=February); B=Site (as in Fig. 1); C=Group (1=female mature, 2=male mature, 3=female juvenile, 4=male juvenile).

The difference in absolute values between seasons was probably due to a seasonal effect, since all fish were leaner in February. Contamination levels within groups of fish varied with the lipid content of the muscle tissue. No pronounced differences in PCB concentrations between groups of fish from the same site were found.

The water quality of the Dutch coastal zone, represented by Site A, largely depends on the effluents of the Rhine, Scheldt and Meuse (Van den Meent *et al.*, 1986). The plume of the river Rhine does not stretch far out into the open sea; sediment associated micropollutants are transported to the north along the coast by the prevailing currents (Eisma and Irion, 1988). Site B (Frisian Front) is a deposition area for water running through the English Channel up to the north and therefore forms a barrier between coastal and Channel water. Site C is considered as a reference area for this study. Site D in the German Bight is a deposition area for coastal water.

CB153 was the dominant CB congener at all stations for all groups of fish during both seasons. Since the concentrations of the measured CB congeners showed high correlations with CB153 (long vectors, small angles) in the bi-plot (Fig. 2), CB153 is a good marker for these congeners. Therefore, the trend in the data is more clearly expressed in Fig. 3A and 3B, where CB153 concentration in muscle tissue for all fish is given. There were no strong site-related differences in the CB-patterns.

In fish, CYP1A can be induced by non-*ortho* congeners (Janz and Metcalfe, 1991; Gooch *et al.*, 1989). Non-*ortho* congeners were not measured during this study, but they were very likely to be present at low levels in muscle tissue of fish. Since PCB patterns in dab did not differ much between sampling stations, differences in concentrations of the measured PCB congeners in muscle tissue of dab between stations, may represent differences in concentrations of non-*ortho* congeners too. De Boer *et al.* (1993) found a correlation of r=0.95 between CB153 concentrations and the CB-TEQ (TCDD toxic equivalents for PCBs) in fish from the Dutch aquatic environment.

Biochemical analyses

The levels of hepatic EROD activity, CYP1A protein (denoted as CYP1A), and ΣP450 in fish from the October survey are presented in Table 2A. EROD activity is considered to be specifically catalyzed by the CYP1A enzyme (Stegeman, 1989). EROD activity and CYP1A were highest in both sexes of mature fish collected from the coastal Site A. Levels at other stations did not differ significantly. No differences were observed for ΣP450 levels. Three-way ANOVA with sex, age, and area as variables revealed that no differences in EROD activity

and CYP1A existed between the sexes. Significant correlations were found between EROD and CYP1A (r=0.67; n=123; p<0.0001), ∑P450 levels and EROD (r=0.48; n=61; p<0.001), and ∑P450 levels and CYP1A (r=0.33; n=61; p<0.01). The poorer correlations with ∑P450 are due to the fact that EROD activity and CYP1A protein were elevated in the coastal area, whereas ∑P450, representing the total set of different P450 isoenzymes, was not higher.

The levels of the biochemical parameters of the February survey are presented in Table 2B. EROD activity and CYP1A were significantly lower in gravid female fish from all sites. The observation of decreased EROD activity and CYP1A in female pre-spawning dab are in

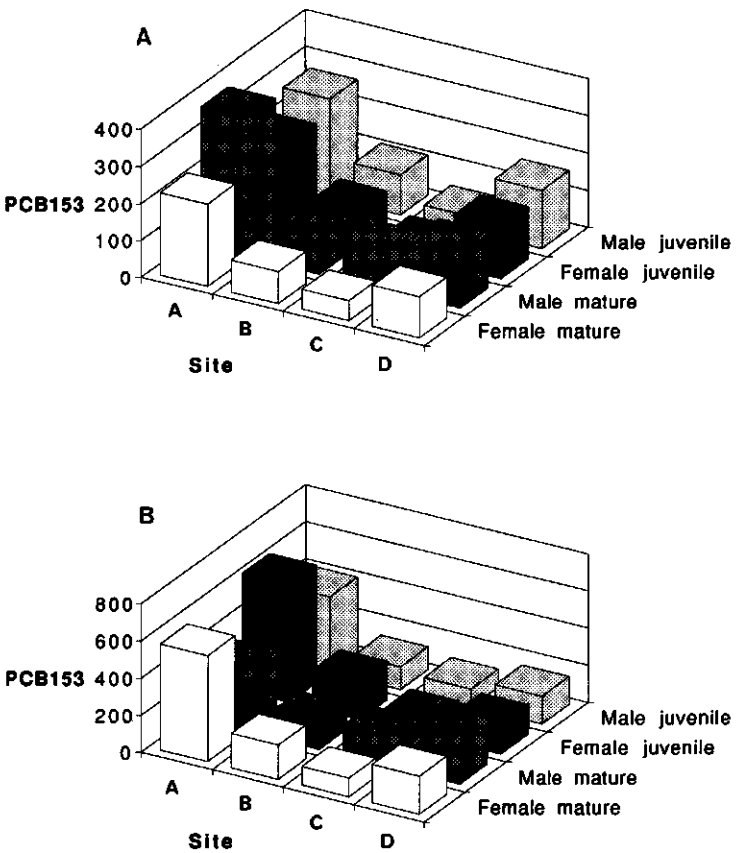


Fig. 3. Concentration of CB153 (in ng/g lipid) in mature and juvenile dab from both sexes caught in two different seasons in the southern North Sea. [A] Fish caught in October, [B] Fish caught in February.

agreement with numerous reports for other fish species (Elskus *et al.*, 1989; Larsen *et al.*, 1992b). The lowered CYP1A levels in these fish have been related to high oestrogen levels (Elskus *et al.* 1989; Larsen *et al.* 1992b).

Statistical analyses (three-way ANOVA) with sex, age, and area as variables revealed an interaction effect of area and age and of sex and age on CYP1A levels in February. This was reflected in higher CYP1A levels at the coastal Site A for juvenile female and mature male dab. The biochemical parameters correlated significantly, r=0.80 for EROD and CYP1A (n=115; p<0.0001), r=0.79 for CYP1A and ∑P450 (n=59; p<0.001), r=0.86 for EROD and ∑P450

Table 2A. Hepatic EROD activity, CYP1A protein, and ∑P450 content of dab from the southern North Sea, caught in October[a]

Group	Site	No of fish	EROD activity[b]	CYP1A protein[c]	∑P450[d]
Female mature	A	8	1.41±1.08	0.70±0.29	0.41±0.10
	B	8	0.40±0.15	0.31±0.12	0.36±0.15
	C	8	0.42±0.35	0.32±0.17	0.29±0.14
	D	8	0.34±0.21	0.28±0.12	0.29±0.10
Male mature	A	5	1.39±1.24	0.70±0.29	0.28±0.15
	B	8	0.44±0.08	0.31±0.05	0.35±0.15
	C	8	0.30±0.14	0.29±0.11	0.33±0.07
	D	8	0.28±0.12	0.19±0.12	0.32±0.07
Female juvenile	A	8	0.53±0.56	0.40±0.17	n.d.[e]
	B	8	0.42±0.26	0.31±0.10	n.d.
	C	8	0.36±0.13	0.28±0.08	n.d.
	D	8	0.41±0.17	0.29±0.10	n.d.
Male juvenile	A	8	0.43±0.23	0.36±0.16	n.d.
	B	8	0.20±0.16	0.25±0.11	n.d.
	C	8	0.30±0.14	0.34±0.12	n.d.
	D	6	0.35±0.22	0.29±0.16	n.d.

[a]Values represent mean ± SD.
[b]EROD = 7-ethoxyresorufin O-deethylase activity, nmol/min/mg microsomal protein.
[c]CYP1A protein = P450 1A ELISA absorbance levels at 492 nm.
[d]∑P450 = total cytochrome P450, nmol/mg microsomal protein.
[e]n.d. = not determined.

(n=59; p<0.0001). In general, the standard deviation in biochemical data was higher during the February survey compared to the October survey; this can be due to a stronger migration in this period. Dab in the southern North Sea show annual migration associated with spawning activities between February and April (Rijnsdorp *et al.*, 1992). It is very probable that all mature fish caught in February consisted of spawning fish. Fish returning from the relatively clean spawning grounds to more polluted areas possibly may still have been influenced by the spawning process.

Table 2B. Hepatic EROD activity, CYP1A protein, and ∑P450 content of dab from the southern North Sea, caught in February[a]

Group	Site	No of fish	EROD activity[b]	CYP1A protein[c]	∑P450[d]
Gravid female	A	3	0.18±0.10	0.30±0.19	0.21±0.05
	B	8	0.07±0.05	0.06±0.01	0.15±0.08
	C	8	0.11±0.10	0.10±0.06	0.20±0.05
	D	8	0.16±0.24	0.12±0.08	0.23±0.17
Male mature	A	8	1.56±1.52	0.97±0.65	0.54±0.29
	B	8	0.87±0.47	0.44±0.19	0.50±0.17
	C	8	0.50±0.34	0.47±0.29	0.35±0.14
	D	8	0.86±0.55	0.62±0.20	0.46±0.15
Female juvenile	A	8	2.10±2.15	1.21±0.40	n.d.[e]
	B	6	1.34±0.81	0.68±0.33	n.d.
	C	5	0.30±0.47	0.31±0.25	n.d.
	D	6	0.40±0.53	0.33±0.26	n.d.
Male juvenile	A	8	0.76±1.10	0.53±0.27	n.d.
	B	7	0.89±0.67	0.44±0.17	n.d.
	C	8	0.16±0.19	0.26±0.15	n.d.
	D	8	0.19±0.36	0.38±0.07	n.d.

[a]Values represent mean ± SD.
[b]EROD = 7-ethoxyresorufin O-deethylase activity, nmol/min/mg microsomal protein.
[c]CYP1A protein = P450 1A ELISA absorbance levels at 492 nm.
[d]∑P450 = total cytochrome P450, nmol/ mg microsomal protein.
[e]n.d. = not determined.

EROD activity was more induced than CYP1A, which is in agreement with other studies (Förlin and Celander, 1993; Van der Weiden *et al.*, 1993). However, in a laboratory study where dab were dosed with the technical PCB mixture Clophen A40 (Sleiderink *et al.*, 1995a) the responses of EROD activity and CYP1A were similar, although the given dose (10 mg/kg) was higher than that occuring in the field.

Even in the more pristine areas of the southern North Sea, such as the Oyster Grounds (Site C), EROD activity and CYP1A in dab were always well above the detection limit. In mature male dab caught on the south coast of Iceland in July, with PCB levels half of that found at Site C (70±42 ng CB153/g lipid), EROD activity was 1.27±1.11 nmol/min/mg protein (unpubl. results). A direct comparison of these Icelandic dab with North Sea dab is difficult to make, since these fish were caught in a different period of the year and also the sea water temperature, which is known to influence CYP1A, differed (7.8°C). This suggests that these fish have been exposed to low concentrations of contaminants which activated the induction response, although differences in dietary factors and photoperiod may have been important too. In hatchery-reared fish species, often very low or even undetectable amounts of CYP1A can be found (Förlin and Celander, 1993), which can be due to species, seasonal or environmental differences.

Correlation of biochemical parameters with PCB concentration

Since in the North Sea, PCB patterns in dab do not differ much between sampling station, differences in concentrations of one single congener may represent differences in concentrations of other congeners too. Since De Boer *et al.* (1993) found a correlation of r=0.95 between CB153 concentrations and the CB-TEQ in fish from the Dutch aquatic environment, CB153 will be used for the correlations with biochemical parameters.

Because water temperature differences between sampling sites were restricted to 1°C, the influence of water temperature could be neglected, in contrast to the situation in spring and summer (Sleiderink *et al.*, 1995c).

For mature male dab caught in October, individual levels of EROD activity and CYP1A correlated significantly with muscle CB153 concentrations (Table 3). However, the significance is determined by data representing part of the coastal population. The correlations for the individual sites only showed significant correlations of ∑P450 for Site A and B; for the correlations of EROD activity and CYP1A, trends in the correlations for Site A can be observed, but the amount of fish sampled was too low, a significant correlation was not observed. No significant correlations between individual muscle CB153 concentrations and

Table 3. Correlations between the logs of muscle concentration of CB153 and EROD activity, CYP1A protein, and ΣP450 in mature male dab from the southern North Sea, caught in October[a,b]

Site	CB153-EROD[c]	CB153-CYP1A[d]	CB153-ΣP450[e]	*N*
A	0.87	0.80	0.99**	5
B	0.14	0.26	0.78*	8
C	0.01	0.47	0.29	8
D	0.24	0.12	0.30	8
all Sites	0.59**	0.42*	0.06	29

[a]Chemical and biochemical analysis was performed in individual samples from the same fish.
[b]*Significant at $p<0.05$, **Significant at $p<0.001$.
[c]EROD = 7-ethoxyresorufin O-deethylase activity, nmol/min/mg microsomal protein
[d]CYP1A protein = P450 1A ELISA absorbance levels at 492 nm
[e]ΣP450 = total cytochrome P450, nmol/mg microsomal protein

CYP1A levels were observed for the off-shore Sites B, C and D. This suggests that only at the coastal area these biochemical parameters can serve as biomarkers for PCB exposure. It should be noted that differences in PCB levels between stations are low and possibly not sufficient to result in differences in CYP1A induction in off-shore areas; other contaminants may have effected the response.

The pooled data of the February survey only show significant correlations between CYP1A and muscle CB153 concentrations in mature males ($r=0.85$; $n=8$; $p<0.01$) and between EROD and CYP1A with muscle PCB153 concentrations in female juveniles ($r=0.72$ and $r=0.75$, respectively; $n=8$; $p<0.05$).

Multiple regression analyses with the combined data of both surveys for mature male dab, with temperature and CB153 as variables influencing CYP1A levels did not show effects. This is probably due to seasonal differences.

The correlations do not imply that CB153 is related to the environmental induction of CYP1A, but the data suggest that PCBs or other contaminants co-varying with PCBs are acting in the North Sea environment. PCDDs, PCDFs, and PAHs are known to induce CYP1A. As for PCBs, the levels of PCDDs and PCDFs in sediments from the southern North Sea, like the Oyster Grounds, are being influenced by the effluents of the river Rhine, Meuse and Scheldt (Evers *et al.*, 1993). Measurements of biliary 1-OH-pyrene, a metabolite of the PAH pyrene, in dab showed that pyrene exposure is also mainly related to the output of polluted sediment of these rivers (Ariese, 1993). Thus, these sediment-associated contaminants show the same

distribution pattern as PCBs. Therefore they might also have contributed to the observed induction of CYP1A, although in the Dutch aquatic environment the toxic potential of PCBs is four times higher than that of PCDDs and PCDFs (de Boer *et al.*, 1993).

The CYP1A induction response in dab as a biomarker for PCBs was sensitive enough to separate the coastal site A from the three more offshore locations B, C, and D for mature male dab. Within the more pristine offshore areas, no correlations between PCB concentrations and CYP1A levels could be observed, although it should be noted that PCB levels in the offshore areas were almost equal and possibly not enough to cause large differences in CYP1A induction by itself. The optimal sampling period for application of these biomarkers seems to be in autumn, since dab migrate during the spawning period in the winter, and in spring and summer, differences in sea water temperature in the North Sea have a strong influence on CYP1A induction in dab (Sleiderink *et al.*, 1995c).

Acknowledgements

This research was supported by a grant from the Royal Dutch Academy of Sciences (KNAW). We are grateful to the crew of the RV *Aurelia* for their help in obtaining dab. Stefan Einarsson is greatfully acknowledged for sampling dab on the south coast of Iceland. We thank Jaap van der Meer for valuable advice about the statistical treatment of the data and Kjersti A. Helgesen and Evaline M. van Weerlee for their technical assistance.

Chapter 3

Influence of temperature and polyaromatic contaminants on CYP1A levels in North Sea dab (*Limanda limanda*)*

Abstract

Mature specimens (n=686) of male dab (*Limanda limanda*) were collected at several stations from the southern North Sea during two surveys in 1991 and 1992. Levels of CYP1A protein and 7-ethoxyresorufin O-deethylase (EROD) activity were measured in liver and heart. Elevated CYP1A levels were observed in dab collected from offshore stations with low bottom water temperatures due to stratification of the water column. Considerably lower CYP1A levels were observed at stations with higher water temperatures. Multiple regression analyses with PCB concentrations in fish and water temperature as independent variables influencing EROD activity or CYP1A protein levels demonstrated significant correlations with both parameters. The water temperature was inversely related to CYP1A levels, whereas PCB concentrations showed a positive relation with CYP1A levels. The effect of water temperature dominated over the effect of PCB contamination. The relationship between water temperature and CYP1A levels was also examined in a laboratory study, where dab were acclimated to 8, 12 and 16°C for 4 weeks. A three-fold increase in EROD activity in the group acclimated to 8°C compared to the group acclimated to 16°C was observed, whereas no differences were observed for CYP1A protein levels. Multiple regression analyses with PCB concentrations and condition factor of fish as independent variables influencing CYP1A levels also demonstrated significant correlations in the field. Thus differences in water temperature and nutritional status of dab between sampling locations obscured the effects of contamination with polyhalogenated aromatic compounds on CYP1A levels. The results indicate that these factors have to be taken into account when employing CYP1A responses in dab as biomarkers for environmental contamination in the North Sea.

* Hedwig M. Sleiderink, Jonny Beyer[1], Enit Scholtens, Anders Goksøyr[1], Joop Nieuwenhuize[2], Jaap M. Van Liere[2], Jan M. Everaarts and Jan P. Boon

[1]Laboratory of Marine Molecular Biology, University of Bergen, Bergen, Norway

[2]Netherlands Institute of Ecology, Centre for Estuarine and Coastal Ecology, Yerseke, The Netherlands

Published in *Aquatic Toxicology* **32** (1995), 189-209

Introduction

The cytochrome P450-dependent monooxygenases belong to a superfamily of structurally related heme proteins, catalyzing a wide variety of endogenous and xenobiotic substrates (Nebert *et al.*, 1991; Nelson *et al.*, 1993). In fish, cytochrome P450 1A (CYP1A) is inducible by a variety of aromatic contaminants including polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polyaromatic hydrocarbons (PAHs) and some polychlorinated biphenyl (PCB) congeners (Goksøyr and Förlin, 1992; Kleinow *et al.*, 1987; Payne *et al.*, 1987). The induction of CYP1A in fish may therefore serve as a biomarker of exposure to especially planar aromatic contaminants in the marine environment (Stegeman and Kloepper-Sams, 1987; Stegeman *et al.*, 1988; Elskus *et al.*, 1989; Van Veld *et al.*, 1990). Apart from the induction by xenobiotics, the activity and induction of P450 enzymes in fish can be influenced by a large number of abiotic and biotic factors, such as water temperature, dietary factors, age, sex and sexual maturity (Andersson and Förlin, 1992).

Although the inductive response of CYP1A to organic pollutants is studied usually in liver tissue, measurement of induction in other organs may give additional information (Stegeman *et al.*, 1989; Van Veld *et al.*, 1990; Husøy *et al.*, 1994). For example, treatment of scup, *Stenotomus chrysops*, with ß-naphthoflavone (BNF) produced a 10-fold induction of spectrally measured P450 (dominated by CYP1A) in cardiac microsomes. Heart microsomes of untreated fish from contaminated environments were shown to contain high levels of CYP1A, indicating that induction, such as that caused by BNF, could occur under field conditions as well (Stegeman *et al.*, 1989).

Since the flatfish dab (*Limanda limanda*) is a demersal fish with a large geographic distribution and abundance throughout the North Sea, it is presently being incorporated in pollution monitoring programmes in the North Sea (e.g. North Sea Task Force; Joint Monitoring Programme). Dab has been shown to be a sensitive species in terms of CYP1A induction responses, e.g. the technical PCB mixture Clophen A40 causes induction of hepatic CYP1A protein and EROD activity (Sleiderink *et al.*, 1995a).

The main objective of the present study was to investigate the applicability of CYP1A responses in dab as indicator of exposure to organic contaminants under field conditions. CYP1A levels were determined by catalytic (7-ethoxyresorufin O-deethylase (EROD) activity) and immunochemical (ELISA) methods in liver and heart.

Materials and methods

Sampling locations

Specimens were obtained during two surveys of the Integrated North Sea Programme, 26 August-13 September 1991 and 18 May-6 June 1992, in the southern North Sea. Stations were chosen along transects from the Dutch coast to the Doggerbank area and along transects perpendicular to the Dutch coast in the Southern Bight (Fig. 1).

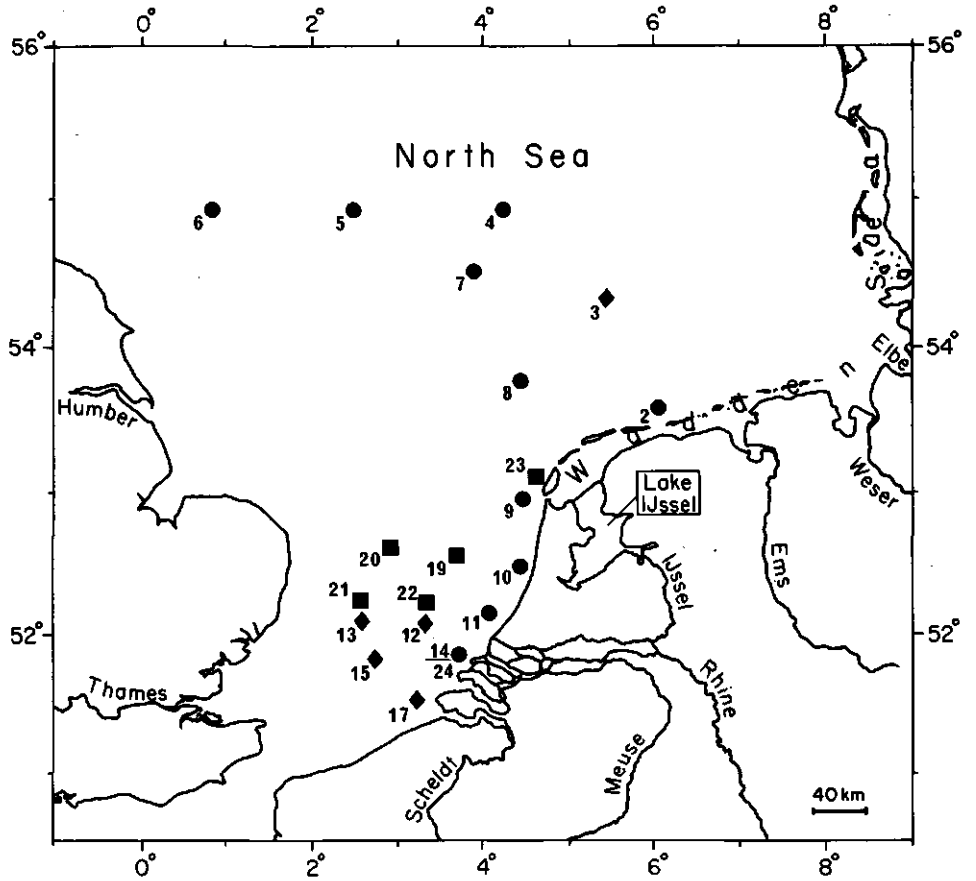


Figure 1. Position of sampling stations during the INP surveys. ◆ Sampled during first survey in 1991; ■ sampled during second survey in 1992; ● sampled during both surveys.

Sampling procedures

Dab were caught by beam trawling (5 m beam) with a trawling time of 15 min. From a single haul maximally five male specimens (length:15-20 cm) were randomly selected. For each station a total of 25 specimens originating from at least five hauls were analysed. When dab densities were lower, the specimens were obtained from more than five hauls.

Directly after capture the fish were measured, weighed and subsequently killed by a cephalic blow and cutting of the spinal cord. Fish did not show visible gonad development, since they were caught out of the spawning season. The livers were excised and rinsed with an ice cold solution of 1.15% KCl to remove remnants of blood and bile. Approximately 0.5 g of liver was homogenized in 4 volumes of 0.1 M Na-phosphate buffer pH 7.4; 0.1 M KCL; 1 mM EDTA with 10% (v/v) glycerol and centrifuged at 13,000g for 15 min. at 4°C. The 13,000g supernatants were analysed for CYP1A protein content, EROD activity and protein content immediately onboard the ship without intermittent freezing of the samples.

During the first survey, about 0.3 g of liver tissue was immediately frozen in liquid nitrogen and stored at -80°C for the preparation of microsomes in the laboratory. For each station, pieces of livers originating from five specimens were pooled. The samples were homogenized in about 3 volumes of 0.1 M Na-phosphate buffer pH 7.4; 0.15 M KCl; 1 mM EDTA; 1 mM DTT with 20% (v/v) glycerol. The homogenate was centrifuged at 10,000g for 20 min at 4°C and the microsomal fraction was isolated by centrifugation of the supernatant at 100,000g for 1 h at 4°C. The microsomes were resuspended in 1.5 ml 0.1 M Na-phosphate buffer pH 7.4, containing 20% (v/v) glycerol, 1 mM EDTA and 1 mM DTT, frozen in liquid nitrogen and stored at -80°C. Levels of EROD activity in the microsomal (100,000g) fraction and the 13,000g supernatants were compared.

The same procedures were followed during the second survey, but additionally half of the 13,000g supernatant was frozen in liquid nitrogen directly after centrifugation and stored at -80°C for EROD measurements at ambient bottom water temperature in the laboratory. No samples were taken for microsomal preparations.

In addition to liver samples, the hearts of 10 individual fish were excised, 13,000g supernatants were prepared and CYP1A protein and EROD activity were measured as described for liver.

Biochemical analyses

All analyses onboard the research vessel were performed directly on 13,000g supernatants. The EROD (7-ethoxyresorufin O-deethylase) activity measurements were performed at room temperature on a Titertek Fluoroskan II plate-reader, according to the method of Eggens and Galgani (1992). The samples were diluted with 0.1% albumin in 0.1 M phosphate buffer (pH 7.4) containing 0.5 µM of 7-ethoxyresorufin (Boehringer Mannheim) and 0.15 mM NADPH. The concentration of the external standard resorufin (Aldrich Chemie) dissolved in 0.1 M phosphate buffer (pH 7.4) was assessed at 572 nm using an extinction coefficient of 73 $mM^{-1}cm^{-1}$ (Klotz *et al.* 1984). An internal laboratory reference material was analysed on every microplate; variance in measurement between assays was kept below 5%.

A semi-quantitative determination of CYP1A protein levels was performed using the indirect CYP1A ELISA technique described by Goksøyr (1991), using heterologous rabbit anti-cod CYP1A IgG as primary antibody. The cross-reactivity of this antibody with dab CYP1A protein has been established (Goksøyr *et al.*, 1991; 1992).

The protein assay was performed according to Bradford (1976), with bovine serum albumin as standard.

Chemical analyses

Laboratory experiments with dab (Sleiderink *et al.*, 1995a) and sole, *Solea solea* (Boon, 1985; Boon *et al.*, 1984) showed that a large part of the differences in PCB concentrations on a wet weight basis between liver and muscle tissue of the same fish can be explained by their differences in lipid content. As a consequence, in this study, PCB concentrations in muscle tissue situated between the dark skin and the skeleton were used as a parameter for the differences in concentrations in the target organ, i.e. the liver, since this had to be used entirely for biochemical measurements. The muscle tissue originated from the same specimens that were used for the CYP1A measurements. When the number of dab caught permitted, each sample contained muscle tissue of five specimens from a single haul. When possible, five such pooled samples were taken at each station.

The samples were freeze-dried during 120 h under a moderate vacuum of 0.2 mBar to prevent evaporation of the more volatile congeners. Further clean-up and gaschromatographic analysis was performed as described previously (Nieuwenhuize and Van Liere, 1988; Duursma *et al.*, 1986) on a Packard Instruments 438A gas chromatograph equipped with a 50 m x 0.15 mm CPSil-19 capillary column (carriergas H_2) and electron capture detection. This allowed for the separation of the most important mono-*ortho* congeners; CB105, -118 and -

156 (De Boer *et al.*, 1992a; Larsen *et al.*, 1992a). The PCB congeners are numbered according to IUPAC rules, as suggested by Ballschmiter and Zell (1980). In every other batch of five samples analysed together, either a blank sample or an aliquot of the certified reference material CRM-349 (EC-BCR) of cod liver oil was analyzed to check for the performance of the analytical procedure. The laboratories currently participate succesfully in an ICES intercalibration exercise on the determination of PCBs (De Boer *et al.*, 1992b).

Laboratory study

Male dab (length: 15-20 cm) were caught on 30 June 1992 on the Doggerbank (station 5, Fig. 1) with a 5-m beam-trawl and transferred to the laboratory. The fish were held in 275 dm^3 glass tanks with running sea water from the Wadden Sea under natural light conditions. The tanks were aerated and contained a thin layer (1-2 cm) of sediment on the bottom. After an acclimation period of four weeks the fish were kept for four weeks at temperatures of 8, 12 and 16°C (n=8 for each group). The fish were fed *ad libitum* three times a week with frozen and fresh shrimps (*Crangon crangon*) and fresh mussel meat (*Mytilus edulis*).

At the end of the acclimation period the fish were weighed and killed by cutting the spinal cord. The fish were kept on ice, the livers were excised and 13,000g supernatants were prepared as described. EROD measurements were performed immediately. CYP1A protein levels were determined at a later stage.

Statistical analyses

To test for differences in the condition factors of the fish and CYP1A levels, statistical analyses were performed on log-transformed data using a least significant difference bands test (LSDB test) and Hochberg's GT2-test for multiple comparisons of means (Sokal and Rohlf, 1981). To examine the correlations between EROD activity, CYP1A protein, PCB concentrations, 1-OH-pyrene and water temperature linear regression analyses were performed on log-transformed data.

To correlate PCB data with biochemical data, EROD and CYP1A protein levels were averaged for the same set of samples that were pooled for the PCB analysis. To correlate 1-OH pyrene and CYP1A levels, averaged values per station were used, due to lack of bile in some of the caught fish.

