Biomarkers responses in juvenile rainbow trout (Oncorhynchus mykiss) after single and combined exposure to low doses of cadmium, zinc, PCB77 and 17beta-estradiol

Selim Ait-Aissa, Olivier Ausseil, Olivier Palluel, Eric Vindimian, Jacqueline Garnier-Laplace, Jean-Marc Porcher

▶ **To cite this version:**

Selim Ait-Aissa, Olivier Ausseil, Olivier Palluel, Eric Vindimian, Jacqueline Garnier-Laplace, et al.. Biomarkers responses in juvenile rainbow trout (Oncorhynchus mykiss) after single and combined exposure to low doses of cadmium, zinc, PCB77 and 17beta-estradiol. Biomarkers, 2003, 8 (6), pp.491-508. 10.1080/13547500310001640037 . ineris-00961882

HAL Id: ineris-00961882

https://hal-ineris.archives-ouvertes.fr/ineris-00961882

Submitted on 20 Mar 2014

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

S. AÏT-AÏSSA[1*], O. AUSSEIL[3], O. PALLUEL[1], E. VINDIMIAN[2], J. GARNIER-LAPLACE[3] and J-M. PORCHER[1]

[1]Ecotoxicological Risks Assessment Unit, [2]National Institute of Industrial Environment and Risks (INERIS), Direction of Chronic Risks, BP 2, f-60550 Verneuil-en-Halatte, France

[3]Laboratory of Experimental Radioecology, Institute of Radioprotection and Nuclear Safety (IRSN), Department of Environmental Protection, BP 1, f-13108 St Paul-Lez-Durance, France

*corresponding author: fax : +33 344556767 ; e-mail: selim.ait-aissa@ineris.fr

ABSTRACT

The objective of this study was to examine (i) biochemical responses of rainbow trout exposed to sublethal water concentrations of metals, cadmium (Cd, 1.5 µg/L) and zinc (Zn, 150 µg/L) and (ii) potential combined effects when applied in mixture (Cd/Zn) with/without co-exposure to model organic chemicals 3,3',4,4'-tetrachlorobiphenyl (PCB77, 1 mg/kg) and 17-β-estradiol (E2, 0.5 mg/kg). After 21 days of exposure, several biomarkers were assessed in the liver [enzymatic and non enzymatic antioxidants, heat shock proteins (HSP70 and 60), ethoxyresorufin-O-deethylase (EROD)] and in the plasma [vitellogenin (Vtg), aminotransferases]. Plasma aminotransferases were not affected by the treatments, whereas other biomarkers showed different patterns of response in function of the treatment. For example, Cd, and Zn to a lesser extent, induced an adaptive response in the liver shown by an increase of antioxidant defences [total glutathione (GSH), superoxide dismutase (SOD), Trolox equivalent antioxidant capacity (TEAC)], without any impairment of GSH redox status nor induction of HSPs. Antagonistic effects were observed on 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 biomarkers, the use of complementary biomarkers is necessary to discriminate the 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 organisation (Huggett *et al.* 1992). In a multi-pollution context, it is now recognised 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 in a multibiomarker approach depends on 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 characterised 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. In any case, providing evidence of the mechanisms involved for each chemical element alone constitutes the first step to understand how they can interfere when they are present in mixtures.

In fish, cellular and molecular responses to contaminants have been characterised for a large number of metals and organic chemicals at sublethal doses. Essential and non essential metals, such as zinc and cadmium 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 metallic pollution of freshwater ecosystems. However, the majority of toxicity studies in fish have been focused on short term exposure, to single chemical, often at relatively high concentrations. Relatively 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 interactive effects of metal/metal and of

metal/organic mixtures on biomarker responses in fish are scarce. Synergistic effects were 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 estrogen receptor (ER) transcriptional activity (Olsson *et al.* 1995; LeGuevel *et al.* 2000). However, these mechanisms were described either *in vivo* after intraperitoneal administration of metals or in *in vitro* studies, which involved 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 possible synergistic effects with two organic chemicals (3,3',4,4'-tetrachlorobiphenyl, PCB77, and 17-β-estradiol, E2), administered either as single chemical or in combination (table 1). 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 estrogen in vertebrate animals, was used as a model inducing compound of estrogenic effect. This hormone has been frequently detected in municipal effluent and in surface waters at biologically active concentrations, within the 10 ng/l-range (Ternes *et al.* 1999). We then measured antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR), as well as non enzymatic antioxidants such as glutathione (GSH), 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 plasmatic vitellogenin (Vtg) as a biomarker of E2 exposure (Sumpter and Jobling 1995). In the plasma, alanine (ALT) and aspartate aminotransferase (AST) were used as indicators of liver dysfunction (Mayer *et al.* 1992). Liver cadmium and zinc measurements were used to link metal accumulation with the explored 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, 145 mg/L HCO_3^-). Throughout the experiment, including the acclimatisation phase, the fish were fed with a standard pellet trout food (160 µq Cd/Kg dw) at 1% per day of the mean fish body weight. For chemical exposure, ten groups of trout (n=45) were allocated in 80 L tanks and were then exposed to chemical treatments for 21 days, as described in table 1. Cd and Zn were added in water at a concentration of 1.5 µg/L and 150 µg/L 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 2 successive intra peritoneal injections at day 0 and 14. Two control groups, one for waterborne exposure and one for corn oil injection (carrier control) were used. To

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

Metal analyses

Cd and Zn contents 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 contents in the liver for five fish were measured after organ digestion in a glass tube with a screw stopper (65% HNO_3, 3 hours, 105°C, Blockdigest). Cadmium was measured by graphite furnace atomic absorption spectrometry (Perkin Elmer 4110 ZL) and zinc by flame atomic absorption spectrometry (Varian, Spectro AA 200). The limits of detection of Cd and Zn were 0.1 µg/L and 10 µg/L, respectively.

