Time- and dose-dependent biomarker responses in flounder (*Platichthys flesus* L.) exposed to benzo[*a*]pyrene, 2,3,3′,4,4′, 5-hexachlorobiphenyl (PCB-156) and cadmium

Jonny Beyer, Morten Sandvik, Janneche U. Skåre, Eliann Egaas, Ketil Hylland, Rune Waagbø and Anders Goksøyr

Responses in flounder (*Platichthys flesus*) towards benzo [*a*]pyrene (B*a*P), 2,3,3′,4,4′,5-hexachlorobiphenyl (PCB-156), and cadmium (Cd) were investigated in time-course and dose–response studies of selected biomarkers. Measurements of biliary fluorescent B*a*P metabolites and hepatic concentrations of PCB-156 and cadmium showed that the injected toxicants were rapidly mobilized from the muscle to the liver, but a depot effect was indicated in the highest dose groups of B*a*P and PCB-156 (12 mg kg^{-1} bodyweight). Clearest biomarker responses were found in the induction of hepatic cytochrome P450 1A (CYP1A) enzymes as a response towards B*a*P and PCB-156 exposure. Maximum induction of CYP1A dependent 7-ethoxyresorufin-O-deethylase (EROD) activity was observed after 2 and 8 days in B*a*P and PCB-156-treated flounder, respectively. Positive dose–effect relationships were observed towards both compounds, but the CYP1A induction was more persistent with PCB exposure than with B*a*P exposure. In Cd-exposed fish, the hepatic level of metallothionein responded more slowly with highest levels observed after 16 days in the time-study. In the combined B*a*P + Cd treatment, the CYP1A induction was only slightly suppressed. Aspartate aminotransferase in serum appeared to be responsive towards B*a*P, but also towards the acetone vehicle in controls in the first part of the exposure period. Hematocrit as well as hepatic activities of aldrin epoxidase, glutathione S-transferase, and UDP-glucuronyl transferase were not responsive to any treatment in the present study. In general, the results demonstrate that selected biomarkers in flounder are responsive to PAH, PCB, and heavy metal pollutant exposure, indicating the applicability of this species in future environmental pollution monitoring programmes.

Keywords: *fish, biomarkers, PAH, PCB, heavy metals.*

Jonny Beyer *and* **Anders Goksøyr** *(author for correspondence) are at the Laboratory of Marine Molecular Biology, University of Bergen, N-5020 Bergen, Norway;* **Morten Sandvik, Janneche U. Skåre, Eliann Egaas** *are at the National Veterinary Institute, N-0033 Oslo, Norway;* **Ketil Hylland** *is at the Norwegian Institute for Water Research, N-0411 Oslo, Norway; and* **Rune Waagbø** *is at the Institute of Nutrition, Ministry of Fisheries, N-5002 Bergen, Norway.*

Introduction

The introduction of various pollution response indicators, or biomarkers, has led to a considerable interest in using teleosts in monitoring aquatic pollution, for reviews see, for example, Stegeman *et al.* (1992) and Goksøyr (1995). However, considerable variation may occur between different fish species both in terms of basal physiological features, but also in response patterns and sensitivity towards chemicals and toxicants. Time- and dose-effects of pollutants on biomarkers should therefore be studied in candidate indicator species. This is important not only for the understanding of pollutant effects observed in the field, but also for the implementation and future use of certain teleost biomarkers as tools in environmental monitoring programmes.

The flounder (*Platichthys flesus*) is common in European estuaries and coastal habitats. Juvenile individuals may also be found in rivers. Flounder typically occur in sediments, where they feed mostly on benthic invertebrates. In studies with feral flounder, environmental pollution exposure has been linked to harmful effects and to certain biomarker responses (von Westernhagen *et al.* 1981, Addison and Edwards 1988, Stegeman *et al.* 1988, Vethaak 1993). Similar links have also been recorded in laboratory studies (Lemaire *et al.* 1994, Eggens and Boon 1996), and also in flounder exposed *in situ* to polyaromatic hydrocarbons (PAHs) by transplant caging (Beyer *et al.* 1996).

The objective of the present study was to provide a basis for better understanding of time- and dose-dependent relationships of pollutant-induced biomarker responses in the flounder. Model pollutants (Figure 1) were administered by intramuscular (i.m.) injections, representing PAHs (benzo[*a*]pyrene, B*a*P), polychlorinated biphenyls (2,3,3′,4,4′,5-hexachlorobiphenyl, PCB-156) and heavy metals (cadmium, Cd). A combined B*a*P + Cd treatment was included as a model of mixed PAH and heavy metal exposure. A suite of biomarkers was measured in the flounder to study the responses in the first weeks after the toxicant administrations. Several biological and physiological markers were measured to monitor the biological status of the flounder during treatment.

MATERIALS AND METHODS

Chemicals

7-Ethoxyresorufin, resorufin and benzo[*a*]pyrene (min. 98%) were purchased from Sigma. PCB-156 was kindly provided by Åke Bergman, Wallenberg Laboratory, University of Stockholm, Sweden. $CdCl_2$ was obtained from May and Baker Ltd, Dagenham, UK. All other chemicals used in preparation and analyses of samples were of analytical grade.

Fish and treatment

The flounder used in the present investigation were collected near the shore in the area of Dale and Tellnes at Sotra, west of Bergen, Norway. Generally, the specimens used were within the range 150–350 g (Table 1). The studies were performed in 1993 at the High-Technology Center in Bergen, in the facilities of the Industrial Laboratory (I-Lab), in June (time-study) and October–November (dose-study), respectively. The flounder were acclimated for 3 weeks prior to the experiments, and fed each second day with frozen krill (time study) or flatfish

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pellet (dose-study) obtained from Norsk Medicinal Union and Felleskjøpet, respectively. The feeding was stopped 3 days before injections, and the flounder were fasted through the experimental period.

Each flounder was injected in the axial muscle on the dark side (Figure 2). BaP and PCB were dissolved in acetone, whereas $CdCl_2$ was dissolved in 9‰ saline. In the time-study, the flounder were injected either with BaP (1 mg kg^{-1} body weight), PCB-156 (2.5 mg kg^{-1} b.w.), or cadmium (1 mg kg^{-1} b.w.), or with BaP and Cd in combination (1 + 1 mg kg^{-1} b.w.). The injection volume was 1 ml kg^{-1} b.w. (1 + 1 ml kg^{-1} b.w. in the BaP + Cd treatment). In the dose-study, the flounder were injected either with BaP or PCB-156 in five different doses (0.02, 0.1, 0.5, 2.5, and 12.5 mg kg^{-1} b.w.). The injection volume was 0.5 ml kg^{-1} kg b.w. After injection, the groups were kept separately in 500 l tanks. The flow rate per tank was a minimum 5 l min^{-1}. The water temperature and salinity during the time-study and dose-study was 8.5 ± 0.2 °C, 34 ± 0.5 ‰ S and 9.4 ± 0.4 °C, 26 ± 2‰ S, respectively. Throughout the dose-study, the salinity was maintained at the low level because of some fin erosion observed during the first part of the acclimation. Each fish tank contained a layer of clean shell sand which allowed the flounder to seek cover.

Samples and preparations

In both experiments, six fish were sampled per treatment per day. Pre-treatment samples (six untreated fish) were also taken. Fish in the time–response study were sampled at days 2, 4, 8, and 16. In the dose–response study, fish were sampled at selected days based on the experience from the time-study; BaP-treated fish were sampled at days 2 and 30, whereas PCB-treated fish were sampled at days 8 and 30. A total number of 294 flounder were sampled altogether.

