BIOMARKERS, VOL. 8, NO. 6 (NOVEMBER–DECEMBER 2003), 491–508

Biomarker responses in juvenile rainbow trout (*Oncorhynchus mykiss*) after single and combined exposure to low doses of cadmium, zinc, PCB77 and 17β-oestradiol

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Received 19 December 2001, revised form accepted 8 October 2003

The objective of this study was to examine (i) the biochemical responses of rainbow trout exposed to sublethal water concentrations of the metals cadmium (Cd) (1.5 $\mu g\,l^{-1}$) and zinc (Zn) (150 $\mu g\,l^{-1}$); and (ii) the potential combined effects when applied in mixture (Cd/Zn) with and without co-exposure to model organic chemicals 3,3′,4,4′-tetrachlorobiphenyl (PCB77) (1 $mg\,kg^{-1}$) and 17β-oestradiol (E2) (0.5 $mg\,kg^{-1}$). After 21 days of exposure, several biomarkers were assessed in the liver (enzymatic and non-enzymatic antioxidants, heat shock proteins [HSP70 and HSP60], ethoxyresorufin-*O*-deethylase [EROD]) and in the plasma (vitellogenin [Vtg], aminotransferases). Plasma aminotransferases were not affected, whereas the other biomarkers showed different patterns of response depending on the treatment. For example, Cd, and Zn to a lesser extent, induced an adaptive response in the liver shown by an increase in antioxidant defences (total glutathione [GSH], superoxide dismutase, Trolox equivalent antioxidant capacity [TEAC]), without any impairment of GSH redox status or induction of heat shock proteins. Antagonistic effects were observed in GSH-related biomarkers after Cd/Zn exposure. PCB77 strongly induced EROD activity, HSP70 and TEAC. Co-exposure with metals did not modulate significantly the effects of PCB77. E2 induced Vtg and inhibited liver antioxidants and basal EROD activity. These inhibitory effects were suppressed in fishes exposed to E2 + Cd/Zn, suggesting additive effects of E2 and metals. In addition, E2-induced Vtg was not altered by metals. Multivariate analyses confirmed some correlation between the biomarkers. The use of complementary biomarkers is necessary to discriminate different treatments and to highlight interactive effects.

Keywords: environmental concentrations, oxidative stress, heat shock protein, cytochrome P450 1A, metal accumulation, principal component analysis, factor correspondence analysis.

Introduction

Molecular and cellular biomarkers measured in aquatic organisms respond rapidly to the stress caused by environmental contaminants, and can be used to assess the health status of organisms and to obtain early-warning signals before irreversible damage occurs at a higher level of biological organization (Huggett *et*

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Biomarkers ISSN 1354-750X print/ISSN 1366-5804 online © 2003 Taylor & Francis Ltd
http://www.tandf.co.uk/journals
DOI: 10.1080/1354750031000164003 7

al. 1992). In a multi-pollution context, it is now recognized that the use of a series of biomarkers is necessary to provide a good understanding of the actual impact of contaminants on organisms. A critical aspect of the multi-biomarker approach is the selection of complementary biomarkers in order to obtain the most complete and reliable information (Cajaraville *et al.* 2000, De Lafontaine *et al.* 2000). The choice of biomarkers to be assessed is most often determined *a priori*, by considering their physiological role and the (eco)toxicological significance of their responses as characterized in laboratory studies. However, there is a lack of information concerning the numerous potential interactions between contaminants involved in the mechanisms responsible for biochemical, cellular or physiological responses. Providing evidence of the mechanisms involved for each chemical element alone constitutes the first step in understanding how they can interfere when they are present in mixtures.

In fish, cellular and molecular responses to contaminants have been characterized for a large number of metals and organic chemicals at sublethal doses. Essential and non-essential metals, such as zinc (Zn) and cadmium (Cd), respectively, are widespread contaminants of the aquatic environment and, as cadmium is a residue of the extraction of zinc, these two metals are frequently associated in the metallic pollution of freshwater ecosystems. However, the majority of toxicity studies in fish have been focused on short-term exposures to single chemicals, often at relatively high concentrations. Little is known about the responses in fish to environmentally relevant chronic exposures to low concentrations of a mixture of chemicals (Dethloff *et al.* 1999). Moreover, data on the interactive effects of metal/metal and of metal/organic mixtures on biomarker responses in fish are scarce. Synergistic effects have been reported for metal mixtures in acute toxicity testing (Newman and McCloskey 1996, Sharma *et al.* 1999), but their combined effects on metal detoxification or tolerance at sublethal doses are still unclear (Dethloff *et al.* 1999, Lange *et al.* 2002). Metals such as Cd interfere with certain biochemical responses induced by organic contaminants, such as ethoxyresorufin-*O*-deethylase (EROD) activity (Beyer *et al.* 1997, Whyte *et al.* 2000) or oestrogen receptor (ER) transcriptional activity (Olsson *et al.* 1995, LeGuevel *et al.* 2000). However, these mechanisms have been described either *in vivo* after intraperitoneal administration of metals or in *in vitro* studies involving concentrations that are often higher than those found in the environment. Hence, there is still a need to increase our understanding of the molecular mechanisms involved in biomarker responses at environmentally relevant concentrations of chemical mixtures.

