Interaction of benzo[*a*]pyrene, 2,3,3´,4,4´,5-hexachlorobiphenyl (PCB-156) and cadmium on biomarker responses in flounder (*Platichthys flesus* L.)

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Interactive effects of a mixed pollutant exposure on biomarker responses were studied in European flounder (*Platichthys flesus* L.). The model chemicals, benzo[*a*]pyrene (BaP, 2.5 mg kg^{-1}), 2,3,3´,4,4´5-hexachlorobiphenyl (PCB-156, 2.5 mg kg^{-1}), and cadmium (cadmium, 1 mg kg^{-1}), were administered to fish by subcutaneous injections. Biomarker responses were quantified both following administration of single chemicals and sequential combinations of the chemicals in pairs. Significant induction of CYP1A protein levels and corresponding ethoxyresorufin-*O*-deethylase (EROD) activities was observed in BaP and PCB-treated flounder after 2 and 8 days, respectively. The strongest induction (44-fold) was caused by BaP. No further induction was observed after additional treatment with PCB-156. CYP1A induction caused by BaP was inhibited (40% compared with BaP treatment alone) in flounder pre-treated with cadmium, whereas induction by PCB-156 appeared to be unaffected by pre-treatment with cadmium. Flounder treated with cadmium only had significantly elevated hepatic levels of metallothionein (MT) after 15 days. Pre-treatment with BaP and PCB prior to cadmium inhibited the MT induction (30–50%) compared with cadmium alone. Furthermore, significantly higher glutathione S-transferase activities were observed in flounder administered cadmium alone, and in flounder treated with BaP or PCB-156 prior to cadmium. GST selenium-independent peroxidase activities appeared to be unaffected by any of the treatments in the present study. The results indicate that chemical mixtures may affect biomarker responses differently from compounds administered alone, and that the sensitivity of both CYP1A and MT are influenced by pollutants other than their primary inducers.

Keywords: *fish, biomarkers, PAH, PCB, cadmium, interactions, CYP1A, metallothionein.*

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Introduction

Relationships between exposure to environmental contaminants and biomarker responses have been well documented in fish. Induction of CYP1A in fish has been associated with exposure to planar aromatic compounds, such as polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and dioxins (Stegeman and Hahn 1994, Bucheli and Fent 1995, Goksøyr 1995). Hence, both in the field and in the laboratory, CYP1A induction has frequently been employed as a biomarker for such pollutants. Metallothionein (MT) is the most widely used biomarker for environmental metal contamination. In fish, tissue levels of MT generally increase with increasing exposure to non-essential metals such as hepatic cadmium and mercury, but may also be induced by high copper and zinc concentrations (George and Olsson 1994).

Polluted areas are generally characterized by a mixture of compounds, rather than by single chemicals, which may result in either synergistic or antagonistic effects on aquatic organisms. Therefore, information regarding such interaction effects clearly is important for a proper interpretation of pollutant-dependent biomarker responses recorded in fish from polluted areas.

The objective of the present study was to evaluate CYP1A and MT as biomarkers in fish in mixed exposure situations. The interactive effects of BaP, PCB-156 and cadmium were studied by sequential administration of the chemicals in pairs compared with administration of the chemicals alone. European flounder (*Platichthys flesus*) was used as model species in the study. This euryhaline flatfish occurs in coastal and estuarine regions throughout Europe, and has therefore frequently been used for the monitoring of pollutant levels and effects in field studies (von Westernhagen *et al.* 1981, Stegeman *et al.* 1988, Beyer *et al.* 1996a, Eggens *et al.* 1996, Hylland *et al.* 1996).

MATERIALS AND METHODS

Chemicals

7-Ethoxyresorufin, resorufin and benzo[*a*]pyrene (BaP) (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. Alkamuls EL-620 (Emulphor oil) was obtained from Rhône-Poulenc Chimie, Paris, France. All other chemicals used in preparation and analyses of samples were of analytical grade.

Animals

The experiments were performed at the High-Technology Centre (HiB) in Bergen, in the laboratory facilities of the Industrial Laboratory. Gonadally-immature flounder (91–376 g) were collected nearshore at Sotra, west of Bergen, Norway. Prior to the experiments, they were acclimated for at least 2 weeks, and fed every second day with commercial flatfish pellet. The feeding was stopped 3 days before the initial injections, and the fish fasted throughout the rest of the experiment. The water temperature was 8.5–9.2 °C, and the salinity was 33.3–34.5 psu during the experimental period. The fish were kept in 500 l tanks, containing a layer of shell sand.