Results

In Table 1, all sampling stations have been characterized in terms of depth and bottom water temperature, the presence of stratification of the water column, and the number of dab examined. During the two surveys in 1991 and 1992, a total of 686 male dab were caught.

Table 1. Number of sampled fish, bottom water temperature (°C), fishing depth (m) and presence (+) or absence (-) of stratification of the water column for sampling stations in the southern North Sea (data from first and second survey).

	First survey (1991)				Second survey (1992)			
Station	No of dab	Water temp.	Fishing depth	Strati-fication	No of dab	Water temp.	Fishing depth	Strati-fication
2	25	18.2	20	-	25	11.7	18	+
3	25	18.0	35	-				
4	25	10.5	50	+	25	8.1	51	+
5	25	17.0	22	-	25	10.6	22	-
6	25	9.5	65	+	25	7.7	63	+
7	25	12.5	46	+	25	8.4	46	+
8	25	17.8	40	-	25	9.5	44	+
9	24	19.0	24	-	25	14.4	24	+
10	4	19.8	20	-	25	14.2	17	-
11	24	19.4	20	-	25	14.0	23	+
12	25	18.5	33	-				
13	14	17.8	46	-				
14	25	19.4	22	-				
15	15	18.4	36	-				
17	15	18.0	53	-				
19					25	12.4	16	-
20					25	11.3	41	-
21					25	11.8	43	-
22					25	12.8	12	-
23					25	12.4	12	-
24					15	15.3	15	-

The average condition factors (determined according to Bagenal and Tesch, 1978) of the fish are summarized in Table 2. In general, condition factors were higher at the end of the summer (1991 survey) compared to the beginning of the summer (1992 survey). Fish from the coastal areas had significantly higher condition factors than fish from the offshore areas.

Table 2. Condition factor (body weight (g) x 100/(length (cm))^3 (Bagenal and Tesch, 1978) per station and average over all stations of dab from the southern North Sea (data from first and second survey). Data represent mean values ± SD. Condition factors indicated by similar letters are not significantly different (p<0.05).

Station	First survey (1991)	Second survey (1992)	Station	First survey (1991)	Second survey (1992)
2	1.02±0.06^{A}	0.92±0.08^{B,C}	13	1.04±0.09^{A,B}	
3	1.01±0.07^{A}		14	1.12±0.08^{B,C}	
4	0.98±0.09^{A}	0.86±0.06^{A,B}	15	1.14±0.21^{A,B,C}	
5	1.11±0.02^{B,C}	0.84±0.05^{A}	17	1.18±0.09^{C}	
6	1.02±0.07^{A}	0.90±0.05^{B}	19		0.89±0.06^{A,B}
7	1.00±0.06^{A}	0.85±0.08^{A,B}	20		0.93±0.10^{B,C}
8	1.15±0.12^{B,C}	0.93±0.06^{B}	21		0.86±0.05^{A,B}
9	1.20±0.11^{C}	1.06±0.17^{C,D}	22		0.87±0.08^{A,B}
10	1.09±0.09^{A}	1.08±0.25^{C,D}	23		0.95±0.11^{B,C}
11	1.10±0.07^{B,C}	1.03±0.24^{B,C,D}	24		1.15±0.08^{D}
12	0.98±0.08^{A}		average	1.06±0.12	0.94±0.15

Chemical analyses

Table 3 gives the lipid content and concentrations of the most important mono-*ortho* chlorine substituted congeners (CB118, -105 and -156), the di-*ortho* chlorine substituted congener CB153 (being the dominant congener in all samples) and the sum of the seven chlorobiphenyl congeners usually selected for international monitoring programmes (CB28, -52, -101, -118, -138, -153, -180).

For the first survey PCB levels at the off-shore stations 5 (Doggerbank), 6 and 8 were the lowest for all congeners, whilst PCB levels around the harbour of Rotterdam (stations 11) and near the Western Scheldt (station 17) were the highest. Differences between highest and lowest PCB levels between stations were approximately 2-fold.

Table 3. Lipid content (%), concentrations (ng/g lipid) of individual CB congeners (indicated according to their IUPAC number), including the mono-*ortho* CB118, -105 and -156 and the sum (Σ7 CB) of seven selected congeners (CB28, -52, -101, -118, -138, -153, -180) in muscle tissue of male mature dab (average values±SD for pooled samples) during the first (August-September 1991) and seconnd survey (May-June 1992). Only 4 fish were caught at station 10 during the 1991 survey, one pooled sample was analysed for PCB concentrations.

	First survey (1991)					
Station	% lipid	CB118	CB153	CB105	CB156	Σ7 CB
2	2.52±0.56	80±20	213±42	29±4	18±13	714±136
3	4.44±0.50	79±28	197±36	35±14	11±3	666±152
4	2.54±2.20	84±36	213±67	29±10	19±11	772±245
5	3.77±2.24	59±25	143±55	25±9	8±3	540±167
6	3.53±1.81	63±24	152±46	29±10	17±18	568±99
7	2.57±0.76	84±14	207±37	27±8	14±4	719±107
8	3.39±0.74	57±13	172±40	23±6	12±4	575±93
9	7.30±3.20	96±47	216±135	37±14	27±19	755±363
10	4.77	86	228	26	10	745
11	3.79±0.80	106±26	322±70	41±7	21±5	952±202
12	2.59±0.41	99±22	217±44	34±4	15±3	737±138
13	2.08±0.40	86±6	215±20	29±3	27±18	734±36
14	3.92±0.98	81±21	217±59	29±5	13±2	695±101
15	2.48±0.50	99±31	217±42	33±11	16±11	737±105
17	4.39±2.39	202±32	225±9	57±9	33±9	1136±72

More pronounced differences in PCB concentrations were found during the second survey (1992) at the beginning of the summer. Levels around the Doggerbank (stations 5 and 6) and the coastal station 24 were lowest for all congeners, whilst levels at the off-shore stations 4 and 7 and the coastal station 9 were the highest. High PCB concentrations were found at the offshore station 4 (903 ng CB153/g lipid versus a range of 174-579 ng CB153/g lipid for the other stations); this is probably due to the very low lipid content of the muscle tissue (0.8% versus a range of 1.1-3.6% for the other stations). Differences between highest and lowest PCB levels between stations were approximately 4-fold.

Table 3. (Continued).

	Second survey (1992)					
Station	% lipid	CB118	CB153	CB105	CB156	Σ7 CB
2	1.52±0.46	156±55	412±149	58±21	28±10	1266±435
4	0.79±0.29	272±203	903±659	87±62	45±31	2555±1770
5	2.48±0.37	77±8	174±11	27±2	11±3	592±42
6	2.17±0.36	88±13	268±45	29±5	13±3	793±120
7	1.05±0.37	179±86	579±401	61±35	28±16	1954±1138
8	1.69±0.37	120±19	337±69	43±7	24±4	1136±607
9	2.44±1.19	195±83	577±241	53±25	22±11	1582±643
10	3.62±0.68	134±14	305±49	39±7	18±1	960±122
11	2.77±0.27	155±21	410±92	44±6	23±3	1184±205
19	2.44±0.61	134±43	281±95	46±16	21±9	932±312
20	1.92±0.19	129±18	276±47	44±6	25±6	993±188
21	1.28±0.13	132±15	293±38	44±6	30±5	1376±250
22	1.34±0.24	205±40	447±100	67±14	42±15	2030±293
23	1.98±0.35	185±36	407±93	52±11	31±9	1757±198
24	3.31±0.21	110±16	283±38	27±4	17±3	1109±84

Biochemical analyses

Analyses were performed on 13,000g supernatants. Mean CYP1A values, as measured in liver tissue of dab obtained from the various sampling stations in the North Sea are presented in Table 4. For the first survey, the EROD activity and CYP1A protein levels at the offshore stations 4, 6 and 7 were significantly higher than at all other 12 stations.

For the second survey, EROD levels at the coastal stations 24, 9 and 10 were significantly lower than those at the other stations. Also, CYP1A protein levels at the mouth of the Western Scheldt (station 24) were significantly lower than levels at the other stations, except for the coastal stations 9 and 10.

Hepatic CYP1A protein and EROD levels were with a few exceptions, generally higher during the 2nd survey (May-June 1992) in comparison with the 1st survey (August-September 1991).

Table 4. Hepatic and cardiac EROD activity, determined at ambient and room temperature (R.T.) (nmol/min/mg protein) and CYP1A protein content (ELISA absorbance 492 nm) in 13,000g fractions of mature male dab from the southern North Sea. Data represent mean values±SD. Levels indicated by similar letters are not significantly different ($p<0.05$).

	First survey (1991) - liver		Second survey (1992) - liver		
Station	EROD (R.T.)	CYP1A protein	EROD (R.T.)	CYP1A protein	EROD (ambient T)
2	0.06 ± 0.04^{CD}	0.35 ± 0.08^{C}	1.46 ± 0.55^{C}	1.08 ± 0.35^{D}	0.68 ± 0.29^{CD}
3	0.04 ± 0.03^{D}	0.39 ± 0.07^{BC}			
4	0.79 ± 0.36^{A}	0.94 ± 0.34^{A}	2.23 ± 0.77^{D}	1.03 ± 0.30^{D}	0.90 ± 0.36^{D}
5	0.11 ± 0.09^{C}	0.25 ± 0.07^{D}	0.95 ± 0.50^{BC}	0.55 ± 0.24^{C}	0.43 ± 0.23^{BC}
6	0.72 ± 0.42^{A}	0.85 ± 0.23^{A}	1.49 ± 0.71^{CD}	1.07 ± 0.45^{D}	0.60 ± 0.37^{BCD}
7	0.64 ± 0.40^{A}	0.99 ± 0.28^{A}	1.46 ± 0.40^{C}	1.21 ± 0.35^{D}	0.61 ± 0.17^{CD}
8	0.12 ± 0.07^{C}	0.10 ± 0.04^{E}	1.34 ± 0.61^{C}	1.29 ± 0.36^{D}	0.61 ± 0.26^{CD}
9	0.07 ± 0.05^{CD}	0.13 ± 0.08^{E}	0.19 ± 0.23^{A}	0.31 ± 0.21^{A}	0.10 ± 0.16^{A}
10	0.15 ± 0.06^{BC}	0.28 ± 0.06^{CD}	0.32 ± 0.37^{A}	0.36 ± 0.30^{AB}	0.17 ± 0.24^{AB}
11	0.24 ± 0.14^{B}	0.28 ± 0.15^{CD}	0.61 ± 0.40^{B}	0.53 ± 0.30^{BC}	0.35 ± 0.30^{B}
12	0.11 ± 0.06^{C}	0.27 ± 0.10^{CD}			
13	0.14 ± 0.08^{BC}	0.37 ± 0.08^{BC}			
14	0.05 ± 0.03^{CD}	0.29 ± 0.10^{CD}			
15	0.14 ± 0.06^{C}	0.47 ± 0.09^{B}			
17	0.07 ± 0.04^{CD}	0.29 ± 0.08^{CD}			
19			1.74 ± 0.80^{CD}	1.02 ± 0.40^{D}	0.98 ± 0.42^{D}
20			1.72 ± 0.82^{CD}	1.18 ± 0.37^{D}	0.81 ± 0.38^{D}
21			1.36 ± 0.90^{C}	1.12 ± 0.49^{D}	0.72 ± 0.51^{BCD}
22			1.22 ± 0.72^{C}	0.91 ± 0.39^{D}	0.83 ± 0.61^{CD}
23			1.14 ± 0.49^{C}	0.70 ± 0.34^{C}	0.65 ± 0.38^{BCD}
24			0.09 ± 0.04^{A}	0.23 ± 0.03^{A}	0.04 ± 0.04^{A}

CYP1A protein and EROD levels were significantly correlated for both surveys ($p<0.0001$), with an overall correlation coefficient of $r=0.59$ ($n=321$) for the first survey and $r=0.90$ ($n=365$) for the second survey.

Table 4. (Continued).

Station	Second survey (1992) - heart	
	EROD (R.T.)	CYP1A protein
2	0.034 ± 0.039^{ABC}	0.045 ± 0.020^{BCD}
3		
4	0.088 ± 0.058^{A}	0.049 ± 0.014^{CD}
5	0.021 ± 0.016^{B}	0.031 ± 0.006^{AB}
6	0.084 ± 0.127^{AB}	0.068 ± 0.046^{D}
7	0.047 ± 0.077^{ABC}	0.052 ± 0.071^{ABCD}
8	0.017 ± 0.010^{BC}	0.037 ± 0.011^{ABCD}
9	0.011 ± 0.007^{BC}	0.025 ± 0.009^{A}
10	0.012 ± 0.010^{BC}	0.027 ± 0.007^{AB}
11	0.011 ± 0.006^{BC}	0.027 ± 0.008^{AB}
19	0.027 ± 0.019^{B}	0.035 ± 0.007^{AB}
20	0.027 ± 0.016^{B}	0.041 ± 0.012^{BCD}
21	0.025 ± 0.017^{B}	0.045 ± 0.013^{CD}
22	0.018 ± 0.013^{B}	0.037 ± 0.009^{ABCD}
23	0.019 ± 0.016^{B}	0.026 ± 0.008^{A}
24	0.005 ± 0.004^{C}	0.029 ± 0.008^{AB}

Hepatic EROD activity in samples from the second survey were also measured in the laboratory at the temperature of the ambient bottom water. EROD levels are presented in Table 4. Levels were lower compared to the analyses carried out at the standard assay temperature.

CYP1A values in heart of dab during the 1992 survey are presented in Table 4. EROD levels at the offshore station 4, where the highest PCB concentrations were found, were significantly higher than levels at all other stations except station 2, 6 and 7. A positive correlation between levels of EROD activity in the liver and in the heart was observed (r=0.51; p<0.0001; n=152). Absolute EROD activity levels in heart were lower by a factor of 16-80 compared to liver. A positive correlation between levels of CYP1A protein levels in liver and in the heart was also observed (r=0.40; p<0.0001; n=152). CYP1A protein levels in heart were lower by a factor of 8-35 compared to liver.

The EROD measurements carried out onboard in the 13,000g supernatants of individual specimens were averaged for the same sets of samples of five specimens that were

pooled for the analysis of EROD in the microsomal fractions. The correlation between EROD activity in the microsomal fractions and EROD activity in 13,000g supernatants was highly significant (r=0.92, p<0.0001; n=73). EROD values in the microsomes were higher by a factor of 2.0±0.8 (mean±SD) compared to the 13,000g supernatants. This is due to the removal of aspecific cytosolic proteins during the ultracentrifugation step in the isolation of microsomes.

Relation of chemical data, condition factor and temperature with CYP1A levels

Table 5 summarizes the results of the multiple regression analyses with concentrations of selected individual CB congeners in fish and water temperature as independent variables influencing hepatic EROD activity or CYP1A protein levels during the 1992 survey. As stated before, PCB data were normalised on lipid weight; the usage of data normalised on wet weight did not yield significant relationships between contaminant concentrations and EROD activity or CYP1A protein.

Table 5. Multiple regression table with temperature and individual PCB congeners as factors influencing EROD activity and CYP1A protein. Data from the 1992 survey. NS = not significant.

CYP1A parameter	CB congener	F-test	Partial F temperature	Partial F CB congener	Probability temperature	Probability CB congener
EROD	CB118	F=41.09 p=0.0001	69.93	10.85	0.0001	0.0016
	CB105	F=46.69 p=0.0001	65.68	16.80	0.0001	0.0001
	CB156	F=50.32 p=0.0001	70.99	20.65	0.0001	0.0001
	CB153	F=35.83 p=0.0001	58.32	5.26	0.0001	0.0248
CYP1A protein	CB118	F=41.09 p=0.0001	56.02	1.22	0.0001	0.27 (NS)
	CB105	F=46.69 p=0.0001	53.60	4.53	0.0001	0.0368
	CB156	F=50.32 p=0.0001	56.77	8.18	0.0001	0.0056
	CB153	F=35.83 p=0.0001	53.21	0.083	0.0001	0.77 (NS)

For the EROD activity all four selected CB congeners fitted significantly in the linear model (p values in Table 5), which can be described by the following relationships:

EROD=3.047-0.190T+0.002[CB118]	(r=0.735, n=73)
EROD=2.861-0.180T+0.008[CB105]	(r=0.756, n=73)
EROD=2.885-0.182T+0.016[CB156]	(r=0.768, n=73)
EROD=3.106-0.183T+4.61E-4[CB153]	(r=0.711, n=73)

As an example, the relationship between water temperature, CB153 and EROD activity is shown in Fig. 2. An inverse relationship between water temperature and EROD activity is present, whereas CB153 concentration is positively related with EROD activity.

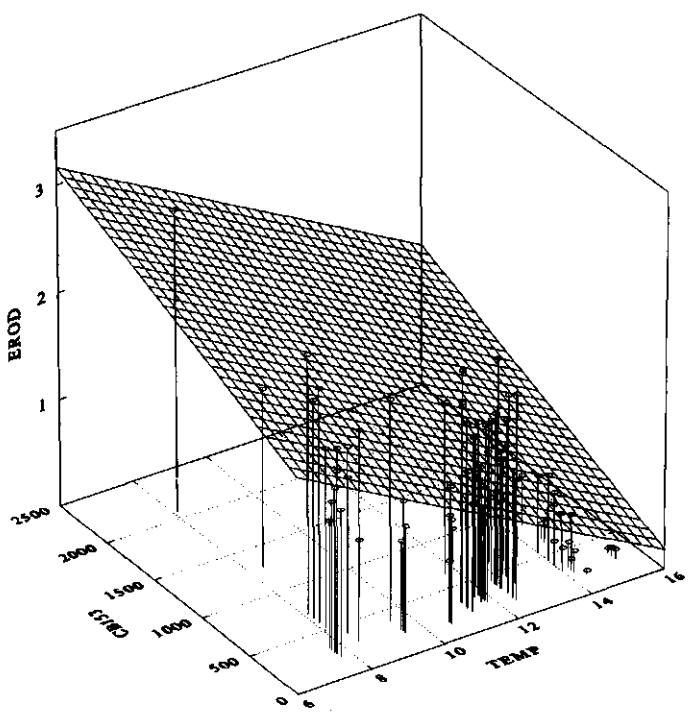


Figure 2. EROD activity (nmol/min/mg protein) of mature male dab in relation to the bottom water temperature and CB153 (ng/g lipid) (data from 1992 survey).

For CYP1A protein, only CB105 and -156 fitted significantly in the linear multiple regression model (p values in Table 5), which can be described by the following relationships:

CYP1A=1.940-0.106T+0.003[CB105] (r=0.690, n=73)

CYP1A=1.911-0.106T+0.006[CB156] (r=0.708, n=73)

Again an inverse relationship between water temperature and CYP1A protein is present, whereas PCB concentrations of individual congeners are positively related to concentrations of CYP1A protein (not shown).

The results of multiple regression analyses with individual PCB congeners in fish and condition factor (k_f) of fish as variables influencing hepatic EROD activity or CYP1A protein levels during the 1992 survey are summarized in Table 6. For the EROD activity all four

Table 6. Multiple regression table with condition factor of fish (k_f) and individual PCB congeners as factors influencing EROD activity and CYP1A protein. Data from the 1992 survey. NS = not significant.

CYP1A parameter	CB congener	F-test	Partial F k_f	Partial F CB congener	Probability k_f	Probability CB congener
EROD	CB118	F=33.96 p=0.0001	56.82	9.38	0.0001	0.0031
	CB105	F=38.17 p=0.0001	51.53	14.22	0.0001	0.0003
	CB156	F=36.01 p=0.0001	47.35	11.73	0.0001	0.0010
	CB153	F=35.96 p=0.0001	58.56	11.68	0.0001	0.0011
CYP1A protein	CB118	F=23.79 p=0.0001	46.05	0.99	0.0001	0.32 (NS)
	CB105	F=26.09 p=0.0001	43.01	3.77	0.0001	0.056 (NS)
	CB156	F=26.33 p=0.0001	56.77	8.18	0.0001	0.048
	CB153	F=24.38 p=0.0001	46.46	1.7	0.0001	0.20 (NS)

selected PCB congeners fitted significantly in the model (p values in Table 6), which can be described by the following relationships:

EROD=4.665-4.068k_f+0.002[CB118]	(r=0.702, n=73)
EROD=4.360-3.808k_f+0.008[CB105]	(r=0.722, n=73)
EROD=4.346-3.739k_f+0.013[CB156]	(r=0.712, n=73)
EROD=4.719-4.071k_f+0.001[CB153]	(r=0.712, n=73)

For CYP1A protein, only CB156 fitted significantly in the multiple regression model (p values in Table 6), which can be described by the following relationship:

CYP1A=2.790-2.202k_f+0.005[CB156]	(r=0.655, n=73)

Since temperature and condition factor correlated significantly (r=0.64, n=73, p<0.0001), no multiple regression analyses, including both parameters influencing, could be conducted.

Data from the 1991 survey were not analysed with multiple regression analysis, because the CYP1A data showed two distinct clusters.

Concentrations of biliary 1-OH pyrene in dab caught during the surveys were measured by Ariese (1993). Levels were shown to be highest in the coastal areas and lower around the Doggerbank and close to the English Channel. Levels of hepatic CYP1A levels did not correlate with biliary 1-OH pyrene levels. Fig. 3A and 3B show the relation between CYP1A levels and 1-OH pyrene during the 1992 survey.

EROD activity in samples from the second survey were also measured in the laboratory at the ambient bottom temperature of the sampling stations. The relationships between the levels of EROD activity, the ambient water temperature and individual PCB congeners, analysed with multiple regression analyses, were significant for CB105, -118 and -156 (Table 7). The relationship between the ambient water temperature and EROD activity at ambient temperature (EROD A.T.) still revealed an inverse correlation, but the slope of the regression became less steep.

EROD A.T.=1.134-0.062T+0.001[CB118]	(r=0.517, n=73)
EROD A.T.=1.037-0.056T+0.004[CB105]	(r=0.550, n=73)
EROD A.T.=1.029-0.057T+0.008[CB156]	(r=0.582, n=73)

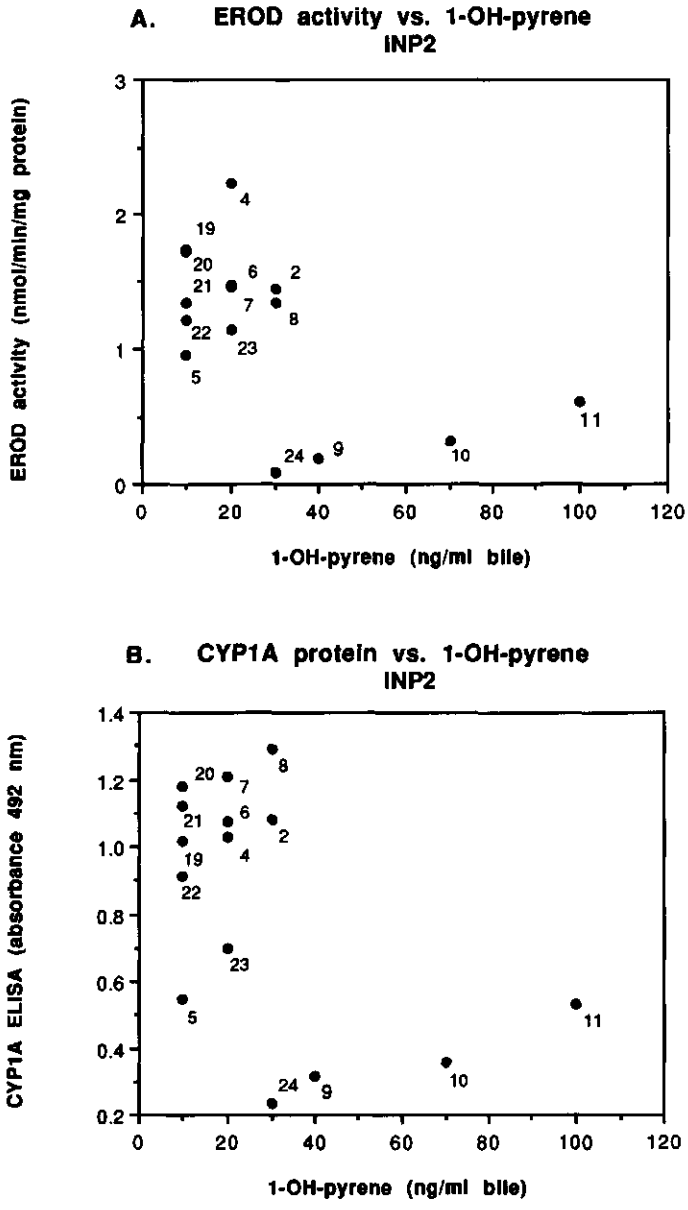


Figure 3. Relation between CYP1A levels and biliary 1-OH pyrene of mature male dab. (A) Hepatic EROD activity (nmol/min/mg protein) versus 1-OH pyrene (ng/ml bile). (B) Hepatic CYP1A protein (ELISA absorbance at 492 nm) versus 1-OH pyrene (ng/ml bile).

Table 7. Multiple regression table with temperature and individual PCB congeners as factors influencing EROD activity measured at ambient temperature. Data from the 1992 survey. NS = not significant.

CYP1A parameter	CB congener	F-test	Partial F temperature	Partial F CB congener	Probability temperature	Probability CB congener
EROD	CB118	F=12.76 p=0.0001	18.76	6.21	0.0001	0.015
	CB105	F=15.19 p=0.0001	16.20	10.09	0.0001	0.0022
	CB156	F=17.92 p=0.0001	17.70	14.45	0.0001	0.0003
	CB153	F=9.84 p=0.0002	15.86	1.55	0.0002	0.22 (NS)

Laboratory study

CYP1A levels in liver of dab obtained from the Doggerbank and kept in the laboratory for a period of 4 weeks at temperatures of 8, 12 and 16°C respectively, are shown in Figure 4. The results show a negative correlation of EROD activity with temperature (r=-0.61; p<0.005; n=24). The groups acclimated to 8 and 16°C differed significantly from each other (p<0.05). CYP1A protein levels, however, did not differ significantly between the three groups.

Discussion

The main objective of the present study was to evaluate the applicability of the CYP1A response to indicate exposure to toxic microcontaminants with capability to induce CYP1A, for the North Sea environment.

The PCB distribution patterns for muscle tissue in dab in general showed slightly higher contaminant levels along the coast and lower levels towards the offshore areas. The PCB data shown in Table 3 are very similar to the results of Knickmeyer and Steinhardt (1993) for dab from the North Sea, where concentrations of Σ7 CB varied in the range 180-2300 ng/g lipid. The absolute values in May (1992 survey) were higher by a factor of 2 compared to the August situation (1991 survey). The difference was very probably due to a seasonal effect, since all fish were leaner in May. Since CB congeners are highly lipophilic, bioaccumulation in

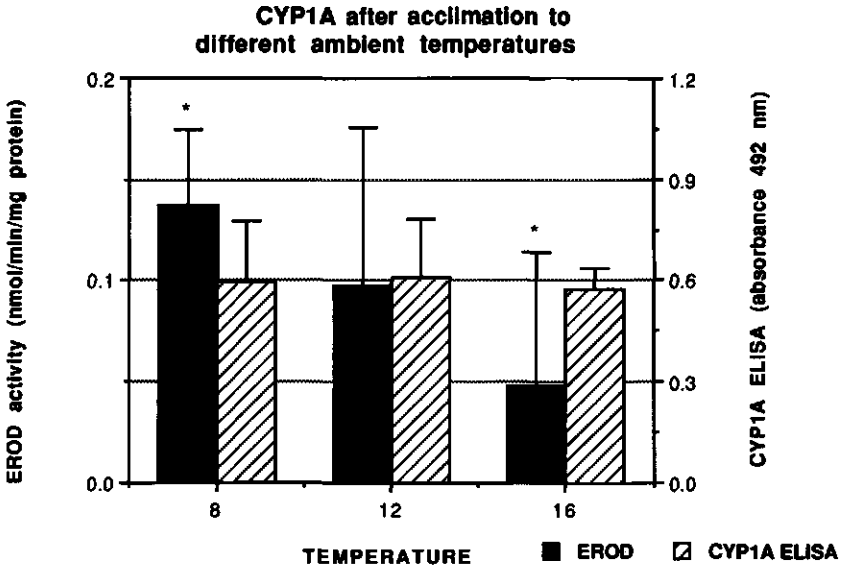


Figure 4. Hepatic EROD activity (nmol/min/mg protein) and CYP1A protein content (ELISA absorbance at 492 nm) of mature male dab from the Doggerbank, acclimated to different water temperatures in the laboratory for a period of 4 weeks, * significantly different at $p<0.05$. Data represent mean values ± SD ($n=8$).

fish is closely related to lipid physiology (Boon, 1985; Boon *et al.*, 1985). An important observation which supports the usage of lipid based data when relating exposure to effects is the fact that PCB data expressed on lipid weight basis correlated significantly with EROD activity and CYP1A levels in the linear multiple regression analysis model, but wet weight-based PCB data did not.

In fish, CYP1A can be induced by non-*ortho* congeners (Gooch *et al.*, 1989; Van der Weiden *et al.*, 1994a). Non-*ortho* CB's were not measured during this study, but they were very likely to be present at low levels in muscle tissue of fish, because they are present in the environment and accumulate in marine organisms (Tanabe *et al.*, 1987b). De Boer *et al.* (1993) found a good correlation between CB153 and non- and mono-*ortho* CB's in fish from the Dutch aquatic environment. Therefore, differences in concentrations of the measured mono- and di-*ortho* CB-congeners in this study may probably represent differences in concentrations of non-*ortho* congeners as well.