The objective of this study was to examine several biochemical and cellular responses in juvenile rainbow trout when exposed to environmentally relevant concentrations of Cd and Zn, and the possible synergistic effects with two organic chemicals, 3,3′,4,4′-tetrachlorobiphenyl (PCB77) and 17β-oestradiol (E2), administered either as single chemicals or in combination. PCB77, a widespread environmental contaminant, is a potent aryl hydrocarbon receptor (AhR) agonist (Safe 1994). It has been shown to induce biotransformation enzymes and to generate oxidative stress in fish liver (Otto *et al.* 1997). E2, an endogenous oestrogen in vertebrate animals, was used as a model of oestrogenic effect. This

hormone has been frequently detected in municipal effluent and in surface waters at biologically active concentrations of around 10 ng l^{-1} (Ternes *et al.* 1999). We measured the antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR), as well as non-enzymatic antioxidants such as glutathione (GSH), the GSH redox status and the total antioxidant capacity, as determined by a modification of the Trolox equivalent antioxidant capacity (TEAC) assay (Miller *et al.* 1993). Besides biomarkers of oxidative stress, further endpoints were measured in the liver, including the molecular chaperons heat shock proteins HSP70 and HSP60 as non-specific biomarkers of proteotoxicity (Ryan and Hightower 1996), hepatic EROD activity as an indicator of PCB77 exposure (Whyte *et al.* 2000), and plasma vitellogenin (Vtg) as a biomarker of E2 exposure (Sumpter and Jobling 1995). In the plasma, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were used as indicators of liver dysfunction (Mayer *et al.* 1992). Liver Cd and Zn measurements were used to link metal accumulation with the investigated biochemical responses.

Materials and methods

Fish maintenance and chemical exposure

Juvenile rainbow trout *Oncorhynchus mykiss* (mean weight 10.04 ± 0.14 g) were obtained from a fish farm (Pisciculture du Petit Large, Vaucluse, France) and were maintained in the laboratory for 2 weeks at 12 ± 0.5°C in 500 l tanks with oxygenated water (pH 7.85, conductivity 421 µS cm^{-1}, 145 mg l^{-1} HCO_3^-). Throughout the experiment, including the acclimatization phase, the fish were fed with standard pellet trout food (160 µg Cd kg^{-1} dry weight) at 1% of the mean fish body weight per day. For the chemical exposure, 10 groups of trout ($n = 45$ for each) were placed in 80 l tanks and exposed to chemical treatments as described in table 1 for 21 days. Cd and Zn were added to the water at concentrations of 1.5 µg l^{-1} and 150 µg l^{-1}, respectively, from aqueous stock solutions of $CdCl_2$ and $ZnCl_2$ (Merck) acidified with 2% HCl. PCB77 and E2 (Promochem) were dissolved in corn oil and administrated by two successive intraperitoneal injections on day 0 and day 14. Two control groups, one for waterborne exposure and one for corn oil injection (carrier control), were used. To allow comparison

Table 1. Organic compound treatments (total dose per kg fish administrated by two intraperitoneal injections on day 0 and day 14 of the experiment) and measured metal concentrations in the water during the experiment.

	Organic compounds			Metals in the water[a]	
	Corn oil (µl g^{-1})	E2 (mg kg^{-1})	PCB77 (mg kg^{-1})	Cd (µg l^{-1})	Zn (µg l^{-1})
Control	–	–	–	–	–
Cd	–	–	–	1.24 ± 0.15	–
Zn	–	–	–	–	158 ± 7
Cd/Zn	–	–	–	1.15 ± 0.10	160 ± 8
Carrier control	4	–	–	–	–
E2	4	0.5	–	–	–
PCB77	4	–	1	–	–
Oil + Cd/Zn	4	–	–	1.17 ± 0.12	157 ± 10
E2 + Cd/Zn	4	0.5	–	1.21 ± 0.09	156 ± 15
PCB77 + Cd/Zn	4	–	1	1.18 ± 0.12	157 ± 11

[a] Time pondered means (±95% confidence intervals) of metal concentrations measured daily throughout the experiment.

between the treatments with organic chemicals and metals, one fish group exposed to the waterborne mixture Cd/Zn received injections of corn oil alone (oil + Cd/Zn). The water in the metal-contaminated and control aquariums was completely renewed three times a week in order to limit alterations to its physicochemical characteristics.

Metal analyses

The Cd and Zn content were determined in the water throughout the experiment (table 1) and in fish liver at the end of the 21 day exposure period. For each experimental condition, the Cd and Zn content in the liver for five fish was measured after organ digestion in a glass tube with a screw stopper (65% HNO_3, 3 h, 105°C, Blockdigest). Cd was measured using graphite furnace atomic absorption spectrometry (Perkin Elmer 4110 ZL) and Zn using flame atomic absorption spectrometry (Varian, Spectro AA 200). The limits of detection of Cd and Zn were 0.1 $\mu g\,l^{-1}$ and 10 $\mu g\,l^{-1}$, respectively.

Plasma and S9 liver sample preparation

After the 21 day chemical exposure period, 10 fishes per treatment were sacrificed and weighed. All steps were carried out on ice or at 4°C. For plasma sample preparation, blood was taken from the caudal vein using a heparinized syringe (lithium heparin plasma microtubes, Sarstedt). Samples were pooled by two in order to obtain enough material for analyses and subjected to a centrifugation (3000 *g* for 15 min at 4°C). The supernatants were frozen in liquid nitrogen and stored at −80°C until use. For liver sample preparation, the fish were opened, and the liver and kidney were excised and weighed to determine liver and kidney somatic indices (LSI and KSI). The liver was rinsed in KCl 150 mM and homogenized for 2 min on ice in 4 volumes of cold homogenization buffer (100 mM Na_2HPO_4, 20% w/v glycerol, 0.2 mM phenylmethylsulphonide fluoride, pH 7.8) using a motor-driven Teflon potter. The homogenate was centrifuged at 10 000 *g* for 15 min at 4°C. The S9 fraction was aliquoted and immediately stored at −80°C until analyses. The total protein content in the samples was determined on the day of dissection using the method of Bradford (1976). All other biochemical assays were carried out within 1 month, except for the GPx and GR assays for injected fishes, which were performed after 3 months of storage.

Plasma biomarkers

AST and ALT were measured by a Cobas Fara automated multi-analyser using commercial kits (Hoffman La Roche, Basel, Switzerland) according to the manufacturer's instructions. Vtg levels were determined by a competitive enzyme-linked immunosorbent assay as previously described (Brion *et al.* 1999), using anti-salmon Vtg antibodies (BN5, Biosense Laboratories, Norway) with purified rainbow trout Vtg as the standard.