At sampling the fish were weighed, length-measured and subjected to external pathological examinations. Blood was drained from the caudal vein by means of a syringe. The haematocrit value (mean of three subsamples) of heparinized blood was obtained within 2 h by a 5 min × 12600 *g* centrifugation. Unheparinized blood was placed overnight in a cooler (4 °C). Serum was prepared from the coagulate by a 10 min × 800 *g* centrifugation, and stored at –80 °C until analysed. The fish was then killed and opened by cutting the spine from the dorsal side with a pair of scissors, and the visceral samples (bile, liver, spleen and gonads) were obtained. The gall bladder was carefully excised and the bile was frozen at –20 °C. The liver was split into three parts; for chemical analyses (frozen at –20 °C), preservation in liquid nitrogen, and further preparation, respectively. The latter part was homogenized in four volumes of ice-cold 0.1 M Na-phosphate buffer (pH 7.4) with 0.15 M KCL, 1 mM EDTA and 1 mM DTT, by means of a Potter-Elvehjem Teflon®- glass homogenizer. The post-mitochondrial supernatant (PMS) was obtained by a 12000 × *g* for 15 min centrifugation, and further subjected to a 100 000 × *g* for 60 min centrifugation for preparation of microsomes and cytosol fractions. The microsomal pellet was resuspended by a mild homogenization in one volume ice-cold 0.1 M Na-phosphate buffer (pH 7.4) with 20 % glycerol (v/v), 1 mM EDTA and 1 mM DTT. The microsomal samples (and cytosol fractions) were frozen in liquid nitrogen and stored at –80 °C. Liver samples in liquid nitrogen were used for metallothionein (MT) measurements. The samples were later homogenized in three volumes (w/v) of ice cold buffer (100 mM Tris–HCl, pH 8.1 with 5 mM 2-mercaptoethanol). The homogenate was centrifuged at 4 °C at 10 000 × *g* for 30 min, and the PMS was frozen and stored at –70 °C.

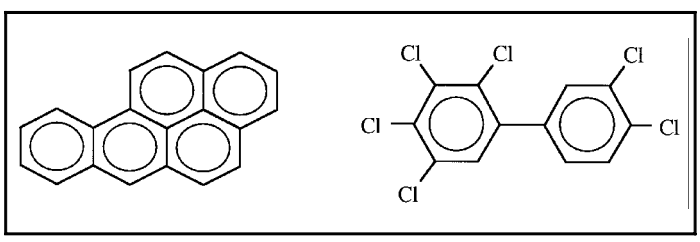

Figure 1. Structural formulae of benzo[*a*]pyrene (BaP) and 2,3,3′,4,4′, 5-hexachlorobiphenyl (IUPAC no. PCB-156), organic toxicants used in the present study.

Sample	Time–response study (*n* = 150) Mean ± SD	Dose–response study (*n* = 144) Mean ± SD
Weight (g)	265 ± 89	268 ± 91
Length (cm)	29 ± 3	28 ± 3
Condition-factor (Fultons)	1.06 ± 0.13	1.21 ± 0.15
Liver somatic index (%)	1.33 ± 0.52	1.73 ± 0.66
Spleen SI (%)	0.09 ± 0.04	0.11 ± 0.05
Gonad SI, males (%)	0.18 ± 0.10	2.00 ± 1.51
Gonad SI, females (%)	1.47 ± 0.43	2.11 ± 1.09
Haematocrit (%)	26 ± 5	23 ± 4
CDNB nmol min^{-1} mg^{-1} protein LC	1454 ± 587	948 ± 264
AE nmol min^{-1} mg^{-1} protein LM	17 ± 7	nm
UGT nmol min^{-1} mg^{-1} protein LM	2.33 ± 0.91	nm
Serum glucose (mM)	0.66 ± 0.44	0.97 ± 0.45
Serum total protein (g l^{-1})	25 ± 6	33 ± 8
Biliary protein (mg ml^{-1})	0.82 ± 0.67	0.75 ± 0.69
Hepatic fat (%)	4.49 ± 1.49	5.44 ± 3.42

Table 1. Overview of biological and physiological markers in the present study and values of biomarkers showing no responses towards any treatments with beno[*a*]pyrene, PCB-156, or cadmium.

Key: SI = somatic index, nm = not measured, LC = liver cytosol, LM = liver microsomes.

Analyses

Unless other methods are noted, all protein analyses were performed according to Bradford (1976) with bovine serum albumin as standard.

Fluorescent metabolites of benzo[*a*]pyrene (BaP-FACs) were measured according to Krahn *et al.* (1980, 1986), and Ariese *et al.* (1991), with modifications as described by Beyer *et al.* (1996). Bile samples were diluted (1:800) in 48 ‰ ethanol, and the fluorescence was measured at 379 nm/425 nm. The biliary BaP-FAC concentration was calculated as follows: BaP-FACs (μg ml^{-1}) = (fluorescence signal × bile-dilution)/2037. The equation was based on a standard curve of biliary BaP-FACs quantitated by HPLC at the NOAA/ECD laboratory in Seattle, US (E. Aas, unpublished data).

Hepatic fat and PCB-156 were measured in the PCB-exposed fish according to Brevik (1978), with modifications as described by Bernhoft and Skaare (1994). PCB-112 was used as internal standard, and the detection limit was 0.005 μg

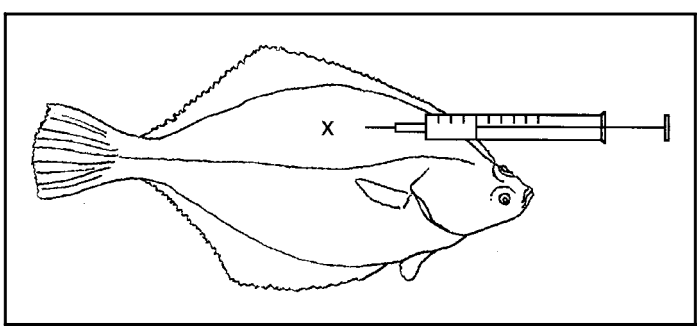

Figure 2. Illustration of the administration procedure used in the present study. The agents, dissolved in either acetone (BaP and PCB-156 treatments) or 9 ‰ saline (Cd treatment), were administered to the flounder by an intramuscular (i.m.) injection into the dorsal part of the right epi-axial muscle.

CB-156 per g (w.w.). The analytical laboratory (at the National Veterinary Institute, Oslo, Norway) successfully in all four phases of the ICES/IOC/OSPARCOM intercomparison exercise on the analyses of PCBs in samples of marine origin.

Liver samples for metal analyses were digested in concentrated nitric acid (Merck suprapure) at 165 °C for 8 h, H_2O_2 subsequently added, and further digested at 140 °C for 2 h, according to the B. Welz protocol of atomic absorption spectroscopy (1985), (ISBN 0-89573-418-4). Cd was measured by graphite furnace atomization (VarianSpectrAA 400), whereas Cu and Zn were measured by flame atomic absorption (Varian SpectrAA 30). The detection limits were 0.01 $\mu g\ g^{-1}$ for Cd, and 0.5 $\mu g\ g^{-1}$ for Cu and Zn. A control system with regular analyses of reference materials was adopted, and all measurements of these samples (NRCC Dogfish DOLT-1 and DORM-1) were close to the certified values.

All analyses of serum constituents were performed according to standardized Technicon protocols at the Institute of Nutrition, Directorate of Fisheries, Bergen using a Technicon RA-1000 analyser (Technicon Instruments Corporation, Tarrytown, NY). The measurements included levels of glucose (Technicon method no. SM4-0143K86), total protein (SM4-0147J84) and aspartate aminotransferase (SM4-0137G85).

CYP1A was measured in hepatic microsomes. The EROD (7-ethoxyresorufin *O*-deethylase) activity was measured as described previously (Burke and Mayer 1974, Stagg and Addison 1995), on a Perkin-Elmer LS-5 spectrofluorometer, with resorufin (Sigma) as internal standard. The assay temperature was 20 °C, with assay pH 7.8 (time-study) or pH 7.4 (dose-study). The pH change was due to an assay-optimization performed between the two studies, but both of these values record similar relative induction levels (fold induction in exposed fish as compared with controls). The EROD activity at pH 7.8 was approx. 70% of the activity at pH 7.4. CYP1A protein levels were measured by using a CYP1A enzyme-linked immunosorbent assay (CYP1A-ELISA) with rabbit-anti cod CYP1A IgG antiserum according to Goksøyr (1991). For both EROD and ELISA analyses, a quality control system was adopted where every sample series included reference samples from untreated and β-naphthoflavone (BNF)-treated cod.

Hepatic cytosolic GST activity towards CDNB (1-chloro-2,4-dinitro-benzene) was measured according to Habig *et al.* (1974). The assay pH was 7.5, and the incubation temperature was 25 °C, as described earlier by Egaas *et al.* (1993).

Hepatic microsomal aldrin epoxidase (AE) activity was measured as described by Moldenke and Terriere (1981), but with some adjustments. A 0.2 M K-phosphate assay buffer (pH 6.7) contained 1 mM EDTA and 0.2 mmol NADPH. After 15 min incubation at 22 °C the reaction was terminated by adding cyclohexane. The dieldrin levels were measured as described by Egaas *et al.* (1988).