Plasma and S9 liver sample preparation

After the 21-day chemical exposure period, 10 fishes per treatment were sacrificed and weighted. All steps were carried out on ice or at 4°C. For plasma sample preparation, blood was taken from the caudal vein using an heparined syringe (Lithium heparine plasma microtubes, Sarstedt). Samples were pooled by two in order to obtain enough material for analyses and subjected to a centrifugation (3000 *g*, 15 min at 4°C). The supernatants were frozen in liquid nitrogen and stored at -80°C until use. For liver sample preparation, fishes were opened, liver and kidney were excised and weighted to determine liver and kidney somatic indices (LSI and KSI). The liver was rinsed in KCl 150 mM and homogenised for 2 min on ice in 4 volumes of cold homogenisation buffer (100 mM Na_2HPO_4, 20 % w/v glycerol, 0.2 mM phenylmethylsulfonide fluoride (PMSF), pH 7.8) by using a motor driven Teflon potter. The homogenate was centrifuged at 10000 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

samples was determined the day of dissection by using the method of Bradford (1976). All other biochemical assays were carried out within one month, except for GPx and GR assays for injected fishes which were performed after three months of storage.

Plasmatic biomarkers

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

Hepatic biomarkers

Glutathione and GSH redox status. Total (tGSH) and oxidised (GSSG) glutathione 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 GSH equivalent, and tGSH. *Antioxidant enzymes*. Total glutathione peroxidase (GPx), superoxide dismutase (SOD), and glutathione reductase (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 Trolox equivalent antioxidant capacity (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 trichloroacetic acid (TCA), centrifuged for 5 min at 4°C and the supernatants were used for the TEAC assay. This step was necessary to avoid high interference of liver proteins with the assay, as noted in preliminary experiments (results not shown). The antioxidant activity was expressed as equivalent Trolox concentration (in mM per mg of 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 SDS-PAGE in 12% polyacrylamide running gels (Laemmli 1970), and electro-transferred 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 C-terminal end of actin (AC-40 clone, 1:600 dilution). The secondary antibody was goat anti-mouse IgG labelled with horseradish peroxidase (BioRad). Immunoreactive band visualisation was performed using ECL reagents (Amersham) and the blots were scanned and analysed by 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 recognises 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) which 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 HSPs signal was normalised by actin signal and the final results were expressed as percent of 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 2 groups according to the type of treatment, *i.e.* waterborne (group 1) or intra peritoneal exposure (group 2), and the effects were analysed by comparing exposed fishes to water control or carrier control, respectively. 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, TEAC ; group

2: GSSG, GR, HSP70a, HSP70b, HSP60 and EROD). Significant effects of chemicals were determined by one-way ANOVA, followed by a unilateral Dunnett's test. Interactive effects of Cd and Zn on biomarkers were determined by a generalised linear model (GLM) with Cd and Zn nominal water concentrations as fixed co-factors. ANOVAs were carried out with the SPSS® v10.1 software.

Multivariate analyses. To explore 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 normalised towards their standard deviation. Then a non centred PCA was used to avoid any further normalisation. 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

The table 2 summarises 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 nine fold that of the control groups. The co-exposure with other chemicals did not significantly alter the level of Cd accumulation. No significant variation of liver Zn content was detected.

Effects on survival, somatic indices, and plasmatic parameters

The different chemical treatments did not alter fish survival nor growth. The table 3 summarises the results for organ somatic indexes and plasmatic parameters. No effect was observed on kidney and liver somatic indices (KSI and LSI), although a slight increase of LSI was noted after E2 and PCB77+CdZn exposure. The plasmatic AST and ALT levels did not differ significantly after chemical treatment as compared to control groups. These results indicate that chemical treatments did not induce acute toxic effect in the liver. Concomitant increases in Vtg levels were measured in fishes treated by E2 and E2+Cd/Zn (table 3), while Vtg was undetectable (*i.e.* < 300 ng Vtg per ml of plasma) in all other groups. Vtg concentrations in E2 and E2+Cd/Zn treated fishes were not statistically different.

Hepatic biomarker responses to waterborne Cd and/or Zn

The effects of metals on hepatic biomarkers are summarised in the table 4. Cd exposure provoked an increase of the non enzymatic (total GSH and TEAC) and enzymatic (SOD, 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 an alteration of the GSH redox status. Zn exposure induced SOD and GR activities, but had no statistically significant effect on GSH levels and 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 on total GSH ($p=0.015$), 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 GLM 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

The table 5 summarises 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 of the pool of GSH (total GSH and GSSG), TEAC and SOD, and an increase of GPx activity. The GSH redox status and GR were not affected by E2. In parallel, a significant inhibition of more than 50% of basal EROD activity is observed while no significant effect on HSPs was noted. When E2 was administrated in presence of Cd and Zn in the water, almost all the inhibitory effects of E2 on antioxidants were removed, resulting to a return to the carrier control level.

Liver biomarker responses to PCB77 and/or metals

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

Multivariate analyses

A PCA was run in order to determine if there was a correlation between the various chemical treatments and alterations in biomarkers. The locations of the biomarkers and chemical treatments on the first factorial plan of the PCA are presented in figure 2 and 3, respectively. The PCA shows that 79% of the overall variance is explained by the first two factors. The first factor (F1) explains 63% of the overall variance comprised of EROD and HSPs, and by SOD, total GSH and TEAC (figure 2). F1 discriminates mainly the effects of PCB77, PCB77+Cd/Zn and oil+Cd/Zn treated groups (figure 3) as these treatments were potent to alter EROD and/or stress proteins. The discrimination between E2, E2+Cd/Zn and oil+Cd/Zn