Hepatic biomarkers

GSH and GSH redox status. Total gluthione (tGSH) and oxidized glutathione (GSSG) were determined after precipitation of S9 proteins with 5% trichloroacetic acid (TCA) according to the 96-well method described by Baker *et al.* (1990). The GSH redox status was expressed as the ratio between GSSG, as the GSH equivalent, and tGSH.

Antioxidant enzymes. Total GPx, SOD and GR were assessed in microplate format according to the methods of Paglia and Valentine (1967), Paoletti *et al.* (1986) and Carlberg and Mannervik (1985), respectively.

Total antioxidant activity. The TEAC was determined according to the method described by Miller *et al.* (1993), with one major modification. The S9 samples were first treated with 5% v/v TCA, centrifuged for 5 min at 4°C, and the supernatants used for the TEAC assay. This step was necessary to avoid high interference of liver proteins with the assay, as noted in preliminary experiments (data not shown). The antioxidant activity was expressed as the equivalent Trolox concentration (in mM mg^{-1} total proteins) eliciting the same antioxidant effect as the sample.

Heat shock proteins. HSP70, HSP60 and β-actin contents in the liver were evaluated by Western blot. In brief, 10 μg of total proteins were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis in 12% polyacrylamide running gels (Laemmli 1970), and electrotransferred onto 0.45 μm nitrocellulose membranes (BioRad Mini Trans-Blot). Probing was performed using a mix of mouse monoclonal antibodies (Sigma) directed against bovine brain HSP70 (BRM-22 clone, 1:5000 dilution), recombinant human HSP60 (LK2 clone, 1:5000 dilution) and the C-terminal of actin (AC-40 clone, 1:600 dilution). The secondary antibody was goat anti-mouse IgG labelled with horseradish peroxidase (BioRad). Immunoreactive band visualization was performed using enhanced chemiluminescence reagents (Amersham) and the blots were scanned and analysed using the ImageMaster® software

(Pharmacia). Three samples per treatment, corresponding to three different fish, and 100 ng of standard HSP70 (H7283, Sigma) were applied to the same gel. The anti-HSP70 monoclonal antibody, which recognizes both the constitutive and inducible forms of HSP70, cross-reacted with two protein bands: an upper band with a relative molecular weight (rMW) of 78 kDa (arbitrarily called HSP70a) and a lower band with a rMW of 72 kDa (called HSP70b) that migrates to the same level as the human inducible HSP72 (figure 1). Similar HSP70 patterns were detected in the dab liver using the same anti-HSP70 antibody (Schröder *et al.* 2000). The heat shock protein (HSP) signal was normalized relative to the actin signal, and the final results were expressed as a percentage of the control value.

EROD activity. EROD was determined using the 96-well plate method described by Flammarion *et al.* (1998).

Statistical analyses

For analyses of variance (ANOVA), the data were divided into two groups according to the type of treatment (group 1, waterborne; group 2, intraperitoneal exposure), and the effects were analysed by comparing the results in the exposed fishes to those in the water and carrier controls. To conform to the normality assumption (Lilliefors test, based on a modification of the Kolmogorov–Smirnov test) and to the homogeneity of the variances (Levene's test), some of the biomarker data were log-transformed (group 1: GSSG, 2GSSG/tGSH, GR and TEAC; group 2: GSSG, GR, HSP70a, HSP70b, HSP60 and EROD). Significant effects of chemicals were determined using one-way ANOVA, followed by a unilateral Dunnett's test. The interactive effects of Cd and Zn on biomarkers were determined by a generalized linear model, with the Cd and Zn nominal water concentrations as fixed co-factors. ANOVAs were carried out using the SPSS® v10.1 software.

Multivariate analyses. To explore the correlation between biomarker variables, a principal component analysis (PCA) was run from a data matrix of 10 liver biomarker variables as columns and 10 treatment groups as lines. Due to scale differences between variables, the variables were centred using their control value and normalized towards their standard deviation. A non-centred PCA was then used to avoid any further normalization. To represent the overall relationships between biomarker variations and treatments on the same graph, a correspondence factor analysis (CFA) was used. The variables were centred to the control value and duplicated using their absolute value as an induction variable (var+) and an inhibition variable (var−) to fit the requirements of positivity in the metric of correspondence analysis. PCA and CFA calculations were carried out using the ADE-4 statistical software (Thioulouse *et al.* 1997).

Results

Bioaccumulation of metals in the liver

Table 2 summarizes the results for Cd and Zn levels in the liver after exposure to the different treatments. All fishes exposed to Cd alone or in combination with other chemicals accumulated this metal up to levels that were nine times those in

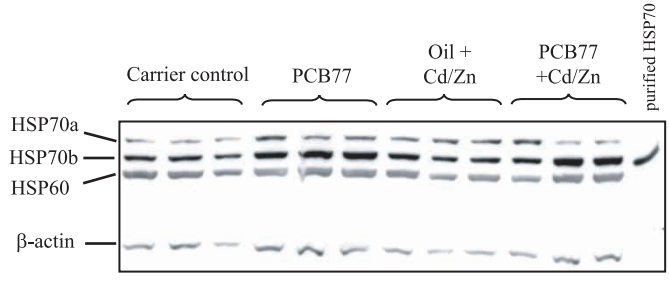

Figure 1. Representative Western blot for liver HSPs and β-actin expression after exposure to carrier, PCB77, oil+Cd/Zn and PCB77+Cd/Zn. Each lane represents one individual fish; 10 μg of total protein were loaded per lane.

Table 2. Cd and Zn concentrations in the trout liver for the different groups after the 21 day exposure period.

Group	Cd (ng g^{-1} fresh weight)	Zn (μg g^{-1} fresh weight)
Control	5±3	32±7
Cd	45±8 *	28±4
Zn	12±9	33±3
Cd/Zn	43±11*	29±8
Carrier control	6±4	27±4
E2	5±3	33±6
PCB	11±7	29±3
Oil+Cd/Zn	39±12*	32±6
E2+Cd/Zn	48±6*	35±9
PCB77+Cd/Zn	50±12*	31±5

Values given are the mean±95% confidence interval ($n = 8$).
* Significantly different from control or carrier control ($p < 0.05$).

the control groups. Co-exposure with other chemicals did not significantly alter the level of Cd accumulation. No significant variation in liver Zn content was detected.