Dab from all stations had very low concentrations of PAHs (Boon *et al.*, 1993), which is very probably due to a rapid biotransformation and excretion of these products (Varanasi *et al.*, 1989). Usually the concentrations were just above or below the limit of detection. The analysis of biliary 1-OH pyrene, which is a pyrene metabolite, gave more information about the amount of pyrene that had been taken up by dab and was used as an index for total PAH exposure. Ariese (1993) found higher pyrene exposure levels along the Dutch coast and lower exposure levels at the offshore areas.

The results of hepatic CYP1A levels in dab described in the present study did not directly correspond in a simple linear manner to these spatial trends in PAH and PCB concentrations. However, PCBs and PAHs are not the only contaminants present in sea water and sediment affecting CYP1A, although in the Dutch aquatic environment the toxic potential of PCBs is about 4 times higher than that of PCDDs and PCDFs (De Boer *et al.*, 1993).

During both surveys the highest CYP1A levels were found at relatively clean off-shore stations with low bottom water temperatures due to stratification of the water column, while considerably lower CYP1A levels were found at stations along the Dutch coast with relatively high bottom water temperatures. Multiple regression analyses with individual PCB congeners and temperature as variables influencing EROD activity or CYP1A protein, indicated that there was a combined influence of water temperature and PCB concentrations on CYP1A levels in fish. The water temperature was inversely related with CYP1A levels, while PCB concentrations were positively related with CYP1A levels. It is apparent that these congeners, or other contaminants that co-vary, influence CYP1A levels. Therefore, the basic concept of using CYP1A responses as a biomarker for environmental contamination is applicable to dab from the North Sea. However, because the partial F values for individual congeners were always lower than those for water temperature (Table 5), the contribution of contamination on the levels of CYP1A was lower than that of temperature.

A laboratory experiment confirmed that EROD activity was indeed inversely proportional to water temperature; a similar relation could not be established for CYP1A protein levels. It has been observed in several studies that hepatic P450-dependent activities in fish respond to acclimation temperature in a compensatory manner, whereby cold-acclimated rates were higher than warm-acclimated rates (Stegeman, 1979; Koivusaari and Andersson, 1984; Andersson and Koivusaari, 1986; Ankley *et al.*, 1985; Blanck *et al.*, 1989). Possible explanations for these higher activities upon cold-acclimation involve changes in microsomal fatty acid composition, which increase membrane fluidity (Hazel, 1984) and an increase in the NADPH cytochrome c reductase/cytochrome P450 ratio, facilitating the reduction of P450

(Blanck *et al.*, 1989). Temperature acclimation may be accompanied with a change in the P450 isoenzyme pattern (Andersson and Förlin, 1992; Carpenter *et al.*, 1990). However, in our laboratory experiment CYP1A protein levels in dab were unchanged with water temperature. During our surveys on the North Sea, however, inverse relationships between water temperature and CYP1A levels seemed to exist. In the laboratory study fish were not induced, therefore additional experiments investigating the induction of CYP1A in dab by contaminants at different temperatures should be conducted.

In the present study, differences in EROD activity between the stations still existed after measuring EROD at ambient temperature, although levels of EROD activity were lower compared to the analyses carried out at the standard assay temperature. Assuming that hepatic EROD activity in dab responds to acclimation temperature in a compensatory manner, this indicates that the differences in EROD activity in the field can only partially be explained by differences in ambient temperature. This is expected, because of the additional effects of exposure to environmental contaminants. The ratio between the partial F values for temperature and individual congeners is lower when EROD activity is measured at ambient temperature (Table 7) compared to the ratio when EROD activity is measured at standard assay temperature (Table 5). This indicates that the contribution of contamination on EROD levels became greater in the multiple regression model when EROD activity was measured at ambient temperature.

Apart from the effects of xenobiotics and temperature, it is known that diet can affect enzymatic systems and monooxygenase activities in some fish species (Jimenez *et al.*, 1987; Lemaire *et al.*, 1992; Goksøyr *et al.*, 1994b). Dab from coastal areas generally showed higher condition factors, varying with the nutritional state of the fish, than dab from the offshore areas, and condition factors were generally higher during the first survey at the end of the summer, compared to the second survey in the middle of the spring. Multiple regression analysis with individual PCB congeners and condition factor of the fish as variables influencing CYP1A levels, indicated that there was a combined influence of both parameters on CYP1A levels, again the contribution of PCBs was less than the condition factor (Table 6). Therefore, care should be taken when trying to compare data from stations with a different nutritional status of dab. Calculations including both condition factor and temperature could not be conducted because these parameters co-varied significantly.

CYP1A levels were generally higher during the second survey compared to the first survey. This observation seems mainly to be explained by the higher contaminant concentrations (on a lipid basis) and generally lower temperatures of the bottom water during the second survey. Differences in hormonal status are not very likely to have influenced the

CYP1A levels, since dab in the southern North Sea spawn between February and April (Rijnsdorp *et al.*, 1992), which means that fish were post-spawning during both surveys (August-September and May-June).

In general CYP1A levels were lower in heart compared to liver, similar results were also reported for the scup, *Stenotomus chrysops* (Stegeman *et al.*, 1982). Cardiac EROD activity measurements were just below or above detection level of the plate reader, therefore the heart seems to be less suitable than the liver as a monitoring organ using the catalytic assay.

In conclusion, the present study showed that differences in CYP1A levels in dab from the North Sea could only be interpreted in terms of exposure to polyaromatic contaminants when the influence of habitat temperature and condition factor of fish were also taken into account.

Acknowledgements

The two surveys were organized under the auspices of the Netherlands Marine Research Foundation (SOZ) of the Netherlands Organization for Scientific Research (NWO). Thanks are due to the crew of the research vessel RV *Pelagia* for their help and all of those involved in the project for their support and enthusiasm. For the information with regard to the statistics applied, the authors owe thanks to Jaap van der Meer.

Chapter 4

Cytochrome P450 1A response in North Sea dab, *Limanda limanda*, from offshore and coastal sites *

Abstract

Dab (*Limanda limanda*) were collected at four stations in the southern North Sea in November 1993. Highest muscle polychlorinated biphenyl (PCB) concentrations were found at a station near the Dutch coast. CB153 was the dominant congener at all stations. Since the concentrations of two mono-*ortho* Cl-substituted congeners covaried strongly with CB153, this congener appears to be a good marker for general differences in PCB concentrations. Mean CB153 concentrations ranged from 50-160 ng/g lipid. Highest cytochrome P450 1A (CYP1A) levels were also found at the coastal station. The extent of induction, measured catalytically (7-ethoxyresorufin O-deethylase, EROD) and immunochemically (CYP1A ELISA), between the less contaminated offshore sites and the coastal site ranged up to five-fold. Both parameters were well correlated with muscle tissue CB concentrations. The data indicate that the CYP1A induction response in dab populations along the Dutch coast is strong enough to separate the Dutch coastal area from more pristine offshore areas of the North Sea. Compared to results of previous studies in other seasons the present sampling period offered the best conditions to investigate the correlation between environmental contamination with PCBs and related compounds, since the variations in natural factors that also interfere, such as water temperature and condition of the fish, were very low.

* Hedwig M. Sleiderink and Jan P. Boon
Published in *Marine Pollution Bulletin* **30** (1995), 660-666

Introduction

The Dutch coastal zone belongs to one of the heaviest polychlorinated biphenyl (PCB) contaminated areas in the world (Lohse, 1991; Klamer *et al.*, 1991; De Boer *et al.*, 1993). The toxic potential of PCBs in fish caught in the Dutch aquatic environment is about four times more important than that of polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) (De Boer *et al.*, 1993). Due to the lipophilic and persistent character of some of these compounds, they can accumulate in fish and other aquatic organisms (Ankley *et al.*, 1992; 1993; Macdonald *et al.*, 1993).

In laboratory studies with fish, especially the planar compounds have been found to cause induction of the cytochrome P450 1A (CYP1A) subfamily (Janz and Metcalfe, 1991; Gooch *et al.*, 1989; Van der Weiden *et al.*, 1994a). This biochemical parameter may therefore serve as a biomarker of exposure to planar environmental contaminants (Goksøyr and Förlin, 1992; Payne *et al.*, 1987). In field studies with fish, CYP1A induction has often been found to correlate with the degree of environmental contamination with PCBs and other halogenated aromatic hydrocarbons with capability to induce CYP1A, like polyaromatic hydrocarbons (PAHs), PCDDs and PCDFs (Elskus and Stegeman, 1989; Monosson and Stegeman, 1994; Van Veld *et al.*, 1990; McMaster *et al.*, 1991; Vindimian *et al.*, 1991; Förlin *et al.*, 1992; Addison *et al.*, 1994). Interpreting the significance of this environmental induction will depend in part of the identity and concentration of inducing aquatic contaminants to which these fish were exposed, and in part of the identification of the several natural abiotic and biotic variables, such as water temperature, dietary factors, age, sex and sexual maturity, influencing the expression of CYP1A (Andersson and Förlin, 1992; Sleiderink *et al.*, 1995b; 1995d).

In pollution monitoring programmes in the North Sea (North Sea Task Force; Joint Monitoring Programme), dab (*Limanda limanda*) is used for measurements of CYP1A activity because it is a demersal fish with a large geographic distribution and abundance. Dab has been shown to be sensitive towards CYP1A inducers, e.g. the technical PCB mixture Clophen A40 caused induction of CYP1A protein and the related enzyme activity, 7-ethoxyresorufin O-deethylase (EROD) (Sleiderink *et al.*, 1995a).

In spring and summer, bottom water temperature differences of up to 10°C can occur between stratified and vertically mixed areas of the southern North Sea. This was previously shown to have a strong effect on CYP1A expresssion in dab, making the response to differences in contaminant concentrations difficult to discern (Sleiderink *et al.*, 1995c). In late autumn and winter, stratification has disappeared resulting in minor differences in temperatures between stations of the same survey. Dab in the southern North Sea show migration associated

with spawning activities peaking between February and April (Rijnsdorp *et al.*, 1992). Thus, this season is also less suitable for monitoring purposes, since specimens are probably not representative for the area of capture, and, moreover, sexual processes also have a strong influence on CYP1A expression, especially in female fish (Sleiderink *et al.*, 1995b). The interference of all these natural factors with CYP1A expression is minimal during the late autumn, when stratification has disappeared and sexual migration has not yet begun. Therefore, the aim of the present study was to examine whether the late autumn is a suitable season for application of CYP1A measurements in dab as indicators of exposure to CB congeners in the southern North Sea.

Materials and methods

Sampling of fish

Mature male dab (length between 15 and 20 cm) were sampled by beam trawling (5 m beam) from four sites in the southern North Sea in November 1993 (Fig. 1). The trawling positions were: Site 1, 4°3'E, 52°5'N; Site 2, 4°0'E, 54°3'N; Site 3, 2°4'E, 54°5'N; Site 4, 4°2'E, 54°5'N. The survey covered two stations (Sites 2 and 4) showing stratification of the water column during spring and summer, resulting in lower bottom water temperatures. Considerably elevated CYP1A levels were measured in dab caught at these offshore stations compared to the coast when stratification was present (Sleiderink *et al.*, 1995c). In late autumn and winter stratification is absent in the southern North Sea resulting in similar values for bottom water temperature. The bottom water temperature and fishing depths were: Site 1, 8.0°C, 22 m; Site 2, 9.8°C, 46 m; Site 3, 8.7°C, 24 m; Site 4, 9.7°C, 50 m.

Dab (n=25) were randomly selected from at least five hauls to eliminate the effect of interhaul-variation. Fish were checked for visible signs of diseases, sexed, measured and killed by a cephalic blow and cutting of the spinal cord. The livers were excised and washed with an ice cold solution of 1.15% KCl to remove remnants of bile and blood. Livers were homogenized in four volumes of 0.1 M Na-phosphate buffer pH 7.4; 0.1 M KCl; 1 mM EDTA with 10% (v/v) glycerol and centrifuged at 13,000g for 15 min. at 4°C. The homogenates were frozen in liquid nitrogen and stored at -80°C until used. The carcasses of the fish were frozen at -25°C for PCB analysis.

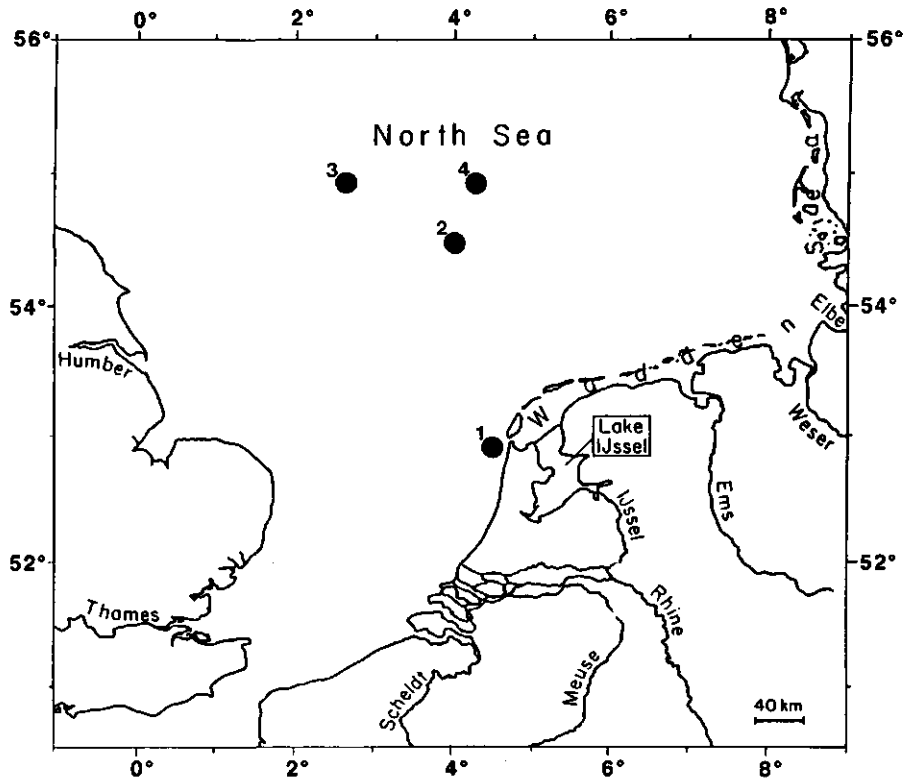


Fig. 1. Map of the southern North Sea showing the location of the sampling stations.

Biochemical analyses

All biochemical analyses were performed on individual livers. EROD activity was measured fluorometrically at room temperature on a platereader using the method of Eggens and Galgani (1992). Resorufin formation was quantified using an extinction coefficient of 73 $mM^{-1}cm^{-1}$ (Klotz *et al.*, 1984). A laboratory reference material was analysed on every plate to obtain an indication for the variance of the results.

Proteins were measured according to Bradford (1976), with bovine serum albumin as reference standard.

CYP1A protein levels were determined semi-quantitatively using an enzyme-linked immunosorbent assay (ELISA) described by Goksøyr (1991), using monoclonal mouse anti-cod CYP1A IgG as primary antibody. The secondary antibody was goat anti-mouse IgG conjugated to horseradish peroxidase.

PCBs analyses

CB congeners were analysed by capillary gas chromatography with electron capture detection (GC-ECD) on a Hewlett Packard 5880-A GC fitted with a 50 m microbore CP-Sil 19 column. Gas chromatographic determination of PCBs have been described before (Boon, 1985; Holden and Marsden, 1969; Boon *et al.*, 1992a; Sleiderink *et al.*, 1995a) and only supplementary information is given here. Lipids and PCBs were extracted from muscle tissue using a modification of the method of Bligh and Dyer (1959), as described by Booij and Van den Berg (1994). A microbore CP-Sil 19 column allows for the analysis of non- and mono-*ortho* congeners (De Boer, 1992a; Larsen *et al.*, 1992a), which cannot be adequately separated with a number of other columns, such as the often used CP-Sil 8. The CB congeners are numbered according to IUPAC rules, as suggested by Ballschmiter and Zell (1980). In every other batch of five samples analysed together, either a blank sample or an aliquot of the certified reference material CRM-349 (EC-BCR) of cod liver oil was analysed to check for the performance of the analytical procedure. The laboratory has recently participated in an ICES intercalibration exercise on PCBs (De Boer *et al.*, 1992b) and currently participates in the EC-based QUASIMEME programme.

Since the entire livers were used for biochemical analyses, PCBs in muscle tissue located between the backbone and the light skin were used as a parameter for qualitative changes occuring in the entire fish. This approach proved to be valid in laboratory experiments with dab (Sleiderink *et al.*, 1995a). Fish were pooled; each sample contained muscle tissue of five specimens.

Statistical analyses

The log-transformed data were analysed statistically by a least significance difference bands test and Hochberg's GT2-test for multiple comparisons of means (Sokal and Rohlf, 1981). To examine the correlations between EROD, CYP1A protein and residue PCB concentrations, linear regression analyses were performed on log-transformed data.

The correlation structure between the concentrations of individual CB congeners for each site was examined by performing a principal components analysis on the correlation matrix of the logarithms of the lipid-based concentrations. The results of such an analysis can be shown in the form of a bi-plot (Gabriel, 1971). The rationale of this method is explained by De Boer *et al.* (1993).

Results

Since all biochemical analyses were performed on individual fish, CYP1A protein and EROD levels could be correlated on an individual basis. For the PCB-analyses muscle tissue of the same fish that were used for biochemical analyses were pooled. Each sample contained the muscle tissue of five specimens and five of such pooled samples were taken at each station. To correlate PCB data with biochemical data, EROD and CYP1A protein levels were averaged for the same five sets of samples that were pooled for the PCB analysis.

Biochemical analyses

Sites from which dab were collected are shown in Fig. 1. Table 1 shows fish weights, condition factors, EROD activity and CYP1A protein content. No differences in fish weights and condition factors between stations were observed. Mean EROD activity was significantly higher (three- to five-fold) in fish from the coastal site (Site 1). Levels of CYP1A protein content were also highest (1.5 fold) in fish from the coastal site compared to fish from the offshore sites. Levels of EROD activity and CYP1A protein were lowest at the offshore Site 4, located east of the Dogger. In general, elevation of the EROD activity and CYP1A protein content followed a similar pattern over the stations; a significant correlation was found (r=0.773; p<0.001; n=100; Fig. 2). This is an indication that in dab EROD activity is specifically catalysed by the CYP1A enzyme as has been found in other studies (Stegeman, 1989).

Table 1. Fish weights, condition factors, EROD activity and CYP1A protein in dab (*Limanda limanda*), n=25, collected from the southern North Sea in November 1993[a, b]

Site	Fish weight (g)	Condition factor (g/cm^3)[c]	EROD (nmol/min/mg)	CYP1A protein (absorbance at 492 nm)
1	62.8 ± 15.7^{A}	1.03 ± 0.09^{A}	0.099 ± 0.072^{A}	0.492 ± 0.098^{A}
2	58.5 ± 10.2^{A}	1.00 ± 0.05^{A}	0.035 ± 0.026^{B}	0.391 ± 0.053^{B}
3	63.0 ± 10.5^{A}	0.98 ± 0.04^{A}	$0.026\pm0.016^{B,C}$	0.368 ± 0.048^{B}
4	61.0 ± 13.5^{A}	0.99 ± 0.06^{A}	0.019 ± 0.014^{C}	0.321 ± 0.043^{C}

[a]Data represent mean values±SD of 25 fish from each site.

[b]Data in the same column indicated by similar letters are not significantly different (p<0.05).

[c]Condition factor, body weight (g) x 100/(length (cm))^3 (Bagenal and Tesch, 1978).

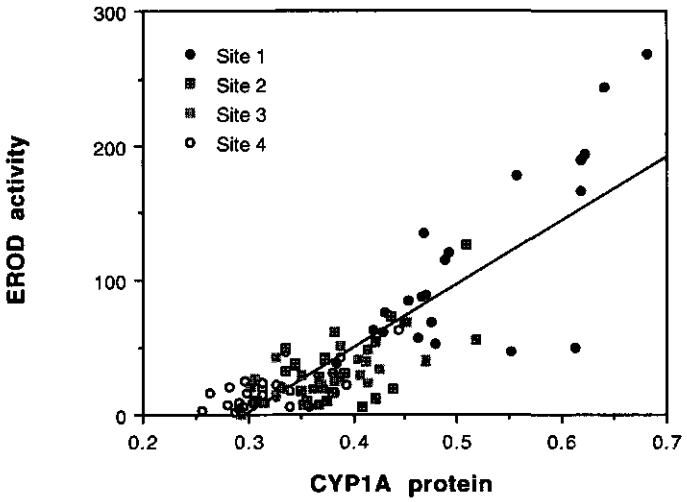


Fig. 2. Correlation between EROD activity (nmol/min/mg protein) and CYP1A protein content (absorbance at 492 nm) in dab from the southern North Sea.

PCB analyses

The lipid-based concentrations of some selected individual CB congeners and $\Sigma 7$ CB in muscle tissue from the same dab as used for biochemical analysis are shown in Table 2. The non-*ortho* congeners CB77 and -126 and the mono-*ortho* congener CB156 were below detection level. An interpretation of the data, facilitating the search for patterns within the CB congeners, is given in the bi-plot of Fig. 3. The first principal component (PC) represents the absolute value of the individual congeners and the second PC represents deviations in the CB patterns. The degree of correlation between two CB congeners is indicated by the product of the cosine of the angle between their vectors and the length of the vectors. Orthogonal vectors therefore indicate a zero correlation. The orthogonal projection of each data point on each vector shows its relative position with respect to the data range for the first and second PC. The length of the vector indicates the reliability of the approximation; if a vector reaches the unit circle then all variance is explained by the sum of the first and second PC.

Dab from the coast (Site 1) always showed significantly higher CB concentrations than dab from the offshore sites. In general PCB levels were lowest at the offshore Site 3 (the Dogger). Differences in PCB levels between stations were approximately three-fold.

Table 2. Lipid content, concentrations of individual CB congeners (indicated according to their IUPAC number) and the sum of seven selected congeners (CB28, -52, -101, -118, -138, -153, -180) in muscle tissue of dab (*Limanda limanda*) collected from the southern North Sea in November 1993[a, b]

Site	Lipid content (%)	CB153 (ng/g lipid)	CB118 (ng/g lipid)	CB105 (ng/g lipid)	Σ7 CB (ng/g lipid)
1	1.19±0.12^{A}	163±32.7^{A}	73.0±18.0^{A}	12.5±1.4^{A}	490±99.0^{A}
2	1.15±0.22^{A}	73.8±16.4^{B,C}	22.4±7.9^{B}	6.2±1.8^{B}	189±46.2^{B,C}
3	0.99±0.13^{A}	51.2±17.6^{C}	28.9±5.6^{B}	6.3±1.1^{B}	155±37.4^{C}
4	0.98±0.45^{A}	86.7±6.4^{B}	26.5±2.9^{B}	7.0±0.6^{B}	223±20.4^{B}

[a]Data represent mean values±SD of five pooled samples, each sample contains muscle tissue of five fish from each site.

[b]Data in the same column indicated by similar letters are not significantly different ($p<0.05$).

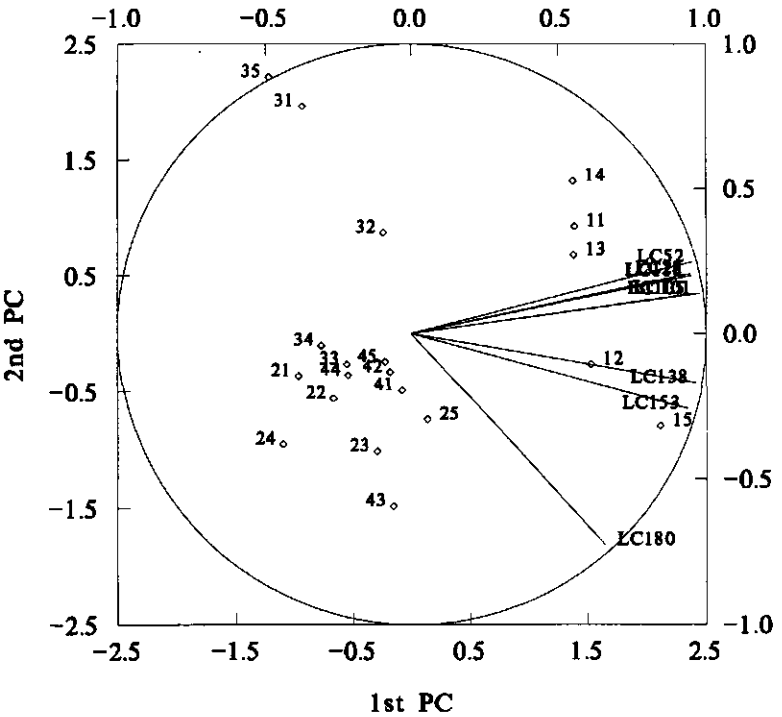


Fig. 3. Bi-plot of principal components analysis of PCB concentrations in samples of mature male dab from the southern North Sea. 1st PC, first principal component; 2nd PC, second principal component. The lower *x*-axis and left *y*-axis apply to the individual data points and show the range of values fo the 1st and 2nd PC respectively. The upper *x*-axis and right *y*-axis apply to the vectors and show the variance explained by the 1st and 2nd PC. LCX=ln(CBX) in ng/g lipid; two digit code of individual samples: first digit, site no. (as in Fig. 1); last digit, sample no. (e.g. 31=Site 3; sample 1).

CB153 was the dominant congener at all stations. Since the concentrations of the measured CB congeners, except CB180, showed high correlations with CB153 (long vectors, small angles) in the bi-plot, CB153 appears to be a good marker for these congeners. CB180 shows a much weaker correlation to the other congeners in the bi-plot (large angle between vectors). Data points with large negative deviations from the population mean especially for this congener stem from the Dogger (Site 3).

Correlation of biochemical parameters with PCBs concentrations

When muscle concentrations of CB congeners (lipid basis) were correlated with hepatic CYP1A parameters (EROD and ELISA), significant correlations were observed (Table 3). For all congeners the significance was determined by the data of the coastal Site 1. When coastal data were omitted from the analysis, no significant correlations between chemical and biochemical parameters were observed for the offshore areas (not shown).

Discussion

The aim of the present study was to examine whether the late autumn is a suitable season for application of CYP1A measurements in dab as indicators of exposure to inducing anthropogenic coumpounds in the southern North Sea. Differences in natural factors like bottom water temperature, condition factors and muscle lipid contents of fish were not observed during this survey, which makes it ideal for an examination of the effects of contaminants on CYP1A.

Table 3. Correlations between hepatic CYP1A protein and EROD activity and muscle tissue levels of selected CB congeners in dab (*Limanda limanda*), collected from the southern North Sea in November 1993[a]

Congener	EROD	CYP1A protein	*N*
CB105	0.572 (0.0085)[b]	0.684 (0.0009)	20
CB118	0.595 (0.0057)	0.698 (0.0006)	20
CB153	0.469 (0.0368)	0.556 (0.0109)	20
∑7 CB	0.551 (0.0118)	0.646 (0.0021)	20

[a]Correlations are made with pooled samples, each sample contains tissue of five fish.
[b]Correlation coefficient *r* (level of significance, $p<0.05$).

The PCB concentrations in muscle tissue of dab showed higher contaminant levels at the coastal site and lower levels at the three offshore sites. The lipid-based PCB concentrations reported in this study are considerably lower than those reported earlier for dab: Knickmeyer and Steinhardt (1990) reported concentrations of hepatic $\Sigma 7$ CB ranging from 180 to 2300 ng g^{-1} lipid for North Sea dab caught in winter 1987, whereas in this study muscle tissue $\Sigma 7$ CB concentrations varied from 155 to 490 ng g^{-1} lipid. The CB153 concentrations were also a factor of two lower compared to muscle CB153 concentrations in dab caught at the same stations in October 1990 (Sleiderink *et al.*, 1995b). Although the inputs of PCBs to the North Sea are contributed by sources like the Atlantic Ocean, the Baltic Sea, Straight of Dover, rivers, sewers and atmospheric deposition, the water quality of the Dutch coastal zone depends largely on the effluents of the highly contaminated effluents of the rivers Rhine, Meuse and Scheldt (Van den Meent *et al.*, 1986; Klamer *et al.*, 1991). Therefore, the lower CB concentrations in dab might reflect a lower input of PCBs from these riverine sources.

In fish studied to date, the non-*ortho* Cl-substituted CB congeners (non-*ortho* CBs) are potent inducers of CYP1A, whereas mono- and di-*ortho* Cl-substituted CB congeners (mono- and di-*ortho* CBs) are weak inducers or inactive (Janz and Metcalfe, 1991; Gooch *et al.*, 1989; Skaare *et al.*, 1991; Van der Weiden *et al.*, 1994a). During this study, PCB body burdens were low, so that non-*ortho* CBs and the mono-*ortho* CB156 were below detection limit. The mono-*ortho* congeners that could be determined (CB105 and -118) showed high correlations with CB153 in the bi-plot. De Boer *et al.* (1993) also found a good correlation of CB153 with these mono-*ortho* substituted CBs and the non-*ortho* CB77, -126 and -169. Since PCB patterns did not differ much between sampling stations, CB153 also appears to be a good marker for relative differences in concentrations between different sites of these congeners.