groups on F1 is explainable by the opposite effects of E2 and metals on non enzymatic antioxidants, as described above (table 5). The second factor (F2; 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 chemical treatments. The figure 4 represents the coordinates of variables and treatments on the first factorial plan of the CFA. The first factor of 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 induction of HSP70A and antioxidants, 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 could be explained by induction of GSSG and GSSG/tGSH, thus illustrating the antagonistic effects of these metals on GSH-related biomarkers, as described above from table 4. Interestingly, 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 explicative 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 "sub-clinical" as none of the chemical treatments affected either survival and growth of 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). Besides, all chemical treatments induced significant biochemical variations resulting in different patterns of biomarker responses in function of the nature of 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 an 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 were not significantly altered, thus suggesting that antioxidant potency in the liver was maintained. To some extent, these results are in line with previous data in fish reporting increase of total GSH in the liver, presumably through an induction of its synthesis, as a primary biochemical response to Cd and/or Zn exposure, before or concomitantly with the induction of metallothioneins (MT) (Kuroshima 1995; Tort *et al.* 1996; Lange *et al.* 2002). In addition, the increase of TEAC by Cd in our study may also reflect the potency of this metal to induce GSH, which is detected in the TEAC assay (Miller *et al.* 1993). Indeed, we observed a linear relationship between total GSH and TEAC ($r^2 = 0.59$, $p<0.001$) and it is likely that this correlation reflected the predominance of GSH amongst 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 of SOD and GR activities may rather reflect a cellular physiological

response of the liver to a transient increase and intracellular redistribution of zinc cellular content 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 antagonised 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 sulfhydryl group oxidation, and the inhibition of 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 on HSPs by waterborne metal exposure (table 4), and especially by cadmium, were thus unexpected since Cd content increased in the liver after exposure (table 2). It is likely that heat shock proteins already present in the liver of control fish, together with the increase of 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 of both HSP70 isoforms by waterborne metals was noted in the oil+Cd/Zn group (table 5), suggesting that corn oil induced an hepatic stress that altered protein homeostasis, leading to a potentiation of metal effect on HSP synthesis. The

use of i.p. injection employing 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 estrogenic effect, E2 inhibited hepatic antioxidants and EROD activity (table 5). The inhibition of cytochrome P450 1A1 by (xeno)estrogens has been several times reported in fish (Arukwe *et al.* 1997; Sole *et al.* 2000; Navas and Segner 2001). Conversely, only few studies have described the (anti)oxidant effects of (xeno)estrogens in fish, although it has been more extensively reported in mammals. In fact, estrogens 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 estrogen metabolites that undergo redox cycling and generate ROS (Liehr and Roy 1990). These estrogen metabolites are able to deplete GSH in the presence of NADPH in rat hepatocytes (Ruiz-Larrea *et al.* 1993) and appear to be involved in the development of liver injury after estrogen treatment (Liehr and Roy 1990). In carp (*Cyprinus carpio*), 17α-ethynilestradiol (EE2) decreased liver P450s and antioxidant enzymes (SOD, Se-GPx and catalase) 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 vitamins consumption, which led to their depletion

in the liver. This antioxidant depletion may trigger accumulation of ROS and subsequent oxidative stress, as suggested by alterations of 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 of 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 ER-mediated response to E2. To our knowledge, no similar result were reported in fish, although interaction between E2 and Cd (but not Zn) on 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 estrogens, which could have some implication for their interpretation in a multipollution 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 PCBs to bind the AhR and to induce CYP1A expression in the liver (Safe 1994; Otto *et al.* 1997). In mammals and fish liver, PCBs are metabolised by P450s to mono- and hydroxyl-metabolites, which can be oxidised to the highly reactive corresponding (semi)quinones. These compounds generate ROS and form adducts to and alter macromolecules such as DNA or proteins (Safe 1994; Srinivasan *et al.* 2001). As a consequence, it is likely that the resulting alteration of 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 antioxidant biomarkers in our study were relatively surprising. Nevertheless, antioxidant responses to coplanar PCBs in fish are not 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 an induction of liver lipoperoxidation and a depletion of tocopherol and retinol were evidenced 6 and 13 weeks, respectively, after injection (Palace *et al.* 1996). These oxidant effects in freshwater fish were obtained for higher doses than those used in our study, which could explain the discrepancies with our data.

The co-exposure to PCB77 and metals did not reveal important joint effect at the biochemical level, the effect of PCB77 appearing predominant on that one of metals. No comparable data using these metal concentrations was found in the literature, although at higher concentrations heavy metals have been shown to inhibit 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 had 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 : (1) EROD and HSP70, (2) SOD, total GSH and TEAC, and (3) GSSG, GSH redox status, GPX and GR (figure 2). Groups 1 and 2 appeared the most explanatory of the variability induced by chemical treatments. Secondly, the CFA, which allowed to distinguish between induction and inhibition of biomarker responses and to explore their correlation with chemical treatments (figure 4), confirmed correlation between certain biomarkers (*e.g.* tGSH, TEAC and SOD induction by metal stress) and highlighted complementarity between others (*e.g.* HSP70 and EROD, both markedly induced by PCB77 and correlated in the PCA). The choice for examining 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 as the most explanatory of oxidant effect for this set of chemicals.

In summary, this study provides further information for the understanding of biomarker responses to low pollutant concentrations in fish. Environmental concentrations of metals induced adaptive biochemical responses by increase of 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 sublethal impact of the selected chemicals in fish in a multipollution context. The measurement of EROD activity, Vtg, HSP70, total GSH (or TEAC ?) and selected antioxidant enzymes may constitute a suitable battery of complementary biomarkers to assess early biochemical responses to this set of chemicals.

ACKNOWLEDGEMENTS

We would like to acknowledge the excellent technical assistance of Chantal Gillet for plasmatic biomarker analyses. Special thanks to Christelle Adam, Isabelle Cavalié and Brigitte Ksas for their precious help during the course of laboratory experiments and to Dr Giles Velarde (University of Manchester) and Pr 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.

REFERENCES

Aït-Aïssa, S., Porcher, J. M., Arrigo, A. P. and Lambre, C. 2000, Activation of the hsp70 promoter by environmental inorganic and organic chemicals: relationships with cytotoxicity and lipophilicity. *Toxicology*, **145**, 147-157.