Effects on survival, somatic indices and plasma parameters

The different chemical treatments did not alter fish survival or growth. Table 3 summarizes the results for organ somatic indexes and plasma parameters. No effect was observed on the KSI and LSI, although a slight increase in the LSI was noted after E2 and PCB77+Cd/Zn exposure. The plasma AST and ALT levels did not differ significantly after chemical treatment compared with the control groups. These results indicate that the chemical treatments did not induce acute toxic effects in the liver. Concomitant increases in Vtg levels were measured in fishes treated with E2 and E2+Cd/Zn (table 3), while Vtg was undetectable (< 300 ng Vtg ml^{-1} plasma) in all other groups. The Vtg concentrations in the E2 and the E2+Cd/Zn treated fishes were not statistically different.

Table 3. Effect of the various chemical treatments on somatic indexes and plasma parameters.

Group	LSI (% total weight)	KSI (% total weight)	AST ($U\,l^{-1}$)	ALT ($U\,l^{-1}$)	Protein (mg ml^{-1})	Vtg (mg ml^{-1})
Control	1.4±0.1	1.0±0.3	521±51	45±10	29±6.4	ND[d]
Cd	1.3±0.1	1.0±0.2	584±38	40±6.4	28±2.6	ND
Zn	1.4±0.1	0.9±0.1	538±96	43±8.3	27±3.2	ND
Cd/Zn	1.3±0.1	0.8±0.1	467±63	39±8.5	25±2.3	ND
Carrier control	1.3±0.1	0.8±0.1	686±81	63±10	30±4.4	ND
E2	1.5±0.2	0.9±0.1	668±50	58±6.4	32±3.7	0.87±0.26
E2+Cd/Zn	1.3±0.1	0.8±0.2	641±39	70±11	35±5.5	0.80±0.14
PCB77	1.4±0.1	0.8±0.1	640±33	54±4.8	26±1.2	ND
PCB77+Cd/Zn	1.5±0.1	0.9±0.1	643±53	61±7.6	24±2.1	ND
Oil+Cd/Zn	1.4±0.2	1.0±0.2	620±40	57±5.2	28±3.4	ND

Values given are the mean ±95% confidence interval ($n = 5$).
ND, not detectable.

Table 4. Effect of a 21 day waterborne metal exposure on trout hepatic biomarkers.

Biomarker	Control	Cd	Zn	Cd/Zn
TGSH (nmol mg^{-1} protein)	16.2±4.1	27.3±5.8**	20.2±3.1	23.3±3.8**
GSSG (nmol mg^{-1} protein)	3.0±2.5	4.4±3.6	5.6±3.3	1.4±0.7
2GSSG/tGSH	0.34±0.28	0.35±0.28	0.55±0.28	0.11±0.05
TEAC (mM mg^{-1} protein)	7.0±1.5	9.1±1.6*	8.9±4.2	10.2±2.6*
SOD (U mg^{-1} protein)	15±5.1	22±5.1*	24±7.2*	29±9.2**
GPx (U g^{-1} protein)	187±55	182±49	151±63	157±59
GR (U g^{-1} protein)	34±11	68±50	49±16*	34±9
HSP70a[a]	1.0±0.5	1.2±1	2.1±1.1*	1.0±0.8
HSP70b[a]	1.0±0.1	1.2±0.2	1.2±0.5	1.1±0.2
HSP60[a]	1.0±0.1	0.8±0.3	1.0±0.5	0.8±0.2
EROD (pmol resorufin min^{-1} mg^{-1} protein)	3.9±2	7.7±3.7*	9.4±3.8**	10.6±5**

Values given are the mean ±SD ($n = 8–10$).
* $p < 0.05$ and ** $p < 0.01$ compared with control.

Hepatic biomarker responses to waterborne Cd and/or Zn

The effects of metals on hepatic biomarkers are summarized in table 4. Cd exposure provoked an increase in the non-enzymatic (tGSH and TEAC) and enzymatic (SOD and GR) antioxidant defences, although the effect on GR was not statistically significant due to a high variability in the Cd group. These antioxidant inductions were not accompanied by alterations in the GSH redox status. Zn exposure induced SOD and GR activities, but had no statistically significant effect on GSH levels or TEAC. Unexpectedly, almost no effect was noted on stress protein induction by both metals, except a significant induction of HSP70a by Zn. Co-exposure to Cd/Zn showed significant interactions between Cd and Zn with regard to tGSH ($p = 0.015$), GSSG ($p = 0.007$), GSH redox status ($p = 0.022$) and GR ($p = 0.013$), as well as on HSP70a ($p = 0.047$), as determined by generalized linear model analysis. These results suggest an antagonistic action of Cd and Zn on the mechanisms responsible for GSH metabolism perturbation by metals. Other biomarkers in the Cd/Zn group were not different from those observed with either Cd or Zn alone. In addition, slight induction of EROD activity (2- to 2.7-fold) by Cd and Zn alone or in mixture was observed.

Liver biomarker responses to E2 and/or metals

Table 5 summarizes the biomarker responses to E2 and/or Cd/Zn. E2 alone caused significant changes in five out of the seven antioxidant parameters. For example, a decrease in the pool of GSH (tGSH and GSSG), TEAC and SOD and an increase in GPx activity were seen. The GSH redox status and GR were not affected by E2. In parallel, a significant inhibition of more than 50% of the basal EROD activity was observed, but no significant effect on HSPs was noted. When E2 was administrated in the presence of Cd and Zn, almost all the inhibitory effects of E2 on antioxidants were abolished, resulting to a return to the carrier control level.

Table 5. Effect of a 21 day exposure to E2 and PCB77, alone and with metals, on trout hepatic biomarkers.