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Treatment

During treatment the fish were randomly selected from the main fish pool, but individuals with fin injuries or other visible abnormalities were not included in the experiments. BaP and PCB were dissolved in 50:50 acetone and Alkamuls, whereas $CdCl_2$ was dissolved in 50:50 filtered seawater and Alkamuls. The toxicants or vehicle (controls) were administered to the fish by subcutaneous injections (1 ml kg^{-1}). At the start, each flounder received a single injection with either PCB-156 (2.5 mg kg^{-1}), BaP (2.5 mg kg^{-1}), cadmium (1 mg kg^{-1}) or the corresponding vehicles. Two days post-injection, the BaP-treated flounder were either sacrificed or re-injected with each of the other toxicants. Similarly, PCB-treated fish were either sacrificed or re-injected after 8 days, whereas cadmium-treated fish were either sacrificed or re-injected after 15 days. The groups subjected to mixed exposure regimes of BaP and PCB, cadmium and BaP, or cadmium and PCB were sacrificed 10, 17, or 23 days after the initial injection, respectively. Samples were obtained from a total of 114 fish. Six treated and six corresponding controls were sacrificed at each sampling point. Before sampling, the size of the fish, and external lesions were recorded. Damaged fish were not used.

Preparation and analyses of samples

The gall bladder was carefully excised and the bile was frozen at –20 °C. Samples of liver for chemical analyses and hepatic subcellular samples (microsomal and cytosol fractions) were prepared as described in Beyer *et al.* (1997a) and frozen at –80 °C.

Analyses of total protein were performed according to Bradford (1976) using bovine serum albumin as standard. Fluorescent biliary compounds of BaP (BaP–FACs) were measured by direct fluorometry as described by Beyer *et al.* (1997a). PCB-156 were measured according to Bernhoft and Skaare (1994). The detection limit of PCB-156 was determined in samples from both PCB-treated and untreated flounder. The detection limit was 44 and 0.88 ng g^{-1} w.w. respectively, due to different dilution of the samples. The Environmental Toxicology Laboratory at the Norwegian College of Veterinary Medicine, Oslo, Norway has participated in all four phases of the ICES/IOC/OCSPARCOM intercomparison exercise on the analyses of PCBs, and the analytical quality has proven acceptable.

Liver samples for metal analyses were digested in concentrated nitric acid (Ultrapure, Merck) 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 (Weltz 1985). Cadmium was measured by graphite furnace atomization (Varian SpectrAA 400). The detection limit was 0.01 μg g^{-1} w.w. A control system with regular analyses of reference materials was adopted, and measurements of these samples (NRCC Dogfish DOLT-1 and DORM-1) were within acceptable limits.

CYP1A protein in hepatic microsomal samples was measured according to Goksøyr (1991) with the use of enzyme-linked immunosorbent assay (ELISA) and rabbit-anti cod CYP1A IgG antiserum. CYP1A-dependent 7-ethoxyresorufin *O*-deethylase (EROD) activity was measured according to Stagg and Addison (1995) with resorufin as internal standard, assay temperature 20 °C, and assay pH 7.4. A control system of the CYP1A measurements was adopted with regular analyses of reference microsome samples from untreated and β-naphthoflavone treated cod.

Hepatic metallothionein (MT) in hepatic cytosol was determined by pulse polarography as described by Olafson and Olsson (1991). Purified flounder MT was used as a standard. The MT concentrations in the standards were quantified by measuring the content of cysteine.

Hepatic cytosolic GST activities towards CDNB (1-chloro-2,4-dinitro-benzene), ETHA (ethacrynic acid) and CU (cumene hydroperoxide) was measured according to Habig *et al.* (1974). The assay conditions for flounder have been described earlier by Egaas *et al.* (1993).

Statistical methods

Sample data were log transformed when necessary in order to allow the use of parametric statistical methods. Parametric tests were always preceded by Bartlett's test for homogeneity of variance. Student's *t*-test was used to test the difference between control and exposed groups. One-way ANOVA and Tukey–Kramer HSD (multiple comparison) tests were used when differences between means of more than two groups were evaluated. The level of statistical significance for rejecting H_0: 'No difference' between groups was set to $p < 0.05$.

Results

General observations

Liver somatic index, condition factor and different sizes were equally distributed between the groups, but the average weight for females was significantly higher than for the males. Analysis of covariance (Draper and Smith 1981) showed that biomarker responses were not significantly affected by sex.