Hepatic microsomal uridine-diphosphate-glucuronosyl transferase (UDP-GT) activity towards *p*-nitrophenol was determined as described by Koivusaari (1983), but with some modifications as described by Anderson *et al.* (1985).

Hepatic metallothionein (MT) was determined using an indirect non-competitive ELISA with polyclonal antiserum raised against perch MT (Hogstrand and Haux 1990, Hylland *et al.* 1994). Partly purified MT from flounder was used as standards in the ELISA. The MT concentration in the standards was quantified by measuring the content of cysteine.

Statistical evaluations

All statistical tests were performed with the use of JMP® software (version 3.1.1) from the SAS institute (1994). Parametric tests were preceded by tests for the normal distribution within each sample of values (per sampling day), and for homogeneity of variance (across treatments and days). Non-normal or non-homogeneous data were subjected to appropriate transformations (log, square-root, or Box-Cox).

The data were tested day-wise by one-way analysis of variance (ANOVA) with 'treatment' as independent variable. Whenever a significant treatment effect was indicated, Dunnett's test for multiple comparisons of means was used to test for differences between exposed and control groups. Multiple linear regression analyses were performed in the dose-study to test for effects on biomarker responses of the contaminant factors (B*a*P-FACs, hepatic PCB-156) and also of relevant biological factors (sex, fish size, condition-factor, serum protein). Only toxicant-treated fish were included (day-wise) in the multiple regression models.

Results

General observations

Before treatment flounder were selected randomly from the main fish pool. Hence, within both studies, the variance of the size (and condition) was more or less similar across treatments and days, but the flounder in the dose-study showed somewhat higher condition-factor, liver somatic index, and gonad somatic index than the flounder in the time-study (Table 1). Apparently, this was caused by seasonal differences.

In all fish that received acetone, a dark spot appeared on the skin in the injection area immediately after the treatment. The spot was present through the whole experiment, though diminishing with time. The saline and saline + Cd fish did not display spots, thus the coloration seems to be a response to the acetone vehicle.

The survival rate in the time– and dose–response studies were 96.1% and 98.3%, respectively. From a total of 354 fish treated in the two experiments, only 10 fish died, and no group trend was observed for this mortality.

Toxico-kinetics of chemicals

After the injection of toxicants in the time–response study, the levels of biliary B*a*P-FACs, hepatic PCB-156, and hepatic Cd increased rapidly up to plateau-like levels (Figure 3). At day 2, the hepatic PCB-156 level was already about 2000-fold higher than the levels measured in three controls (shown as day 0 level in Figure 3(B). Hence, further PCB analyses in controls were not performed. In the B*a*P + Cd group, the accumulation patterns of the two chemicals did not differ significantly from those of the chemicals given alone.

As judged from the dose-study, the levels of B*a*P-FACs and hepatic PCB-156 appeared in general to be linearly correlated to the doses injected, but a delay, most probably a depot effect, appeared to be caused by the highest doses of both B*a*P and PCB. Particularly at the first sampling point, this effect gave lower levels than should be expected from the relationship observed in the lower dose groups (Figure 4).

In the time-study, the hepatic levels of Zn appeared generally to be lower in the two Cd-exposed groups than in the saline controls, although this decrease not proved to be statistically significant when day-wise tested (not shown). A similar effect was not recorded for the hepatic Cu levels. This may indicate that the Cd treatment to some extent impaired the Zn homeostasis in the flounder, whereas the Cu homeostasis was not affected.

Time-and dose-trends of biomarkers

In the time-study, both B*a*P-exposed groups displayed a peak in serum AST levels already after 2 days (Figure 5). However

the other acetone-treated groups also showed elevated serum AST levels in the first period after the injection treatment. In all groups the AST levels were returned to control levels by day 8. Linear regression analyses of results from the dose-study indicated positive dose-dependent relationship between B*a*P-FACs and AST at day 2, but also the size of the fish appeared to correlate (negatively) with AST in the same model (not shown). Similar correlation trends between PCB-156 (and fish size) and the AST levels were recorded in PCB-exposed flounder, but these relationships were not statistically significant on either sampling day in the dose study.

Both hepatic CYP1A parameters investigated (EROD, CYP1A protein) were induced by the B*a*P and PCB treatments. The CYP1A protein showed a similar trend as for the EROD, and validated therefore the activity measurements. In the time–response study, highest EROD activity and CYP1A protein levels were found after 2 and 8 days in B*a*P- and PCB-treated fish, respectively (Figure 6). A negative effect of Cd on the CYP1A induction response was indicated in the time–response study. The EROD activity, and partly CYP1A protein level, was generally lower in the B*a*P + Cd group than in the group receiving B*a*P alone. However, the suppression was not very clear (statistically significant only for CYP1A protein at day 2). No CYP1A responses were recorded in flounder treated with Cd alone (data not shown).

The multiple regression analysis of results from the dose-study demonstrated that CYP1A induction was dose-dependent towards B*a*P and PCB exposure. In B*a*P-treated flounder at day 2, the biliary B*a*P-FAC level was positively correlated ($p < 0.001$) both to the EROD activity (Figure 7(A)) and CYP1A protein level (not shown), whereas no correlation was recorded at day 30. The biological factors (sex, size, condition, serum protein) did not influence the model at day 2. In PCB-treated flounder at day 8, a significant correlation ($p < 0.05$)