Arukwe, A., Förlin, L. and Goksoyr, A. 1997, Xenobiotic and steroid biotransformation enzymes in atlantic salmon (*Salmo salar*) liver treated with an estrogenic compound, 4-nonylphenol. *Environmental Toxicology and.Chemistry*, **16**, 2576-2583.

Ausseil, O., Adam, C., Garnier-Laplace, J., Baudin, J. P., Casellas, C. and Porcher, J. M. 2002, Influence of metal (Cd and Zn) waterborne exposure on radionuclide (^{134}Cs, ^{110}mAg and ^{57}Co) bioaccumulation by rainbow trout (*Oncorhynchus mykiss*): a field and laboratory study. *Environmental Toxicology and.Chemistry*, **21**, 619-625.

Baker, M. A., Cerniglia, G. J. and Zaman, A. 1990, Microtiter plate assay for the measurement of glutathione disulfide in large numbers of biological samples. *Analytical Biochemistry*, **190**, 360-365.

Beyer, J., Sandvik, M., Skave, J. U., Egaas, E., Hylland, K., Waagbo, R. and Goksoyr, A. 1997, Time- and dose-dependent biomarker responses in flouder (Platichthys flesus L.) 2,3,3',4,4', 5-hexachlorobiphenyl (PCB-156) and cadmium. *Biomarkers*, **2**, 35-44.

Bradford, M. M. 1976, A rapid sensitive method for the quantitation of micrograms quantities of proteins utilizing the principe of protein-dye binding. *Analytical Biochemistry*, **72**, 248-254.

Brion, F., Flammarion, P., Aït-Aïssa, S., Garric, J., Thybaud, E. and Porcher, J. M. (1999) Vitellogenin measurement in juvenile rainbow trout after nonylphenol treatment : establishment and validation of an ELISA. *9th Annual meeting of SETAC Europe*, Leipzig.

Cajaraville, M. P., Bebianno, M. J., Blasco, J., Porte, C., Sarasquete, C. and Viarengo, A. 2000, The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian peninsula: a practical approach. *Science of the Total Environment*, **247**, 295-311.

Carlberg, I. and Mannervik, B. 1985, Glutathion reductase. In *Methods in enzymology*: (Academic Press) pp. 484-490.

De Lafontaine, Y., Gagné, F., Blaise, C., Costan, G., Gagnon, P. and Chan, H. M. 2000, Biomarkers in zebra mussels (Dreissenia polymorpha) for the assessment and monitoring of water quality of the St Lawrence river (Canada). *Aquatic Toxicology*, **50**, 51-71.

Dethloff, G., Schlenk, D., Hamm, J. T. and Bailey, H. C. 1999, Alterations in physiological parameters of rainbow trout (*Oncorhynchus mykiss*) with exposure to copper and copper/zinc mixtures. *Ecotoxicology and Environmental Safety*, **42**, 253-264.

Dilworth, C. and Timbrell, J. A. 1998, An investigation into the sensitivity of heat shock proteins as markers of cellular damage: a comparative study of hydrazine and cadmium chloride in primary rat hepatocyte culture. *Biomarkers*, **3**, 177-190.

Flammarion, P., Migeon, B. and Garric, J. 1998, Statistical analysis of cyprinid ethoxyresourfin-*o*-deethylase data in a large french watershed. *Ecotoxicology and Environmental Safety*, **40**, 144-153.

Grosvik, B. E. and Goksoyr, A. 1996, Biomarker protein expression in primary cultures of salmon (*Salmo salar* L.) hepatocytes exposed to environmental pollutants. *Biomarkers*, **1**, 45-53.

Huggett, R., Kimerle, R., Mehrle, P. and Bergman, H. 1992, Biomarkers: biochemical, physiological and histological markers of anthropogenic stress (Boca Raton, FL, Lewis).

Iwama, G. K., Thomas, P. T., Forsyth, R. B. and Vijayan, M. M. 1998, Heat shock protein expression in fish. *Reviews on Fish Biology and Fisheries*, **8**, 35-56.

Klinger, W., Lupp, A., Karge, E., Baumbach, H., Eichhorn, F., Feix, A., Fuldner, F., Gernhardt, S., Knels, L., Kost, B., Mertens, G., Werner, F., Oettel, M., Romer, W., Schwarz, S., Elger, W. and Schneider, B. 2002, Estradiol, testosterone, dehydroepiandrosterone and androstenedione: novel derivatives and enantiomers. Interactions with rat liver microsomal cytochrome P450 and antioxidant/radical scavenger activities in vitro. *Toxicology Letters*, **128**, 129-144.

Kuroshima, R. 1995, Hepatic metallothionein and glutathione levels in Red Sea bream. *Comparative Biochemistry and Physiology*, **110C**, 95-100.

Laemmli, U. K. 1970, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685.

Lange, A., Ausseil, O. and Segner, H. 2002, Alterations of tissue glutathione levels and metallothionein mRNA in rainbow trout during single and combined exposure to cadmium and zinc. *Comparative Biochemistry and Physiology*, **131C**, 231-243.

LeGuevel, R., Petit, F. G., LeGoff, P., Metivier, R., Valotaire, Y. and Pakdel, F. 2000, Inhibition of rainbow trout (Oncorhynchus mykiss) estrogen receptor activity by cadmium. *Biology of Reproduction*, **63**, 259-266.

Liehr, J. G. and Roy, D. 1990, Free radical generation by redox cycling of estrogens. *Free Radical Biology and Medicine*, **8**, 415-423.

Mayer, F. L., Versteeg, D. J., McKee, M. J., Folmar, L. C., Graney, R. L., McDume, D. C. and Rattner, B. A. 1992, Physiological and non specific biomarkers. In *Biochemical, physiological and histological markers of anthropogenic stress.*, R. J. Hugget, M. A. Kimerle, P. M. Mehrle and H. L. Bergman, eds (Chelsea, MI, USA: Lewis publishers), pp. 5-86.