Correlations of CB congeners with CYP1A parameters were significant, but the significance was largely determined by the data representing the coastal population. Significant correlations were not observed when coastal data were omitted from the analysis. Probably, differences in PCB levels at offshore areas were too low to result in differences in CYP1A induction. To test this hypothesis the mean EROD values of the present study (measured in 13,000g supernatants) were compared with results from another study of dab in the southern North Sea (measured in microsomal fractions) (Sleiderink *et al.*, 1995b). Since EROD values were higher by a factor of 2.0 in microsomes compared to 13,000g supernatants in North Sea dab (Sleiderink *et al.*, 1995c), the microsomal data were divided by a factor of two to allow for a comparison. All data together (r=0.884; p<0.0001; n=12; Fig. 4) show that differences in PCB body burdens at offshore sites are indeed too low to result in differences in CYP1A

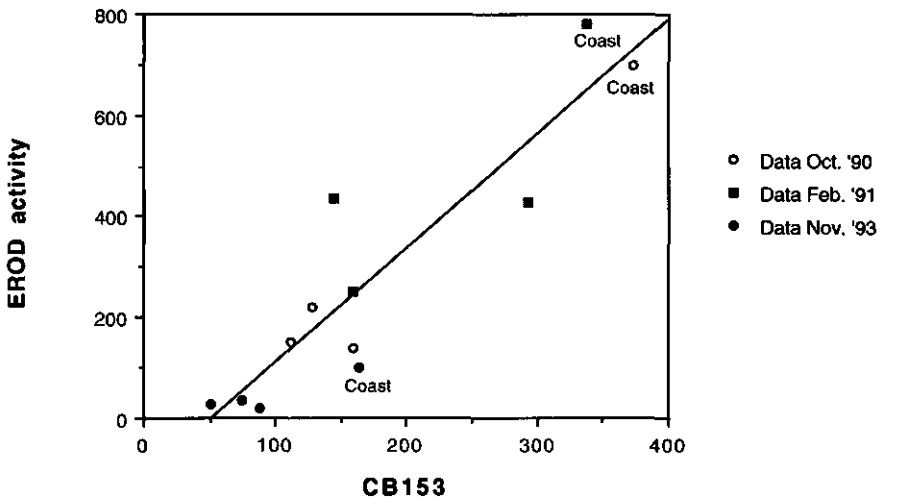


Fig. 4. Relation between mean EROD activity (pmol/min/mg protein) vs mean CB153 (ng/g lipid) in dab from the southern North Sea. Data presented are from this study (data November 1993) and from Sleiderink *et al.* (1995b) (data October 1990 and February 1991). Coastal sites are indicated, other data points are offshore sites.

levels. This supports our previous conclusion (Sleiderink *et al.*, 1995b) that the sensitivity of these biochemical parameters is enough to distinguish the more contaminated coastal area from the offshore areas in the North Sea, but the observed variations in contamination are too small to show differences between the lattter.

Clearly, environmental contaminants other than PCBs such as PAHs, PCDDs and PCDFs might have contributed to the observed environmental induction of CYP1A. However, PAHs are rapidly metabolized in fish, so that a strong positive relationship between PAH residues and induction cannot be expected. Even a relationship between the metabolite 1-OH-pyrene in bile and induction of CYP1A in liver could not be established for North Sea dab (Sleiderink *et al.*, 1995c). Furthermore, the gradients in levels of PCDDs, PCDFs and PAHs are also related to the effluents of the rivers Rhine, Meuse and Scheldt, as for PCBs (Ariese, 1993; Evers *et al.*, 1993). Thus these contaminants are likely to show a similar distribution pattern to PCBs in the North Sea.

The Dogger (Site 3) can be considered remote from the contaminated coastal zone. Exposure to CYP1A inducing compounds is low. PCB levels in dab from the Dogger are among the lowest from the southern North Sea (Sleiderink *et al.*, 1995c) and measurements of

biliary 1-OH pyrene in North Sea dab showed that PAH exposure also is low on the Dogger (Ariese, 1993). Detection of CYP1A in wild fish from relatively uncontaminated sites has been reported before (Förlin and Celander, 1993; Monosson and Stegeman, 1994) and this observation is in contrast with hatchery reared fish where CYP1A is often very low or even undetectable. This suggests that in wild fish low exposure to inducing agents, which may include naturally occuring chlorinated compounds (Gribble, 1994), already activated the induction response. The biological significance and long-term consequences of environmental induction of CYP1A at higher levels of biological organisation is still uncertain.

The responsiveness of EROD measurements was higher than the CYP1A ELISA. This has also been reported for other field studies (Förlin and Celander, 1993; Sleiderink *et al.*, 1995b) and can be due to higher background absorbances of the primary antibody.

In conclusion, the data indicate that the CYP1A induction response in dab populations along the Dutch coast was strong enough to separate the coast from the more pristine offshore areas. Offshore areas differences in PCB body burdens were too small to result in differences in CYP1A levels. The autumn seems the optimal season for application of these biomarkers, since in spring and summer differences in water temperature have a strong effect on CYP1A expression (Sleiderink *et al.*, 1995c), whilst dab show a strongly increased migration during the spawning period in the winter (Rijnsdorp *et al.*, 1992).

Acknowledgements

We are grateful to the crew of the RV *Pelagia* and to Johan Jol (RIKZ-RWS) for his assistance during collection of the fish.

Part II

Laboratory studies

Chapter 5

Hepatic cytochrome P450 1A induction in dab (*Limanda limanda*) after oral dosing with the polychlorinated biphenyl mixture Clophen A40 *

Abstract

The flatfish dab (*Limanda limanda*) serves as an indicator species in pollution monitoring programmes in the North Sea. The present study investigated the induction response of the monooxygenase system and haematological changes in female dab after multiple administrations of a technical mixture of polychlorinated biphenyls (PCBs). Mature female dab were dosed with 1 mg of the PCB mixture Clophen A40 (Clo A40) in sunflower oil every 6 weeks, with a maximum of three doses per fish. In all PCB-administered groups, levels of cytochrome P450 1A (CYP1A) protein, measured with a semi-quantitative ELISA method, and 7-ethoxyresorufin O-deethylase (EROD) activity showed a three to nine-fold induction 14 d after dosing compared with control groups, smaller but also significant increases were observed in total cytochrome P450 (ΣP450) levels. Although the PCB concentrations and the corresponding toxic equivalent (TEQ) value in muscle tissue still increased after administration of the second and third dose of Clo A40, maximum responses of the EROD activity were already reached after the first dose at a TEQ value for chlorinated biphenyls (CB-TEQ) of 2 ng/g lipid. The PCB patterns of liver and muscle tissue of female dab from the central North Sea were found to be virtually identical. Hence, the use of PCB concentrations in muscle as a qualitative model for changes in the liver appears legitimate. Haemoglobin concentrations were elevated after the third dose of Clo A40, whilst haematocrit values and the mean corpuscular haemoglobin concentration (MCHC) between treated and control groups did not differ.

* Hedwig M. Sleiderink, Jan M. Everaarts, Anders Goksøyr[1] and Jan P. Boon

[1]Laboratory of Marine Molecular Biology, University of Bergen, Bergen, Norway

Published in *Environmental Toxicology and Chemistry* **14** (1995), 679-687

Introduction

Halogenated aromatic hydrocarbons such as polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), and dibenzofurans (PCDFs) are major environmental pollutants. Their residues have been found in almost every compartment of the world's ecosystem (Safe, 1984; 1990). The lipophilic character of these compounds make them subject to accumulation in fish from estuarine areas, shelf seas, and - in the case of PCBs - even oceanic and polar marine environments (Muir *et al.*, 1988; Norstrom *et al.*, 1988).

Polychlorinated hydrocarbons can be divided in subclasses depending on their chlorine substitution. Congeners that are either non- or mono-*ortho* substituted can attain a planar configuration (Safe, 1990). Their primary toxic effects are exerted through a common mode of action, which involves binding of the planar congeners to a specific cytosolic receptor, the Ah receptor (Safe, 1990; Poland and Knutson, 1982). The receptor-ligand complex is translocated into the nucleus where it binds to responsive elements on the DNA and thereby causes changes in gene expression. As a result, both induction of cytochrome P450 1A (CYP1A) and toxic responses are observed (Poland & Knutson, 1982). CYP1A belongs to the P450 gene superfamily, which includes several enzymes that catalyse monooxygenase reactions of a wide variety of endogenous and xenobiotic substrates (Nelson *et al.*, 1993).

The potency of polyaromatic hydrocarbons to cause induction of CYP1A and toxic effects relative to the most toxic compound, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, can be expressed by a toxic equivalency factor (TEF) (Safe, 1990). In this way, the toxic potency of complex mixtures can be expressed by a single toxic equivalency (TEQ) value, by multiplying the concentration of each congener by its TEF value and summing the products (Safe, 1990). Only toxic effects that are mediated through the Ah receptor mechanism are included in the TEF approach (Ahlborg *et al.*, 1992). The TEQ concept developed for mammalian studies is now widely applied in human and environmental risk assessment (Ahlborg *et al.*, 1992).

In fish, CYP1A can be induced by planar halogenated aromatic hydrocarbons (Andersson and Förlin, 1992; Goksøyr and Förlin, 1992). Elevated levels of CYP1A protein and/or activity have been found in several field studies (Stegeman and Kloepper-Sams, 1987; Elskus *et al.*, 1989; Goksøyr *et al.*, 1991). Such environmental induction has been associated with increased concentrations of xenobiotic compounds.

The Dutch coastal zone, which is influenced by the effluents of the rivers Rhine, Meuse and Scheldt, belongs to one of the most heavily PCB-contaminated areas in the world (Klamer *et al.*, 1991; De Boer *et al.*, 1993). Since the TEQs of PCBs (CB-TEQs) are substantially

higher than those of the PCDDs and PCDFs in fish caught in the Dutch environment (De Boer *et al.*, 1993), the present study focused on the effects of PCBs. Induction of CYP1A, as measured with catalytic and immunochemical methods in dab, *Limanda limanda*, is already in use as a biological effect technique for monitoring in the North Sea (North Sea Task Force; Joint Monitoring Programme; ICES/IOC Workshop on Biological Effect Techniques in Bremerhaven). The present study was designed to complement the field studies with a mechanistic background and was aimed to investigate the CYP1A induction response in dab after multiple oral administrations of a technical PCB mixture. Because specimens are exposed to complex mixtures in the environment, a PCB mixture was chosen instead of individual congeners. The apparent toxicity of PCBs probably increases with multiple doses (Safe, 1984); therefore, dab were administered multiple doses. In addition, some haematological parameters were investigated because changes in relation to contamination have been reported (Andersson *et al.*, 1988; Boon *et al.*, 1992a). Furthermore, PCB patterns in liver and muscle tissue from female dab from the North Sea were compared to investigate whether the use of PCB concentrations in muscle tissue as a qualitative model for the changes in the liver appears legitimate.

Materials and methods

Fish and treatment

Mature female dab, *Limanda limanda* (length 15-20 cm; mean body weight 70 g) were caught on November 29, 1990, by beam trawling on the Doggerbank, a central part of the North Sea relatively clean with respect to PCBs. Care was taken to avoid damage to the fish during capture: the trawling-time was only 5 min, and after hauling, the net was opened under water in a container on deck. The fish were transported to the laboratory within 1 d.

In the laboratory, dab were kept in tanks (8 fish in each) with running sea water from the Wadden Sea under natural light conditions. The tanks were aerated and contained a thin layer (1-2 cm) of sediment on the bottom. The fish were acclimated at 5 °C for 3 weeks prior to treatment. Throughout the experiment the water temperature was kept at 5 to 6 °C. They were fed *ad libitum* three times a week with frozen shrimp (*Crangon crangon*) from the Wadden Sea before and during the experiment.

Dab (n=7 or 8/group) were administered orally a gelatin capsule filled with 150 µl of a solution containing 1.0 mg of the technical PCB mixture Clophen A40 (Clo A40; Bayer,

Leverkusen, Germany) dissolved in sunflower oil (±10 mg/kg) every six weeks, starting on December 20, 1990, with a maximum of three doses Clo A40 per fish. Control fish were administered the sunflower oil vehicle alone. Because it was established that the biological half-times for elimination of CB congeners from Clo A40 in the juvenile flatfish sole (*Solea solea*) were in the order of 2 to 6 weeks (Boon, 1985), dab were administered Clo A40 every 6 weeks. During administration the fish were not anaesthetized because of the possible interaction of the drug with P450 activity (Kleinow *et al.*, 1986).

Two weeks after each injection, one treated and one control group were killed by a cephalic blow, measured, and weighed; the tail was cut off and blood was sampled in capillaries for determination of haematological parameters. After cutting the spinal cord the livers were excised, rinsed with an ice cold solution of 1.15% KCl (w/v) to remove remnants of blood and bile, dried with tissue paper, and weighed. The liver was homogenized in 0.5 volume of 99% glycerol using an Ultra-turrax TP 18/10. The homogenate was frozen into liquid nitrogen and stored at -80°C for preparation of microsomes. The carcass of the fish was frozen at -25°C for PCB analysis at a later stage.

Clo A40 contains mostly tri-, tetra-, and pentachlorobiphenyls; its detailed composition is given by Schulz *et al.* (1989). The PCDF and PCDD analysis (carried out by RITOX, Utrecht, The Netherlands) of Clo A40 gave the following concentrations of PCDFs: 33 to 100 ng/g 2,3,7,8-TCDF, 36 to 100 ng/g 1,2,3,7,8-PnCDF, 86 ng/g 2,3,4,7,8-PnCDF, 42 ng/g 2,3,4,6,7,8-HxCDF, 36 ng/g 1,2,3,6,7,8-HxCDF, 97 ng/g 1,2,3,4,7,8-HxCDF, 6.4 ng/g 1,2,3,4,6,7,8-HpCDF, 0.9 ng/g 1,2,3,4,7,8,9-HpCDF, 4.3 ng/g OCDF. Clo A40 did not contain PCDDs. The PCDF-TEQ value of Clo A40 was calculated as 65 to 75 ng TCDD eq/g using NATO/CCMS-TEFs as proposed by Safe (1990).

Preparation of microsomes

The preparation of microsomes was performed at 4°C, following the method of Rutten *et al.* (1987) with some modifications. The samples were homogenized in 0.1 M Na-phosphate buffer pH 7.4; 0.15 M KCl; 1 mM EDTA; 1 mM dithiothreitol (DTT) with 20% (v/v) glycerol. The homogenate was centrifuged at 10,000g for 20 min at 4°C. The microsomal fraction was isolated by subsequent centrifugation of the supernatant at 100,000g for 1 h at 4°C in a MSE Prepspin 50 ultracentrifuge with a 8 x 14 ml rotor. The microsomes were resuspended in 0.1 M Na-phosphate buffer pH 7.4, containing 20% (v/v) glycerol, 1mM EDTA and 1mM DTT, frozen in liquid nitrogen, and stored at -80°C.

Biochemical assays

The EROD (7-ethoxyresorufin O-deethylase) activity was measured fluorometrically at 20°C in samples diluted with 0.1% albumin in phosphate buffer. Assays were performed in 0.1 M phosphate-buffer (pH 7.4) containing 0.5 µM 7-ethoxyresorufin (Boehringer Mannheim GmbH, Mannheim, Germany) and 0.15 mM NADPH (Boehringer Mannheim GmbH, Mannheim, Germany). The concentration of the external standard resorufin (Aldrich Chemie, Steinheim, Germany) was assessed, using an extinction coefficient of 73 $mM^{-1}cm^{-1}$ (Klotz *et al.*, 1984). Fluorometric measurements were performed on a Titertek Fluoroskan II plate-reader (Flow Laboratories, Lugano, Switzerland), according to the method of Eggens and Galgani (1992). A laboratory reference material was analyzed on every microplate to obtain an indication for the variance of the results. Data are expressed as nmoles of resorufin released per min per mg protein.

The protein assay was performed according to Bradford (1976), with bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as standard. A spectrophotometer plate reader (Bio-Rad Laboratories, Richmond, California) was used for measurements at the absorption wavelength of 595 nm.

The total amount of cytochrome P450 (∑P450) was measured spectrophotometrically using dithionite-reduced difference spectra of CO-treated microsomes according to the method by Omura and Sato (1964), as adapted by Rutten *et al.* (1987), using a Perkin Elmer Lambda 15 UV/VIS spectrophotometer equipped with a scattered-transmission accessory in the scan-mode.

Immunoblotting (Western blotting) was performed essentially as described by Goksøyr *et al.* (1987), using heterologous rabbit anti-cod CYP1A IgG as primary antibody. The CYP1A protein content of the microsomes was analyzed with the semi-quantitative CYP1A ELISA technique (Goksøyr, 1991), using the same antibodies.

PCB analysis

Since the entire livers were used for biochemical analyses, PCBs were analyzed in muscle of fish; the dorsal part between the backbone and the light skin was used for this purpose. All analyses were done on individual fish. The PCB analysis was carried out according to Boon (1985), as further adapted by Boon *et al.* (1992a). Only modifications are described here. Determination of PCBs with capillary gas chromatograph (GC) with electron capture detection (ECD) were done on a Carlo Erba 5160 GC equipped with a microbore 50 m x 0.15 mm (i.d.) x 0.30 µm (film thickness of stationary phase) CP-Sil 8 CB column

(Chrompack, Middelburg, the Netherlands) and on a Hewlett Packard 5880-A GC equipped with a microbore 50 m x 0.15 mm x 0.20 µm CP-Sil 19 CB column (Chrompack, Middelburg, the Netherlands). Both columns were operated with hydrogen as carrier gas; pressure 350 kPa, linear gas velocity 30 $cm.s^{-1}$. Injection $1mm^3$ splitless; temperature of the injector 250°C; detector ^{63}Ni, 340°C; temperature program isothermal phases at 90°C (2 min), 215°C (25 min), 270°C (22 min) with intermediate temperature increase rates of 10° and 5°C min^{-1}, respectively. Both identification and quantification was carried out on the basis of a synthetic mixture prepared from standards of the individual CB congeners. This external mixture was injected at three different concentrations to calibrate the instruments and to compensate for a non-linear response of the electron capture detector (De Boer *et al.*, 1992b). Peaks quantified had a height that differed less than a factor of three from the highest or lowest concentration of these external mixtures; CB112 was added as an internal standard. Concentrations of the other CBs were corrected for their recovery. In every other batch of five samples analyzed together, either a blank sample or an aliquot of the certified reference material CRM-349 (EC-BCR) of cod liver oil was analyzed to check for the performance of the analytical procedure. The laboratory currently participates in an ICES intercalibration exercise on PCBs in sediments and seal blubber (De Boer *et al.*, 1992b).

Use of a CP-Sil 19 column allowed for the analysis of some non- and mono-*ortho* CB congeners (CB77, -105 and -156; IUPAC numbers) (Ballschmiter and Zell, 1980), which cannot be adequately separated from some other congeners on a CP-Sil 8 column (De Boer *et al.*, 1992a; Larsen *et al.*, 1992a). Fractionation of samples by HPLC and final determination by GC/MS following the method of De Boer *et al.* (1993) for determination of non-*ortho* CBs confirmed the fact that the CP-Sil 19 column can be used for the analysis of CB77, since this congener was well separated from other congeners. The non-*ortho* congeners CB126 and -169 were below detection limit in Clo A40 (Schulz *et al.*, 1989) and they are not reported. Congeners CB97, -151, -132, and 185 also gave better separation on a CP-Sil 19 column. Data on concentrations of other congeners (CB28, -52, -49, -44, -74, -70, -101, -99, -136, -149, -118, -153, -141, -187, -183, -128, -177, -170, -195, -194) were taken from the CP-Sil 8 column, but data for these congeners obtained by analysis on the CP-Sil 19 column were within 10% of the data from the CP-Sil 8 column.

Calculation of CB patterns

To allow for a comparison of the CB pattern between liver and muscle tissue, additional analyses were performed in liver and muscle of female dab (n=5) from the central part of the

North Sea. The CB patterns in liver and muscle tissue of dab were calculated from the data on lipid-based concentrations by dividing the concentrations of all congeners in a given chromatogram by the concentration of a persistent reference congener (CB153). Boon *et al.* (1992b) described the rationale for the selection of the reference congener.

Toxic equivalency value

The toxic potency of complex mixtures can be expressed by a TEQ value by multiplying the concentration of each congener by its TEF value and summing the products (Safe, 1990). In this study we have used the mammalian TEFs of Safe (1990), which are 0.01 for CB77 and 0.001 for CB105, -118 and -156, to calculate the CB-TEQs in muscle tissue.

Haematology

Blood was sampled and the haematocrit values and the haemoglobin concentrations were measured as described earlier (Everaarts, 1978). The mean corpuscular haemoglobin concentration (MCHC, in mM) was calculated as MCHC=[Hb]/Ht x 100%, where [Hb] is the haemoglobin concentration in mM and Ht is the haematocrit value in % cellular fraction of total blood volume.

Statistics

The log-transformed data sets were analyzed statistically by a two-way ANOVA, a least significant difference bands test, and Hochberg's GT2 test for multiple comparisons of means (Sokal and Rohlf, 1981). To examine the correlations between EROD, CYP1A protein, and ΣP450 content, linear-regression analyses were performed on log-transformed data. This was carried out with the Statview Software on a Macintosh computer.

Results

General condition of control and treated fish

No mortality or visible diseases occurred during the experiment, which lasted from the middle of December 1990 until the end of March 1991. Although the weight, length, and condition factor (Bagenal and Tesch, 1978) became higher during the experiment due to favourable feeding conditions, no gross treatment effects could be observed (Table 1). The liver somatic index (LSI) was significantly ($p<0.05$) higher from the corresponding control after the third dose of Clo A40 (Table 1).

Cytochrome P450 induction

Western blotting analysis showed that polyclonal anti-cod CYP1A IgG cross-reacted with a single protein band from both treated and control dab (not shown). The induction response as detected with a semi-quantitative ELISA, with the same anti-cod CYP1A antibody as probe revealed significantly ($p<0.05$) higher CYP1A protein content in Clo A40-treated fish compared to the control (Table 2). EROD activity and $\sum$P450 were also significantly higher in Clo A40-treated fish than in control fish (Table 2). After the first dose of Clo A40 the EROD activity was induced approximately ninefold, CYP1A protein sevenfold, and the $\sum$P450 twofold compared to the corresponding control values. The EROD activity and CYP1A protein

Table 1. Body weight, length, liver somatic index (LSI), and the condition factor of dosed and control dab[a]

Treatment	Body weight (g)	Length (cm)	Liver somatic index (%)[b]	Condition factor[c]	*N*
PCB dose 1	77.8±18.1	19.1±1.4	2.82±0.32	1.10±0.08	7
Control 1	74.0±16.3	18.8±1.1	2.97±0.49	1.09±0.05	8
PCB dose 2	93.8±35.3	19.5±2.0	3.40±0.74	1.22±0.12	8
Control 2	83.9±15.5	18.9±1.3	3.04±0.65	1.23±0.09	8
PCB dose 3	104.9±33.5	20.5±1.9	3.17±0.61[d]	1.19±0.11	8
Control 3	93.4±22.3	20.0±1.2	2.27±0.75	1.15±0.11	8

[a]Data expressed as mean ± SD.
[b]Percentage liver per body weight.
[c]Condition factor, body weight (g) x 100/(length (cm))^3 (Bagenal and Tesch, 1978).
[d]Significantly different from corresponding control, $p<0.05$.

were decreased to threefold induction after the second and third dose, whereas ΣP450 stayed constant at twofold induction compared to controls over three doses. During the experiment, the absolute EROD activity stayed constant and the absolute CYP1A protein content showed a very slight increase after the third dose, while the absolute ΣP450 levels showed slight increases over the second and third dose (Table 2). In the control groups the EROD activity became significantly higher during the 18 weeks of the experiment. The CYP1A protein content and ΣP450 of the control groups showed a slight increasing trend during this period; however, this was not statistically significant. The catalytic activity of CYP1A, which can be expressed as EROD activity/CYP1A protein, did not show differences upon treatment (data not shown). Both control and treated groups showed equally high levels; therefore, no inhibition at the active site of the enzyme seem to have occurred.

Comparison of CB patterns between organs and CB exposure level

PCB concentrations in muscle tissue of the experimental fish were used as a parameter for the qualitative changes in the target organ (i.e., the liver), which was used entirely for biochemical measurements. This approach proved to be valid for experiments with sole (Boon, 1985; Boon *et al.*, 1984). Additional analyses in dab from the Doggerbank area also showed

Table 2. Hepatic cytochrome P450 levels in control and dosed female dab[a,b]

Treatment	*N*	ΣP450 (nmol/mg)	EROD activity (nmol/min/mg)	CYP1A (absorbance at 492 nm)
PCB dose 1	7	0.39 ± 0.09^{A}	1.90 ± 0.64^{A}	2.05 ± 0.25^{A}
Control 1	8	0.21 ± 0.05^{C}	0.22 ± 0.13^{B}	0.31 ± 0.11^{C}
PCB dose 2	8	0.42 ± 0.15^{A}	1.83 ± 1.11^{A}	$2.05\pm0.25^{A,B}$
Control 2	8	$0.28\pm0.06^{A,C}$	$0.51\pm0.28^{B,C}$	0.66 ± 0.30^{C}
PCB dose 3	8	0.55 ± 0.09^{B}	1.95 ± 0.46^{A}	2.25 ± 0.12^{B}
Control 3	8	$0.27\pm0.08^{A,C}$	0.65 ± 0.47^{C}	0.66 ± 0.37^{C}
Two-way ANOVA[c]		PCB, dose ($p<0.05$)	PCB x dose ($p<0.05$)	PCB x dose ($p<0.05$)

[a]Data expressed as mean ± SD.

[b]Values indicated by similar letters are not significantly different (LSDB and GT2-test; $p<0.05$).

[c]Results of two-way ANOVA analysis; significant main effects (PCB, dose) or interaction effects are given.

that the relative changes ([CB-*X*]/[CB153]) in liver and muscle tissue are almost similar (Figure 1), although the absolute concentrations based on lipid content were slightly higher in muscle. Lipid content varied from 0.42 to 0.65% in muscle and from 3.1 to 5.0% in liver. A very good correlation was found between [CB-*X*]/[CB153] in the liver and [CB-*X*]/[CB153] in muscle (r=0.98, n=27, p<0.0001).

In Table 3 the increase in concentrations of non- and mono-*ortho* CB congeners 77, 105, 118 and 156, the sum of 27 well-separated congeners, and the CB-TEQs, based on TEFs suggested by Safe (1990), in muscle tissue are given for each group. CB77 is the main contributor to the CB-TEQ in all samples. A linear relationship between the Clo A40 dose and the resulting concentration increases of CBs in muscle tissue was established. Figure 2 shows that even 14 d after injection with Clo A40, there was a significant correlation between the absolute increase in the concentration of a given congener in muscle tissue and its percentage in Clo A40 (n=23, r=0.98/0.95/0.95 for the 1st/2nd/3rd dose, respectively, p<0.05). The absolute increase in concentration of a given chlorobiphenyl congener was defined as its concentration in the Clo A40-injected group minus the concentration in the corresponding control group.

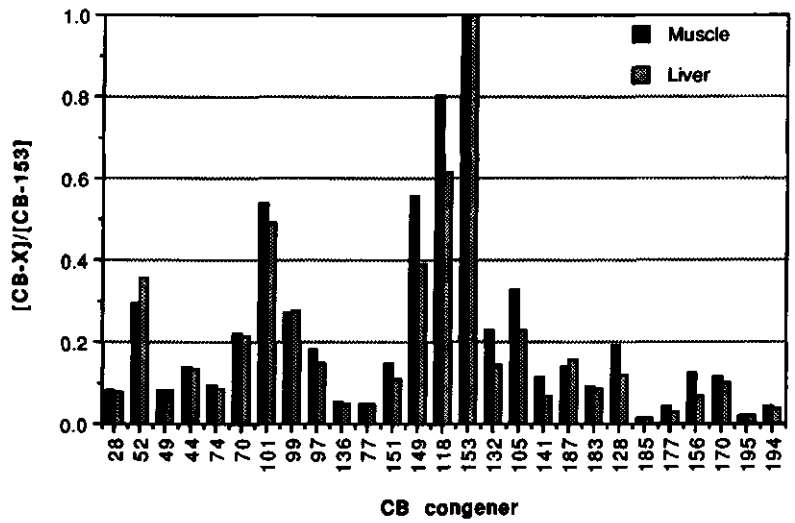


Fig. 1. Patterns of chlorobiphenyls, expressed as the ratio of the concentration of each congener to the concentration of CB153, in muscle tissue and liver of mature female dab *Limanda limanda* from the central part of the North Sea. Congeners are given in sequence of elution from the GC column.

Table 3. Concentrations (ng/g lipid) of individual non- and mono-*ortho* CB congeners (identified by their IUPAC numbers), the sum of 27 selected CBs (µg/g lipid) and toxic equivalents (CB-TEQ) in muscle tissue of female dab[a,b]

CB no.	PCB - Dose 1	PCB - Dose 2	PCB - Dose 3	% in Clo A40[c]
Non-*ortho* (ng/g lipid)				
77	174±71.4	367±146	428±72.1	0.66
Mono-*ortho* (ng/g lipid)				
105	649±306	1,346±411	1,857±371	1.43
118	867±419	1,744±636	2,449±550	2.47
156	54.7±31.0	63.4±16.7	74.5±12.5	0.23
Sum CBs (µg/g lipid)				
Σ^{27}CB[d]	18.1±8.0	41.5±15.2	55.9±11.8	46.4
CB-TEQ (ng/g lipid)				
CB-TEQ	3.31±1.44	6.83±2.51	8.66±1.55	

[a]Data expressed as mean ± SD.

[b]Data for groups of control fish varied between 4.0 and 4.6 ng CB77/g lipid, 17 and 21 ng CB105/g lipid, 57 and 77 ng CB118/g lipid, 10 and 17 ng CB156/g lipid, 1.8 and 3.6 µg ΣCB/g lipid, and 0.13-0.16 ng CB-TEQ/g lipid.

[c]After Schulz *et al.* (1989).

[d]Values include concentrations of CB77, -105, -118 and -156.