Approximately 5% of the flounder died during the acclimation period. Most of the mortalities were presumably due to tail fin injuries. Similar tail fin injuries were observed in some individuals during the exposure period. However, such individuals were excluded, since physiological stress of the fin-injury could have influenced the responses measured. Overall, less than 3% of the treated flounder (4 of 144) died during the exposure period. Interestingly, three out of four fish died in the PCB-exposed group re-injected with cadmium, whereas no mortality occurred in the group injected with cadmium before PCB-156.

Tissue levels of chemicals

The levels of biliary BaP-FACs, hepatic PCB-156, and hepatic cadmium following subcutaneous administration of the toxicants alone or in different pairs are shown in Table 1. No apparent interference in the accumulation of the chemicals was observed when groups were compared using one-way ANOVA. Regression analysis showed that biliary BaP–FAC levels were positively correlated both to EROD activity and CYP1A levels only when BaP was administered alone. No correlation was observed between hepatic PCB levels and CYP1A induction. The MT induction was not positively correlated to cadmium exposure either.

CYP1A

Interactions on the biomarker effects following administration of the toxicants alone or in different pairs were investigated on timepoints derived from earlier time-course studies (Figure 1). In BaP-treated flounder, a significant induction of EROD activity and CYP1A protein levels (44- and-2 fold relative to controls, respectively) was observed 2 days after the administration of BaP (Figures 2 and 3).

A similar response to BaP was observed in the group pre-treated with PCB-156 8 days earlier (Figure 2). In the group receiving BaP following cadmium pre-treatment 15 days earlier, however, the CYP1A induction (EROD 3-fold of control) appeared to be somewhat inhibited by the cadmium pre-treatment. The CYP1A induction in this group appeared to be lower than in the other BaP-expos[illegible]

Treatment (day)	Sampling day	Tissue levels (μg BaP ml^{-1})	(μg PCB-156 g^{-1})	(μg Cd g^{-1})
None	0	2.7 ± 1.1 (6)	0.001[a]	Not analysed
BaP (0)	2	256 ± 34 (5)		
PCB-156 (0)	8		4.3 ± 1.1 (6)	
Cadmium (0)	15			32 ± 5 (6)
PCB-156 (0)+BaP (8)	10	194 ± 45 (6)	4.8 ± 1.6 (6)	
BaP (0)+PCB-156 (2)	10	157 ± 39 (5)	4.1 ± 1.8 (6)	
Cadmium (0)+BaP (15)	17	214 ± 26 (6)		24 ± 2 (6)
BaP (0)+cadmium (2)	17	401 ± 230 (5)		25 ± 5 (6)
PCB-156 (0)+cadmium (8)	23		7.2 ± 0.4 (3)	17 ± 3 (3)
Cadmium (0)+PCB-156 (15)	23		9.2 ± 2.7 (5)	25 ± 6 (5)

Table 1. Hepatic levels of PCB-156 and cadmium and biliary BaP levels in flounder (*Platichthys flesus*) following subcutaneous administration of the toxicants alone or in different sequential combinations.

Each flounder received a single subcutaneous injection with either PCB-156 (2.5 mg kg^{-1}), BaP (2.5 mg kg^{-1}) or cadmium (1 mg kg^{-1}) at the start of the experiment. BaP-treated fish were either sacrificed or re-injected with one of the other toxicants 2 days post-injection. PCB-treated fish were either sacrificed or re-injected with one of the other toxicants 8 days post-injection. Cadmium-treated fish were either sacrificed or re-injected with one of the other toxicants 15 days post-injection. Flounder treated with BaP and PCB-156 were sacrificed 10 days after the first injection. Flounder treated with cadmium and BaP were sacrificed 17 days after the first injection, whereas fish treated with cadmium and PCB-156 were sacrificed after 23 days. Values are presented as mean±SEM (*n*).

[a] Detected in only one control fish.

EROD nor CYP1A protein levels were significantly different from the corresponding control group levels. However, this apparent inhibitory effect of cadmium was not statistically significant when the groups were compared using one-way ANOVA.

In PCB-156-treated flounder, a significant induction of EROD activity and CYP1A protein levels (3- and 1.5-fold of controls, respectively) was observed 8 days after the PCB injection (Figures 2 and 3, respectively). More or less similar responses to the PCB exposure were recorded in the two groups pre-treated with either BaP 2 days earlier or cadmium 5 days earlier. Thus, CYP1A induction in PCB-treated fish, was not affected by BaP or cadmium pre-treatment.