Miller, N. J., Rice-Evans, C., Davies, M. J., Gopinathan, V. and Milner, A. 1993, A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clinical Science*, **84**, 407-412.

Navas, J. M. and Segner, H. 2001, Estrogen-mediated suppression of cytochrome P4501A (CYP1A) expression in rainbow trout hepatocytes: role of estrogen receptor. *Chemico-Biological Interactions*, **138**, 285-298.

Newman, M. C. and McCloskey, J. T. 1996, Predicting relative toxicity and interactions of divalent metal ions: Microtox bioluminescence assay. *Environmental Toxicology and Chemistry*, **15**, 275-281.

Olsson, P. E., Kling, P., Petterson, C. and Silversand, C. 1995, Interaction of cadmium and oestradiol-17 beta on metallothionein and vitellogenin synthesis in rainbow trout (*Oncorhynchus mykiss*). *Biochemical Journal*, **307**, 197-203.

Otto, D. M. E. and Moon, T. W. 1995, 3,3',4,4'-Tetrachlorobiphenyl effects on antioxidant enzymes and glutathione status in different tissues of Rainbow trout. *Pharmacology & Toxicology*, **77**, 281-287.

Otto, D. M. E., Sen, C. K., Casley, W. L. and Moon, T. W. 1997, Regulation of 3,3',4,4'-tetrachlorobiphenyl induced cytochrome P450 metabolism by thiols in tissues of rainbow trout. *Comparative Biochemistry and Physiology*, **117C**, 299-309.

Paglia, D. E. and Valentine, W. N. 1967, Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *Journal of Laboratory and Clinical Medicine*, **70**, 158-169.

Palace, V. P., Evans, R. E., Wautier, K., Baron, C. L., Werner, J., Klaverkamp, J. F., Kidd, K. A. and Dick, T. A. 2001, Altered distribution of lipid-soluble antioxidant vitamins in juvenile sturgeon exposed to waterborne ethynylestradiol. *Environmental Toxicology and Chemistry*, **20**, 2370-2376.

Palace, V. P., Klaverkamp, J. F., Lockhart, W. L., Metner, D. A., Muir, D. C. G. and Brown, S. B. 1996, Mixed-function oxidase enzyme activity and oxidative stress in lake trout (*Salvelinus namaycush*) exposed to 3,3',4,4',5-pentachlorobiphenyl (PCB-126). *Environmental Toxicology and Chemistry*, **15**, 955-960.

Paoletti, F., Aldinucci, D., Mocali, A. and Caparrini, A. 1986, A sensitive spectrophotometric method for the determination of superoxide dismutase activity in tissue extracts. *Analytical Biochemistry*, **154**, 536-541.

Ruiz-Larrea, M. B., Garrido, M. J. and Lacort, M. 1993, Estradiol-induced effects on glutathione metabolism in rat hepatocytes. *Journal of Biochemistry*, **113**, 563-567.

Ryan, J. A. and Hightower, L. E. 1996, Stress proteins as molecular biomarkers for environmental toxicology. In *Stress-Inducible Cellular Responses*, U. Feige, R. I.

Morimoto, I. Yahara and B. Polla, eds (Birkhäuser Verlag Basel/Switzerland.), pp. 411-424.

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.

Schlezinger, J. J., Keller, J., Verbrugge, L. A. and Stegeman, J. J. 2000, 3,3',4,4'-Tetrachlorobiphenyl oxidation in fish, bird and reptile species: relationship to cytochrome P450 1A inactivation and reactive oxygen production. *Comparative Biochemistry and Physiology*, **125C**, 273-286.

Schröder, H. C., Batel, R., Hassanein, H. M. A., Lauenroth, S., StJenke, H., Simat, T., Steinhart, H. and Muller, W. E. G. 2000, Correlation between the level of the potential biomarker, heat-shock protein, and the occurrence of DNA damage in the dab, *Limanda limanda*: a field study in the North Sea and the English Channel. *Marine Environmental Research*, **49**, 201-215.

Schröder, H. C., Batel, R., Lauenroth, S., Hassanein, H. M. A., Lacorn, M., Simat, T., Steinhart, H. and Muller, W. E. G. 1999, Induction of DNA damage and expression of heat shock protein HSP70 by polychlorinated biphenyls in the marine sponge *Suberites domuncula Olivi. Journal of Experimental Marine Biology and Ecology*, **233**, 285-300.

Sharma, S. S., Schat, H., Vooijs, R. and VanHeerwaarden, L. M. 1999, Combination toxicology of copper, zinc, and cadmium in binary mixtures: concentration-dependent antagonistic, nonadditive, and synergistic effects on root growth in *Silene vulgaris*. *Environmental Toxicology and Chemistry*, **18**, 348-355.

Sies, H. 1999, Glutathione and its role in cellular functions. *Free Radical Biology and Medicine*, **27**, 916-921.

Sole, M., Porte, C. and Barcelo, D. 2000, Vitellogenin induction and other biochemical responses in carp, *Cyprinus carpio*, after experimental injection of 17α-ethynylestradiol. *Archives of Environmental Contamination and Toxicology*, **38**, 494-500.

Srinivasan, A., Lehmler, H. J., Robertson, L. W. and Ludewig, G. 2001, Production of DNA strand breaks *in vitro* and reactive oxygen species *in vitro* and in HL-60 cells by PCB metabolites. *Toxicological Sciences*, **60**, 92-102.

Stohs, S. J. and Bagchi, D. 1995, Oxidative mechanisms in the toxicity of metal ions. *Free Radical Biology and Medicine*, **18**, 321-336.

Sumpter, J. P. and Jobling, S. 1995, Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environmental Health Perspectives*, **103**, 173-178.

Ternes, T. A., Stumpf, M., Mueller, J., Haberer, K., Wilken, R.-D. and Servos, M. 1999, Behavior and occurrence of estrogens in municipal sewage treatment plants -- I. Investigations in Germany, Canada and Brazil. *Science of the Total Environment*, **225**, 81-90.