Biomarker	Carrier (oil) control	Oil+Cd/Zn	E2	E2+Cd/Zn	PCB77	PCB77+Cd/Zn
TGSH (nmol mg^{-1} protein)	21.5±6.6	24.6±8.4	12.1±4.9**	21.9±6.6	26.9±6.8	21.8±3.7
GSSG (nmol mg^{-1} protein)	2.5±0.6	1.7±2.3	1.7±0.6**	1.0±0.4**	2.7±1.5	0.5±0.4**
2GSSG/tGSH	0.26±0.11	0.12±0.09**	0.26±0.10	0.09±0.05**	0.19±0.11	0.05±0.03**
TEAC (mM mg^{-1} protein)	6.7±1.6	10.5±2.9**	4.5±1.0*	8.0±1.7	9.3±2.7*	9.1±1.0*
SOD (U mg^{-1} protein)	23±5.4	20±7.6	17±1.9**	18±5.3	25±5.1	19±5.8
GPx (U g^{-1} protein)	48±18	80±41*	92±35*	94±34**	63±45	73±11*
GR (U g^{-1} protein)	29±9	41±26	25±7	25±5	47±25	31±11
HSP70a[a]	1.0±0.4	3.1±2.3*	0.9±0.3[a]	1.6±0.6[a]	1.4±0.7	2.7±2.6
HSP70b[a]	1.0±0.2	1.5±0.4*	0.5±0.2[a]	1.2±0.2[a]	1.8±0.4**	2.4±1.1**
HSP60[a]	1.0±0.3	1.2±0.3	0.8±0.3[a]	0.9±0.3[a]	1.3±0.7	1.4±0.8
EROD (pmol resorufin min^{-1} mg^{-1} protein)	8.6±4.2	6.2±3.7	3.1±2.4**	8.0±3.9	264.4±44**	208±107**

Values given are the mean ±SD ($n = 6–10$, except for [a]$n = 3$).
*$p < 0.05$ and **$p < 0.01$ compared with control.

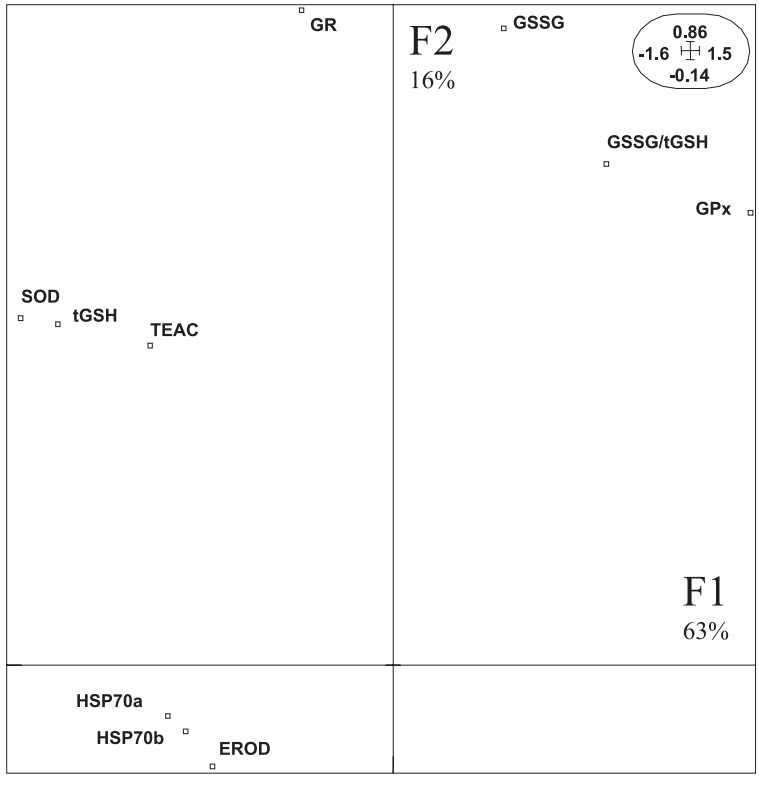

Figure 2. Representation of the biomarkers in the first factorial plane of the PCA. The variables were centred using their control value and normalized towards their standard deviation. A non-centred PCA was then used to avoid any further normalization.

Liver biomarker responses to PCB77 and/or metals

PCB77 induced EROD 30-fold compared with controls, as well as HSP70 (table 5 and figure 1). Co-exposure with metals did not significantly modulate the effect of PCB77 on either HSP70 or EROD. PCB77 alone had virtually no effect on antioxidant defence biomarkers, except a significant increase in the non-enzymatic antioxidant capacity TEAC (table 5). After co-exposure with metals (PCB77 + Cd/Zn), some joint effects were noted on the glutathione pool in favour of the reduced form, with a significant decrease in the GSSG concentration and GSH redox status.

Multivariate analyses

A PCA was run to determine whether there was a correlation between the various chemical treatments and the observed alterations in biomarkers. The locations of the biomarkers and chemical treatments on the first factorial plan of the PCA are presented in figures 2 and 3, respectively. The PCA shows that 79% of the overall variance is explained by the first two factors. The first factor (F1), which was responsible for 63% of the overall variance, is comprised of EROD, the HSPs,