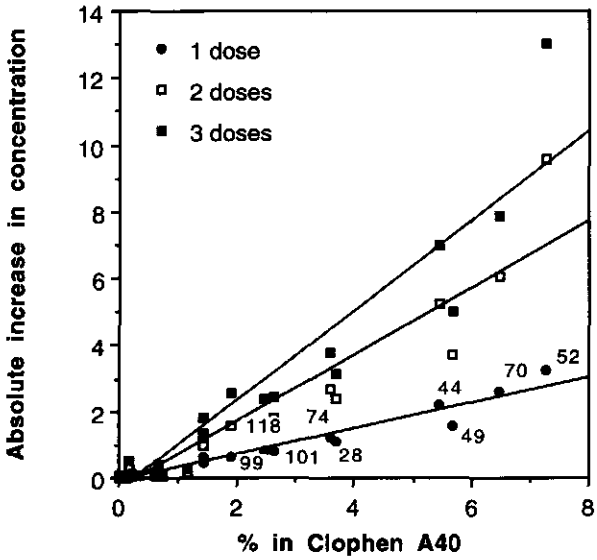


Fig. 2. Correlation between percentage contribution of CB congeners in Clophen A40 and the absolute increase in concentrations (ng/g lipid) in muscle of female dab after administration of 1, 2 or 3 doses of Clophen A40 (1 mg/fish/dose).

Relation between CB-TEQ and CYP1A parameters

In Figure 3 the relationships between the CB-TEQ value in muscle tissue and both the EROD activity and CYP1A ELISA protein content for individual fish from the treated and

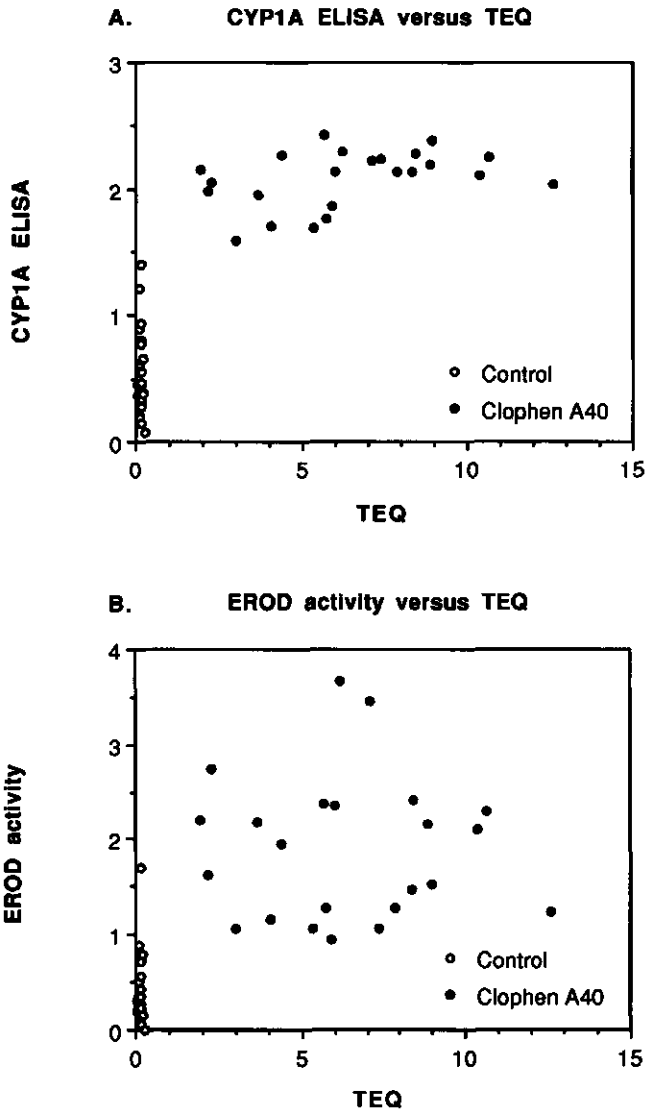


Fig. 3. Relation between (A) EROD activity (nmol/min/mg protein) and TEQ (ng/g lipid) and (B) CYP1A protein (absorbance at 492 nm) and TEQ (ng/g lipid).

control groups are given. These relationships are not linear; there are no dose-response relationships for the CB-TEQ and EROD activity or CYP1A, since all dosed fish were already maximally induced regardless of dose. Both parameters showed a plateau, which begins at some unknown CB-TEQ less than 2 ng/g lipid. The EROD activity data show a higher variability than the immunochemical determined CYP1A protein content.

Haematology

Haematocrit (Ht) values were not influenced by treatment with Clo A40 (Table 4). ANOVA analysis with PCB and dose as variables revealed a significant interaction effect of PCB x dose ($p<0.05$) for the haemoglobin concentrations ([Hb]). Because there was no response in the quotient of [Hb] and Ht - the mean corpuscular haemoglobin content (MCHC) - elevated levels after the third injection were caused by the presence of increased erythrocyte concentrations.

Table 4. Haematological parameters in control and dosed female dab[a]

Treatment	*N*	Haemoglobin (mM)	Haematocrit (%)	MCHC (mM)
PCB dose 1	7	0.75±0.06	20.9±4.2	3.65±0.57
Control 1	8	0.78±0.04	21.3±3.1	3.69±0.42
PCB dose 2	8	0.67±0.07	19.9±3.9	3.33±0.55
Control 2	8	0.63±0.05	18.8±2.4	3.38±0.26
PCB dose 3	8	0.73±0.04[c]	21.2±1.7	3.49±0.31
Control 3	8	0.65±0.06	20.7±3.3	3.19±0.33
Two-way ANOVA[b]		PCB x dose ($p<0.05$)	Not significant	Not significant

MCHC, mean corpuscular haemoglobin concentration.
[a]Data expressed as mean ± SD.
[b]Results of two-way ANOVA analysis; significant main effects (PCB, dose) or interaction effects are given.
[c]Significantly different from corresponding control, $p<0.05$.

Discussion

The present study clearly demonstrates that Clo A40 is an effective inducer of CYP1A in dab liver. After the first injection of Clo A40, EROD activity was induced ninefold and CYP1A protein sevenfold. Although the induction of both biochemical parameters decreased to threefold induction after the second and third dose compared to controls, there were no indications for a decreased catalytic activity as has been observed after treatment of plaice (*Pleuronectes platessa*) with Clo A40 (Boon *et al.*, 1992a). Both CYP1A protein and EROD activity responded analogously to Clo A40 treatment, whereas ΣP450 remained at twofold induction after the second and third dose. It is possible that repeated administration may have caused inhibition of the induction of CYP1A, but it cannot be excluded that P450 forms other than CYP1A may have been induced by Clo A40 treatment.

In fish, most studies have found that CYP1A can be induced by non-*ortho* substituted CB congeners, which do have a high affinity for the cytosolic Ah receptor, but not by mono-*ortho* congeners (Gooch *et al.*, 1989). However, in rainbow trout (*Oncorhynchus mykiss*) injection of the mono-*ortho* CB-118 did result in induction of CYP1A (Skaare *et al.*, 1991), indicating that this mono-*ortho* congener had a (low) affinity for the Ah receptor too. CB77 is the only non-*ortho* congener and CB105, -118 and -156 are the only mono-*ortho* congeners present above detection level in Clo A40. Based on estimates of the concentrations of those congeners in Clo A40 (Schulz *et al.*, 1989) and a mean fish weight in our experiment of 95 g, a dose of 1 mg Clo A40 per fish would yield a dose of 70 µg/kg of CB77, 150 µg/kg of CB105, 260 µg/kg of CB118, and 25 µg/kg of CB156. In scup (*Stenotomus chrysops*), CB77 caused significant CYP1A induction at doses of 0.1 mg/kg (Gooch *et al.*, 1989). In rainbow trout aryl hydrocarbon hydroxylase induction was found at a dose of 58 µg/kg CB77 (Janz and Metcalfe, 1991). Skaare *et al.* (1991) found induction of CYP1A in rainbow trout by CB118 at doses of 30 mg/kg, whereas Gooch *et al.* (1989) did not find CYP1A induction in scup for CB105 and CB118 at doses of 15 and 5 mg/kg, respectively. Apart from species differences it is very likely that these mono-*ortho* CBs possess a very low affinity for the Ah receptor and that their inducing capacity is therefore very low. It appears very likely that CB77 was the most important congener causing induction effects in this experiment. Additional studies with individual congeners can help to determine which compounds in the technical mixture are responsible for the observed induction of CYP1A in dab.

Additionally, PCDFs were present in Clo A40. The PCDF-TEQ value of Clo A40 was 75 ng TCDD eq/g, while CB-TEQ value of Clo A40 was 107 µg/g, which is a factor 1,400

higher. With a dose of 1 mg Clo A40 per fish and a mean fish weight of 95 g, the PCDFs present would yield a dose of 0.79 ng TCDD eq/kg. Van der Weiden *et al.* (1994b) found that the lowest-observed-effect level for CYP1A induction in the carp (*Cyprinus carpio*) was 0.06 µg TCDD/kg, while a five times higher dose was required for the rainbow trout (Van der Weiden *et al.*, 1992). It seems very unlikely that PCDFs caused the observed induction of CYP1A in dab.

In the experiment, CB77 was the main contributor to the CB-TEQ value and determined about 50 to 55% of the TEQ values. The CYP1A parameters showed a plateau at all CB-TEQ values above 2 ng/g lipid. The used TEFs are based on mammalian studies (Safe, 1990), which can be dissimilar from those obtained in fish (Janz and Metcalfe, 1991; Walker and Peterson, 1991; Van der Weiden *et al.*, 1994a). Based on early-life-stage mortality in rainbow trout, TEFs of 0.00016 for CB77 and <0.00007 for CB105 and -118 were found (Walker and Peterson, 1991). Janz and Metcalfe (1991) based their TEF, 0.002 for CB77, on aryl hydrocarbon hydroxylase induction in rainbow trout. Van der Weiden *et al.* (1994a) found a TEF based on CYP1A induction of 0.0004 for CB118 in the mirror carp. In general, these TEFs are lower than those proposed for risk assessment; this can result in a lower CB-TEQ at which a plateau for CYP1A induction occurs.

Both EROD activity and CYP1A protein levels are decreased in female pre-spawning dab in the North Sea (Sleiderink *et al.*, 1995b), which is in agreement with numerous reports for other fish species (Elskus *et al.*, 1989; 1992; Larsen *et al.*, 1992b). Low CYP1A levels in these fish have been related to high oestrogen levels (Elskus *et al.*, 1989; 1992). The female dab that were used in the present study were pre-spawning until the end of February/beginning of March, when eggs were striped off for an investigation of egg quality and larval development (Fonds *et al.*, 1995). Possibly, the absolute increases in CYP1A levels observed in the controls and dosed fish are the result of low levels in pre-spawning fish in January. In addition, although the administered Clo A40 resulted in increased PCB levels in the eggs, there appeared to be no significant differences in egg production, egg quality, fertilization rate, hatching rate, and survival of larvae between eggs of Clo A40-exposed females and eggs from the reference groups (Fonds *et al.*, 1995).

Relative CB concentrations on a lipid basis between liver and muscle tissue of dab were virtually identical, which is in agreement with other reports (Boon, 1985; Boon *et al.*, 1984). Although absolute concentrations differed between different organs and tissues, the relative changes are similar. Therefore, analysis of PCBs in muscle tissue as a model for the qualitative changes occuring in the entire fish seems legitimate (Boon *et al.*, 1992a).

The high correlation between the absolute increase of a given congener in muscle tissue and its percentage in Clo A40 indicates that the PCB-patterns in the dosed fish were not much influenced by changes due to differential elimination rates between congeners. This leads to the conclusion that, in general, biotransformation did not play a major role in the kinetics of CB-congeners in this study.

Increased haemoglobin concentrations in Clo A40-treated groups were found after administration of the third dose; possibly this is an indication for an increased oxygen demand. Increased [Hb] levels were also found in fish exposed to a pollution gradient of bleached kraft mill effluent (Andersson *et al.*, 1988). On the contrary, Boon *et al.* (1992a) found decreased [Hb] levels in plaice after Clo A40 treatment. Future experiments should clarify the influence of halogenated aromatic hydrocarbons on blood parameters.

In summary, induction of CYP1A was observed in dab liver following Clo A40 exposure. Biochemical effects were related to increases in concentrations of total and specific planar CB congeners and toxic equivalents in muscle. In all treated groups $\sum$P450 levels, EROD activity, and CYP1A ELISA were higher than the control groups. The maximum for EROD activity, CYP1A, and $\sum$P450 was reached after the first dose of Clo A40, although the CB concentrations in muscle tissue became higher after administration of a second and third dose of Clo A40.

Acknowledgements

This research was supported by a grant from the Royal Dutch Academy of Sciences (KNAW). The authors would like to thank the RV *Aurelia* crew for help in obtaining the dab. Mark Fonds assisted during injection of the fish and Peter van der Puyl took care of feeding. Jaap van der Meer gave valuable advice about the statistical treatment of the data. Kjersti A. Helgesen, M. Theo J. Hillebrand, and Evaline M. van Weerlee are greatly acknowledged for their technical assistance. We are grateful to Ruud W. Nieboer for the PCDD/PCDFs analysis of Clo A40.

Chapter 6

Temporal induction pattern of hepatic cytochrome P450 1A in thermally acclimated dab (*Limanda limanda*) treated with 3,3',4,4'-tetrachlorobiphenyl (CB77) *

Abstract

Mature male dab (*Limanda limanda*) acclimated at 10° and 16°C were orally administered a single dose of 0.5 mg/kg 3,3',4,4'-tetrachlorobiphenyl (CB77). At both temperatures, levels of cytochrome P450 1A (CYP1A) protein and 7-ethoxyresorufin O-deethylase (EROD) activity showed a two to six fold induction 40 days after CB77 treatment compared to control groups. Maximum responses of both EROD activity and CYP1A protein for the warm-acclimated fish were observed at 5 days after treatment. For the cold-acclimated fish a slow, progressive elevation for both EROD activity and CYP1A protein was observed and maximum responses were measured 40 days after treatment. Absolute EROD activity and CYP1A protein levels of fish from both temperatures were equally high at 40 days after treatment. Since in the control groups EROD activity and CYP1A protein levels were higher in the cold-acclimated fish, the magnitude of induction was higher in the warm acclimated ones. The highest concentrations of CB77 in muscle of fish from both temperatures were found at 5 and 10 days after treatment. The liver somatic index (LSI) showed 1.5 fold significantly higher values for the fish acclimated at 10°C.

* Hedwig M. Sleiderink and Jan P. Boon
Published in *Chemosphere* **32** (1996), 2335-2344.

Introduction

In the aquatic environment, polychlorinated biphenyls (PCBs) are ubiquitous environmental contaminants. Due to their lipophilic and persistent character they are subject to accumulation in fish and other aquatic organisms, although the individual congeners differ in their persistence. The toxicity of a specific congener depends upon both its degree of chlorination and the position of its chlorine atoms (Safe, 1984). Congeners that can attain a planar configuration belong to the most toxic. Their primary toxic effects appear to be exerted through a common mode of action, which involves binding to the Ah receptor (Safe, 1990; Poland and Knutson, 1982). The planar CB congener 3,3',4,4'-tetrachlorobipenyl (IUPAC CB77; Ballschmiter and Zell, 1980) is widely used as a model Ah-receptor ligand and inducer of cytochrome P450 1A (CYP1A) in fish (Melancon and Lech, 1983; Monosson and Stegeman, 1991; Gooch *et al.*, 1989; Wirgin *et al.* 1992; Hahn *et al.*, 1993; Lindström-Seppä *et al.*, 1994). At high concentrations, CB77 can also act as an inhibitor of CYP1A in fish (Gooch *et al.*, 1989).

In the field, induction of CYP1A in fish has often been reported to correlate with environmental exposure to halogenated aromatic hydrocarbons including PCBs (Goksøyr and Förlin, 1992; Kleinow *et al.*, 1987; Payne *et al.*, 1987). This environmental induction depends in part of the identity and concentration of the inducing aquatic contaminants and in part to the influences of several abiotic and biotic variables, like water temperature, age, sex and sexual maturity (Andersson and Förlin, 1992; Sleiderink and Boon, 1995; Sleiderink *et al.*, 1995b,c).

The flatfish dab is target species for monitoring programmes in the North Sea. Dab showed induction of hepatic CYP1A after dosing with the technical PCB mixture Clophen A40 (Sleiderink *et al.*, 1995a). Furthermore, previous results on the influences of environmental temperature on basal CYP1A levels in the laboratory and CYP1A expression in the field, reveal that basal hepatic CYP1A activity in dab responds to water temperature in a compensatory manner, whereby cold-acclimated rates were higher than warm-acclimated rates (Sleiderink *et al.*, 1995c). The aim of the present study was to investigate the temperature dependence of the induction pattern of hepatic CYP1A in dab, *Limanda limanda*. To this end, dab was acclimated at two different temperatures (10 and 16°C) and kept at these temperatures for a period of 40 days after intragastrical administration of a single dose of CB77.

Materials and methods

Fish, treatment and preparation of microsomes

Mature specimens of male dab, *Limanda limanda*, were collected by beam trawling in the end of May 1993, from the Doggerbank (54°46'N, 2°39'E), a relatively clean part of the North Sea with respect to PCBs. The trawling-time was 5 min. and after hauling the net was opened under water in a container on deck. Fish were transported to the laboratory within one day.

In the laboratory, the fish were kept in glass aquaria with running sea water at 12°C under natural light conditions. The tanks were aerated and contained a thin layer of sediment on the bottom. Fish were acclimated to these conditions during four weeks. One week prior to treatment temperatures were changed to 10 and 16°C respectively, the experimental temperatures. Before and during the experimental period the fish were fed *ad libitum* three times a week with frozen shrimps (*Crangon crangon*).

Groups of fish (n=5) were administered once intragastrically a gelatin capsule filled with a solution containing 0.03 mg of CB77 dissolved in 10% DMSO/90% sunflower oil (±0.5 mg/kg body weight). Control fish were given the vehicle alone. During administration the fish were not anaesthetized, because of the possible interaction of the drug with P450 activity (Kleinow *et al.*, 1986). At days 0, 5, 10 and 40, control and dosed fish of both temperatures were sampled. They were terminated after weighing by a cephalic blow and cutting of the spinal cord. Livers were excised and rinced with an ice cold 1.15 % KCl solution. The liver was homogenized in 0.5 volume of 99% glycerol with an Ultra-turrax. The homogenate was frozen into liquid nitrogen and stored at -80°C for preparation of microsomes. The carcass of the fish was frozen at -25°C for PCB analysis. The microsome preparation was performed as described by Sleiderink *et al.* (1995a).

Protein and enzyme assay

All biochemical analyses were performed on individual livers. 7-ethoxyresorufin O-deethylase (EROD) activity was measured fluorometrically at room temperature on a platereader using the method of Eggens and Galgani (1992). Resorufin formation was quantified using an extinction coefficient of 73 $mM^{-1}cm^{-1}$ (Klotz *et al.*, 1984). A laboratory reference material of dab liver was analysed on every plate to obtain an indication for the long term variance of the results.

Proteins were measured according to Bradford (1976), with bovine serum albumin as reference standard.

CYP1A protein levels were determined semi-quantitatively using an enzyme-linked immunosorbent assay (ELISA) described by Goksøyr (1991), using monoclonal mouse anti-cod CYP1A IgG as primary antibody. The secondary antibody was goat anti-mouse IgG conjugated to horseradish peroxidase.

CB77 analysis

CB77 was analyzed by capillary gas chromatography with electron capture detection (GC-ECD) on a Hewlett Packard 5880-A GC fitted with a 50 m microbore CP-Sil 19 column. Gas chromatographic determination of PCBs have been described before (Boon, 1985; Holden and Marsden, 1969; Boon *et al.*, 1992a; Sleiderink *et al.*, 1995a) and only supplementary information is given here. Lipids and PCBs were extracted from muscle tissue using a modification of the method of Bligh and Dyer (1959), as described by Booij and van den Berg (1994). Use of a CP-Sil 19 column allowed for the analysis of non- and mono-*ortho* congeners (De Boer *et al.*, 1992a; Larsen *et al.*, 1992a). Fractionation of samples by HPLC and final determination by GC/MS following the method of De Boer *et al.* (1993) for determination of non-*ortho* CBs confirmed the fact that the peak established as CB77 was caused exclusively by this compound. Identification and quantification was carried out on basis of a synthetic external standard mixture prepared from standards of individual CB congeners. This mixture was injected at 3 different concentrations to calibrate the instruments and to compensate for a non-linear response of the detector (De Boer *et al.*, 1992b). In every other batch of five samples analyzed together, either a blank sample or an aliquot of the certified reference material CRM-349 (EC-BCR) of cod liver oil was analyzed to check for the performance of the analytical procedure. The laboratory currently participates in an ICES intercalibration exercise on PCBs (De Boer *et al.*, 1992b; 1994).

Since the entire livers were used for biochemical analyses, CB77 in muscle tissue located between the backbone and the light skin was used as a parameter for qualitative changes occuring in the entire fish. This approach proved to be valid in laboratory experiments with dab (Sleiderink *et al.*, 1995a). Fish were pooled; each sample contained muscle tissue of five specimens.

The CB77 levels were only determined in CB77 treated fish. CB77 content in control fish were not determined, since they were assumed to be below 6 ng/g lipid (Sleiderink *et al.*, 1995a, c); which is lower by a factor of 10^6 compared to the dosed fish .

Statistics

The log transformed data sets were analyzed statistically by a 3-way ANOVA, a least significant difference bands test, and Hochberg's GT2 test for multiple comparisons of means (Sokal and Rohlf, 1981). To examine the correlations between EROD, CYP1A protein and residue CB77 concentrations, linear regression analyses were performed on log transformed data as well.

Results

General condition and liver parameters of control and dosed fish

During the entire experiment no mortality or visible diseases occurred. Although no significant differences in body weight and length could be established, there was a significant effect of temperature on the condition factor of the fish (Table 1). The condition factors were higher in fish acclimated at 10°C compared to those acclimated at 16°C. Statistical analyses (three-way ANOVA) with CB77, temperature and time as independent variables revealed an interaction effect of CB77 and time, and an effect of temperature on the liver somatic index (LSI). This was reflected in lower LSI values for dosed dab at 16°C at days 5 and 10 and for dab at 10°C at day 10 compared to the corresponding controls. Furthermore, LSI values were higher in cold-acclimated fish compared to the warm-acclimated ones (Table 1).

Cytochrome P450 1A induction

The levels of hepatic EROD activity and CYP1A protein are presented in Figures 1A and 1B. Treatment of the control group with DMSO/sunflower oil did not cause elevation of CYP1A levels over time. There was a tendency of higher basal CYP1A levels, expressed as EROD activity as well as CYP1A protein levels, in 10°C fish compared to 16°C fish (Table 2).

Statistical analyses (three-way ANOVA) with CB77, temperature and time as independent variables influencing EROD activity, revealed an effect of CB77. This was reflected in higher EROD levels in dosed fish compared to control fish (Table 2). The induction responses, measured as EROD activity, were approximately higher by a factor of 2 for 10°C fish and a factor of 4 for 16°C fish compared to the corresponding control fish.

Statistical analyses (three-way ANOVA) with CB77, temperature and time as variables influencing CYP1A protein levels, showed an interaction effect of temperature and CB77, and an effect of time. Maximum increases in CYP1A protein levels in 16°C fish were already

observed 5 days after treatment, while for the 10°C fish the maximal response was observed 40 days after treatment (Table 2). The magnitude of the maximum response did not differ with temperature. The induction response measured as CYP1A protein was approximately higher by a factor of 3 to 4 for 10°C CB77 treated fish and a factor of 6 for 16°C CB77 treated fish compared to the corresponding control fish.

Table 1. Body weight, length, liver somatic index (LSI), and the condition factor of CB77 dosed and control fish[a]

Treatment	Body weight (g)	Length (cm)	LSI (%)[b]	Condition factor[c]
10°C				
Control-d0	61.0±4.8	18.6±0.5	2.09±0.23	0.95±0.04
Control-d5	63.7±14.4	18.9±1.2	2.22±0.61	0.94±0.04
Control-d10	62.8±11.6	18.9±1.5	2.33±0.70	0.93±0.09
Control-d40	64.1±18.9	18.8±1.6	2.49±0.79	0.94±0.04
CB77-d5	65.1±18.3	18.7±1.5	2.25±0.40	0.98±0.08
CB77-d10	54.0±15.3	18.0±1.2	2.12±0.49	0.91±0.06
CB77-d40	59.6±23.9	17.8±2.0	2.67±0.37	1.01±0.07
16°C				
Control-d0	65.8±14.8	19.2±1.3	1.76±0.25	0.92±0.03
Control-d5	59.5±9.0	18.6±0.9	1.48±0.33	0.92±0.09
Control-d10	67.9±8.0	19.3±0.7	2.19±0.19	0.94±0.07
Control-d40	68.5±13.2	19.6±1.2	1.63±0.45	0.90±0.03
CB77-d5	60.1±18.6	18.8±1.9	1.34±0.34	0.87±0.04
CB77-d10	59.9±7.7	19.0±0.7	1.38±0.31[d]	0.88±0.07
CB77-d40	64.9±12.2	19.0±1.2	2.31±0.71	0.93±0.07
Three-way ANOVA[e]	No effect	No effect	CB77 x time ($p<0.05$) temperature ($p<0.05$)	temperature ($p<0.05$)

[a]Data expressed as mean±SD ($n=5$).
[b]Percentage liver per unit body weight.
[c]Condition factor, body weight (g) x 100/(length (cm))^3 (Bagenal and Tesch, 1978).
[d]Significantly different from corresponding control, $p<0.05$.
[e]Results of three-way ANOVA analysis; significant main effects (CB77, temperature, time) or interaction effects are given.

The CYP1A protein content parallelled more or less the EROD activity. As a result the EROD activity and CYP1A protein content are well correlated for both temperatures; r=0.69 for cold-acclimated fish (n=35; p<0.0001), r=0.65 for warm-acclimated fish (n=35; p<0.0001).

In general, the 16°C fish reached maximal CYP1A responses 5 days after treatment and they remained at a plateau till day 40, whereas for 10°C fish there was a slow, progressive elevation for both EROD activity and CYP1A protein over the course of 40 days after treatment (Figure 1A and 1B). Nevertheless, after 40 days for both warm- and cold-acclimated fish the absolute values of EROD activity and CYP1A protein were equally high.

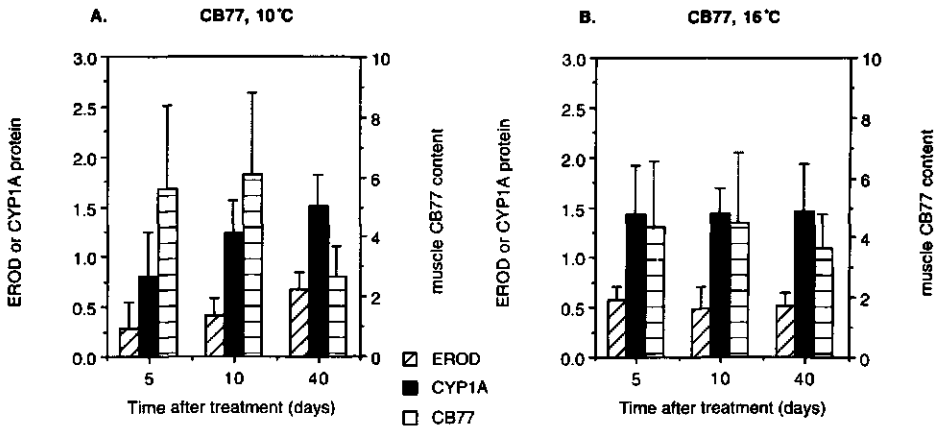


Fig. 1A-B. EROD activity (nmol/min/mg protein), CYP1A protein (absorbance at 405 nm) and muscle CB77 concentrations (mg/g lipid) in dab acclimated at 10 and 16°C, 5, 10 and 40 days after administration of a single dose of CB77.

Concentration of CB77

The muscle CB77 concentrations are listed in Table 2. The CB77 concentrations in muscle tissue were used as as a parameter for the qualitative changes in the target organ (i.e., the liver), which had to be entirely used for biochemical measurements. This approach proved to be valid in previous experiments with dab (Sleiderink *et al.*, 1995a), but also for the flatfish sole, *Solea solea* (Boon, 1985; Boon *et al.*, 1984). The highest CB77 concentrations were found in the first 10 days after treatment. The CB77 concentrations were obviously lower 40 days after treatment.

No significant correlations were observed between muscle CB77 concentrations and both EROD activity and CYP1A protein levels.

Table 2. Hepatic EROD activity and CYP1A protein levels and muscle % lipid and CB77 concentrations[a]

Treatment	EROD activity (nmol/min/mg)	CYP1A protein (absorbance 492 nm)	% lipid	CB77 concentrations (µg/g lipid)
10°C				
Control-d0	0.15±0.18	0.30±0.05		
Control-d5	0.23±0.24	0.30±0.09		
Control-d10	0.31±0.22	0.38±0.14		
Control-d40	0.34±0.45	0.42±0.32		
CB77-d5	0.29±0.25	0.81±0.44	0.90±0.10	5579±2773
CB77-d10	0.41±0.18	1.23±0.33[B]	0.78±0.15	6113±2700
CB77-d40	0.67±0.17	1.51±0.32[B]	0.85±0.27	2664±1002
16°C				
Control-d0	0.17±0.32	0.25±0.02		
Control-d5	0.14±0.09	0.22±0.05		
Control-d10	0.05±0.08	0.26±0.02		
Control-d40	0.15±0.14	0.26±0.07		
CB77-d5	0.58±0.12[B]	1.44±0.48[B]	0.77±0.03	4314±2205
CB77-d10	0.49±0.22[B]	1.43±0.26[B]	0.82±0.17	4503±2321
CB77-d40	0.52±0.12[B]	1.47±0.47[B]	0.73±0.08	3622±1165
Three-way ANOVA[c]	CB77 ($p < 0.05$)	temp. x CB77 ($p < 0.05$) time ($p < 0.05$)		

[a]Data expressed as mean±SD (n=5).
[b]Significantly different from corresponding control, p<0.05.
[c]Results of three-way ANOVA analysis; significant main effects (CB77, temperature, time) or interaction effects are given.