Thioulouse, J., Chessel, D., Dolédec, S. and Olivier, J. M. 1997, ADE-4: a multivariate analysis and graphical display software. *Statistics and Computing*, **7**, 75-83.

Toborek, M., Barger, S., Mattson, M. P., Espandiari, P., Robertson, L. W. and Hennig, B. 1995, Exposure to polychlorinated biphenyls causes endothelial cell dysfunction. *Journal of Biochemical Toxicology*, **10**, 219-226.

Tort, L., Kargacin, B., Torres, P., Giralt, M. and Hidalgo, J. 1996, The effect of cadmium exposure and stress on plasma cortisol, metallothionein levels and oxidative status in rainbow trout (*Oncorhynchus mykiss*) liver. *Comparative Biochemistry and Physiology*, **114C**, 29-34.

Werner, I. and Nagel, R. 1997, Stress proteins HSP60 and HSP70 in three species of amphipods exposed to cadmium, diazinon, dieldrin and fluoranthene. *Environmental Toxicology and Chemistry*, **16**, 2393-2403.

Whyte, J. J., Jung, R. E., Schmitt, C. J. and Tilitt, D. E. 2000, Ethoxyresorufin-*O*-deethylase (EROD) activity in fish as a biomarker of chemical exposure. *Critical Reviews in Toxicology*, **30**, 347-570.

Williams, J. H., Farag, A. M., Stansbury, M. A., Young, P. A., Bergman, H. L. and Petersen, N. S. 1996, Accumulation of HSP70 in juvenile and adult rainbow trout gill exposed to metal-contaminated water and/or diet. *Environmental Toxicology and Chemistry*, **15**, 1324-1328.

Winston, G. W. and DiGiulio, R. T. 1991, Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquatic Toxicology*, **19**, 137-161.

Table 1. Organic compound treatments (total dose per kg fish administrated by 2 i.p. injections at day 0 and day 14 of the experiment) and measured metal concentrations in the water during the experiment.

	Organic compounds (i.p.)			Metals in the water[a]	
	Corn oil (µl/g)	E2 (mg/kg)	PCB77 (mg/kg)	Cd (µg/L)	Zn (µg/L)
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.

Table 2. Cadmium and Zinc concentrations in the trout liver for the different groups after the 21-day exposure period.

Fish Groups	Cd (ng/g fw)	Zn (µg/g fw)
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

Means ± 95% confidence intervals (8 replicates). *: significantly different from control or carrier control ($p<0.05$).

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

Parameters	Control	Cd	Zn	Cd/Zn	Carrier control	E2	E2 + Cd/Zn	PCB77	PCB77 + Cd/Zn	Oil + Cd/Zn
LSI[a]	1.4 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.5 ± 0.2	1.3 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	1.4 ± 0.2
KSI[a]	1.0 ± 0.3	1.0 ± 0.2	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.2	0.8 ± 0.1	0.9 ± 0.1	1.0 ± 0.2
AST[b]	521 ± 51	584 ± 38	538 ± 96	467 ± 63	686 ± 81	668 ± 50	641 ± 39	640 ± 33	643 ± 53	620 ± 40
ALT[b]	45 ± 10	40 ± 6.4	43 ± 8.3	39 ± 8.5	63 ± 10	58 ± 6.4	70 ± 11	54 ± 4.8	61 ± 7.6	57 ± 5.2
Protein[c]	29 ± 6.4	28 ± 2.6	27 ± 3.2	25 ± 2.3	30 ± 4.4	32 ± 3.7	35 ± 5.5	26 ± 1.2	24 ± 2.1	28 ± 3.4
Vtg[c]	ND[d]	ND	ND	ND	ND	0.87 ± 0.26	0.80 ± 0.14	ND	ND	ND

Data represent mean ± 95% confidence interval (n = 5). [a] : Liver and Kidney somatic indexes, expressed as % of total weight ; [b] : U/L ; [c] : mg/ml ; [d] : not detectable.

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

Biomarkers	Control	Cd	Zn	Cd/Zn
tGSH[a]	16.2 ± 4.1	27.3 ± 5.8**	20.2 ± 3.1	23.3 ± 3.8**
GSSG[a]	3.0 ± 2.5	4.4 ± 3.6	5.6 ± 3.3	1.4 ± 0.7
2×GSSG/tGSH	0.34 ± 0.28	0.35 ± 0.28	0.55 ± 0.28	0.11 ± 0.05
TEAC[b]	7.0 ± 1.5	9.1 ± 1.6*	8.9 ± 4.2	10.2 ± 2.6*
SOD[c]	15 ± 5.1	22 ± 5.1*	24 ± 7.2*	29 ± 9.2**
GPx[d]	187 ± 55	182 ± 49	151 ± 63	157 ± 59
GR[d]	34 ± 11	68 ± 50	49 ± 16*	34 ± 9
HSP70a[e]	1.0 ± 0.5	1.2 ± 1	2.1 ± 1.1*	1.0 ± 0.8
HSP70b[e]	1.0 ± 0.1	1.2 ± 0.2	1.2 ± 0.5	1.1 ± 0.2
HSP60[e]	1.0 ± 0.1	0.8 ± 0.3	1.0 ± 0.5	0.8 ± 0.2
EROD[f]	3.9 ± 2	7.7 ± 3.7*	9.4 ± 3.8**	10.6 ± 5**

Data represent means ± SD (n = 8 to 10). a : nmol/mg protein ; b : mM/mg protein ; c : U/mg protein ; d : U/g protein ; e: HSP/β-actin ratio relative to control level; f : pmol resorufin/min/mg protein. * $p<0.05$ and ** $p<0.01$ as compared to control.