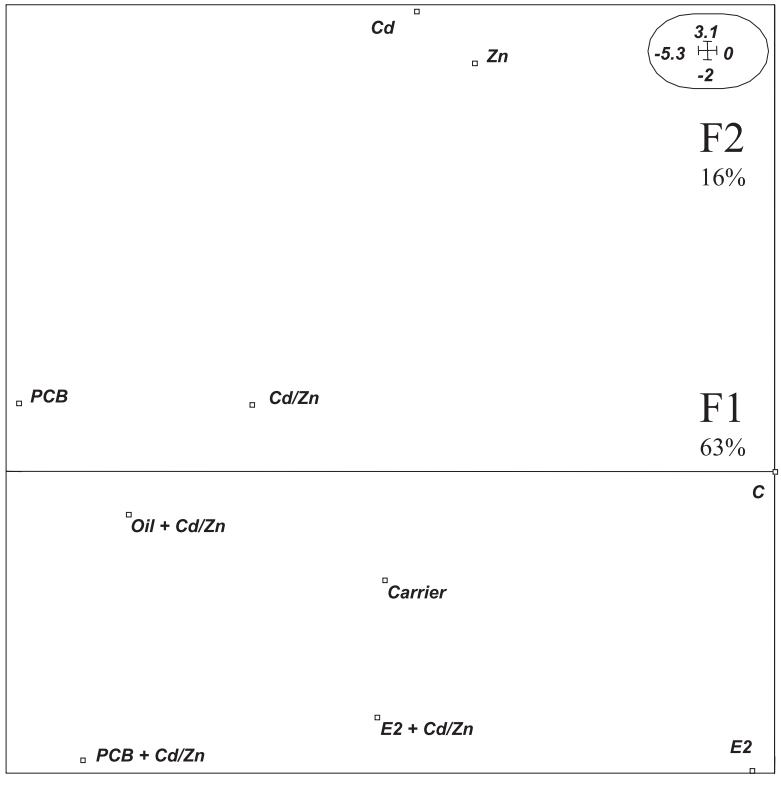

Figure 3. Representation of the treatments in the first factorial plane of the PCA. The variables were centred using their control value and normalized towards their standard deviation. A non-centred PCA was then used to avoid any further normalization. In this way the control value (*C*) lies in the centre of gravity of the plane.

SOD, tGSH and TEAC (figure 2). F1 discriminates mainly the effects of PCB77, PCB77 + Cd/Zn and oil + Cd/Zn (figure 3), as these treatments were potent in altering EROD and/or the stress proteins. The discrimination between the E2, E2 + Cd/Zn and oil + Cd/Zn groups on F1 can be explained by the opposite effects of E2 and metals on non-enzymatic antioxidants, as described above (table 5). The second factor (F2), which was responsible for 16% of the overall variance, explains the effects on GR, GSSG, GPX and GSH redox status, and also discriminates SOD, total GSH and TEAC from EROD and HSP70 (figure 2). Cd and Zn, which induced antioxidants in the liver, are clearly distinguished by F2 (figure 3).

A CFA was run to analyse in a single graph the overall relationships between biomarker variations, i.e. inhibition or induction relative to control, and the various chemical treatments. Figure 4 represents the coordinates of variables and treatments on the first factorial plan of the CFA. The first factor of the CFA (F1; 48% of overall variance) distinguishes the inhibitory effects of E2 on five biomarkers from other variables and treatments, including the E2 + Cd/Zn group. The second factor (F2; 27% of overall variance) clearly demonstrates the relationships between metal exposure and the induction of HSP70a and anti-

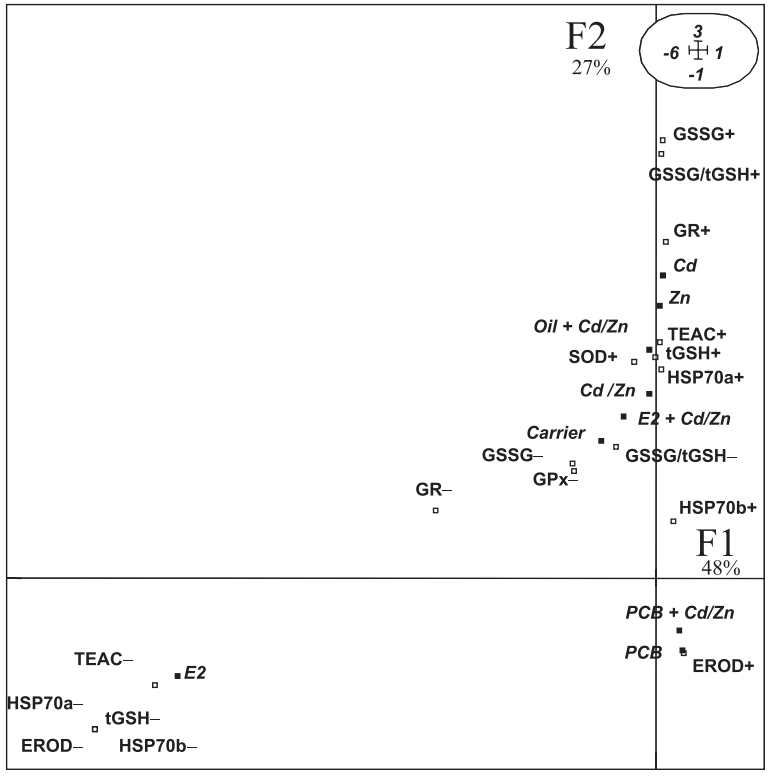

Figure 4. Representation of the treatments (italic, filled squares) and variables (empty squares) on the first factorial plane of the CFA. The variables were centred to the control value and duplicated using their absolute value as an induction variable (var+) and an inhibition variable (var −) to fit the requirements of positivity in the metric of correspondence analysis.

oxidants, among which tGSH, TEAC and SOD appeared to be closely related to the metal treatment. On this axis, discrimination of Cd/Zn from single Cd and Zn exposures can be explained by the induction of GSSG and GSSG/tGSH, thus illustrating the antagonistic effects of these metals on GSH-related biomarkers, as described above (table 4). Interestingly, the CFA also discriminates HSP70b from HSP70a, which were correlated in the PCA factorial plan, as well as EROD from HSP70b induction, the latter being less closely associated with of PCB77 exposure.

Discussion

The sublethal impact of environmentally relevant concentrations of Cd and Zn, and their potential interactive effects with two model organic compounds (E2 and PCB77), were examined in juvenile rainbow trout. The exposure conditions were clearly 'subclinical', as none of the chemical treatments affected either survival and growth of the fish or organ somatic indices (table 3). Moreover, AST and ALT enzymatic activities in the plasma were not altered, suggesting that no severe cellular toxicity occurred in liver or muscle (Mayer *et al.* 1992). All the chemical

treatments induced significant biochemical variations resulting in different patterns of biomarker responses that depended on the nature of the chemical treatment.