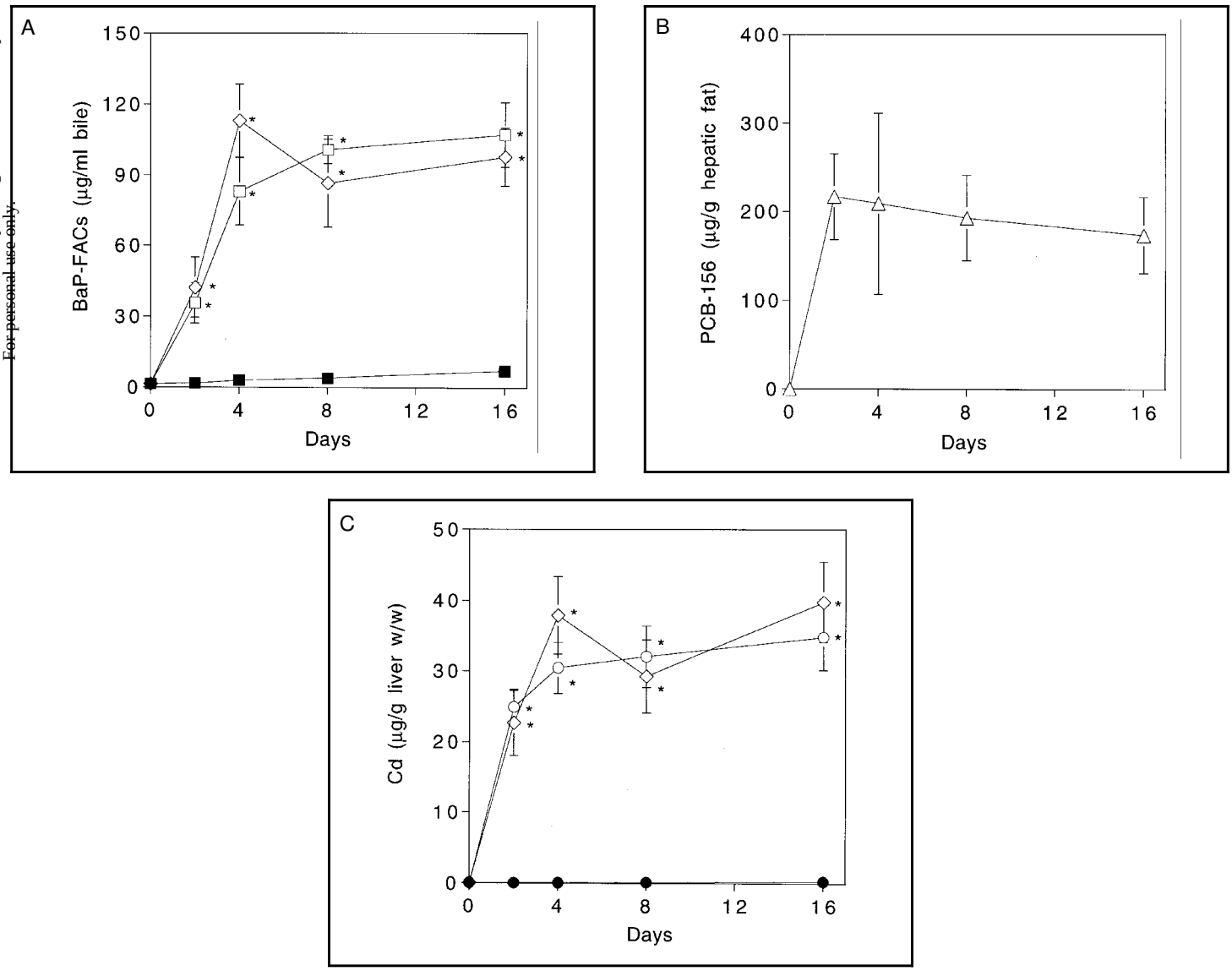

Figure 3. Time trends of contaminants in flounder: (A) biliary fluorescent aromatic compounds of benzo[*a*]pyrene metabolites (BaP-FACs); (B) hepatic levels of 2,3,3′4,4′, 5-hexachlorobiphenyl (PCB-156); (C) hepatic cadmium (Cd) levels. The fish were exposed to either 1 mg kg^{-1} body weight of B*a*P (□), 2.5 mg kg^{-1} PCB-156 (△), 1 mg kg^{-1} Cd (○) or 1 mg kg^{-1} B*a*P + 1 mg kg^{-1} Cd (◇). Control fish were exposed to acetone (■) or to 9‰ saline (●). Toxicant levels in pre-treatment fish are shown as day 0 levels in figures. All other data are shown as the group mean ($n = 6$) with standard error. * Significantly different ($p < 0.05$) from control group (Dunnett's test of means).

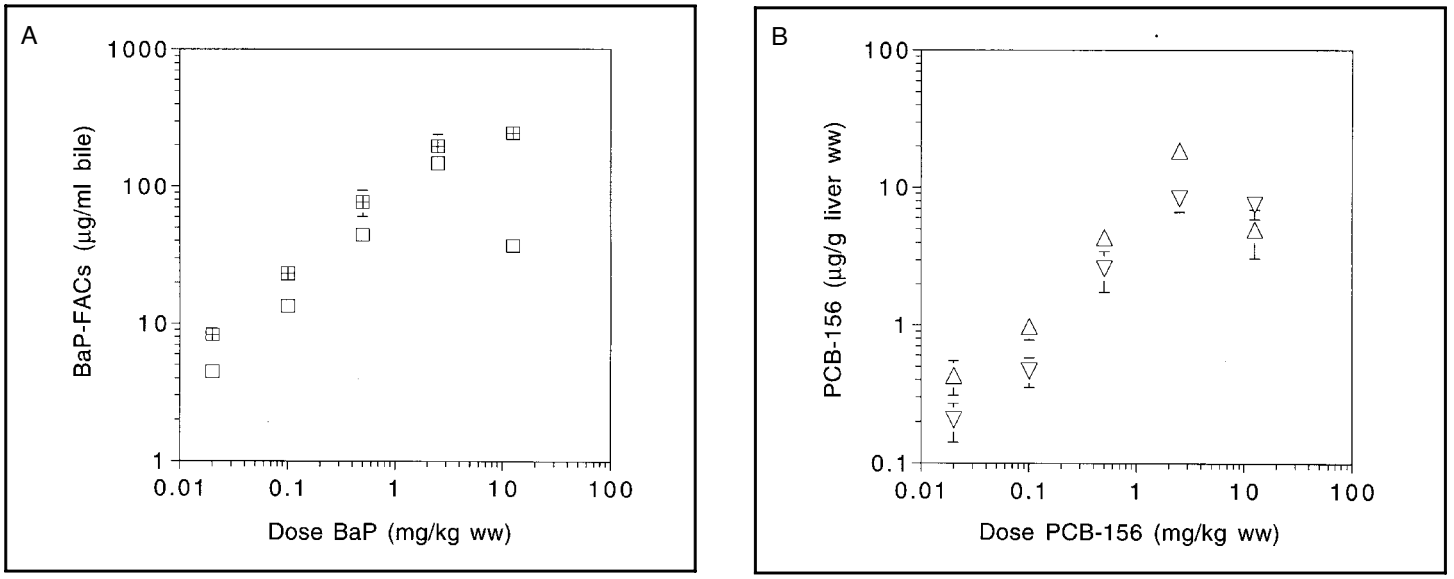

Figure 4. Correlation of i.m. injected toxicant dose and recorded levels of: (A) biliary BaP-FACs in flounder after 2 days (□) and after 30 days (⊞); (B) hepatic PCB-156 in flounder after 8 days (△) and after 30 days (▽). The injected doses of B*a*P or PCB-156 were 0.02, 0.1, 0.5, 2.5, and 12.5 mg kg^{-1} body weight. Control values were 1.5 and 4.2 µg ml^{-1} (2 and 30 days, respectively) for B*a*P-FACs, and around 0.005 µg g^{-1} (w/w) for hepatic PCB-156 (not measured in all controls). All data are shown as the group mean (n = 6) with standard error.

was recorded between hepatic PCB-156 level and EROD activity (Figure 7(B)), but, also sex displayed a significant effect ($p < 0.05$) on EROD in this model (i.e. lowered EROD activity in females). Within these females (n = 19) the gonad-somatic index was negatively correlated to the EROD activity (not shown). The CYP1A protein level did not correlate significantly to the PCB-156 level on either sampling day.

In the time-study, an increase of the hepatic MT level appeared to occur in both Cd-treated groups in the latter part of the exposure period, i.e. at day 16 (Figure 8). The increase was not strong (1.6 and 1.5-fold of control for Cd and B*a*P + Cd groups, respectively), neither was it statistically significant (p = 0.14, ANOVA). However, the MT increase occurred in parallel in both Cd-treated groups, and the MT increase became statistically significant when the Cd-treated fish were regarded as one group in a comparison with the control group ($p < 0.05$, ANOVA). The B*a*P did not appear to influence the MT response in the B*a*P + Cd group. The MT response was delayed as compared to the hepatic Cd levels, which were significantly elevated already 2 days after injection in the two Cd-treated groups.

Parameters showing no-response

Several of the parameters investigated gave no response in any of the treatment groups (Table 1). These parameters included liver and spleen somatic indices, haematocrit, serum glucose, and hepatic activities of glutathione-S-transferase (CDNB), aldrin epoxidase (AE), and UDP-glucuronosyl transferase (UDP-GT). AE and UDP-GT activities were not measured in the dose-study.

Discussion

Knowledge of time- and dose-dependent relationships of pollutant responses in flounder is an important basis for the use of this species in pollutant effect monitoring. In the present investigation, we have established such relationships for several biomarkers towards the model pollutants B*a*P, PCB-156, and Cd (Cd, time–response only). Except for the recent study by Eggens and Boon (1996), no previous laboratory studies have investigated similar time- and dose-relationships of biomarker responses in the flounder.

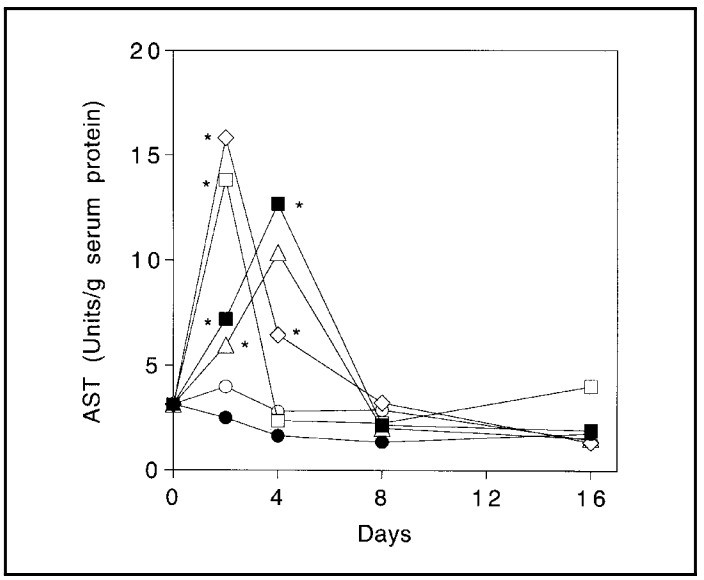

Figure 5. Changes in serum aspartate aminotransferase (AST) levels in flounder exposed to saline or acetone (controls), B*a*P, PCB-156, Cd, or B*a*P + Cd. The AST levels are normalized against the serum total protein content. The data are shown as the group mean (n = 6), error bars are not included in the figure. * Significantly different (p<0.05) from control group (Dunnett's test of means). Treatments and symbols: 1 mg kg^{-1} B*a*P (□), 2.5 mg kg^{-1} PCB-156 (△), 1 mg kg^{-1} Cd (○), 1 mg kg^{-1} B*a*P + 1 mg kg^{-1} Cd (◇), acetone (■), 0% saline (●).