Discussion

In the present study the LSI showed an inverse relation with acclimation temperature. This has been observed before in another temperature study with dab (Sleiderink and Boon, unpublished data). Other studies have reported similar results (Egaas and Varanasi, 1982; Ankley *et al.*, 1985). A higher LSI due to CB77 exposure was not observed in this study, although several contaminants, like PCBs and PAHs, are known to cause liver enlargement.

The present study showed that treatment of dab with CB77 resulted in elevated levels of EROD activity and CYP1A protein, indicating that CB77 is an inducer of CYP1A in dab, as in other fish species like rainbow trout (Melancon and Lech, 1983), winter flounder (Monosson and Stegeman, 1991), scup (Gooch *et al.*, 1989) and fathead minnow (Lindström-Seppä *et al.*, 1994). In those studies, a decline in EROD activity at higher doses has been observed, while CYP1A protein levels were still elevated. Melancon and Lech (1983) found that the doses associated with the decline in activity were above 1 mg/kg. Gooch *et al.* (1989) also observed inhibition of EROD activity in scup at doses of 5 and 10 mg/kg, but not at 1 mg/kg. Kinetic mechanisms like competitive inhibition, could explain much of this EROD suppression. In our study with dab, we used a concentration of approximately 0.5 mg/kg, therefore it can not be excluded that some inhibition of catalytic activity occurred. Clearly, additional dose-response studies with CB77 will help to elucidate this.

Being poikilothermic animals, the body temperature of fish corresponds closely to the ambient temperature. Fish are subject to seasonal temperature cycles, which means that many species of fish undergo quite extensive adaptive processes, like changes in enzymatic activity (Hazel and Prosser, 1974). Fish exposed to decreased environmental temperatures can respond with increased enzymatic activities, which in turn offset lower reaction rates that would normally be inherent with these lower temperatures (Ankley *et al.*, 1985). This type of response has been termed positive temperature compensation (Hazel and Prosser, 1974). P450-dependent enzymes in fish acclimated to different temperatures, often respond to acclimation temperature in a positive compensatory manner (Koivusaari and Andersson, 1984; Ankley *et al.*, 1985; Blanck *et al.*, 1989). For dab, the causal relationship between water temperature and basal EROD levels was investigated in a laboratory experiment with fish from the Doggerbank, a relatively clean part of the North Sea with respect to PCBs (Sleiderink *et al.*, 1995c): EROD activity proved to be inversely proportional with water temperature. This was confirmed by the present study; basal EROD levels in the control groups were a factor of 2 higher in the cold-acclimated fish compared to the warm-acclimated ones. Possible explanations involve effects on membrane fatty acid composition (Hazel, 1984) and changes in NADPH-cytochrome c reductase levels (Blanck *et al.*, 1989).

Temperature acclimation may be accompanied with a change in the P450 isoenzyme pattern (Andersson and Förlin, 1992). In the present study, there is a trend that the cold-acclimated control dab showed a slightly higher content of CYP1A protein. This is in contrast to earlier results with dab, in which no differences in CYP1A content in fish acclimated to different temperatures were found (Sleiderink *et al.*, 1995c). However, Carpenter *et al.* (1990)

found a higher CYP1A (LM4B) content in cold-acclimated untreated rainbow trout. The fact that elevated levels of CYP1A protein upon cold-acclimation were not observed during the earlier study with dab might be due to the fact that the amount of fish used were too low to detect the relatively small differences.

EROD activity and CYP1A protein were induced to the same absolute values in the 10°C as in the 16°C CB77-treated fish, although the relative increases in EROD activity and CYP1A protein were higher for warm-acclimated fish. This was due to lower basal CYP1A levels for the warm-acclimated dab. Also in BNF-treated rainbow trout, cold-acclimated fish achieved an EROD activity equal to that in warm-acclimated fish within 7 days after exposure (Förlin *et al.*, 1984). There are, however, studies which report higher absolute CYP1A values in warm acclimated fish than in cold-acclimated ones (Andersson and Koivusaari, 1985; Kloepper-Sams and Stegeman, 1992).

In the present study, the inductive response, measured as EROD activity and CYP1A protein came faster in the warm-acclimated fish than in the cold-acclimated ones. This is a known phenomenon; Egaas and Varanasi (1982) also found that low temperature influences the process of monooxygenase induction by increasing the time necessary to reach the maximal enzyme activity after exposure.

Very poor correlations between muscle CB77 concentrations and both CYP1A parameters were observed. Differences in residue concentrations and biochemical parameters for fish acclimated at 16° C were too low to result in significant correlations. For fish acclimated at 10° C residue concentrations were lowest 40 days after treatment, while on the contrary both EROD activity and CYP1A protein were highest 40 days after treatment. This is in agreement with an earlier study of rainbow trout treated with PCBs (Celander and Förlin, 1995). A possible explanation is that the concentration of CB77 is still high enough to persist at active inducing levels 40 days after treatment.

In conclusion, the results show that exposure of dab to the non-*ortho* chlorinated CB77 lead to induction of both EROD activity and CYP1A protein. Furthermore, the acclimation temperature influenced the basal CYP1A levels and the kinetics of the effect of CB77 to be evident.

Acknowledgements

The authors wish to thank Coen Fischer, Eligius Everaarts and Hans Kralt for their assistance. We are grateful to the crew of the RV *Pelagia* for their help in obtaining dab. We thank Dr. Jacob de Boer (Netherlands Institute for Fisheries Research) for the determination of CB77 with GC/MS.

Chapter 7

Effects of non- and mono-*ortho* substituted chlorinated biphenyls on cytochrome P450 1A expression in dab (*Limanda limanda*) *

Abstract

The effects of the non- and mono-*ortho* chlorine substituted 3,3',4,4',5-pentachlorobiphenyl (CB126), 2,3,3',4,4'-pentachlorobiphenyl (CB105), 2,3',4,4',5-pentachlorobiphenyl (CB118) and 2,3,3',4,4',5-hexachlorobiphenyl (CB156) on cytochrome P450 1A (CYP1A) expression in dab (*Limanda limanda*) were studied. Mature male dab were dosed intragastrically with 0.01, 0.1, 1 or 10 mg/kg of the non-*ortho* substituted CB126 and 10, 20 and 20 mg/kg of the mono-*ortho* substituted CB118, CB105 and CB156 respectively. Treatment of dab with these different non- and mono-*ortho* chlorinated CB congeners did not result in statistically significant increases in hepatic 7-ethoxyresorufin O-deethylase (EROD) activity, CYP1A protein levels or total P450 ($\sum$P450) content. A large inter-individual variation in especially the reference group was partly responsible for this. Exposure to CB126 even caused a dose-dependent inhibition of EROD activity, but not of CYP1A protein levels or $\sum$P450 content, at dose levels of 1 and 10 mg/kg. Dab exposed to 10 mg CB126/kg showed a significantly higher liver somatic index.

* Hedwig M. Sleiderink and Jan P. Boon

Introduction

Polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are widespread in the environment. In fish, especially the planar compounds cause induction of the cytochrome P450 1A (CYP1A) subfamily (Goksøyr and Förlin, 1992; Kleinow *et al.*, 1987; Payne *et al.*, 1987; Stegeman *et al.*, 1992). Therefore, induction of CYP1A is increasingly used as a marker for environmental contaminants that can induce CYP1A. Elevated levels of CYP1A have often been found to correlate with exposure to these compounds (Förlin and Celander, 1993; Monosson and Stegeman, 1994; Sleiderink and Boon, 1995; Sleiderink *et al.*, 1995b). For the interpretation of the significance of the CYP1A induction, it is important to evaluate the influence of different environmental factors (e.g. water temperature, sex, dietary factors, CYP1A inhibiting contaminants) which are known to influence CYP1A expression (Andersson and Förlin, 1992; Boon *et al.*, 1992a; Sleiderink *et al.*, 1995b, c) besides the identity and concentration of the inducing compounds.

In the Dutch aquatic environment the toxic potential of PCBs is estimated to be four times more important than that of PCDDs and PCDFs (De Boer *et al.*, 1993), therefore the present study focused on the effects of PCBs. Individual CB congeners differ in their toxicological mechanism and toxicity, depending on their degree of chlorination and ring substitution pattern (McFarland and Clarke, 1989). The most toxic congeners tend to be sterically similar to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, showing no chlorine substitution at *ortho* positions (coplanar PCBs), like 3,3',4,4'-tetraCB (CB77) and 3,3',4,4',5-pentaCB (CB126). Introduction of one chlorine atom at an *ortho* position results in a decreased coplanarity, e.g. 2,3,3',4,4'-pentaCB (CB105); 2,3,3',4,4',5-hexaCB (CB156), mono-*ortho* chlorine substituted analogues of CB77 and CB126 respectively, and 2,3',4,4',5-pentaCB (CB118). These congeners still show dioxin-type toxicity in mammals (Safe, 1990).

In this study we examined the effects of a non-*ortho* (CB126) and three mono-*ortho* substituted CBs (CB105, -118, -156) on CYP1A induction in dab (*Limanda limanda*), a marine flatfish. The effects of CB77 have been reported in a previous paper (Sleiderink and Boon, 1996).

Materials and methods

Fish, treatment and preparation of microsomes

Male mature dab (*Limanda limanda*) were caught by beam-trawling in the end of September on the Doggerbank (54°4'N, 2°4'E); a relatively clean area in the North Sea with respect to PCBs and related compounds. The fish were kept in indoor glass basins supplied with flowing seawater at 8-11°C under natural light conditions. The basins contained a thin layer of sediment on the bottom. The fish were acclimated to these conditions for 3 to 6 months prior to treatment. During the acclimation and experimental period fish were fed *ad libitum* with frozen shrimp (*Crangon crangon*).

The study consisted of two experiments; the first experiment with CB126 was carried out in January 1994 and the second one with the mono-*ortho* chlorinated CBs in April 1994. The fish weights in the first and second experiment were 77.6±12.8 and 81.9±20.9 gram, respectively. In the first experiment, groups of fish (n=8) were adminstered once intragastrically a gelatin capsule filled with different concentrations of CB126 dissolved in 20% iso-octane/80% sunflower oil (approximately 0, 0.01, 0.1, 1 or 10 mg/kg). In the second experiment groups of fish (n=7-8) were administered once intragastrically a gelatin capsule filled with 10% iso-octane/90% sunflower oil (controls) and different concentrations of CB105, CB156 and CB118 (approximately 20, 20 and 10 mg/kg). All dissolved in 10% iso-octane/90% sunflower oil. One positive control group (n=8 or 6 for first and second experiment, respectively) per experiment received a capsule with the technical PCB mixture Clophen A40 in sunflower oil (approximately 10 mg/kg).

The fish were sacrificed 10 and 7 days post-administration for CB126 and the mono-ortho chlorinated CBs respectively, whereupon their livers were excised and prepared as previously described (Sleiderink *et al.*, 1995a). The microsomal fractions were isolated as described by Sleiderink *et al.* (1995a).

Biochemical analyses

The protein content was determined according to Bradford (1976), with bovine serum albumine as reference standard. The total P450 ($\sum$P450) content was measured spectrofotometrically using dithionite-reduced difference spectra of CO-treated microsomes according to the method by Omura and Sato (1964), as adapted by Rutten *et al.* (1987). CYP1A protein levels were determined semi-quantitatively using an enzyme-linked immunosorbent assay (ELISA) according to the method of Goksøyr (1991), using monoclonal

mouse anti-cod IgG as primary antibody. The secondary antibody was goat anti-mouse IgG conjugated to horseradish peroxidase. 7-Ethoxyresorufin-O-deethylase (EROD) acitivity was determined as described previously (Sleiderink *et al.*, 1995a). All biochemical assays were performed on individual livers.

Statistics

The log-transformed data sets were analyzed statistically by a least significant difference bands test, and Hochberg's GT2 test for multiple comparisons of means (Sokal and Rohlf, 1981). To examine the correlations between EROD, CYP1A protein, and ∑P450, linear regression analyses were performed on log-transformed data.

Results

CB126 experiment

During the experimental period, one dab exposed to 1 mg CB126/kg died. At dose levels of 1 and 10 mg CB126/kg a lack of food intake occurred and dab in the 10 mg CB126/kg dose group showed lethargic behaviour.

The levels of the liver somatic index (LSI), EROD activity, CYP1A protein, and ∑P450 content are summarized in table 1. The LSI was significantly higher after a single dose of 10 mg CB126/kg. No significant differences in LSI were observed after administration of lower doses of CB126 or Clophen A40. At dose levels of 1 and 10 mg CB126/kg lowered levels of EROD activity were observed, respectively 4 to 8 times the control levels. There were no significant differences between EROD activity after administration of lower doses of CB126 or Clophen A40. No differences in CYP1A protein or ∑P450 content compared to control values were observed.

Mono-ortho chlorinated CBs experiment

No mortality or visible diseases occurred during the experimental period. The levels of the liver somatic index (LSI), EROD activity, CYP1A protein, and ∑P450 content are summarized in table 2. No differences in LSI upon treatment with mono-*ortho* chlorinated CBs or Clophen A40 were observed. EROD activity was significantly lower in CB105 treated dab compared to dab treated with CB118 or CB156. However, the enzyme activity was not lower compared to control values, this was due to the high standard deviations observed in control

Table 1. Liver somatic index (LSI), EROD activity, CYP1A protein, and ΣP450 content after intragastrically dosage of the non-*ortho* chlorinated CB126 and Clophen A40 (10 mg/kg)[a,b]

Treatment	LSI (%)[c]	EROD activity (nmol/min/mg)	CYP1A protein (absorbance at 492 nm)	ΣP450 (nmol/mg)	*N*
Control	2.21±0.44^{A}	0.46±0.69^{B}	1.11±0.49^{A,B}	0.52±0.29^{A,B}	8
0.01 mg/kg	2.29±0.31^{A}	0.70±0.15^{B}	1.25±0.40^{A,B}	0.28±0.11^{A,B}	8
0.1 mg/kg	2.82±0.58^{A,B}	0.61±0.20^{B}	1.64±0.21^{B}	0.42±0.13^{B}	8
1 mg/kg	2.89±0.83^{A,B}	0.10±0.065^{A}	1.24±0.24^{A}	0.22±0.13^{A}	7
10 mg/kg	3.08±0.65^{B}	0.056±0.064^{A}	1.36±0.20^{A,B}	0.31±0.16^{A,B}	8
Clophen A40	2.34±0.23^{A}	0.77±0.28^{B}	1.52±0.29^{A,B}	0.44±0.15^{B}	8

[a]Data expressed as mean±SD.
[b]Values indicated by similar letters are not significantly different (LSDB and GT2 test; $p<0.05$).
[c]Percentage liver per body weight.

fish. EROD activity was signicant higher in Clophen A40 treated dab compared to control values. No differences in CYP1A protein or total P450 levels upon treatment with mono-*ortho* chlorinated CBs or Clophen A40 were observed.

Discussion

In the present study fish exposed to 10 mg CB126/kg showed significantly higher LSI values compared to fish from the control group. Higher LSI values upon treatment with CB105, -118 and 156 were not observed. It is well known that several PCBs are known to cause liver enlargement (McConnell, 1980). This is dependent of the dose and the CB congener used; dab exposed to 0.5 mg CB77/kg did not show liver enlargement (Sleiderink and Boon, 1996).

Single intragastrically administration of the different non- and mono-*ortho* chlorinated CB congeners in dab did not result in increased hepatic EROD activity, CYP1A protein, or ΣP450 content. For the non-*ortho* chlorinated CB126 this is in sharp contrast with earlier studies were CB126 was found to be a potent inducer of CYP1A in fish (Melancon and Lech, 1983; Janz and Metcalfe, 1991; Van der Weiden *et al.*, 1994a; Newsted *et al.*, 1995). In the present study higher EROD levels in the 0.01 and 0.1 mg CB126/kg dose groups compared to

Table 2. Liver somatic index (LSI), EROD activity, CYP1A protein and ΣP450 content after intragastrically dosage of the mono-*ortho* chlorinated CB105 (20 mg/kg), CB118 (10 mg/kg) and CB156 (20 mg/kg) and Clophen A40 (10 mg/kg)[a,b]

Treatment	LSI (%)[c]	EROD activity (nmol/min/mg)	CYP1A protein (absorbance at 492 nm)	ΣP450 (nmol/mg)	*N*
Control	3.51±0.64[A]	0.37±0.48[A,B]	1.10±0.61[A]	0.24±0.27[A]	8
CB105	3.27±0.82[A]	0.042±0.052[A]	1.33±0.34[A]	0.26±0.15[A]	7
CB118	3.16±0.75[A]	0.33±0.22[B]	1.36±0.58[A]	0.48±0.25[A]	8
CB156	2.96±0.98[A]	0.29±0.15[B]	1.22±0.21[A]	0.28±0.15[A]	7
Clophen A40	3.70±1.34[A]	0.69±0.29[C]	1.61±0.36[A]	0.49±0.16[A]	6

[a]Data expressed as mean±SD.
[b]Values indicated by similar letters are not significantly different (LSDB and GT2 test; $p<0.05$).
[c]Percentage liver per body weight.

the control group were observed; these were however not statistically significant. In addition, the CYP1A levels from the positive control group (dosed with Clophen A40), were also not significantly higher from the control group. A possible explanation is that the individual variation in EROD activity, but also in CYP1A protein, and ΣP450 content, within the control group was found to be extremely large. The reason for this is not known. As a result of this large individual variation, the mean CYP1A levels of the 0.01 and 0.1 mg CB126/kg and Clophen A40 dose groups were not statistically different from the control group. It can however not be exluded that the fact that fish were pre-spawning during the experiment might have influenced the inducibility of CYP1A.

A decline in EROD activity at doses of 1 and 10 mg CB126/kg was found in the present study, whereas the CYP1A protein and ΣP450 content were not reduced. Reduced CYP1A catalytic activity in the presence of not reduced CYP1A protein or mRNA contents has been observed before in studies with fish at higher doses of PCBs (Melancon and Lech, 1983; Gooch *et al.*, 1989; Boon *et al.*, 1992a; Hahn *et al.*, 1993; Newsted *et al.*, 1995). The response to CB126 however, is contradictory in literature. In rainbow trout, a decline in EROD activity at a dose of 100 µg CB126/kg was observed (Newsted *et al.*, 1995). In contrast, Janz and Metcalfe (1991) did not find a decline in AHH activity in the same fish species at doses of up to 2 mg CB126/kg. In the mirror carp, *Cyprinus carpio*, doses of up to 56 µg/kg did not inhibit EROD activity (Van der Weiden *et al.*, 1994a).

One hypothesis for the decrease in CYP1A catalytic activity is the inhibition of substrate metabolism by residual CB126 present in microsomes competing with 7-ethoxyresorufin for sites on the CYP1A enzymes (Gooch *et al.*, 1989; Hahn *et al.*, 1993). Another hypothesis is the reduction of the CYP1A protein synthesis through a negative feedback mechanism (Hahn *et al.*, 1993; Tysklind *et al.*, 1995). Results from our study seem to support the first hypothesis, since a decline in EROD activity was not accompanied by a decline in CYP1A protein levels.

Non of the mono-*ortho* chlorinated CBs in our study increased the CYP1A activity in dab. The response of CYP1A in fish to mono-*ortho* chlorinated congeners seems to depend of fish species and type of congener used. Newsted *et al.* (1995) did not find any CYP1A induction in rainbow trout upon treatment with CB105, -118 and -156 with doses up to 4.5 mg/kg. In contrast, a higher dose of 10 mg CB105/kg did induce CYP1A in the rainbow trout, but was not capable to induce CYP1A in cod, *Gadus morhua* (Bernhoft *et al.*, 1994). In addition, 30 mg CB118/kg caused higher EROD and CYP1A levels in rainbow trout (Skaare *et al.*, 1991) and 21 mg CB118/kg induced CYP1A in the mirror carp (Van der Weiden *et al.*, 1994a). In contrast, in scup, *Stenotomus chrysops*, no induction was found after treatment with CB105 and -118 with doses up to 15 and 5 mg/kg, respectively (Gooch *et al.*, 1989).

In the present study, there was a trend of lowered EROD activity levels in dab treated with CB105, whereas CYP1A protein and ∑P450 content were unchanged. This was however not statistically significant, because of the high standard deviations in the control group. CB105 is a mono-*ortho* chlorine analogue of the non-*ortho* chlorinated CB77, which is known to inhibit CYP1A catalytic at doses above 1 to 5 mg/kg in several fish species (Melancon and Lech, 1983; Gooch *et al.*, 1989; Monosson and Stegeman, 1991). Gooch *et al.* (1989) observed that the mono-*ortho* chlorinated CB156 inhibited CYP1A activity in hepatic microsomes. In our study, EROD activity levels from dab dosed with CB156 were also lower compared to the control group, but this was again not statistically significant. It must however be remarked that for each of the mono-*ortho* chlorinated congeners one single dose was used; this might not be enough to reveal inhibition or induction processes. Clearly, additional dose-response studies are needed to reveal the effects of these congeners.

In conclusion, the results show that exposure of dab to the non- and mono-*ortho* chlorinated CB126, -105, -118 and -156 did not lead to induction of CYP1A. Exposure to CB126 caused inhibition of CYP1A activity at higher dose levels.

Acknowledgements

The authors thank the crew of the RV *Pelagia* for their help in obtaining dab. The assistence of Coen Fisher during injection and sampling of fish is gratefully acknowledged.

Part III

Summary and concluding remarks

Chapter 8

Summary and concluding remarks

The objective of this thesis was to investigate whether the CYP1A response in dab could be used as a biological marker of exposure to PCBs and related compounds in monitoring programmes in the southern North Sea. Only because of its wide occurrence in the North Sea, dab is already playing an important role in international contamination monitoring programmes, but no attempt has been made to investigate the suitability of the organism for monitoring purposes so far. During the field studies and laboratory experiments, emphasis has been laid on the study of PCBs, since they are by far the most important class of compounds capable of inducing CYP1A in the southern North Sea.

In chapter 2 the effects of maturity and sex on CYP1A expression in the field were investigated. Mature and juvenile dab of both sexes were collected from different areas of the southern North Sea with varying levels of PCB contamination. In all cases, muscle PCB concentrations were highest near the Dutch coast. The highest CYP1A levels, measured as EROD activity and CYP1A protein, were also found in this area for mature fish of both sexes in autumn. The same was found during the spawning season in winter for juvenile females and mature males. During this season gravid females showed significantly lowered contents of CYP1A protein and EROD activity compared to mature males and juveniles. Muscle PCB concentrations and both biochemical parameters were positively correlated for mature males, and not for other groups of dab, during both seasons. It was concluded that the sensitivity of CYP1A induction in dab as a biomarker for PCBs and related compounds was highest in mature males.

In chapter 3 it was shown that bottom water temperature differences of up to 10°C, occuring between stratified and vertically mixed areas during spring and summer, have a strong effect on CYP1A expression in dab. Highly elevated CYP1A levels were observed in mature male dab collected from off-shore stations with low bottom water temperatures due to stratification whereas considerably lower CYP1A levels were observed at stations with higher water temperatures in vertically mixed areas, including coastal stations. Statistical analyses of the data indicated that water temperature was inversely related to CYP1A levels, whereas PCB concentrations showed a positive correlation with CYP1A levels. The effect of water

temperature, however, dominated over the effect of PCB contamination. A laboratory study confirmed that EROD activity was inversely proportional to water temperature. Furthermore, it was shown that differences in the nutritional status of dab, reflected by the condition factor, also obscured the effects of PCB contamination on CYP1A levels during these seasons.

Because it was demonstrated that spring and summer are less suitable seasons for monitoring purposes and next to this, dab in the southern North Sea show considerably more migration during the spawning period in winter, it was investigated whether the most suitable period for annual monitoring would be the late autumn. Chapter 4 describes a final field study during which differences in interfering natural factors, like water temperature, condition factor of the fish and percentage lipid in muscle tissue proved to be minimal. The results showed that the CYP1A induction response in male dab was strong enough to separate the Dutch coastal area from three more pristine offshore areas of the North Sea. Thus, the general conclusion of all field studies is that the late autumn indeed offers the best conditions to investigate the relation between environmental contamination with PCBs and related compounds, since disturbing influences of factors other than PHAH contamination are minimal.

The induction capacity of CYP1A by PCBs has been studied in laboratory experiments. Chapter 5 describes an experiment in which mature female dab were dosed with 1 mg of the technical PCB mixture Clophen A40 every 6 weeks, with a maximum of three doses per fish. Induction of CYP1A was observed following this Clophen A40 exposure. Biochemical effects were related to increases in concentrations of total PCBs and specific congeners and consequently to the corresponding toxicity equivalencies of these PCBs (CB-TEQs) in muscle. In all treated groups the EROD activity, CYP1A protein and total CYP1A levels were higher than those of the control groups. The maximum for these biochemical parameters was already reached after the first single dose, although the CB concentrations in muscle tissue increased further after administration of a second and third dose. It was concluded that the CYP1A system of dab is sensitive towards PCBs.

Since PCB congeners differ in potency to induce CYP1A, the biochemical effects of several non- and mono-*ortho* substituted CB congeners were investigated. Exposure to the non-*ortho* chlorinated CB77 resulted in induction of CYP1A (chapter 6). On the contrary, exposure to three different mono-*ortho* chlorinated CB congeners and the non-*ortho* chlorinated CB126 did not result in increases of CYP1A levels (chapter 7). For CB126 this is in sharp contrast with earlier studies in fish where this congener was found to be a potent inducer of CYP1A. A large inter-individual variation in especially the reference group was held partly responsible for this. Exposure to CB126 even caused a dose-dependent inhibition of EROD activity at higher concentrations.

In chapter 6 the influence of temperature on the temporal induction pattern of CYP1A was investigated. Mature males were exposed to a single dose of a non-*ortho* chlorinated CB congener (CB77). The fish were acclimated at two different temperatures (10 and 16°C) and kept at these temperatures for a period of 40 days. At both temperatures, CYP1A protein and EROD activity were induced 40 days after dosing. Maximum responses of both EROD activity and CYP1A protein for the warm-acclimated fish were observed 5 days after treatment. For the cold-acclimated fish a slow, progressive elevation for both biochemical parameters was observed and maximum responses were measured 40 days after treatment. In the control groups EROD activity and CYP1A protein levels were higher in the cold-acclimated fish, which is in accordance with the study described in chapter 3. In the dosed groups, however, absolute biochemical levels were equally high at 40 days after treatment. It is therefore concluded that the magnitude of induction was higher in warm-acclimated fish.

Since CYP1A is a biomarker of exposure it does not provide knowledge of adverse (toxic) effects. The question can thus be raised whether enzyme induction reflects deleterious effects on dab. In the study where dab were exposed to Clophen A40 (chapter 5), no effects on reproductive parameters were found (Fonds *et al.*, 1995). It is very likely that the concentrations of contaminants needed for effects at higher levels of biological organisation will seldom be reached in the marine environment.

From the present research, it has become clear that dab is a suitable organism to monitor hepatic CYP1A, indicating exposure to PHAHs, in the North Sea. Dab has shown to be sensitive towards PCBs. However, when applying the CYP1A measurement in dab, several interfering co-factors, like seawater temperature, maturity and sex, have to be taken into account. As a consequence, monitoring with mature males in the autumn seems to be preferable. With respect to monitoring programmes, the CYP1A measurement could very well serve as a quick and cheap screening method for contamination of the aquatic environment. The joint impact of a whole group of chemicals, the PHAHs, on CYP1A can be assessed. Although there is ample evidence for the existence of a dose-response relationship between PHAHs and CYP1A, direct linear correlations will not always be found in the environment. For instance, concentrations of PAHs in the North Sea are too low to induce CYP1A in dab. Therefore measurement of CYP1A can never fully replace chemical analyses, but it can give complementary information about inducing PHAH compounds. Application of CYP1A seems to be useful to discrimate between estuarine and/or coastal areas and the open North Sea, which

implies that increased concentrations of PHAHs reaching the marine environment via the outflow of contaminated rivers can be detected.

References

Addison, R.F., D.E. Willis and M.E. Zinck, 1994. Liver microsomal mono-oxygenase induction in winter flounder (*Pseudopleuronectes americanus*) from a gradient of sediment PAH concentrations at Sydney Harbour, Nova Scotia. *Mar. Environ. Res.* **37**, 283-296.

Ahlborg, U.G., A. Brouwer, M.A. Fingerhut, J.L. Jacobson, S.W. Jacobson, S.W. Kennedy, A.A.F. Kettrup, J.H. Koeman, H. Poiger, C. Rappe, S.H. Safe, R.F. Seegal, J. Tuomisto and M. van den Berg, 1992. Impact of polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls on human and environmental health, with special emphasis on application of the toxic equivalency factor concept. *Eur. J. Pharmacol.* **228**, 179-199.

Andersson, T. and U. Koivusaari, 1985. Influence of environmental temperature on the induction of xenobiotic metabolism by ß-naphthoflavone in rainbow trout, *Salmo gairdneri*. *Toxicol. Appl. Pharmacol.* **80**, 43-50.

Andersson, T. and U. Koivusaari, 1986. Oxidative and conjugative metabolism of xenobiotics in isolated liver cells from thermally acclimated rainbow trout. *Aquat. Toxicol.* **8**, 85-92.

Andersson, T. and L. Förlin, 1992. Regulation of the cytochrome P450 enzyme system in fish. *Aquat. Toxicol.* **24**, 1-20.

Andersson, T., L. Förlin, J. Härdig and A. Larsson, 1988. Physiological disturbances in fish living in coastal waters polluted with bleached kraft pulp mill effluents. *Can. J. Fish. Aquat. Sci.* **45**, 1525-1536.

Ankley, G.T., R.E. Reinert, A.E. Wade and R.A. White, 1985. Temperature compensation in the hepatic mixed function oxidase system of bluegill. *Comp. Biochem. Physiol.* **81**C, 125-129.