Effects of single and combined metal exposure

Metals ions are known to be toxic through oxidative mechanisms (Stohs and Bagchi 1995). The liver biomarker responses to metal exposure suggest an adaptive response in the liver to the oxidative action of metals, as shown by induction of enzymatic (SOD and GR by Cd and Zn) and non-enzymatic (tGSH and TEAC by Cd) antioxidant defences (table 4). Such biochemical responses may be sufficient to counteract the molecular toxic effects of Cd and Zn, as the GSH redox status was not significantly altered, suggesting that antioxidant potency in the liver was maintained. To some extent, these results are in line with previous data in fish reporting an increase in tGSH in the liver, presumably through induction of its synthesis, as a primary biochemical response to Cd and/or Zn exposure, before or concomitantly with the induction of metallothioneins (Kuroshima 1995, Tort *et al.* 1996, Lange *et al.* 2002). In addition, the increase in TEAC caused by Cd in our study may also reflect the potency of this metal to induce GSH, which is detected by the TEAC assay (Miller *et al.* 1993). Indeed, we observed a linear relationship between tGSH and TEAC ($r^2 = 0.59$, $p < 0.001$), and it is likely that this correlation reflected the predominance of GSH amongst the total non-enzymatic antioxidants in the liver (Sies 1999).

In contrast to Cd, Zn is an essential element of cell metabolism, and impairment of cellular Zn homeostasis is a critical factor for its toxicity (Stohs and Bagchi 1995). Similarly to previous data reported in rainbow trout exposed to equivalent Zn concentrations (Dethloff *et al.* 1999, Ausseil *et al.* 2002), Zn did not accumulate in fish liver in our study, suggesting that the metabolic process responsible for its intracellular regulation was not altered by the treatment. Hence, the increase in SOD and GR activities may reflect a cellular physiological response of the liver to a transient increase and intracellular redistribution of Zn cellular content rather than a toxic effect of this metal.

In fish exposed to the metal mixture, the pattern of antioxidant biomarker responses appeared at first glance similar to that of fish exposed to Cd alone, suggesting that the effect of Cd was greater than the overall effect of Zn. This agrees with previous reported data on the combination effects of metal mixtures such as Cd/Zn (Lange *et al.* 2002) or Cu/Zn (Dethloff *et al.* 1999) in fish. Nevertheless, the co-exposure to both metals showed low but significant combined effects on GSSG and GSH redox status, in favour of reducing conditions and, as a consequence, on GR activity (table 4). This may indicate that Zn antagonized the oxidative impact of Cd on intracellular thiols. This could be explained by the known protective action of Zn against the toxicity of redox cycling compounds and other bivalent metals such as Cd (Stohs and Bagchi 1995). The mechanism may involve the prevention of sulphydryl group oxidation, and the inhibition of reactive oxygen species (ROS) production by transition metals by the displacement of redox metal ions from site-specific loci (Stohs and Bagchi 1995).

Heavy metals are known to induce HSPs at sublethal doses in a wide range of organisms either *in vitro* (Dilworth and Timbrell 1998, Aït-Aïssa *et al.* 2000) or *in*

vivo (Williams *et al.* 1996, Werner and Nagel 1997), although only few data have been reported *in vivo* in fish (Iwama *et al.* 1998). The weak effects of waterborne metal exposure, especially Cd, on HSPs (table 4) were thus unexpected, since Cd content increased in the liver after exposure (table 2). It is likely that HSPs already present in the liver of control fish, together with the increased antioxidant defences, are sufficient to repair and/or prevent the alteration of cellular proteins by metals without necessarily enhancing further HSP synthesis. However, a significant increase in both HSP70 isoforms by waterborne metals was noted in the oil+ Cd/Zn group (table 5), suggesting that corn oil induced hepatic stress that altered protein homeostasis, leading to a potentiation of the metal effect on HSP synthesis. The use of intraperitoneal injection using a carrier is critical in environmental studies. However, this route of exposure did not readily influence overall biomarker responses to chemicals, except the HSP response.

EROD and antioxidant inhibition by E2: modulation by metals

The induction of Vtg by E2 (table 3) was an expected outcome, in line with previous data in fish (Sumpter and Jobling 1995). In addition to its oestrogenic effect, E2 inhibited hepatic antioxidants and EROD activity (table 5). The inhibition of cytochrome P450 1A1 by (xeno)estrogens has been reported several times in fish (Arukwe *et al.* 1997, Sole *et al.* 2000, Navas and Segner 2001). Conversely, only a few studies have described the (anti)oxidant effects of (xeno)estrogens in fish, although it has been more extensively reported in mammals. Oestrogens are known to have antioxidant properties (Klinger *et al.* 2002), but they can also exert oxidant effects at higher concentrations *in vivo* through their metabolism by cytochrome P450 enzymes to catechol and quinone oestrogen metabolites that undergo redox cycling and generate ROS (Liehr and Roy 1990). These oestrogen metabolites are able to deplete GSH in the presence of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) in rat hepatocytes (Ruiz-Larrea *et al.* 1993) and appear to be involved in the development of liver injury after oestrogen treatment (Liehr and Roy 1990). In carp (*Cyprinus carpio*), 17α-ethinyloestradiol (EE2) decreased liver P450 and antioxidant (SOD, Se-Gpx and catalase) enzymes after 8 days, although the latter effect did not reach statistical significance (Sole *et al.* 2000). In juvenile sturgeon, waterborne EE2 exposure for 25 days altered the levels of vitamins A and E in kidney and liver (Palace *et al.* 2001). In our study, it is likely that oxidative metabolism of E2 by cytochrome P450 was responsible for EROD inactivation, probably through inhibition of P450 oxidase function by ROS release, and for increased GSH and vitamin consumption, leading to their depletion in the liver. This antioxidant depletion may trigger accumulation of ROS and subsequent oxidative stress, as suggested by alterations in the SOD and GPx activities.