In cadmium-treated flounder, no CYP1A induction was recorded in the groups pre-treated with BaP or PCB, as compared with the respective controls (Figures 2 and 3).

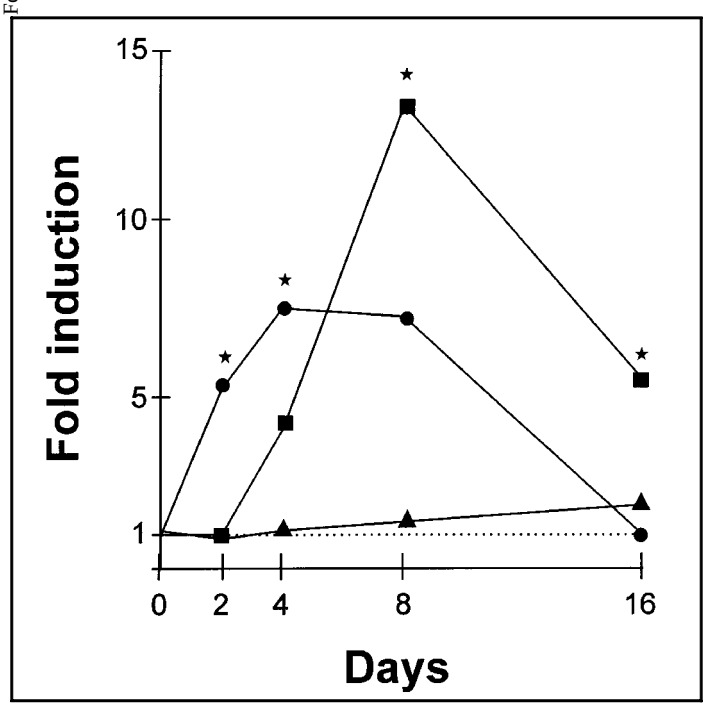

Figure 1. Temporal hepatic CYP1A induction (EROD activity) following intramuscular administration to BaP (●), PCB-156 (■), and induction of hepatic MT levels (▲) following cadmium exposure to flounder. ★: Significantly different from corresponding control group as determined with Student's *t* test. Adapted from Beyer *et al.* (1996a).

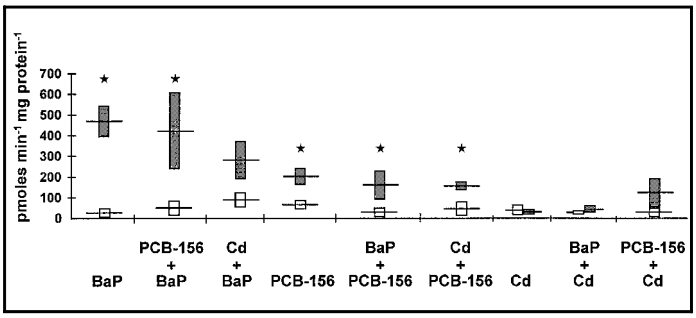

Figure 2. Hepatic 7-ethoxyresorufin-*O*-deethylase (EROD) in flounder following subcutaneous administration of BaP, PCB-156 or cadmium, either singly or in different combinations. Treated fish are represented by closed bars (means±SEM (6)). Open bars represent corresponding controls injected with the vehicles only. ★: Significantly different from corresponding control group as determined with Student's *t* test. Treatment: BaP: Administration at day 0 and sampling at day 2. PCB-156+BaP: Administration of PCB-156 at day 0, administration of BaP at day 8 and sampling at day 10. Cd+BaP: Administration of cadmium at day 0, administration of BaP at day 15 and sampling at day 17. PCB-156: Administration at day 0, and sampling at day 8. BaP+PCB-156: Administration of BaP at day 0, administration of PCB-156 at day 2 and sampling at day 10. Cd+PCB-156: Administration of cadmium at day 0, administration of PCB-156 at day 15 and sampling at day 23. Cd: Administration at day 0 and sampling at day 15. BaP+Cd: Administration of BaP at day 0, administration of cadmium at day 2 and sampling at day 17. PCB-156+Cd: Administration of PCB-156 at day 0, administration of cadmium at day 8 and sampling at day 23.