Ankley, G.T., P.M. Cook, A.T. Carlson, D.J. Call, D.J. Swenson, H.F. Corcoran and R.A. Hoke, 1992. Bioaccumulation of PCBs from sediments by oligochaetes and fishes: Comparison of laboratory and field studies. *Can. J. Fish. Aquat. Sci.* **49**, 2080-2085.

Ankley, G.T., G.J. Niemi, K.B. Lodge, H.J. Harris, D.L. Beaver, D.E. Tillitt, T.R. Schwartz, J.P. Giesy, P.D. Jones and C. Hagley, 1993. Uptake of planar polychlorinated biphenyls and 2,3,7,8-substituted polychlorinated dibenzofurans and dibenzo-*p*-dioxins by birds nesting in the lower Fox river and Green Bay, Wisconsin, USA. *Arch. Environ. Contam. Toxicol.* **24**, 332-344.

Ariese, F., 1993. Shpol'skii spectroscopy and synchronous fluorescence spectroscopy. (Bio)monitoring of polycyclic aromatic hydrocarbons and their metabolites. PhD Thesis. Free University of Amsterdam, pp. 143-163.

Bagenal, T.B. and F.W. Tesch, 1978. Methods for assessment of fish production in fresh waters. In *Age and Growth*, ed. T.B. Bagenal. Blackwell Scientific, Oxford, UK, pp. 101-136.

Ballschmiter, K. and M. Zell, 1980. Analysis of polychlorinated biphenyls (PCB) by glass capillary gas chromatography. Composition of technical Aroclor and Clophen-PCB mixtures. *Z. Anal. Chem.* **302**, 20-31.

Berndtson, A.K. and T.T. Chen, 1994. Two unique CYP1 genes are expressed in response to 3-methylcholanthrene treatment in rainbow trout. *Arch. Biochem. Biophys.* **310**, 187-195.

Bernhoft, A., H. Hektoen, J.U. Skaare and K. Ingebrigtsen, 1994. Tissue distribution and effects on hepatic xenobiotic metabolising enzymes of 2,3,3',4,4'-pentachlorobiphenyl (PCB105) in cod (*Gadus morhua*) and rainbow trout (*Oncorhynchus mykiss*). *Environ. Poll.* **85**, 351-359.

Blanck, J., P. Lindström-Seppä, J.J. Ågren, O. Hänninen, H. Rein and K. Ruckpaul, 1989. Temperature compensation of hepatic microsomal cytochrome P-450 activity in rainbow trout. **I**. Thermodynamic regulation during water cooling in autumn. *Comp. Biochem. Physiol.* **93**C, 55-60.

Bligh, E.G. and W.J. Dyer, 1959. A rapid method of total lipid extraction and purification. *Can. J. Physiol.* **37**, 911-917.

Bolle, L., R. Dapper, J.IJ. Witte and H.W. van der Veer, 1994. Nursery grounds of dab (*Limanda limanda* L.) in the southern North Sea. *Neth. J. Sea Res.* **32**, 299-307.

Booij, K. and C. Van den Berg, 1994. Comparison of techniques for the extraction of lipids and PCBs from benthic invertebrates. *Bull. Environ. Contam. Toxicol.* **53**, 71-76.

Boon, J.P., 1985. Uptake, distribution and elimination of selected PCB components of Clophen A40 in juvenile sole (*Solea solea*) and effects on growth. In *Marine Biology of Polar Regions and Effects of Stress*

on Marine Organisms, eds. J.S. Gray and M.E. Christiansen. John Wiley & Sons, New York, NY, pp. 493-512.

Boon, J.P., 1992. PCB-138 concentration in benthic worms. In *North Sea Atlas for Netherlands policy and management*. ICONA, Amsterdam, map no. 88.

Boon, J.P., R.C.H.M. Oudejans and J.C. Duinker, 1984. Kinetics of individual polychlorinated biphenyl (PCB) components in juvenile sole (*Solea solea*) in relation to their concentrations in food and to lipid metabolism. *Comp. Biochem. Physiol.* **79**C, 131-142.

Boon, J.P., M.B. van Zantvoort, M.J.M.A. Govaert and J.C. Duinker, 1985. Organochlorines in benthic polychaetes (*Nephtys* spp.) and sediments from the southern North Sea. Identification of individual PCB components. *Neth. J. Sea Res.* **19**, 93-109.

Boon, J.P., F. Eijgenraam, J.M. Everaarts and J.C. Duinker, 1989. A structure-activity relationship (SAR) approach towards metabolism of PCBs in marine animals from different trophic levels. *Mar. Environ. Res.* **27**, 159-176.

Boon, J.P., J.M. Everaarts, M.T.J. Hillebrand, M.L. Eggens, J. Pijnenburg and A. Goksøyr, 1992a. Changes in levels of hepatic biotransformation enzymes and haemoglobin levels in female plaice (*Pleuronectes platessa*) after oral administration of a technical polychlorinated biphenyl mixture (Clophen A40). *Sci. Total Environ.* **114**, 113-133.

Boon, J.P., E. van Arnhem, S. Jansen, N. Kannan, G. Petrick, D.E. Schulz, J.C. Duinker, P.J.H. Reijnders and A. Goksøyr, 1992b. The toxicokinetics of PCBs in marine mammals with special reference to possible interactions of individual congeners with the cytochrome P450-dependent monooxygenase system - An overview. In *Persistent Pollutants in Marine Ecosystems*, eds. C.H. Walker and D.R. Livingstone. Pergamon Press, Oxford, UK, pp. 119-159.

Boon, J.P., J.M. Nieuwenhuize, J. van Liere, S. Wilhelmsen and J. Klungsøyr, 1993. Concentrations of PCBs and PAHs in dab muscle. NIOZ-Rapport 1993-10, pp. 101-103.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation for microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Bright, D.A., S.L. Grundy and K.J. Reimer, 1995. Differential bioaccumulation of non-*ortho*-substituted and other PCB congeners in coastal arctic invertebrates and fish. *Environ. Sci. Technol.* **29**, 2504-2512.

Bruggeman, W.A., 1983. Bioaccumulation of polychlorobiphenyls and related hydrophobic chemicals in fish. Ph. D. thesis. Univ. of A'dam, pp. 151-158.

Bucheli, T.D. and K. Fent, 1995. Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. *Crit. Rev. Environ. Sci. Technol.* **25**, 201-268.

Burgeot, T., G. Bocquene, G. Pingray, D. Godefroy, J. Legrand, J. Dimeet, F. Marco, F. Vincent, Y. Henocque, H. Oger Jeanneret and F. Galgani, 1994. Monitoring biological effects of contamination in marine fish along French coasts by measurement of ethoxyresorufin-O-deethylase activity. *Ecotoxicol. Environ. Saf.* **29**, 131-147.

Carpenter, H.M., L.S. Fredrickson, D.E. Williams, D.R. Buhler and L.R. Curtis, 1990. The effect of thermal acclimation in the activity of arylhydrocarbon hydroxylase in rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol.* **97**C, 127-132.

Celander, M. and L. Förlin, 1995. Decreased responsiveness of the hepatic cytochrome P450 1A1 system in rainbow trout (*Oncorhynchus mykiss*) after prolonged exposure to PCB. *Aquat. Toxicol.* **33**, 141-153.

Cullen, J.M. and K.L.E. Kaiser, 1984. An examination of the role of rotational barriers in the toxicology of PCBs. In *QSAR in Environmental Toxicology*, ed. K.L.E. Kaiser. Reidel Publishing Company, pp. 39-66.

Daan, N., P.J. Bromley, J.R.G. Hislop and N.A. Nielsen (1990). Ecology of North Sea fishes. *Neth. J. Sea Res.* **26**, 343-386.

De Boer, J., 1995. Analysis and biomonitoring of complex mixtures of persistent halogenated micro-contaminants. PhD thesis. Free Univ. of Amsterdam.

De Boer, J., Q.T. Dao and R. van Dortmond, 1992a. Retention times of fifty one chlorobiphenyl congeners on seven narrow bore capillary columns coated with different stationary phases. *J. High Resolut. Chromatogr.* **15**, 249-255

De Boer, J., J.C. Duinker, J.A. Calder and J. van der Meer, 1992b. Interlaboratory study on the analysis of chlorobiphenyl congeners. *J. AOAC International* **75**, 1054-1062.

De Boer, J., C.J.N. Stronck, W.A. Traag and J. van der Meer, 1993. Non-*ortho* and mono-*ortho* substituted chlorobiphenyls and chlorinated dibenzo-p-dioxins and dibenzofurans in marine and freshwater fish and shellfish from the Netherlands. *Chemosphere* **26**, 1823-1842.

De Boer, J., J. van der Meer, L. Reutergårdh and J.A. Calder, 1994. Interlaboratory study on the determination of chlorobiphenyls in cleaned-up seal blubber and marine sediment extracts. *J. AOAC International* **77**, 1411-1422.

Duursma, E.K., J. Nieuwenhuize, J.M. van Liere and M.T.J. Hillebrand, 1986. Partitioning of organochlorines between water, particulate matter and some organisms in estuarine systems of the Netherlands. *Neth. J. Sea Res.* **20**, 239-251.

Egaas, E. and U. Varanasi, 1982. Effects of polychlorinated biphenyls and environmental temperature on *in vitro* formation of benzo[a]pyrene metabolites by liver of trout (*Salmo gairdneri*). *Biochem. Pharmacol.* **31**, 561-566.

Eggens, M.L. and F. Galgani, 1992. Ethoxyresorufin-O-deethylase (EROD) activity in flatfish: Fast determination with a fluorescence plate-reader. *Mar. Environ. Res.* **33**, 213-221.

Eggens, M.L., F. Galgani, J. Klungsøyr and J. Everts, 1992. Hepatic EROD activity in dab *Limanda limanda* in the German Bight using an improved plate-reader method. *Mar. Ecol. Prog. Ser.* **91**, 71-75.

Eggens, M.L., A. Bergman, A.D. Vethaak, M.E.J. Van der Weiden, M. Celander and J.P. Boon, 1995. Cytochrome P450 1A indices as biomarkers of contaminant exposure: results of a field study with plaice, *Pleuronectes platessa* and flounder, *Platichthys flesus*, from the southern North Sea. *Aquat. Toxicol.* **32**, 211-225.

Eisma, D. and G. Irion, 1988. Suspended matter and sediment transport. In *Pollution of the North Sea, an assessment*, eds. W. Salomons, B.L. Bayns, E.K. Duursma and Förstner. Springer Verlag, Heidelberg, Germany.

Elskus, A. A. and J.J. Stegeman, 1989. Induced cytochrome P-450 in *Fundulus heteroclitus* associated with environmental contamination by polychlorinated biphenyls and polynuclear aromatic hydrocarbons. *Mar. Environ. Res.* **27**, 31-50.

Elskus, A.A., J.J. Stegeman, L.C. Susani, D. Black, R.J. Pruell and S.J. Fluck, 1989. Polychlorinated biphenyl concentration and cytochrome P-450E expression in winter flounder from contaminated environments. *Mar. Environ. Res.* **28**, 25-30.

Elskus, A.A., R. Pruell and J.J. Stegeman, 1992. Endogenously-mediated, pretranslational suppression of cytochrome P4501A in PCB-contaminated flounder. *Mar. Environ. Res.* **34**, 97-101.

Evans, M.S., G.E. Noguchi and C.P. Rice, 1991. The biomagnification of polychlorinated biphenyls, toxaphene, and DDT compounds in a Lake Michigan offshore food web. *Arch. Environ. Contam. Toxicol.* **20**, 87-93.

Everaarts, J.M., 1978. The haemoglobin of the herring, *Clypea harengus*. *Neth. J. Sea Res.* **12**, 1-57.

Evers, E.H.G., H.J.C. Klamer, R.W.P.M. Laane and H.A.J. Govers, 1993. Polychlorinated dibenzo-*p*-dioxin and dibenzofuran residues in estuarine and coastal North Sea sediments: sources and distribution. *Environ. Toxicol. Chem.* **12**, 1583-1598.

Fent, K. and J.J. Stegeman, 1991. Effects of tributyltin chloride in vitro on the hepatic microsomal monooxygenase system in the fish *Stenotomus chrysops*. *Aquat. Toxicol.* **20**, 159-168.

Fent, K. and J.J. Stegeman, 1993. Effects of tributyltin in vivo on hepatic cytochrome P450 forms in marine fish. *Aquat. Toxicol.* **24**, 219-240.

Fonds, M., E. Casal, D. Schweizer, J.P. Boon and H. van der Veer, 1995. Effects of PCB contamination on the reproduction of the dab *Limanda limanda* L. under laboratory conditions. *Neth. J. Sea Res.* **34**, 0-8.

Förlin, L., 1980. Effects of Clophen A50, 3-methylcholanthrene, pregnenolone-16a-carbonitrile, and phenobarbital on the hepatic microsomal cytochrome P-450-dependent monooxygenase system in rainbow trout, *Salmo gairdneri*, of different age and sex. *Toxicol. Appl. Pharmacol.* **54**, 420-430.

Förlin, L. and C. Haux, 1989. Sex differences in hepatic cytochrome P-450 monooxygenase activities in rainbow trout during an annual reproductive cycle. *J. Endocr.* **124**, 207-213.

Förlin, L. and M. Celander, 1993. Induction of cytochrome P450 1A in teleosts: environmental monitoring in Swedish fresh, brackish and marine waters. *Aquat. Toxicol.* **26**, 41-56.

Förlin, L., T. Andersson, U. Koivusaari and T. Hansson, 1984. Influence of biological and environmental factors on hepatic steroid and xenobiotic metabolism in fish: interaction with PCB and ß-naphthoflavone. *Mar. Environ. Res.* **14**, 47-58.

Förlin, L., C. Haux, L. Karlsson-Norrgren, P. Runn and A. Larsson, 1986. Biotransformation enzyme activities and histopathology in rainbow trout, *Salmo gairdneri*, treated with cadmium. *Aquat. Toxicol.* **8**, 51-64.

Förlin, L., L. Balk, M. Celander, S. Bergek, M. Hjelt, C. Rappe, C. De Wit and B. Jansson, 1992. Biotransformation enzyme acitvities and PCDD/PCDF levels in pike caught in a Swedish lake. *Mar. Environ. Res.* **34**, 169-173.

References

Förlin, L., A. Goksøyr and A.-M. Husøy, 1994. Cytochrome P450 monooxygenase as indicator of PCB/dioxin like compounds in fish. In *Biomonitoring of coastal waters and estuaries*, ed. K.J.M. Kramer. CRC Press, Boca Raton, Florida, pp. 135-150.

Gabriel, K.R., 1971. The biplot graphic display of matices with application to principal components analysis. *Biometrika* **58**: 453-467.

Galgani, F., G. Bocquene, M. Lucon, D. Grzebyk, F. Letrouit and D. Claisse, 1991. EROD measurements in fish from the Northwest part of France. *Mar. Poll. Bull.* **22**, 494-500.

George, S.G., 1989. Cadmium effects on plaice liver xenobiotic and metal detoxication systems: dose-response. *Aquat. Toxicol.* **15**, 303-310.

George, S. and J. Henderson, 1992. Influence of dietary PUFA content on cytochrome P450 and transferase activities in Atlantic salmon (*Salmo salar*). *Mar. Environ. Res.* **34**, 127-131.

George, S., P. Young, M. Leaver and D. Clarke, 1990. Activities of pollutant metabolising and detoxication systems in the liver of the plaice, *Pleuronectes platessa*: sex and seasonal variations in non-induced fish. *Comp. Biochem. Physiol.* **96C**, 185-192.

Goksøyr, A., 1991. A semi-quantitative cytochrome P-450IA1 ELISA: a simple method for studying the monooxygenase induction response in environmental monitoring and ecotoxicological testing of fish. *Sci. Tot. Environ.* **101**, 255-262.

Goksøyr, A. and L. Förlin, 1992. The cytochrome P-450 system in fish, aquatic toxicology and environmental monitoring. *Aquatic. Toxicol.* **22**, 287-312.

Goksøyr, A., T. Andersson, T. Hansson, J. Klungsøyr, Y. Zhang and L. Förlin, 1987. Species characteristics of the hepatic xenobiotic and steroid biotransformation systems of two teleost fish, Atlantic cod (*Gadus morhua*) and rainbow trout (*Salmo gairdneri*). *Toxicol. Appl. Pharmacol.* **89**, 347-360.

Goksøyr, A., A.-M. Husøy, H.E. Larsen, J. Klungsøyr, S. Wilhelmsen, A. Maage, E.M. Brevik, T. Andersson, M. Celander, M. Pesonen and L. Förlin, 1991. Environmental contaminants and biochemical responses in flatfish from the Hvaler Archipelago in Norway. *Arch. Environ. Contam. Toxicol.* **21**, 486-496.

Goksøyr, A., H.E. Larsen, S. Blom and L. Förlin, 1992. Detection of cytochrome P450 1A1 in North Sea dab liver and kidney. *Mar. Ecol. Prog. Ser.* **91**, 83-88.

Goksøyr, A., J. Beyer, A.-M. Husøy, H.E. Larsen, K. Westrheim, S. Wilhelmsen and J. Klungsøyr, 1994a. Accumulation and effects of aromatic and chlorinated hydrocarbons in juvenile Atlantic cod (*Gadus morhua*) caged in a polluted fjord (Sørfjorden, Norway). *Aquat. Toxicol.* **29**, 21-35.

Goksøyr, A., M. Bjørnevik and A. Maage, 1994b. Effects of dietary iron concentrations on the cytochrome P450 system of Atlantic salmon (*Salmo salar*). *Can. J. Fish. Aquat. Sci.* **51**, 315-320.

Gooch, J.W., A.A. Elskus, P.J. Kloepper-Sams, M.E. Hahn and J.J. Stegeman, 1989. Effects of *ortho*- and non-*ortho*-substituted polychlorinated biphenyl congeners on the hepatic monooxygenase system in scup (*Stenotomus chrysops*). *Toxicol. Appl. Pharmacol.* **98**, 422-433.

Gribble, G.W.,1994. The natural production of chlorinated compounds. *Environ. Sci. Technol.* **28**, 310A-317A.

Hahn, M.E., T.M. Lamb, M.E. Shultz, R.M. Smolowitz and J.J. Stegeman, 1993. Cytochrome P4501A induction and inhibition by 3,3',4,4'-tetrachlorobiphenyl in an Ah receptor-containing fish hepatoma cell line (PLHC-1). *Aquat. Toxicol.* **26**, 185-208.

Hahn, M.E., A. Poland, E. Glover and J.J. Stegeman, 1994. Photoaffinity labeling of the Ah receptor: phylogenetic survey of diverse vertebrate and invertebrate species. *Arch. Biochem. Biophys.* **310**, 218-228.

Haux, C. and L. Förlin, 1988. Biochemical methods for detecting effects of contaminants on fish. *Ambio* **17**, 376-380.

Hazel, J.R., 1984. Effects of temperature on the structure and metabolism of cell membranes in fish. *Am. J. Physiol.* **246**, 460-470.

Hazel, J.R. and C.L. Prosser, 1974. Molecular mechanisms of temperature compensation in poikilotherms. *Physiol. Rev.* **54**, 620-673.

Heilmann, L.J., Y.-Y. Sheen, S.W. Bigelow and D.W. Nebert, 1988. Trout P-450IA1: cDNA and deduced protein. Sequence, expression in liver and evolotionary significance. *DNA* **7**, 379-387.

Holden, A.V. and K. Marsden, 1969. Single stage clean-up of animal tissue extracts for organochlorine residue analysis. *J. Chrom.* **44**, 481-449.

Holdway, D.A., S.E. Brennan and J.T. Ahokas, 1994. Use of hepatic MFO and blood enzyme biomarkers in sand flathead (*Platycephalus bassensis*) as indicators of pollution in Port Phillip Bay, Australia. *Mar. Poll. Bull.* **28**, 683-695.

Hong, C.S., B. Bush and J. Xiao, 1992. Coplanar PCBs in fish and mussels from marine and estuarine waters of New York State. *Ecotoxicol. Environ. Safety* **23**, 118-131.

Huggett, R.J., R.A. Kimerle, P.M. Mehrle and H.L. Bergman, 1992. Biomarkers: Biochemical, physiological, and histological markers of anthropogenic stress. Lewis Publishers, Boca Raton, Florida.

Husøy, A.-M., M.S. Myers, M.J. Willis, T.K. Collier, M. Celander and A. Goksøyr, 1994. Immunohistochemical localization of CYP1A and CYP3A isozymes in hepatic and extrahepatic tissues of Atlantic cod. *Toxicol. Appl. Pharmacol.* **129**, 294-308..

Janz, D.M. and C.D. Metcalfe, 1991. Relative induction of aryl hydrocarbon hydroxylase by 2,3,7,8-TCDD and two coplanar PCBs in rainbow trout (*Onchorhynchus mykiss*). *Environ. Toxicol. Chem.* **10**, 917-923.

Jimenez, B.D., C.P. Cirmo and J.F. McCarthy, 1987. Effects of feeding and temperature on uptake, elimination and metabolism of benzo(a)pyrene in bluegill sunfish (*Lepomis macrochirus*). *Aquat. Toxicol.* **10**, 41-58.

Kannan, N., S. Tanabe, M. Ono and R. Tatsukawa, 1989. Critical evaluation of polychlorinated biphenyl toxicity in terrestial and marine mammals: Increasing impact of non-*ortho* and mono-*ortho* coplanar polychlorinated biphenyls from land to ocean. *Arch. Environ. Contam. Toxicol.* **18**, 850-857.

Klamer, J., R.W.P.M. Laane and J.M. Marquenie, 1991. Sources and fate of PCBs in the North Sea: A review of available data. *Water Sci. Technol.* **24**, 77-85.

Kleinow, K.M., M.L. Haasch and J.J. Lech, 1986. The effect of tricaine anaesthesia upon induction of select P-450 dependent monooxygenase activities in rainbow trout (*Salmo gairdneri*). *Aquat. Toxicol.* **8**, 231-241.

Kleinow, K.M., M.J. Melancon and J.J. Lech, 1987. Biotransformation and induction: implications for toxicity, bioaccumulation and monitoring of environmental xenobiotics in fish. *Environ. Health Perspect.* **71**, 105-119.

Kloepper-Sams, P.J. and J.J. Stegeman, 1992. Effects of temperature acclimation on the expression of hepatic cytochrome P4501A mRNA and protein in the fish *Fundulus heteroclitus*. *Arch. Biochem. Biophys.* **299**, 38-46.

Kloepper-Sams, P.J. and E. Benton, 1994. Exposure of fish to biologically treated bleached-kraft effluent. 2. Induction of hepatic cytochrome P4501A in mountain whitefish (*Prosopium williamsoni*) and other species. *Environ. Toxicol. Chem.* **13**, 1483-1496.

Klotz, A.V., J.J. Stegeman and C. Walsh, 1984. An alternative 7-ethoxy-resorufin O-deethylase activity assay: A continuous visible spectrophotometric method for measurement of cytochrome P-450 monooxygenase activity. *Anal. Biochem.* **140**, 138-145.

Knickmeyer, R. and H. Steinhart, 1990. Patterns of cyclic organochlorine contamination in liver of male pleuronectiformes from the North Sea, Winter 1987. *Mar. Poll. Bull.* **21**, 187-189.

Koivusaari, U. and T. Andersson, 1984. Partial temperature compensation of hepatic biotransformation enzymes in juvenile rainbow trout (*Salmo gairdneri*) during water warming in spring. *Comp. Biochem. Physiol.* **78**B, 223-226.

Larsen, B., S. Bøwadt and R. Tilio, 1992a. Congener specific analysis of 140 chlorobiphenyls in technical mixtures on five narrow-bore GC columns. *Int. J. Environ. Anal. Chem.* **47**, 47-68.

Larsen, H.E., M. Celander and A. Goksøyr, 1992b. The cytochrome P450 system of Atlantic salmon (*Salmo salar*): II. Variations in hepatic catalytic activities and isozyme patterns during an annual reproductive cycle. *Fish. Physiol. Biochem.* **10**, 291-301.

Lemaire, P., A. Mathieu, J. Giudicelli and M. Lafaurie, 1992. Effect of diet on the responses of hepatic biotransformation enzymes to benzo(a)pyrene in the European sea bass (*Dicentrarchus labrax*). *Comp. Biochem. Physiol.* **102**C, 413-420.

Lindström-Seppä, P., P.J. Korytko, M.E. Hahn and J.J. Stegeman, 1994. Uptake of waterborne 3,3',4,4'-tetrachlorobiphenyl and organ and cell-specific induction of cytochrome P4501A in adult and larval fathead minnow *Pimephales promelas*. *Aquat. Toxicol.* **28**, 147-167.

Livingstone, D.R., 1993. Biotechnology and pollution monitoring: use of molecular biomarkers in the aquatic environment. *J. Chem. Tech. Biotechnol.* **57**, 195-211.

Livingstone, D.R., P. Lemaire, A. Matthews, L. Peters, D. Bucke and R.J. Law, 1993. Pro-oxidant, antioxidant and 7-ethoxyresorufin O-deethylase (EROD) activity responses in liver of dab (*Limanda limanda*) exposed to sediment contaminated with hydrocarbons and other chemicals. *Mar. Poll. Bull.* **26**, 602-606.

Lohse, J., 1991. Distribution of organochlorine pollutants in North Sea sediments. *Wat. Sci. Tech.* **24**, 107-113.

Lorenzen, A. and A.B. Okey, 1990. Detection and characterization of (3H)2,3,7,8-tetrachlorodibenzo-*p*-dioxin binding to Ah receptor in a rainbow trout hepatoma cell line. *Toxicol. Appl. Pharmacol.* **106**, 53-62.

References

Luxon, P.L., P.V. Hodson and U. Borgmann, 1987. Hepatic aryl hydrocarbon hydroxylase activity of lake trout (*Salvelinus namaycush*) as an indicator of organic pollution. *Environ. Toxicol. Chem.* **6**, 649-657.

MacDonald, C. R., C.D. Metcalfe, G.C. Balch and T.L. Metcalfe, 1993. Distribution of PCB congeners in seven lake systems: Interactions between sediment and food-web transport. *Environ. Toxicol. Chem.* **12**, 1991-2003.

McCarthy, J.F. and L.R. Shugart, 1990. Biomarkers of environmental contamination. Lewis Publishers, Boca Raton, Florida.

McConnell, 1980. Acute and chronic toxicity, carcinogenesis, reproduction, teratogenesis and mutagenesis in animals. In *Halogenated Biphenyls, Terphenyls, Naphtalenes, Dibenzodioxins and Related Products*, ed. R.D. Kimbrough. Elsevier Biomedical Press, NY, pp. 109-150.

McFarland, V.A. and J.U. Clarke, 1989. Environmental occurrence, abundance, and potential toxicity of polychlorinated biphenyl congeners: Considerations for a congener-specific analysis. *Environ. Health Perspect.* **81**, 225-239.

McMaster, M. E., G.J. Van der Kraak, C.B. Portt, K.R. Munkittrick, P.K. Sibley, I.R. Smith and D.G. Dixon, 1991. Changes in hepatic mixed-function oxygenase (MFO) activity, plasma steroid levels and age at maturity of a white sucker (*Catostomus commersoni*) population exposed to bleached kraft pulp mill effluent. *Aquat. Toxicol.* **21**, 199-218.

Melancon, M.J. and J.J. Lech, 1983. Dose-effect relationship for induction of hepatic monooxygenase activity in rainbow trout and carp by Aroclor 1254. *Aquat. Toxicol.* **4**, 51-61.

Miranda, C.L., M.C. Henderson, J.-L. Wang, H.-S. Chang, J.D. Hendricks and D.R. Buhler, 1992. Differential effects of 3,4,5,3',4',5'-hexachlorobiphenyl (HCB) on interrenal steroidogenesis in male and female rainbow trout *Oncorhynchus mykiss*. *Comp. Biochem. Physiol.* **103C**, 153-157.

Monosson, E. and J.J. Stegeman, 1991. Cytochrome P450E (P450 1A) induction and inhibition in winter flounder by 3,3',4,4'-tetrachlorobiphenyl: comparison of response in fish from Georges Bank and Narragansett Bay. *Environ. Toxicol. Chem.* **10**, 765-774.

Monosson, E. and J.J. Stegeman, 1994. Induced cytochrome P4501A in winter flounder, *Pleuronectes americanus* from offshore and coastal sites. *Can J. Fish. Aquat. Sci.* **51**, 933-941.

Muir, D.C.G., R.J. Norstrom and M. Simon, 1988. Organochlorine contaminants in arctic food chains: Accumulation of specific polychlorinated biphenyls and chlordane-related compounds. *Environ. Sci. Technol.* **22**, 1071-1079.

Munkittrick, K.R., G.J. van der Kraak, M.E. McMaster, C. B. Portt, M.R. van den Heuvel and M.R. Servos, 1994. Survey of receiving-water environmental impacts associated with discharges from pulp mill. 2. Gonad size, liver size, hepatic EROD activity and plasma sex steroid levels in white sucker. *Environ. Toxicol. Chem.* **13**, 1089-1101.

Nebert, D.W., D.R. Nelson, M.J. Coon, R.W. Estabrook, R. Feyereisen, Y. Fujii-Kuriyama, F.J. Gonzalez, F.P. Guengerich, I.C. Gunsalus, E.F. Johnson, J.C. Loper, R. Sato, M.R. Waterman and D.J. Waxman, 1991. The P450 superfamily: update on new sequences, gene mapping, and recommended nomenclature. *DNA Cell Biol.* **10**, 1-14.

Nelson, D.R., T. Kamataki, D.J. Waxman, F.P. Guengerich, R.W. Estabrook, R. Feyereisen, F.J. Gonzalez, M.J. Coon, I.C. Gunsalus and O. Gotoh, 1993. The P450 superfamily - update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol.* **12**, 1-51.