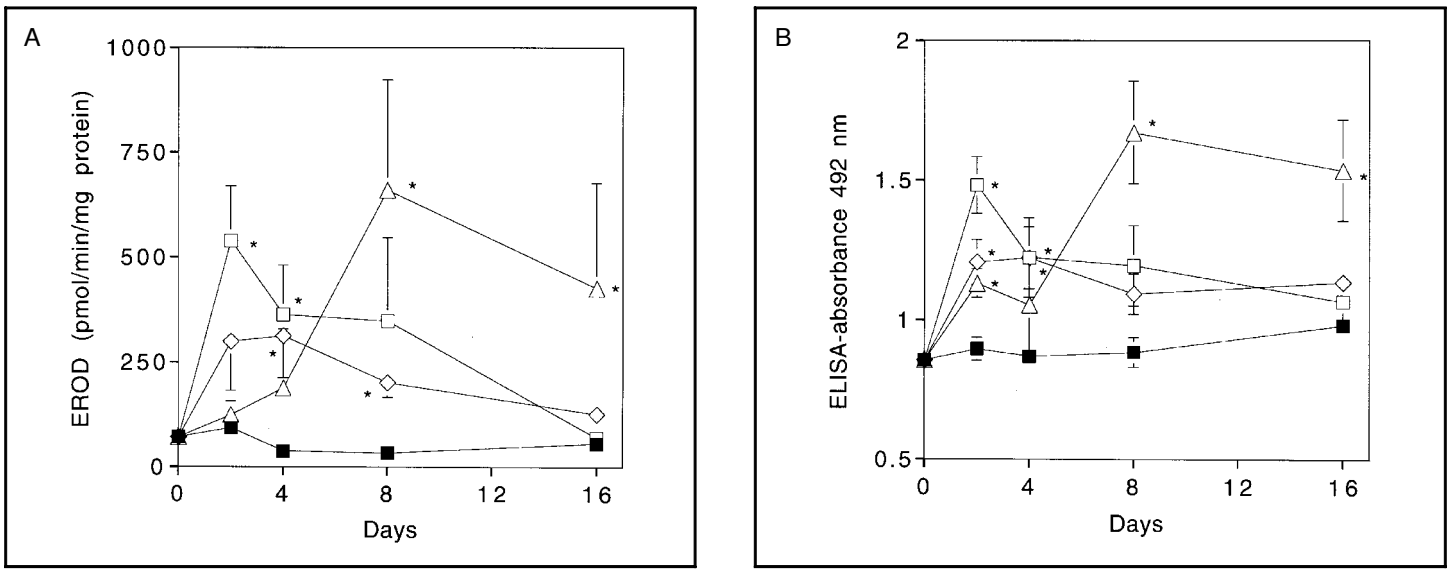

Figure 6. Changes in (A) 7-ethoxyresorufin *O*-deethylase (EROD) activity and (B) CYP1A protein levels (detected by ELISA) in hepatic microsomal samples of flounder exposed to acetone (control), BaP, PCB-156, or BaP + Cd. The data are shown as the group mean ($n = 6$) with standard error. * Significantly different ($p < 0.05$) from control group (Dunnett's test of means). Treatments and symbols: 1 mg kg^{-1} BaP (□), 2.5 mg kg^{-1} PCB-156 (△), 1 mg kg^{-1} BaP + 1 mg kg^{-1} Cd (◇), acetone (■).

Exposure regime

Intramuscular (i.m.) administration, instead of the more commonly used intraperitoneal (i.p.) route, were used to avoid the possibility of contamination of visceral samples (liver and bile) by remnants of injected chemicals. By the i.m. procedure, the chemicals have to be mobilized into the blood and re-distributed before they can induce any effect in the liver. The levels of toxicants detected in liver and bile should therefore be a good reflection of the *in vivo* exposure level or body burden. Rapid mobilization of all three toxicants injected was demonstrated in the time-study, and a linear relationship between injected and (visceral-) accumulated dose was recorded in the dose-study. These findings clearly demonstrate the applicability of i.m. exposure of fish in pollutant studies. However, a depot effect apparently occurred at the highest doses of both B*a*P and PCB-156. This depot effect might have been caused by crystallization of B*a*P and PCB-156 in the muscle tissue in the highest dose groups. Histopathological examination of the injection area could have contributed to explaining the depot effect observed, e.g. whether muscle

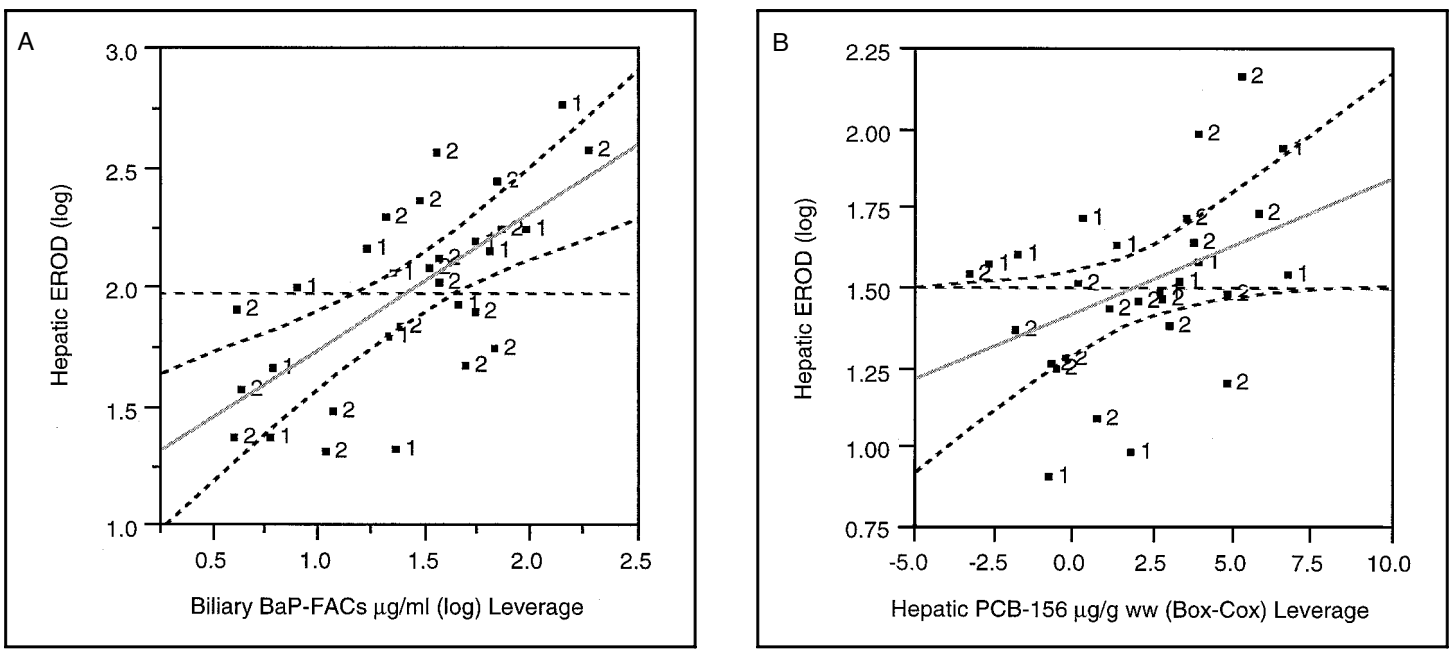

Figure 7. Selected leverage plots of multiple linear regression models: (A) biliary BaP-FACs and EROD activity in BaP-exposed flounder ($n = 30$) after 2 days; (B) hepatic PCB-156 levels and EROD activity in PCB-156 exposed flounder ($n = 29$) after 8 days. The confidence curves shows the 95% confidence region for the line of fit. Each multiple regression model also included sex, fish size, condition-factor, and serum protein as factors (leverage plots not shown). In the figures, the sex of each individual flounder is indicated by the numbers 1 (male) or 2 (female).