Interestingly, exposure to the E2–metal mixture suppressed the inhibitory action of E2 on antioxidants and EROD activity (table 5). The former effects seem to be additive and suggest that E2 and the tested metals disrupt redox status through independent mechanisms that lead to opposite responses in the antioxidant biomarkers. Moreover, the inability of the tested metals to modulate E2-induced Vtg (table 3) indicates that, at these concentrations, they do not interfere with the

ER-mediated response to E2. To our knowledge, no similar results have been reported in fish, although interaction between the effects of E2 and Cd (but not Zn) on the transcriptional activity of ER has been described in rainbow trout (Olsson *et al.* 1995, LeGuevel *et al.* 2000). However, this inhibition was observed at much higher Cd doses than those used in our study, which may explain the discrepancy with our results. Overall, while further research will be necessary to understand the mechanisms underlying the observed joint effects, our data show that oxidative stress biomarker responses to environmental concentrations of metals are influenced by oestrogens, which could have implications for their interpretation in a multi-pollution context *in situ*.

EROD and HSP70 induction by PCB77

The strong induction of EROD activity by PCB77 (table 5) is in line with the well-described potency of coplanar polychlorinated biphenyls (PCBs) to bind to the AhR and to induce CYP1A expression in the liver (Safe 1994, Otto *et al.* 1997). In mammals and fish liver, PCBs are metabolized by the P450 enzymes to mono- and hydroxyl metabolites, which can be oxidized to the highly reactive corresponding (semi)quinones. These compounds generate ROS and form adducts with and alter macromolecules such as DNA and proteins (Safe 1994, Srinivasan *et al.* 2001). As a consequence, it is likely that the resulting alteration in cellular proteins, by either activated PCB adducts and/or ROS attack, was the trigger for the induction of HSP70 in our study (table 5). The potency of PCB77 to induce HSP70, reported here for the first time in rainbow trout, correlates with previous studies describing HSP70 induction in marine sponge exposed to PCB77, PCB118 and PCB153 congeners (Schröder *et al.* 1999) and in salmon hepatocytes exposed to PCB156 (Grosvik and Goksoyr 1996).

Since there is growing evidence that PCB metabolism by CYP1A is correlated with oxidative stress (Toborek *et al.* 1995, Schlezinger *et al.* 2000), the weak effects of PCB77 on the antioxidant biomarkers in our study were relatively surprising. Nevertheless, antioxidant responses to coplanar PCBs in fish have not been completely elucidated. In PCB77-treated rainbow trout, induction of several GSH-related biomarkers has been reported in the liver (Otto and Moon 1995). Conversely, no effect on hepatic antioxidant enzymes (catalase, GPx and SOD) was observed in lake trout injected with 6.3 mg PCB126/kg, whereas induction of liver lipoperoxidation and depletion of tocopherol and retinol were seen 6 and 13 weeks, respectively, after injection (Palace *et al.* 1996). These oxidant effects in freshwater fish were produced using higher doses than those in our study, which could explain the discrepancies with our data.

Co-exposure to PCB77 and metals did not reveal important interactions at the biochemical level, the effect of PCB77 appearing to predominate that of the metals. No comparable data using similar metal concentrations was found in the literature, although at higher concentrations heavy metals have been shown to inhibit the EROD activity induced by dioxin-like compounds (Beyer *et al.* 1997). Again, the absence of such inhibition in our study may be attributed to the low concentrations of metals used and thus confirmed that these metal concentrations have a weak toxic impact in rainbow trout liver.

Overall relationships between biomarker responses

This study was aimed at identifying complementary biomarkers in fish by assessing the effect of low concentrations of single and combined model chemicals on several biochemical variables. On the basis of their correlation in the PCA, the biomarkers could be divided into three groups: (i) EROD and HSP70; (ii) SOD, tGSH and TEAC; and (iii) GSSG, GSH redox status, GPX and GR (figure 2). The first two groups appeared to be most explanatory of the variability induced by chemical treatments. Secondly, the CFA, which allowed the induction and inhibition of biomarker responses to be distinguished and their correlation with chemical treatments to be explored (figure 4), confirmed the correlation between certain biomarkers (e.g. tGSH, TEAC and SOD induction by metal stress) and highlighted the complementarity between others (e.g. HSP70 and EROD, both markedly induced by PCB77 and correlated in the PCA). The decision to examine several biomarkers of oxidative stress was driven by the complex and non-specific character of their responses (Winston and DiGiulio 1991). Since all of the measured antioxidants were significantly altered by the different treatments in our study, all may be considered as relevant biomarkers for detecting changes in redox status in fish. However, the multivariate analyses suggested that certain biomarkers, i.e. tGSH, TEAC and SOD, appeared to be the most representative of the oxidant effect for this set of chemicals.

In summary, this study provides further information concerning the biomarker responses to low pollutant concentrations in fish. Environmental concentrations of metals induced adaptive biochemical responses by increasing antioxidant defences, which prevented severe oxidative stress. These responses were readily influenced by co-exposure to E2 or PCB77, thereby highlighting potential interactive effects between chemicals. This study supports the view that a combination of several biomarkers is necessary to assess the sublethal impact of the selected chemicals in fish in a multi-pollution context. The measurement of EROD activity, Vtg, HSP70, tGSH (or possibly TEAC) and selected antioxidant enzymes may constitute a suitable battery of complementary biomarkers to assess the early biochemical responses to this set of chemicals.

Acknowledgements

We would like to acknowledge the excellent technical assistance of Chantal Gillet for the plasma biomarker analyses. Special thanks are due to Christelle Adam, Isabelle Cavalié and Brigitte Ksas for their help during the course of the laboratory experiments, and to Dr Giles Velarde (University of Manchester) and Professor Coulomb (University of Utah State) for reviewing the manuscript.

This work was supported by the Budget Civil de Recherche et de Développement (BCRD 00-102) to INERIS and by the Région Provence-Alpes-Cote d'Azur to IRSN.

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