Table 5. Effect after a 21-day period of E2 and PCB77, alone and with metals, on trout hepatic biomarkers

Biomarkers	Carrier (oil) control	Oil + Cd/Zn	E2	E2 + Cd/Zn	PCB77	PCB77 + Cd/Zn
TGSH[a]	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[a]	2.5 ± 0.6	1.7 ± 2.3	1.7 ± 0.6**	1.0 ± 0.4**	2.7 ± 1.5	0.5 ± 0.4**
2×GSSG/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[b]	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[c]	23 ± 5.4	20 ± 7.6	17 ± 1.9**	18 ± 5.3	25 ± 5.1	19 ± 5.8
GPx[d]	48 ± 18	80 ± 41*	92 ± 35*	94 ± 34**	63 ± 45	73 ± 11*
GR[d]	29 ± 9	41 ± 26	25 ± 7	25 ± 5	47 ± 25	31 ± 11
HSP70a[e]	1.0 ± 0.4	3.1 ± 2.3*	0.9 ± 0.3[#]	1.6 ± 0.6[#]	1.4 ± 0.7	2.7 ± 2.6
HSP70b[e]	1.0 ± 0.2	1.5 ± 0.4*	0.5 ± 0.2[#]	1.2 ± 0.2[#]	1.8 ± 0.4**	2.4 ± 1.1**
HSP60[e]	1.0 ± 0.3	1.2 ± 0.3	0.8 ± 0.3[#]	0.9 ± 0.3[#]	1.3 ± 0.7	1.4 ± 0.8
EROD[f]	8.6 ± 4.2	6.2 ± 3.7	3.1 ± 2.4**	8.0 ± 3.9	264.4 ± 44**	208 ± 107**

Data represent means ± SD (n = 6 to 10, except for # : n = 3). a : nmol/mg protein ; b : mM/mg protein ; c : U/mg protein ; d : U/g protein ; e: HSP/β-actin ratio relative to control level ; f : pmol resorufin/min/mg protein. * $p<0.05$ and ** $p<0.01$ as compared to control.

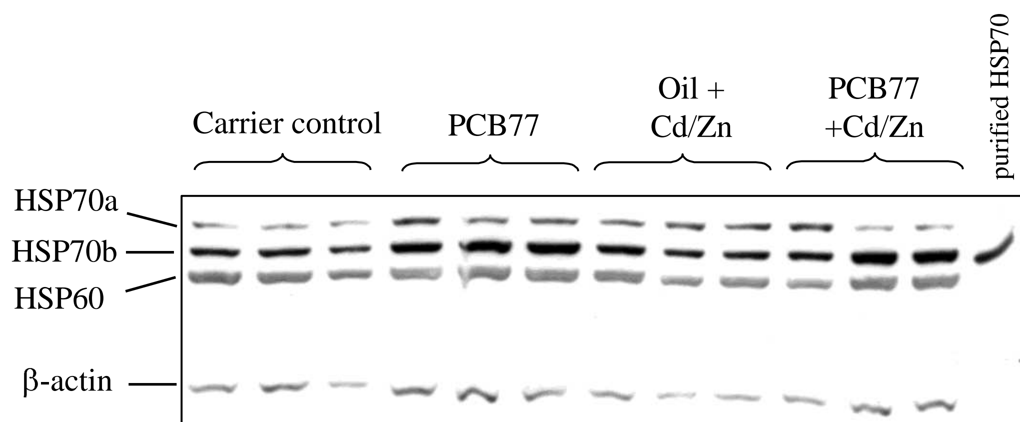
Figure captions

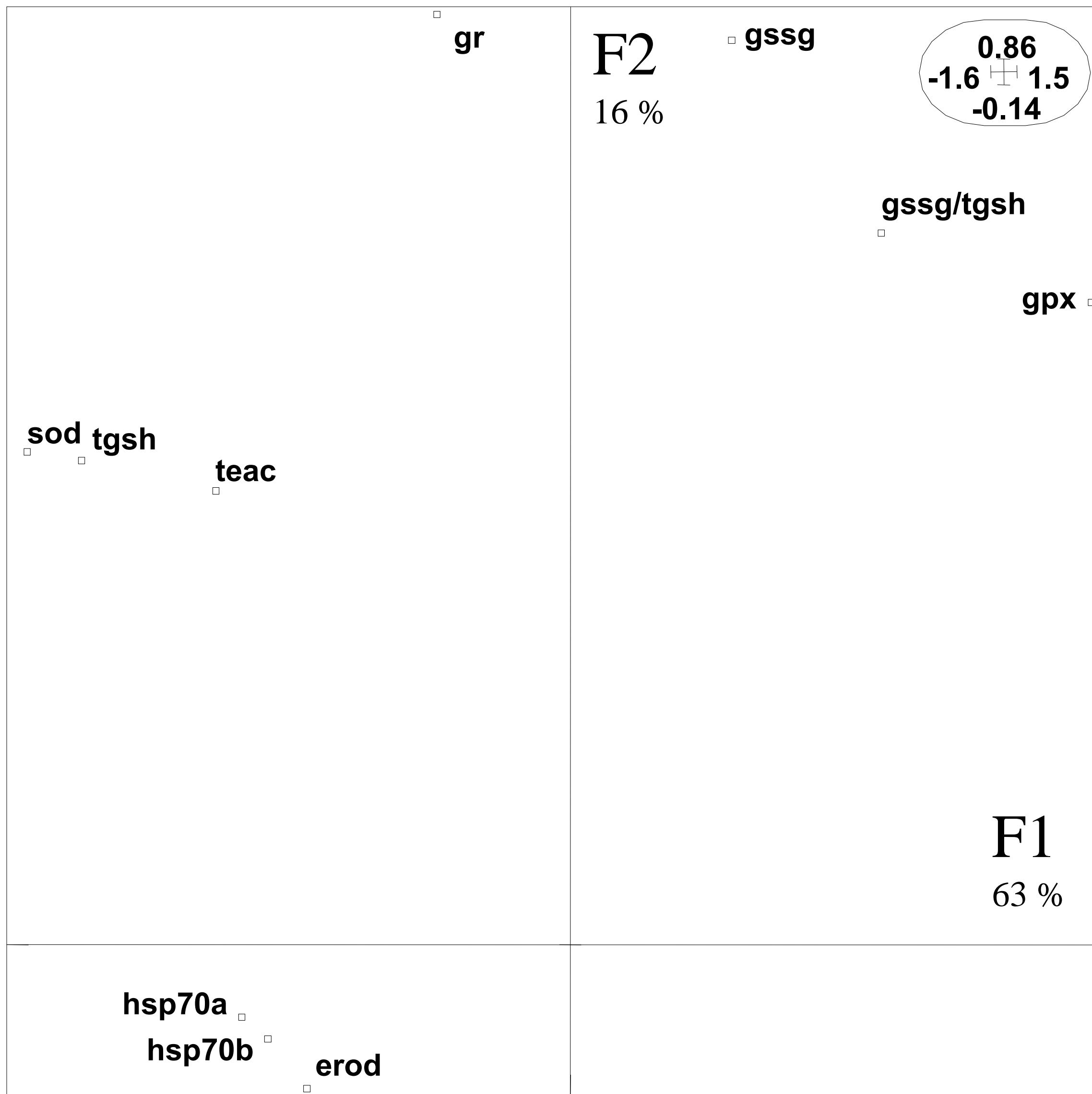
Figure 1. Representative western blot result for liver heat shock proteins and actin expression after exposure to carrier, PCB77, Oil+Cd/Zn and PCB77+Cd/Zn. Ten µg of total protein were loaded per lane, each lane represent one individual fish.