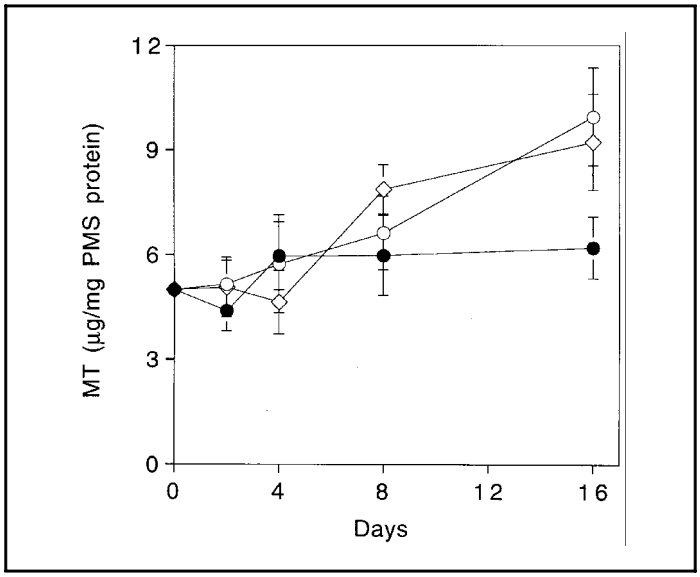

Figure 8. Changes in hepatic metallothionein levels in flounder exposed to saline (control), Cd, or BaP + Cd. The post-mitochondrial supernatant (PMS) was used for MT measurements. The data are shown as the group mean ($n = 6$) with standard error. Treatments and symbols: 1 mg kg^{-1} Cd (○), 1 mg kg^{-1} BaP + 1 mg kg^{-1} Cd (△), 9‰ saline (●).

necrosis was caused by the highest doses, but such examinations were not performed. Evidently, the depot effect was not caused by the acetone vehicle, since all fish received equal volumes of the carrier. Interestingly, a similar depot effect of B*a*P at the same dose-range was observed in i.p. injected flounder by Eggens and Boon (1996).

BaP exposure and CYP1A induction

In several teleost species, CYP1A has been shown to respond rapidly towards B*a*P treatment, with CYP1A induction peaking within 1–3 days, depending on parameter measured (EROD activity, CYP1A protein) (Smolowitz *et al.* 1992, Levine *et al.* 1994, van der Weiden *et al.* 1994). In flounder exposed to B*a*P by single i.p. injections, Eggens and Boon (1996) observed the highest levels of hepatic EROD and CYP1A protein after 1 and 3 days, respectively. They also recorded positive correlations between B*a*P dose and hepatic CYP1A levels, and observed that both CYP1A markers were almost returned to control levels after 7 days. These findings all correspond with the CYP1A responses recorded in B*a*P-exposed flounder in the present study.

PCB exposure and CYP1A induction

To our knowledge, PCB-156 has not previously been used in studies of CYP1A induction in fish, but several investigators have used other mono-*ortho* PCBs (e.g. Skaare *et al.* 1991, Bernhoft *et al.* 1994, Eggens and Boon 1996). PCB-156 is the mono-*ortho* substituted analogue of the ultimate toxic non-*ortho* 3,3′,4,4′,5-pentachlorobiphenyl (PCB-126). Based on quantitative structure–activity studies in mammalian systems, the toxic equivalence factors (TEFs) of PCB-126 and PCB-156 compared with 2,3,7,8-tetrachloro-*p*-dioxin have been estimated to be 0.1 and 0.0003, respectively (Safe 1994). Thus, as compared with PCB-126, the *ortho*-chlorine lowers the toxicity (and CYP1A-inducing potency) of PCB-156 dramatically, due to the restricted ability of the molecule for adapting a planar configuration, and hence bind to the cytosolic aryl hydrocarbon (Ah)-receptor (Safe *et al.* 1985, Safe 1994). However, since mono-*ortho* PCBs (such as PCBs-156, -105, -118) occur in higher concentrations than the non-*ortho* PCBs in the environment, their ecotoxicological impact is considered to be significant (Tanabe *et al.* 1989, De Voogt *et al.* 1990).

In the present time study, a delayed and more persistent CYP1A induction was recorded in PCB-exposed flounder compared with B*a*P-exposed fish, with highest CYP1A levels (19-fold of control) after 8 days in the time-study. This response time corresponds with the observations of Eggens and Boon (1996). They recorded a five-fold increase in hepatic EROD activity in flounder 7 days after i.p. injections of the mono-*ortho* substituted PCB-105 (2,3,3′,4,4′-pentachlorobiphenyl; 0.5 mg kg^{-1} b.w.).

Inhibitory effects on CYP1A induction

In nature, CYP1A-inducing compounds may occur together with compounds which may have inhibitory effects on the teleost CYP1A system, such as tributyltin (TBT), certain PCBs, arsenite, and cadmium (Stegeman and Hahn 1994, Bucehli and Fent 1995). In plaice (*Pleuronectes platessa*), both EROD activity and CYP1A protein synthesis were inhibited by Cd in a dose-dependent manner, with 1 mg Cd kg^{-1} b.w. resulting in 90% inhibition of the EROD activity (George and Young 1986, George 1989). However, the opposite effect of Cd has been recorded in European eel (*Anguilla anguilla*) and European sea bass (*Dicentrarchus labrax*), i.e. enhanced hepatic EROD induction in B*a*P-exposed fish pre-treated with Cd (Lemaire-Gony and Lemaire 1992, Lemaire-Gooney *et al.* 1995). Indeed, in sea bass, the treatment alone caused a 10-fold increase of the EROD activity. Such discrepancy clearly illustrates the pit-falls of expecting similar biomarker responses in different teleost species. In the present time-study, no CYP1A induction was recorded in the Cd-treated group, but a slight suppression of the CYP1A induction response seemed to occur in the B*a*P + Cd-treated fish. Thus, CYP1A induction responses in flounder appear to be less susceptible to the interactive influence of Cd than in plaice, eel and sea bass. However, further biomarker studies should be performed in mixed exposure situations with flounder, and such studies are presently being performed in our laboratories. Evidently, these issues are important in interpreting pollutant effects in different fish species, especially in mixed pollution situations in the field.

CYP1A responses in teleosts may also be influenced by inhibitory effects of the inducing compound itself. Such inhibition has previously been observed in several teleosts expose to the non-*ortho* 3,3′,4,4′-tetrachlorbiphenyl (PCB-77) (Melancon and Lech 1983, Gooch *et al.* 1989, Monosson and Stegeman 1991, Lindström-Seppä *et al.* 1994). Similar inhibition effects may explain the lack of CYP1A induction recorded by Eggens and Boon in PCB-77-exposed flounder (Eggens and Boon 1996). It should be noted that PCB-77 at low concentrations has been demonstrated as a potent CYP1A

inducer in other teleosts (Melancon and Lech 1983, Smolowitz *et al.* 1991, Lindström-Seppä *et al.* 1994). Inhibition or suppression of CYP1A-mediated responses have also been observed in teleosts exposed to high levels of PCB mixtures (Melancon and Lech 1983, Boon *et al.* 1992). It is therefore apparent that the possibility of inhibitory influence always has to be considered when discussing dose–response relationships, TEFs and quantitative structure–activity relationships (QSAR) of PCBs in different teleost species. In the present dose-study, PCB inhibition of CYP1A responses was not observed. However, in the time-study, inhibition of EROD activity 2 days after PCB injection was indicated by induced CYP1A protein levels and no change in EROD activity. Apparently, the lower induction in the highest dose-group was not caused by inhibition, but rather by the depot effect, leading to lower *in vivo* exposure level in this group than in the second highest dose group.

Serum AST

Aspartate aminotransferase (AST) is a non-specific cytosolic and mitochondrial enzyme found in a variety of tissues including liver, skeletal muscle, cardiac muscle, and kidney (Verma *et al.* 1981). Determinations of transaminases, such as AST, in teleost serum or plasma have proved useful as a biomarker in the diagnosis of liver-pathology or hepatocyte dysfunction, e.g. Maita *et al.* (1984) (see also Gingerich (1982) and Foster *et al.* (1992) for reviews). However, the AST activity may vary considerably among different fish species, e.g. Goel *et al.* (1981). In environmental monitoring, AST may be useful in field situations where hepatotoxic pollutants occur. In flounder exposed by caging to high PAH levels in Sørfjorden (SW-Norway), AST was significantly correlated to the levels of biliary B*a*P-FACs (Beyer *et al.* 1996). In the present time–response study, the serum AST was most responsive in the first part of the exposure period, but in chronically-exposed situations more persistent elevations of the transaminase level could be expected. Evidently, the AST responded towards the acetone vehicle used in the present investigation, but a dose-dependent response towards B*a*P was also indicated. However, the influence of fish size should also be considered in the utilization of AST as a biomarker tool in teleosts. For biomonitoring purposes, AST and other transaminase assays have the advantage of already being established in a broad range of analytical laboratories, also in clinical institutions. They can also be used as non-destructive biomarkers since blood is the biological sample used.