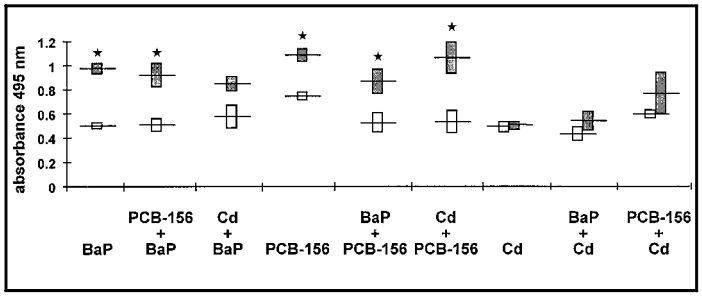

Figure 3. Hepatic CYP1A protein levels in flounder following subcutaneous administration of BaP, PCB-156 or cadmium, either singly or in different combinations. Treated fish are represented by closed bars (means±SEM (6)). Open bars represents corresponding controls injected with the vehicles only. ★: Significantly different from corresponding control group as determined with Student's *t* test. Treatment: BaP: Administration at day 0 and sampling at day 2. PCB-156+BaP: Administration of PCB-156 at day 0, administration of BaP at day 8 and sampling at day 10. Cd+BaP: Administration of cadmium at day 0, administration of BaP at day 15 and sampling at day 17. PCB-156: Administration at day 0, and sampling at day 8. BaP+PCB-156: Administration of BaP at day 0, administration of PCB-156 at day 2 and sampling at day 10. Cd+PCB-156: Administration of cadmium at day 0, administration of PCB-156 at day 15 and sampling at day 23. Cd: Administration at day 0 and sampling at day 15. BaP+Cd: Administration of BaP at day 0, administration of cadmium at day 2 and sampling at day 17. PCB-156+Cd: Administration of PCB-156 at day 0, administration of cadmium at day 8 and sampling at day 23.

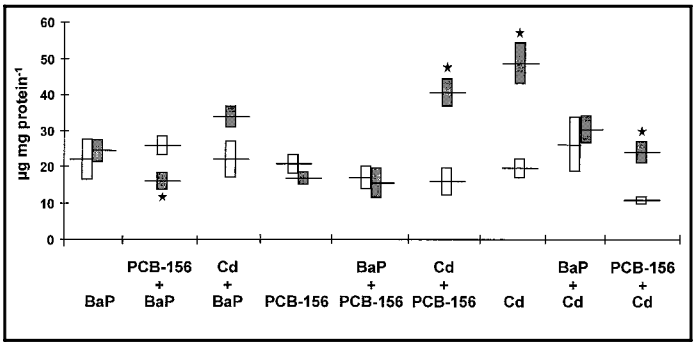

Figure 4. Hepatic metallothionein (MT) levels in flounder following subcutaneous administration of BaP, PCB-156 or cadmium, either singly or in different combinations. Treated fish are represented by closed bars (means±SEM (6)). Open bars represents corresponding controls injected with the vehicles only. ★: Significantly different from corresponding control group as determined with Student's *t* test. Treatment: BaP: Administration at day 0 and sampling at day 2. PCB-156+BaP: Administration of PCB-156 at day 0, administration of BaP at day 8 and sampling at day 10. Cd+BaP: Administration of cadmium at day 0, administration of BaP at day 15 and sampling at day 17. PCB-156: Administration at day 0, and sampling at day 8. BaP+PCB-156: Administration of BaP at day 0, administration of PCB-156 at day 2 and sampling at day 10. Cd+PCB-156: Administration of cadmium at day 0, administration of PCB-156 at day 15 and sampling at day 23. Cd: Administration at day 0 and sampling at day 15. BaP+Cd: Administration of BaP at day 0, administration of cadmium at day 2 and sampling at day 17. PCB-156+Cd: Administration of PCB-156 at day 0, administration of cadmium at day 8 and sampling at day 23.

MT

Administration of cadmium alone caused significantly elevated concentrations of hepatic MT (2.5-fold) 15 days after injection (Figure 4).

A significant reduction (37%) in MT levels was observed when BaP was injected prior to cadmium, as compared with treatment with cadmium alone, and the MT level was not significantly different from the corresponding control group (Figure 4). Similarly, flounder exposed to cadmium 15 days prior to re-injection with BaP had 30% lower MT levels than in fish injected with cadmium alone (Figure 4).

In the group pre-treated with PCB-156 and then injected with cadmium, the MT level was significantly lower (50%) than in the group receiving cadmium only, but the levels were still significantly elevated (2.5-fold) as compared with the corresponding control group (Figure 4). The same relative induction was also observed when cadmium was administered before PCB-156 (Figure 4).