Newsted, J.L., J.P. Giesy, G.T. Ankely, D.E. Tillitt, R.A. Crawford, J.W. Gooch, P.D. Jones and M.S. Denison, 1995. Development of toxic equivalency factors for PCB congeners and the assessment of TCDD and PCB mixtures in rainbow trout. *Environ. Toxicol. Chem.* **14**, 861-871.

Nieuwenhuize, J. and J.M. van Liere, 1988. PCBs in oystercatcher liver. Chrompack Application note 337-GC, Middelburg, the Netherlands, 2 pp.

Niimi, A.J. and B.G. Oliver, 1989. Assessment of relative toxicity of chlorinated dibenzo-*p*-dioxins, dibenzofurans and biphenyls in Lake Ontario salmonids to mammalian systems using toxic equivalent factors (TEF). *Chemosphere* **18**, 1413-1423.

Norstrom, R.J., M. Simon, D.C.G. Muir and R.E. Schweinsburg, 1988. Organochlorine contaminants in arctic marine food chains: Identification, geographical distribution and temporal trends in polar bears. *Environ. Sci. Technol.* **22**, 1063-1071.

Okey, A.B., D.S. Riddcik and P.A. Harper, 1994. The Ah receptor: mediator of the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds. *Toxicol. Lett.* **70**, 1-22.

Omura, T. and R. Sato, 1964. The carbon monoxide-binding pigment of liver microsomes: I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239**, 2370-2378.

Payne, J.F., L.L. Fancey, A.D. Rahimtula and E.L. Porter, 1987. Review and perspective on the use of mixed-function oxygenase enzymes in biological monitoring. *Comp. Biochem. Physiol.* **86C**, 233-245.

Peakall, D.B. and L.R. Shugart, 1993. Biomarkers: Research and application in the assessment of environmental health. Springer Verlag Berlin, Heidelberg.

Peters, L.D., C. Porte, J. Albaiges and D.R. Livingstone, 1994. 7-Ethoxyresorufin O-deethylase (EROD) and antioxidant enzyme activities in larvae of sardine (*Sardina pilchardus*) from the North coast of Spain. *Mar. Poll. Bull.* **28**, 299-304.

Poland, A. and C. Knutson, 1982. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: Examination of the mechanism of toxicity. *Annu. Rev. Pharmacol. Toxicol.* **22**, 517-554.

Rappe, C., R. Andersson, P.-A. Bergqvist, C. Brohede, M. Hansson, L.-O. Kjeller, G. Lindström, S. Marklund, M. Nygren, S.E. Swansson, M. Tysklind and K. Wiberg, 1987. Overview on environmental fate of chlorinated dioxins and dibenzofurans. Sources, levels and isomeric pattern in various matrices. *Chemosphere* **16**, 1603-1618.

Renton, K.W. and R.F. Addison, 1992. Hepatic microsomal mono-oxygenase activity and P450IA mRNA in North Sea dab *Limanda limanda* from contaminated sites. *Mar. Ecol. Prog. Ser.* **91**, 65-69.

Rijnsdorp, A. D., A.D. Vethaak and P.I. Van Leeuwen, 1992. Population biology of dab *Limanda limanda* in the southeastern North Sea. *Mar. Ecol. Prog. Ser.* **91**, 19-35.

Ritchie, W. and M. O'Sullivan, 1994. Health of wild fish: dabs. In *The environmental impact of the wreck of the Braer*, eds. W. Ritchie and M. O'Sullivan. The Scottisch Office, Edinburgh, UK, pp. 90-93.

Rutten, A.A.J.J.L., H.E. Falke, J.F. Catsburg, R. Topp, B.J. Blaauboer, I. Van Holsteijn, L. Doorn and F.X.R. van Leeuwen, 1987. Interlaboratory comparison of total cytochrome P-450 and protein determinations in rat liver microsomes. *Arch. Toxicol.* **61**, 27-33.

Safe, S., 1984. Polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs): Biochemistry, Toxicology, and Mechanism of Action. *CRC Crit. Rev. Toxicol.* **13**, 319-393.

Safe, S., 1990. Polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs) and related compounds: Environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). *Crit. Rev. Toxicol.* **31**, 51-88.

Safe, S., 1994. Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment. *CRC Crit. Rev. Toxicol.* **24**, 87-149.

Schulz, D.E., G. Petrick and J.C. Duinker, 1989. Complete characterization of polychlorinated biphenyl congeners in commercial Aroclor and Clophen mixtures by multidimensional gas chromatography-electron capture detection. *Environ. Sci. Technol.* **23**, 852-859.

Schulz-Bull, D.E., G. Petrick and J.C. Duinker, 1991. Polychlorinated biphenyls in North Sea water. *Mar. Chem.* **36**, 365-384.

Skaare, J.U., E. Gram Jensen, A. Goksøyr and E. Egaas, 1991. Response of xenobiotic metabolizing enzymes of rainbow trout (*Oncorhynchus mykiss*) to the mono-*ortho* substituted polychlorinated PCB congener 2,3',4,4',5-pentachlorobiphenyl, PCB118, detected by enzyme activities and immunochemical methods. *Arch. Environ. Contam. Toxicol.* **20**, 349-352.

Sleiderink, H.M. and J.P. Boon, 1995. Cytochrome P450 1A response in North Sea dab, *Limanda limanda*, from offshore and coastal sites. *Mar. Poll. Bull.* **30**, 660-666.

Sleiderink, H.M. and J.P. Boon, 1996. Temporal induction pattern of hepatic cytochrome P450 1A in thermally acclimated dab (*Limanda limanda*) treated with 3,3',4,4'-tetrachlorobiphenyl (CB77). *Chemosphere*, **32**, 2335-2344.

Sleiderink, H.M., J.M. Everaarts, A. Goksøyr and J.P. Boon, 1995a. Hepatic cytochrome P450 1A induction in dab (*Limanda limanda*) after oral dosing with the polychlorinated biphenyl mixture Clophen A40. *Environ. Toxicol. Chem.* **14**, 679-687.

Sleiderink, H.M., I. Oostingh, A. Goksøyr and J.P. Boon, 1995b. Sensitivity and cytochrome P450 1A induction in dab (*Limanda limanda*) of different age and sex as a biomarker for environmental contaminants in the southern North Sea. *Arch. Environ. Contam. Toxicol.* **28**, 423-430.

Sleiderink, H.M., J. Beyer, E. Scholtens, A. Goksøyr, J. Nieuwenhuize, J.M. Van Liere, J.M. Everaarts and J.P. Boon, 1995c. Influence of temperature and polyaromatic contaminants on CYP1A levels in North Sea dab (*Limanda limanda*). *Aquat. Toxicol.* **32**, 189-209.

Sleiderink, H.M., J. Beyer, J.M. Everaarts and J.P. Boon, 1995d. Influence of temperature on cytochrome P4501A in dab (*Limanda limanda*) from the southern North Sea: Results from field surveys and a laboratory study. *Mar. Environ. Res.* **39**, 67-71.

References

Smith, L.M., T.R. Schwartz, K. Feltz and T.J. Kubiak, 1990. Determination and occurrence of AHH-active polychlorinated biphenyls, 2,3,7,8-tetrachloro-*p*-dioxin and 2,3,7,8-tetrachlorodibenzofuran in Lake Michigan sediment and biota. The question of their toxicological significance. *Chemosphere* **21**, 1063-1085.

Snegaroff, J. and J. Bach, 1990. The effects of temperature on the basal activity of cytochrome P-450 in rainbow trout (*Salmo gairdneri*). *Comp. Biochem. Physiol.* **95B**, 515-519.

Sokal, R.R. and F.J. Rohlf, 1981. Biometry. W.H. Freeman and Co., San Francisco, California.

Spies, R.B. and D.W. Rice, 1988. Effects of organic contaminants on reproduction of the starry flounder *Platichthys stellatus* in San Francisco Bay. II. Reproductive success of fish captured in San Francisco Bay and spawned in the laboratory. *Mar. Biol.* **98**, 191-200.

Stegeman, J.J., 1979. Temperature influence on basal activity and induction of mixed function activity in *Fundulus heteroclitus*. *J. Fish. Res. Board Can.* **36**, 1400-1405.

Stegeman, J.J., 1989. Cytochrome P450 forms in fish: Catalytic, immunological, and sequence similarities. *Xenobiotica* **19**, 1093-1110.

Stegeman, J.J., 1992. Nomenclature for hydrocarbon inducible cytochrome P450 in fish. *Mar. Environ. Res.* **34**, 133-138.

Stegeman, J.J. and P.J. Kloepper-Sams, 1987. Cytochrome P-450 isoenzymes and monooxygenase activity in aquatic animals. *Environ. Health Perspect.* **71**, 87-95.

Stegeman, J.J. and J.J. Lech, 1991. Cytochrome P-450 monooxygenase systems in aquatic species: carcinogen metabolism and biomarkers for carcinogen and pollutant exposure. *Environ. Health Perspect.* **90**, 101-109.

Stegeman, J.J., B.R. Woodin, A.V. Klotz, R.W. Wolke and N.R. Orme-Johnson, 1982. Cytochrome P450 and monooxygenase activity in cardiac microsomes from the fish *Stenotomus chrysops*. *Mol. Pharmacol.* **21**, 517-526.

Stegeman, J.J., F.Y. Teng and E.A. Snowberger, 1987. Induced cytochrome P450 in winter flounder (*Pseudopleuronectes americanus*) from coastal Massachusetts evaluated by catalytic assay and monoclonal antibody probes. *Can. J. Fish. Aquat. Sci.* **44**, 1270-1277.

Stegeman, J.J., B.R. Woodin and A. Goksøyr, 1988. Apparent cytochrome P450 induction as an indication of exposure to environmental chemicals in the flounder *Platichthys flesus*. *Mar. Ecol. Progr. Ser.* **46**, 55-60.

Stegeman, J.J., M.R. Miller and D.E. Hinton, 1989. Cytochrome P-4501A1 induction and localization in endothelium of vertebrate (Teleost) heart. *Molec. Pharmacol.* **36**, 723-729.

Stegeman, J.J., K.W. Renton, B.R. Woodin, Y.-S. Zhang and R.F. Addison, 1990. Experimental and environmental induction of cytochrome P450E in fish from Bermuda waters. *J. Exp. Mar. Biol. Ecol.* **138**, 49-67.

Stegeman, J.J., M. Brouwer, R.T. Di Giulio, L. Förlin, B.A. Fowler, B.M. Sanders and P.A. Van Veld, 1992. Molecular responses to environmental contamination: enzyme and protein systems as indicators of chemical exposure and effect. In *Biomarkers: Biochemical, Physiological, and Histological Markers of Anthropogenic Stress*, eds. R.J. Huggett, R.A. Kimerle, P.M. Mehrle, Jr. and H.L. Bergman. Lewis Publishers, Boca Raton, Florida, pp. 235-335.

Stein, J.E., T.K. Collier, W.L. Reichert, E. Casillas, T. Hom and U. Varanasi, 1992. Bioindicators of contaminant exposure and sublethal effects: studies with benthic fish in Puget Sound, Washington. *Environ. Toxicol. Chem.* **11**, 701-714.

Tanabe, S., N. Kannan, A. Subramanian, S. Watanabe, M. Ono and R. Tatsukawa, 1987a. Occurrence and distribution of toxic coplanar PCBs in biota. *Chemosphere* **16**, 1965-1970.

Tanabe, S., N. Kannan, A. Subramanian, S. Watanabe and R. Taksukawa, 1987b. Highly toxic coplanar PCBs: Occurrence, source, persistency and toxic implications to wildlife and humans. *Environ. Poll.* **47**, 147-163.

Tanabe, S., N. Kannan, M. Ono and R. Tatsukawa, 1989. Toxic threat to marine mammals: increasing toxic potential of non-*ortho* coplanar PCBs form land to ocean. *Chemosphere* **18**, 485-490.

Thomas, P., 1989. Effects of Aroclor 1254 and cadmium on reproductive endocrine function and ovarian growth in Atlantic Croaker. *Mar. Environ. Res.* **28**, 499-503.

Tysklind, M., A.T.C. Bosveld, P. Andersson, E. Verhallen, T. Sinnige, W. Seinen, C. Rappe and M. van den Berg, 1995. Phorphyrin accumulation by PCBs and TCDD downregulates CYP1A1 activity in chicken hepatocytes. *Environ. Sci. Poll. Res.*, submitted.

Van den Meent, D., H.A. Den Hollander, W.G. Pool, M.J. Vredenbregt, H.A.M. Van Oers, E. De Greef and J.A. Luiten, 1986. Organic micropollutants in Dutch coastal waters. *Water Sci. Technol.* **18**, 73-81.

Van der Oost. R., H. Heida, A. Opperhuizen and N.P.E. Vermeulen, 1991. Interrelationhips between bioaccumulation of organic trace pollutants (PCBs, organochlorine pesticides and PAHs), and MFO-induction in fish. *Comp. Biochem. Physiol.* **100**C, 43-47.

Van der Weiden, M.E.J., J. van der Kolk, R. Bleumink, W. Seinen and M. van den Berg, 1992. Concurrence of P450 1A induction and toxic effects in the rainbow trout (*Oncorhynchus mykiss*) after administration of a low dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Aquat. Toxicol.* **24**, 123-142.

Van der Weiden, M.E.J., M. Celander, W. Seinen, M. Van den Berg, A. Goksøyr and L. Förlin, 1993. Induction of cytochrome P450 1A in fish treated with 2,3,7,8-tetrachorodibenzo-*p*-dioxin, TCDD, or chemically contaminated sediment. *Environ. Toxicol. Chem.* **12**, 989-999.

Van der Weiden, M.E.J., L.P. de Vries, K. Fase, M. Celander, W. Seinen and M. van den Berg, 1994a. Relative potencies of polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs) and biphenyls (PCBs), for cytochrome P450 1A induction in the mirror carp (*Cyprinus carpio*). *Aquat. Toxicol.* **29**, 163-182.

Van der Weiden, M.E.J., R. Bleumink, W. Seinen and M. van den Berg, 1994b. Concurrence of P450 1A induction and toxic effects in the mirror carp (*Cyprinus carpio*), after administration of a low dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Aquat. Toxicol.* **29**, 147-162.

Van Veld, P. A., D. J. Westbrook, B. R. Woodin, R. C. Hale, C. L. Smith, R. J. Huggett and J. J. Stegeman, 1990. Induced cytochrome P-450 in intestine and liver of spot (*Leiostomus xanthurus*) from a polycyclic aromatic hydrocarbon contaminated environment. *Aquat. Toxicol.* **17**, 119-132.

Varanasi, U., J.E. Stein, M. Nishimoto, W.L. Reichert and T.K. Collier, 1987. Chemical carcinogenesis in feral fish: uptake, activation, and detoxication of organic xenobiotics. *Environ. Health Perspect.* **71**, 155-170.

Varanasi, U., J.E. Stein and M. Nishimoto, 1989. Biotransformation and disposition of polycyclic aromatic hydrocarbons (PAH) by fish. In *Metabolism of polycyclic aromatic hydrocarbons in the aquatic environment*, ed. U. Varanasi. CRC Press, Inc., Boca Raton, Florida, pp. 93-149.

Vindimian, E., P. Namour, B. Migeon and J. Garric, 1991. In situ pollution induced cytochrome P450 activity of freshwater fish: barbel (*Barbus barbus*), chub (*Leuciscus cephalus*) and nase (*Chondrostoma nasus*). *Aquat. Toxicol.* **21**, 255-266.

Von Westernhagen, H., V. Dethlefsen, P. Cameron and D. Janssen, 1987. Chlorinated hydrocarbon residues in gonads of marine fish and effects on reproduction. *Sarsia* **72**, 419-422.

Walker, C.H., 1990. Review: Persistent pollutants in fish-eating sea birds-bioaccumulation, metabolism and effects. *Aquat. Toxicol.* **17**, 293-324.

Walker, M.K. and R.E. Peterson, 1991. Potencies of polychlorinated dibenzo-*p*-dioxin, dibenzofuran, and biphenyl congeners, relative to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, for producing early life stage mortality in rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.* **21**, 219-238.

Wirgin, I.I., G.-L. Kreamer, C. Grunwald, K. Squibb, S.J. Garte and S. Courtenay, 1992. Effects of prior exposure history on cytochrome P4501A mRNA induction by PCB congener 77 in atlantic tomcod. *Mar. Environ. Res.* **34**, 103-108.

Samenvatting en conclusies

De doelstelling van dit proefschrift was na te gaan of de CYP1A meting in schar uit de Noordzee als een biologische indicator voor de blootstelling aan PCBs en verwante stoffen in monitoringsprogramma's gebruikt kan worden. Alleen vanwege zijn algemene voorkomen in de Noordzee speelt de schar al een belangrijke rol in vervuilingsmonitoringsprogramma's. Tot nu toe is echter niet vastgesteld of de soort hier wel geschikt voor is. Bij de veldstudies en laboratoriumexperimenten lag de nadruk op PCBs, omdat deze tot de belangrijkste groep stoffen in de Noordzee behoren die CYP1A kunnen induceren.

In hoofdstuk 2 werden in een veldstudie de effecten van maturatie en geslacht op CYP1A bestudeerd. Op verschillende lokaties in de Noordzee, met variaties in PCB vervuiling, werden volwassen en juveniele scharren van beide geslachten verzameld. In alle gevallen waren de PCB concentraties in spierweefsel van deze vissen het hoogste langs de Nederlandse kust. In de herfst werden hier ook de hoogste CYP1A niveaus (gemeten als EROD activiteit en CYP1A eiwit), bij zowel volwassen mannetjes als vrouwtjes gevonden. Hetzelfde werd tijdens de paaitijd in de winter gemeten voor juveniele vrouwtjes en volwassen mannetjes. Paairijpe vrouwtjes vertoonden tijdens dit seizoen lagere CYP1A niveaus vergeleken met volwassen mannetjes en juveniele vissen. Zowel in de herfst als de winter, correleerden de PCB concentraties in spierweefsel en de beide biochemische parameters significant bij volwassen mannnetjes. Voor de andere scharren was deze correlatie niet significant. Er werd dan ook geconcludeerd dat de gevoeligheid van CYP1A voor PCBs en verwante stoffen het grootste was als gebruik werd gemaakt van volwassen mannetjes.

In hoofdstuk 3 werd aangetoond dat temperatuurverschillen van het bodemwater, die gedurende het voorjaar en de zomer kunnen voorkomen tussen gestratificeerde en verticaal gemengde watermassa's, een grote invloed hebben op CYP1A in de schar. In volwassen mannetjes die in de open Noordzee op lokaties met lage bodemwatertemperaturen werden gevangen, werden verhoogde CYP1A niveaus gemeten, terwijl op lokaties met verticaal gemengde watermassa's en kustlokaties aanzienlijk lagere CYP1A niveaus werden gemeten. De watertemperatuur bleek negatief met de CYP1A niveaus te correleren, terwijl de PCB concentraties juist een positieve correlatie met de CYP1A niveaus vertoonden. Het effect van de watertemperatuur bleek echter veel sterker dan het effect van PCBs. In een

laboratoriumexperiment werd bovendien bevestigd dat de watertemperatuur negatief met de EROD activiteit is gecorreleerd. Bovendien bleek uit de veldstudie dat, gedurende het voorjaar en de zomer, verschillen in voedingsgewoonten van de schar een sterker effect op CYP1A hebben dan verschillen in PCB concentraties.

Besloten werd dat het voorjaar en de zomer minder geschikte seizoenen zijn om te monitoren vanwege het temperatuureffect. De winter is geen gunstig seizoen omdat de schar dan, gedurende de paaitijd, veel meer trekt. Daarom is onderzocht of de herfst het meest geschikte seizoen om te monitoren is. In hoofdstuk 4 wordt een veldstudie beschreven waarbij verschillen in natuurlijke factoren die CYP1A beïnvloeden, zoals de watertemperatuur, de conditiefactor van de vis en het vetpercentage in het spierweefsel, minimaal waren. De CYP1A niveaus in mannetjes die langs de Nederlandse kust werden gevangen waren aanzienlijk hoger dan in mannetjes die in de open Noordzee werden gevangen, hetgeen samenging met hogere PCB concentraties langs de kust. Samenvattend kan dan ook geconcludeerd worden dat de herfst inderdaad het beste seizoen is om de CYP1A meting toe te passen, omdat de invloed van storende factoren dan minimaal is.

Het vermogen van PCBs om CYP1A in scharren te induceren is in verschillende laboratoriumexperimenten onderzocht. In hoofdstuk 5 wordt een experiment beschreven waarbij volwassen vrouwtjes om de zes weken 1 mg van het technische PCB mengsel Clophen A40 toegediend kregen. Er werden maximaal drie doses per schar toegediend. De blootstelling aan Clophen A40 leidde tot inductie van CYP1A. De EROD activiteit, het CYP1A eiwit en het totale CYP gehalte waren in de gedoseerde groepen hoger dan in de controle groepen. Het maximum werd al na 1 dosis bereikt, alhoewel de PCB concentraties in het spierweefsel na de tweede en derde dosis nog verder toenamen. Het CYP1A systeem van de schar blijkt dus gevoelig voor PCBs.

Aangezien PCB congeneren verschillen in hun vermogen CYP1A te induceren, werden de biochemische effecten van verschillende non- en mono-*ortho* gesubstitueerde CB congeren onderzocht. Blootstelling aan de non-*ortho* gechloreerde CB77 leidde to inductie van CYP1A (hoofstuk 6). Blootstelling aan de non-*ortho* gechloreerde CB126 en drie verschillende mono-*ortho* gechloreerde CB congeneren leidde echter niet tot significante inductie van CYP1A (hoofdstuk 7). Voor CB126 staat dit in scherp contrast met voorgaande studies waarbij deze congeneer juist wel voor een sterke inductie van CYP1A zorgde; een grote variatie tussen verschillende individuen van de referentiegroep werd verantwoordelijk voor dit effect gehouden. Blootstelling aan CB126 zorgde zelfs voor een dosis-afhankelijke remming van de EROD activiteit bij de hoogste concentraties.

In hoofdstuk 6 werd de invloed van de temperatuur op de inductie van CYP1A in de tijd bestudeerd. Volwassen mannetjes kregen op dag 0 een enkele dosis van CB77 toegediend, waarna twee groepen vissen gedurende 40 dagen op verschillende temperaturen (10 and 16°C) werden gehouden. Zowel het CYP1A eiwit als de EROD activiteit waren op de veertigste dag na de blootstelling verhoogd. De maximale respons van het CYP1A eiwit en de EROD activiteit werd voor schar die bij 16°C werd gehouden al na 5 dagen gemeten. Voor schar die bij 10°C werd gehouden werd de maximale respons na 40 dagen gemeten. Na 40 dagen waren deze parameters in beide blootgestelde groepen even hoog. De EROD activiteit en de CYP1A eiwit niveaus waren bij de controlegroepen het hoogste in schar die bij 10°C werd gehouden, hetgeen in overeenstemming is met de studie die in hoofdstuk 3 werd beschreven. Er werd dan ook geconcludeerd dat de mate van inductie het grootste was bij vissen die bij 16°C werden gehouden.

CYP1A is een blootstellingsbiomarker en geeft derhalve geen informatie over negatieve (toxische) effecten. De vraag kan dan ook gesteld worden of enzyminductie schadelijke effecten op de schar weergeeft. In het experiment waarbij scharren aan Clophen A40 werden blootgesteld (hoofdstuk 5), werden geen effecten op reproductieparameters waargenomen (Fonds *et al.*, 1995). Het is dan ook zeer waarschijnlijk dat de contaminantconcentraties die nodig zijn om effecten op deze biologische niveaus te bewerkstelligen, zelden worden bereikt in het milieu.

Uit het hier beschreven onderzoek is duidelijk geworden dat de schar een geschikte vissoort is om te gebruiken in monitoringsprogramma's, waarbij CYP1A in de lever als een biologische indicator voor de blootstelling aan PCBs en verwante stoffen toegepast kan worden. Schar is gevoelig voor PCBs. Als de CYP1A meting wordt toepast moet er echter rekening worden gehouden met verschillende factoren die CYP1A beinvloeden, zoals water temperatuur, mate van maturatie en geslacht van de vis. Monitoren in de herfst met volwassen mannetjes heeft de voorkeur. De CYP1A meting kan goed dienst doen als een snelle en goedkope screening methode om de vervuiling van het aquatische milieu vast te stellen. Het gezamenlijke effect van een groep stoffen, de PHAHs, op CYP1A kan ermee worden bepaald. Alhoewel er genoeg bewijs is dat er dosis-respons relaties tussen deze stoffen en CYP1A bestaan, worden er in het milieu niet altijd lineaire verbanden gevonden. De PAK concentraties in de Noordzee zijn bijvoorbeeld te laag om CYP1A in de schar te induceren. Als gevolg daarvan kan de CYP1A meting de chemische analyse nooit volledig vervangen, maar hij kan wel informatie over stoffen die CYP1A induceren geven. De CYP1A meting kan worden

toegepast om estuaria en/of kustwateren van de open Noordzee te onderscheiden. Een toename van PHAHs in het mariene milieu zou met deze meting kunnen worden ontdekt.

Curriculum vitae

Hedwig Sleiderink werd op 5 februari 1966 geboren in Zutphen. In 1984 behaalde zij het Atheneum B diploma aan de Thomas a Kempis scholengemeenschap in Zwolle. In hetzelfde jaar begon zij de studie moleculaire wetenschappen aan de Landbouwuniversiteit Wageningen. Zij koos voor de afstudeervakken biokatalyse bij de vakgroep organische chemie en microbiële genetica bij de vakgroep microbiologie. De stageperiode werd volbracht bij de groep organische chemie van het Unilever Research Laboratorium in Vlaardingen. In maart 1990 behaalde zij het doctoraaldiploma moleculaire wetenschappen, chemische en biologisch-chemische oriëntaties.

Van september 1990 tot maart 1995 werkte zij als onderzoeker in opleiding bij de groep organische microverontreiningingen van de afdelingen chemische oceanografie & zeevervuiling en beleidsgericht onderzoek (thans de groep toxicologie van de afdeling mariene biogeochemie en toxicologie) van het Nederlands Instituut voor Onderzoek der Zee. Het resultaat staat in dit proefschrift beschreven. Daarnaast volgde zij in deze periode de postdoctorale opleiding toxicologie.

Vanaf april 1995 is zij nog enige tijd aan de afdeling verbonden geweest als projectonderzoeker.

Dankwoord

Iedereen die de afgelopen jaren voor langere of kortere tijd bij mij is geweest, bij wie ik gelogeerd heb, waarmee ik heb gevaren en zeeziek ben geworden, toneel heb gespeeld, chocola heb gegeten, heb gekleid, in bus, trein, veerboot of vliegtuig heb gereisd, koffie heb gedronken, gediscussieerd, gelachen, gekibbeld, huizen gesloopt en weer opgebouwd, gesnorkeld en gedoken, gehuild, risk gespeeld, congressen bezocht, eindeloos getelefoneerd, schar heb gevangen, over het strand gelopen, film heb gekeken, geborreld en in kroegen heb gezeten wil ik van harte bedanken voor zijn of haar bijdrage bij het tot stand komen van dit proefschrift. Bovenal wil ik de volgende mensen bedanken:

Jan Boon heeft op heldhaftige wijze het project begeleid; hij heeft steeds trouw en razendsnel 1000 en 1 versies van mijn manuscripten van commentaar voorzien. Het was een waar genoegen! *Jan Koeman* wil ik bedanken voor de bereidheid als promotor op te treden. De hulp en constructieve kritiek tijdens de eindfase heb ik als inspirerend ervaren. Mijn afdelingsgenoten *Jan Everaarts*, *Kees Booij*, *Theo Hillebrand*, *Evaline van Weerlee* en natuurlijk mijn kamergenoot *Berend Mensink* wil ik bedanken voor hun hulp, gezelligheid en interesse tijdens het geploeter (analyses waar geen eind aan leek te komen), het drinken van honderden kopjes koffie en het nuttigen van (vooral) Hema-taart, hoestsnoepjes en Ricola-bonbons.

Harry Besselink, *Martin Eggens*, *Martine van der Weiden* en *Ron van der Oost* wil ik bedanken voor de stimulerende 'P4501A' discussies. De inzet van *Enit Scholtens* tijdens haar stageperiode werd zeer gewaardeerd. I wish to thank *Anders Goksøyr*, *Jonny Beyer* and *Kjersti Helgesen* for their help and hospitality during those four weeks in Bergen (including the Julefest, which I will never forget). It was great!

De bemanningen van de schepen *Aurelia* en *Pelagia* wil ik graag bedanken voor de inzet bij het vangen van honderden scharren. *Mark Fonds* gaf mij raad en hulp bij het in leven houden van deze vissoort. *Peter van der Puyl*, *Hans Kralt* en *Coen Fischer* voerden de vissen als ik op cursus was. *Jaap van der Meer* wil ik bedanken voor zijn adviezen over statistiek. *Henk Hobbelink* heeft me geholpen met de omslag. Verder zijn er natuurlijk de medewerkers

van de bibliotheek, de repro, de administratie, het magazijn, de werkplaatsen en de kantine. Bedankt voor al die copyproofs, pipetpunten, overdrukjes, tosties en kopjes koffie!

Taco de Bruin wil ik graag bedanken voor het uitlenen van zijn Mac en zijn hulp bij talloze Macprobleempjes. Naast al die andere collega-OIO's, wil ik *Corina Brussaard* bedanken voor de vriendschap, de koekjes en de gedeelde smart.

Ineke Keet was gedurende de gehele promotietijd een ongelooflijke steun. Ik wil haar graag bedanken voor haar wijsheid en geduld om m'n zoektocht in positieve banen te leiden. Mijn ouders ben ik erkentelijk voor hun ondersteuning en voortdurende belangstelling. En tot slot een olijke knipoog naar *Ramses* en *Yenthe*. Het is gelukt!

Hedwig Sleiderink
23 juli 1996