Metallothionein

Several studies have shown that teleost metallothionein is responsive to heavy metal exposure, e.g. George and Young (1986), George (1989), Hogstrand and Haux (1991), for reviews see also Stegeman *et al.* (1992), Livingstone (1993), and George and Olsson (1994). However, metal exposure seldom increases the teleost MT level more than a few fold. In the present time–response study, a slight increase of the MT level occurred after 16 days in both Cd-exposed groups (1.5- and 1.6-fold of control). Possibly, a stronger MT induction response could have been found with longer exposure, since the MT curves in both groups were rising when the 16 days' exposure period ended. Ongoing studies in our laboratory support this hypothesis; flounder exposed to Cd by subcutaneous injections displayed significantly increased hepatic MT levels after 15 days and 23 days (results not shown). Thus, in future investigations of MT induction after Cd treatment in flounder, the exposure period should be extended to 3 weeks or more.

Summary

The present study has shown that biomarkers in flounder are responsive to PAH, PCB, and heavy metal pollutant exposure. Clearest responses were found in hepatic CYP1A levels as a dose-dependent biomarker of the organic toxicants, especially B*a*P. Different time–response trends were seen for the three chemicals, with B*a*P and Cd giving the fastest and slowest responses, respectively. The biomarker responses in flounder seem to be reasonably sensitive and reliable, and possibly also less influenced by associated pollutants as compared with those found in other teleost species. Its coastal, estuarine, and benthic habitat preferences make flounder interesting for monitoring of pollutant effects. Effects at higher levels of biological organization, such as impaired disease resistance and reproduction competence, need further investigation, e.g. by employing ecologically relevant exposure regimes (more realistic doses and long-term exposure). Standardized protocols for biomarker investigations will in general facilitate these studies, and such standards will also be necessary for the future application of teleost biomarkers in environmental pollution monitoring programmes.

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References

ADDISON, R. F. AND EDWARDS, A. J. (1988) Hepatic microsomal mono-oxygenase activity in flounder *Platichthys flesus* from polluted sites in Langesundfjord and from mesocosms experimentally dosed with diesel oil and copper. *Marine Ecology Progress Series*, **46**, 51–54.

ANDERSSON, T., PESONEN, M. AND JOHANSSON, C. (1985) Differential induction of cytochrome P-45-dependent monooxygenase, epoxide hydrolase, glutathione transferase and UDP glucuronosyltransferase activities in the liver of the rainbow trout by β-naphthoflavone or Clophen A50. *Biochemical Pharmacology*, **34**, 3309–3314.

ARIESE, F., KOK, S. J., VERKAIK, M., GOOIJER, C., VELTHORST, N. H. AND HOFSTRAAT, J. W. (1991) Monitoring of PAH exposure levels in the marine environment using synchronous scanning fluorescence analysis of 1-hydroxy pyrene in fish bile. *13th International Symposium on PAH*, Bordeaux, 1991.

Bernhoft, A. and Skaare, J. U. (1994) Levels of selected individual polychlorinated biphenyls in different tissues of harbour seals (*Phoca vitulina*) from the southern coast of Norway. *Environmental Pollution,* **86**, 99–107.

Bernhoft, A., Hektoen, H., Skaare, J. U. and Ingebrigtsen, K. (1994) Tissue distribution and effects on hepatic xenobiotic metabolizing enzymes of 2,3,3′,4,4′-pentachlorobiphenyl (PCB-105) in cod (*Gadus morhua*) and rainbow trout (*Oncorhynchus mykiss*). *Environmental Pollution*, **85**, 351–359.

Beyer, J., Sandvik, M., Hylland, K., Fjeld, E., Egaas, E., Aas, E., Skåre, J. U. and Goksøyr, A. (1996) Contaminant accumulation and biomarker responses in flounder (*Platichthys flesus* L.) and Atlantic cod (*gadus morhua* L.) exposed by caging to polluted sediments in Sørfjorden, Norway, *Aquatic Toxicology* (in press).

Boon, J. P., Everaarts, J. M., Hillebrand, M. T. J., Eggens, M. L., Pijnenburg, J. and Goskøyr, A. (1992) Changes in levels of hepatic biotransformation enzymes and haemoglobin levels in female plaice (*Pleuronectes platessa*) after oral administration of a technical PCB mixture (Clophen A40). *Science of the Total Environment*, **114**, 113–133.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. *Analytical Biochemistry*, **72**, 248–254.

Brevik, E. M. (1978) Gas chromatographic method for the determination of organochlorine pesticides in human milk. *Bulletin of Environmental Contamination and Toxicology*, **19**, 281–286.

Bucheli, T. D. and Fent, K. (1995) Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. *Critical Reviews in Environmental Science and Technology*, **25**, 201–268.

Burke, M. D. and Mayer, R. T. (1974) Ethoxyresorufin: direct flurometric assay of a microsomal *O*-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metabolism and Disposition*, **2**, 583–588.

De voogt, P., Wells, D. E., Reutergårdh, L. and Brinkman, U. A. t. (1990) Biological activity, determination and occurrence of planar, mono- and di-*ortho* PCBs. *International Journal of Environmental Analytical Chemistry*, **40**, 1–46.

Eggas, E., Gram Jensen, E. and Skaare, J. U. (1988). Activities of some microsomal enzymes of the yellow mealworm, *Tenebrio molitor* (Linné). *Pesticide Biochemistry and Physiology*, **30**, 35–39.

Eggas, E., Skaare, J. U., Svendsen, N. O., Sandvik, M., Falls, J. G., Dauterman, W. C., Collier, T. K. and Netland, J. (1993) A comparative study of effects of atrazine on xenobiotic metabolizing enzymes in fish and insect, and of the *in vitro* Phase II atrazine metabolism in some fish, insects, mammals and one plant species. *Comparative Biochemistry and Physiology*, **106C**, 141–149.

Eggens, M. L. and Boon, J. P. (1996). Induction of CYP1A in flounder, *Platichthys flesus*, after administration of individual PAHs and PCBs and a technical PCB mixture (Clophen A40). *Biomarkers* (accepted).

Foster, L. M., Versteeg, D. J., Mckee, M. J., Folmar, L. C., Graney, R. L., Mccume, D. C. and Rattner, B. A. (1992) Physiological and nonspecific biomarkers. In *Biomarkers: Biochemical, Physiological and Histological Markers of Anthropogenic Stress*, R. J. Huggett, R. A. Kimerle, P. M. Mehrle Jr and H. L. Bergman, eds (Lewis Publishers, Chelsea, MI, USA), pp. 5–85.

George, S. G. (1989) Cadmium effects on plaice liver xenobiotic and metal detoxication systems: dose response. *Aquatic Toxicology*, **15**, 303–310.

George, S. G. and Olsson, P.-E. (1994) Metallothioneins as indicators of trace metal pollution. In *Biomonitoring of Coastal Waters and Estuaries* , K. J. M. Kramer, ed. (CRC Press, Inc., London.), pp. 151–171.

George, S. G. and Young, P. (1986) The time course of effects of cadmium and 3-methylcholanthrene on activities of enzymes of xenobiotic metabolism and metallothionein levels in the plaice, *Pleuronects platesa. Comparative Biochemistry and Physiology*, **83C**, 37–44.

Gingerich, W. H. (1982) Hepatic toxicology of fishes. In *Aquatic Toxicology*, L. J. Weber, ed. (Raven Press, New York), pp. 55–105.

Goel, K. A., Awasthi, A. K. and Tyagi, S. K. (1981) Comparative haematological studies in some fresh water Indian fishes. *Zeitschrift für Tierphysiologie, Tierenahrung und Futtermittelkontrolle*, **46**, 202–206.

Goksøyr, A. (1991) A semi-quantitative cytochrome P450IA1 ELISA: a simple method for studying the monoxygenase induction response in environmental monitoring and ecotoxicological testing of fish. *Science of the Total Environment*, **101**, 255–262.

Goksøyr, A. (1995) Use of cytochrome P450 1A (CYP1A) in fish as a biomarker of aquatic pollution. *Archives of Toxicology*, Supplement 17, 80–95.