The mixed treatment with PCB and BaP appeared to depress the MT level when PCB was administered prior to BaP (Figure 4), but not when the toxicants were administered in the reverse order (Figure 4). However, neither BaP nor PCB-156 appeared to influence the MT response when these chemicals were administered alone (Figure 4).

GST

Hepatic glutathione S-transferase activities towards CDNB and ETHA were significantly elevated (2-fold) in flounder administered cadmium, either alone or following pre-treatment with BaP or PCB-156 (Figure 5). However, induction was abolished when cadmium was administered prior to BaP or PCB-156. GST activities in controls injected with acetone:Alkamuls (controls for BaP and PCB injections) after filtered seawater:Alkamuls (controls for cadmium injection), were higher (significant at day 23), than in controls injected with the same vehicles in reverse order (results not shown). The selenium-independent GST peroxidase activity towards CU appeared to be unaffected by any treatment in the present study (Figure 5).

Discussion

Exposure regime

In previous studies with flounder given intramuscular injections, occasionally a smaller fraction of the injected dose was leaking out of the injection spot. Subcutaneous administration seemed to solve this problem and was chosen as an alternative exposure regime. Hepatic levels of cadmium and biliary levels of BaP were similar following both routes of exposure. Levels of PCB-156 after 8 days, however, appeared to be lower following subcutaneous rather than intramuscular administration (4.3±1.1 and 18.6±2.7 µg g^{-1} liver w.w., respectively).

CYP1A

The present study demonstrated that CYP1A in flounder was responsive to treatment with both BaP and PCB-156. The differences in temporal induction patterns following treatment with BaP or PCB-156 have been established earlier (Beyer *et al.* 1997b), where the observed maximum induction occurred 2 and 8 days after intramuscular injections with BaP and PCB

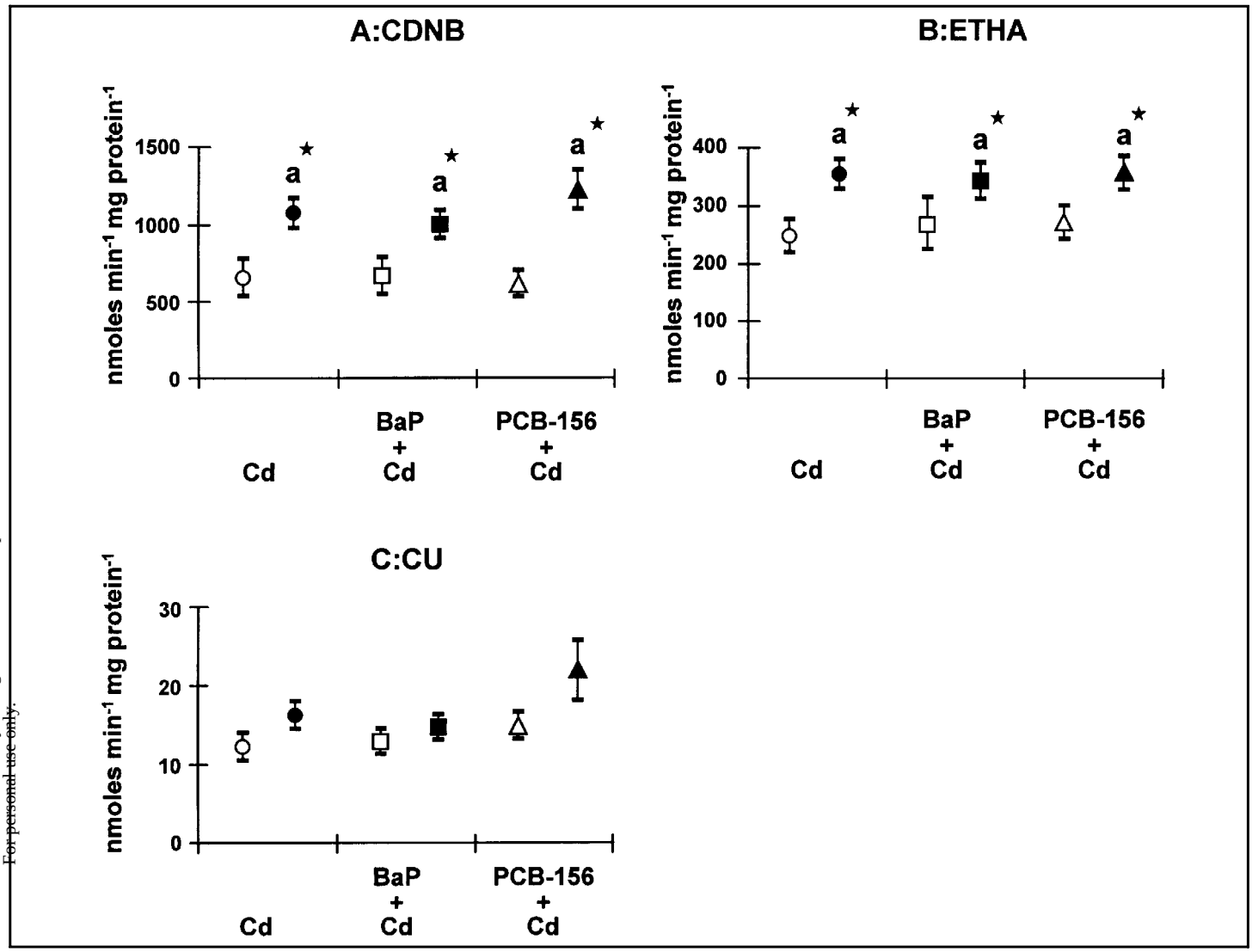

Figure 5. Hepatic glutathione-S-transferase (GST) activities towards 1-chloro-2,4 dinitrobenzene (CDNB) (A), ethacrynic acid (ETHA) (B) and GST selenium independent peroxidase activities towards cumene hydroperoxide (CU) (C) following subcutaneous administration of cadmium alone (●), injection of BaP 2 days prior to cadmium injection (■) or injection of PCB-156 8 days prior to cadmium injection (▲). The flounder were sacrificed 15 days after the cadmium treatment. Open symbols represents corresponding controls injected with the vehicles only. Values are means±SEM (6). ★: Significantly different from corresponding control group as determined with Student's *t* test.

respectively. The results are generally in accordance with previous studies (Skaare *et al.* 1991, Bernhoft and Skaare 1994, Levine *et al.* 1994, van der Weiden *et al.* 1994). The PCB congener used in the present study (2,3,3′,4,4′,5-PCB, IUPAC no. 156) is a mono-*ortho* substituted analogue of PCB-126, one of the ultimate toxic PCBs in the non-*ortho* class. The single extra *ortho*-chlorine of PCB-156 lowers both the toxicity and CYP1A-inducing potency considerably as compared with PCB-126. However, because mono-*ortho* PCBs (e.g. PCB-105, -118, and -156) occur in higher environmental concentrations than their non-*ortho* counterparts, their environmental impact is considered to be highly significant (Tanabe 1992, De Voogt *et al.* 1990).

A less than additive effect in the CYP1A response was observed in the mixed BaP–PCB treatment groups. When BaP was injected prior to PCB, the lack of contribution from BaP may be explained by the short duration of CYP1A induction following BaP exposure, as was observed in the previous time-course study with flounder (Beyer *et al.* 1996b). However, in the same study, the CYP1A induction of PCB-156 was more persistent. The CYP1A activity was still significantly elevated 16 days following PCB-156 treatment, but there was no apparent contribution from the PCB treatment when re-injected with BaP. The CYP1A levels in groups treated with either BaP or PCB-156, singly or in any combination, were not significantly different when compared using one-way ANOVA and Tukey–Kramers HSD test. This may indicate that enzyme saturation was already reached by administration of both compounds alone.

In the present study, there was no influence of cadmium exposure alone on the CYP1A in flounder, but EROD induction by BaP was significantly suppressed by prior exposure to cadmium, whereas CYP1A protein levels remained unchanged.

Inhibition of CYP1A by environmental chemicals such as some PCBs and metals on CYP1A has been demonstrated earlier in fish (Fent and Stegeman 1993, Stegeman and Hahn 1994). Both CYP1A levels and associated catalytic activities strongly decreased following administration of tributyltin (Fent and Stegeman 1993) and cadmium (George and Young 1986, George 1989). The inhibition caused by cadmium was dose-dependent, at 1 mg kg^{-1} a 90% decrease was observed in plaice (*Pleuronectes platessa*). However, induction of CYP1A has also been observed with cadmium in European eel (*Anguilla anguilla*) and European sea bass (*Dicentrarchus labrax*) (Lemaire-Gony *et al.* 1995). In sea bass, cadmium treatment caused a 10-fold increase in EROD activity. The contradictory nature of such observations illustrates the need for obtaining knowledge about the specific species to be used in monitoring. The abolished CYP1A induction of 3-MC by coinjection with cadmium in plaice suggested that this was due to a decrease in enzyme protein rather than direct inhibition of activity by cadmium (George 1989). Furthermore, Means *et al.* (1979) have shown that the decrease in CYP protein levels in cadmium-treated rats was due to both a decrease in protein synthesis and increased degradation of the CYP enzyme by a haem oxygenase.