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

Habig, W. H., Pabst, M. J. and Jakoby, W. B. (1974) Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry*, **249**, 7130–7139.

Hogstrand, C. and Haux, C. (1990) A radioimmunoassay for perch (*Perca fluviatilis*) metallothionein. *Toxicology and Applied Pharmacology*, **103**, 56–65.

Hogstrand, C. and Haux, C. (1991) Binding and detoxification of heavy metals in lower vertebrate with reference to metallothionein. *Comparative Biochemistry and Physiology*, **100C**, 137–141.

Hylland, K., Hogstrand, C., Haux, C., Sletten, K. and Andersen, R. A. (1994) Properties of cod metallothionein, its presence in different tissues and effects of Cd and Zn treatment. *Fish Physiology and Biochemistry*, **13**, 81–91.

Koivusaari, U. (1983) Thermal acclimatization of hepatic polysubstrate monooxygenase and UDP-glucuronyltransferase of mature rainbow trout. *Journal of Experimental Zoology*, **227**, 35–42.

Krahn, M. M., Brown, D. W., Collier, T. K., Friedman, A. J., Jenkins, R. G. and Malins, D. C. (1980) Rapid analysis of naphthalene and its metabolites in biological systems: determination by high-performance liquid chromatography/fluorescence detection and by plasma desorption/chemical ionization mass spectrometry. *Journal of Biochemical and Biophysical Methods*, **2**, 233–246.

Krahn, M. M., Moore, L. K. and Macleod, W. D. J. (1986) Standard Analytical Procedures of the NOAA National Analytical Facility: Metabolites of aromatic compounds in fish bile. National Technical Information Service, US Dept. of Commerce, Springfield, VA, USA.

Lemaire, P., Matthews, A., Förlin, L. and Livingstone, D. R. (1994) Stimulation of oxyradical production of hepatic microsomes of flounder (*Platichthys flesus*) and perch (*Perca fluviatilis*) by model and pollutant xenobiotics. *Archives of Environmental Contamination and Toxicology*, **26**, 191–200.

Lemaire-Gony, S. and Lemaire, P. (1992) Interactive effects of cadmium and benzo(a)pyrene on cellular structure and biotransformation enzymes of the liver of the European eel *Anguilla anguilla*. *Aquatic Toxicology*, **22**, 145–160.

Lemaire-Gony, S., Lemaire, P. and Pulsford, A. L. (1995) Effects of cadmium and benzo(a)pyrene on the immune system, gill ATPase and EROD activity of European sea bass *Dicentrarchus labrax*. *Aquatic Toxicology*, **31**, 297–313.

Levine, S. L., Oris, J. T. and Wissing, T. E. (1994) Comparison of P-4501A1 monooxygenase induction in gizzard shad (*Dorosoma cepedianum*) following intraperitoneal injection or continuous waterborne-exposure with benzo(a)pyrene: temporal and dose-dependent studies. *Aquatic Toxicology*, **30**, 61–75.

Lindström-Seppä, P., Korytko, P. J., Hahn, M. and Stegeman, J. J. (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*. *Aquatic Toxicology*, **28**, 147–167.

Livingstone, D. R. (1993) Biotechnology and pollution monitoring: use of molecular biomarkers in the aquatic environment. *Journal of Chemical Technology and Biotechnology*, **57**, 195–211.

Maita, M., Shiomitsu, K. and Ikeda, Y. (1984) Health assessment by the climogram of hemochemical constituents in cultured yellowtail. *Bulletin of the Japanese Society for Scientific Fisheries*, **51**, 205–211.

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

Moldenke, A. F. and Terriere, L. C. (1981) Cytochrome P-450 in insects. Increase in substrate binding by microsomes from phenobarbital-induced house flies. *Pesticide Biochemistry*, **16**, 222–230.

Monosson, E. and Stegeman, J. J. (1991) Cytochrome P450E (P450IA) induction and inhibition in winter flounder by 3,3′4,′4-tetrachlorobiphenyl: comparison of response in fish from Georges Bank and Narragansett Bay. *Environmental Toxicology and Chemistry*, **10**, 765–774.

SAFE, S. H. (1994) Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment. *Critical Reviews in Toxicology*, **24**, 87–149.

SAFE, S., BANDIERA, S., SAWYER, T., ROBERTSON, L., SAFE, L., PARKINSON, A., THOMAS, P. E., RYAN, D. E., REIK, L. M., LEVIN, W., DENOMME, M. A. AND FUJITA, T. (1985) PCBs: structure–function relationship and mechanism of action. *Environmental Health Perspectives*, **60**, 47–56.

SAS (1994) *Statistics made Visual™, JMP® statistics for the Apple® Macintosh from SAS Institute Inc.* (SAS Institute Inc., Cary, NC, USA).

SKAARE, J. U., GRAM JENSEN, E., GOKSØYR, A. AND EGAAS, E. (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, PCB-118, detected by enzyme activities and immunochemical methods. *Archives of Environmental Contamination and Toxicology*, **20**, 349–352.

SMOLOWITZ, R. M., HAHN, M. E. AND STEGEMAN, J. J. (1991). Immunohistochemical localization of cytochrome P-450IA1 induced by 3,3′,4,4′-tetrachlorobiphenyl and by 2,3,7,-tetrachlorodibenzo-furan in liver and extrahepatic tissues of the teleost *Stenotomus chrysops* (scup). *Drug Metabolism and Disposition*, **19**, 113–123.

SMOLOWITZ, R. M., SCHULTZ, M. E. AND STEGEMAN, J. J. (1992) Cytochrome P4501A induction in tissues, including olfactory epithelium, of topminnows (*Poeciliopsis spp,*) by waterborne benzo(*a*)pyrene. *Carcinogenesis*, **13**, 2395–2402.

STAGG, R. M. AND ADDISON, R. F. (1995) An inter-laboratory comparison of measurements of ethoxyresorufin *O*-deethylase activity in dab (*Limanda limanda*) liver. *Marine Environmental Research*, **40**, 93–108.

STEGEMAN, J. J. AND HAHN, M. E. (1984) Biochemistry and molecular biology of monooxygenases: current perspectives on forms, functions and regulation of cytochrome P450 in aquatic species. In *Aquatic Toxicology: Molecular, Biochemical, and Cellular Perspectives*, D. C. Malins and G. K. Ostrander, eds (Lewis Publishers, Boca Raton, FL, USA), pp. 87–204.

STEGEMAN, J. J., WOODIN, B. R. AND GOKSØYR, A. (1988) Apparent cytochrome P-450 induction as an indication of exposure to environmental chemicals in the flatfish *Platichthys flesus*. *Marine Ecology Progress Series*, **46**, 55–60.

STEGEMAN, J. J., BROUWER, M., RICHARD, T. D. G., FÖRLIN, L., FOWLER, B. A., SANDERS, B. M. AND VAN VELD, P. A. (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*, R. J. Huggett, R. A. Kimerle, P. M. Mehrle Jr and H. L. Bergman, eds (Lewis Publishers, Chelsea, MI, USA), pp. 235–335.

TANABE, S., KANNAN, N., ONO, M. AND TATSUKAWA, R. (1989) Toxic threat to marine mammals: increasing toxic potential of non-*ortho* and mono-*ortho* coplanar PCBs from land to ocean. *Chemosphere*, **18**, 485–490.

VAN DER WEIDEN, M. E. J., HANEGRAAF, F. H. M., EGGENS, M. L., CELANDER, M., SEINEN, W. AND VAN DER BERG, M. (1994) Temporal induction of cytochrome P450 1A in the mirror carp (*Cyprinus carpio*) after administration of several polycyclic aromatic hydrocarbons. *Environmental Toxicology and Chemistry*, **13**, 797–802.

VERMA, S. R., TINK, I. P., GUPTA, A. K. AND DALELA, R. C. (1981) Role of ascorbic acid in the toxicity of pesticides in a freshwater teleost. *Water, Air and Soil Pollution*, **16**, 107–114.

VETHAAK, A. D. (1993) Fish disease and marine pollution: a case study of the flounder (*Platichthys flesus*) in Dutch coastal and estuarine waters. University of Amsterdam, The Netherlands.

VON WESTERNHAGEN, H., ROSENTHALM, H., DETHLEFSEN, V., ERNST, W., HARMS, U. AND HANSEN, P.-D. (1981) Bioaccumulating substances and reproductive success in Baltic flounder *Platichthys flesus*. *Aquatic Toxicology*, **1**, 85–99.

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