CYP1A induction by PCB-156 appeared to be unaffected by pre-treatment with cadmium. The mechanisms responsible for this difference are unknown. Halogenated aromatic hydrocarbons, such as PCBs, are slowly metabolized and they continue to induce synthesis of CYP1A mRNA and protein over time (Hahn and Stegeman 1994), while readily metabolized inducers like BaP do not produce continued transcriptional activation as they are metabolized. Other mechanisms such as differential protein stabilization or enhanced translation of a minor mRNA pool, might contribute to persistence of CYP1A (Kloepper-Sams and Stegeman 1994).

Metallothionein

The presently observed MT induction in flounder following injection of cadmium alone confirms earlier studies with other teleost species, which have demonstrated that the hepatic MT level is induced (in a dose-dependent manner) following metal exposure (Roch and McCarter 1984, George 1989, Hogstrand and Haux 1990). Furthermore, the hepatic MT level in fish appears not to be induced by environmental pollutants other than metals (Overnell and Abdullah 1988, Sulaiman *et al.* 1991).

As in the present study, where both BaP and PCB-156 pre-treatment exerted an inhibitory effect upon the MT induction response towards cadmium, inhibitory influences of mixed exposure regimes on fish MT responses have been recorded by several other investigators. For example, in plaice (*Pleuronectes platessa* L.), George and Young (1986) injected a mixture of 3-methylcholanthrene and cadmium, and found that induction of hepatic MT was delayed 1 week compared with when cadmium was administrated alone.

Inhibition of MT induction, as presently observed when PCB was injected prior to cadmium, is likely to increase the metal toxicity. Such interaction effects may explain the mortality observed in this treatment group.

GST

Hepatic glutathione S-transferase activities towards CDNB and ETHA in control flounder were in the range reported by George (1989). The results indicated that BaP and PCB-156 had no effect on glutathione S-transferase activities in flounder. Treatment with 3-methylcholanthrene (3-MC) depressed GST activities in flounder (Scott *et al.* 1992), whereas no effect was seen in Fathead minnows and Sheepshead minnows (James *et al.* 1988). In contrast, induction by 3-MC has been observed in plaice (George and Young 1986). Similar interspecies variations have been observed following treatment with commercial PCB mixtures such as Arochlor 1254 and Clophen A50 (Andersson *et al.* 1985, Scott *et al.* 1992). It is possible, as in mammals, that CYP1A inducers have different effects on various GST isoforms. CDNB activity is an integration of the activity of nearly all of the different GST isoenzymes and does not provide information on the isoform composition. Quantification of the various GST subunits using high performance liquid chromatography has proven more informative as regards isoform composition than either activity measurements or immunohistochemistry (Ostlund Farrants *et al.* 1987, Parola *et al.* 1993, Egaas *et al.* 1994).

GST activities towards CDNB and ETHA were significantly elevated in flounder administered cadmium, either alone or following pre-treatment with BaP or PCB-156. Intraperitoneal coinjection of BaP and cadmium produced a significant increase in GST activities in black sea bass (*Centropristis striata*), whereas the induction was abolished when cadmium was injected prior to BaP (Fair 1986). In our study, the induction was suppressed when cadmium was administered prior to BaP or PCB-156. GST activities in controls injected with acetone:Alkamuls after filtered seawater:Alkamuls, were higher (significant at day 23) than in controls injected with the same vehicles in reverse order, suggesting a possible acetone effect on CDNB activity in flounder.

Summary

Given the widespread pollution of the marine environment by mixtures of both organic pollutants and heavy metals, and their potential to modify the function and induction of biomarkers, further studies of pollutant interaction effects should be conducted in fish. The present interaction study with flounder has investigated such effects for three model pollutants (BaP, PCB-156, cadmium) and three biomarkers (CYP1A, metallothionein and GST). These experiments have demonstrated that chemical mixtures may affect biomarker responses differently from compounds given alone, and that both CYP1A and metallothionein are influenced by pollutants other than their primary inducers. Also, the sequence of exposures may be of importance for the biomarker signal. A number of other pollutants (and biomarkers) are candidates for similar investigations. Such information is indispensible for the application and implementation of fish biomarkers for pollution studies in natural fish populations inhabiting polluted coastal areas.

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