Figure 2. Representation of the biomarkers in the first factorial plane of the principal component analysis (PCA). The variables were centred using their control value and normalised towards their standard deviation. Then a non centred PCA was used to avoid any further normalisation.

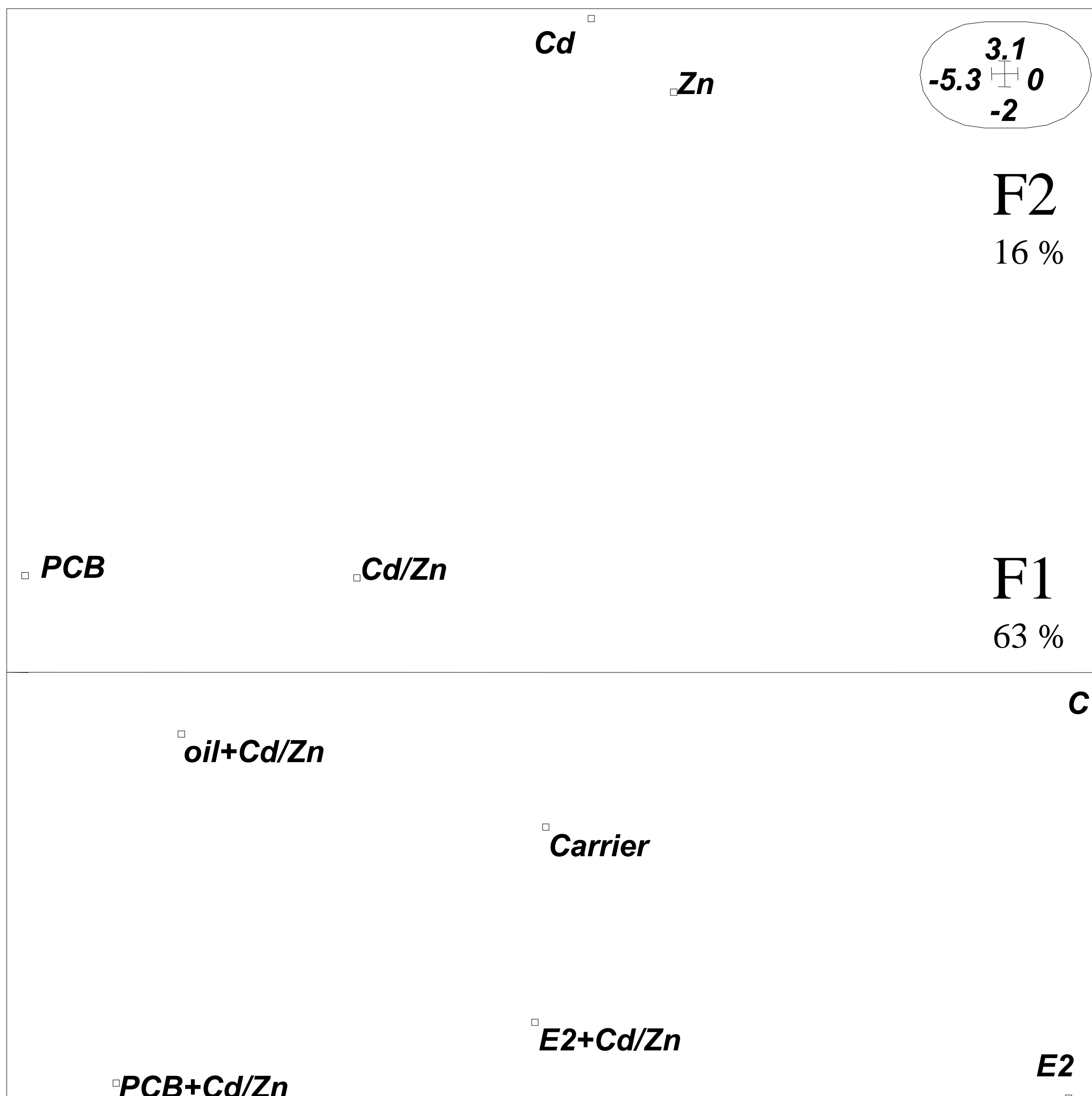
Figure 3. Representation of the treatments in the first factorial plane of the principal component analysis (PCA). The variables were centred using their control value and normalised towards their standard deviation. Then a non centred PCA was used to avoid any further normalisation. By this means the control value (noted C) lies in the centre of gravity of the plane.

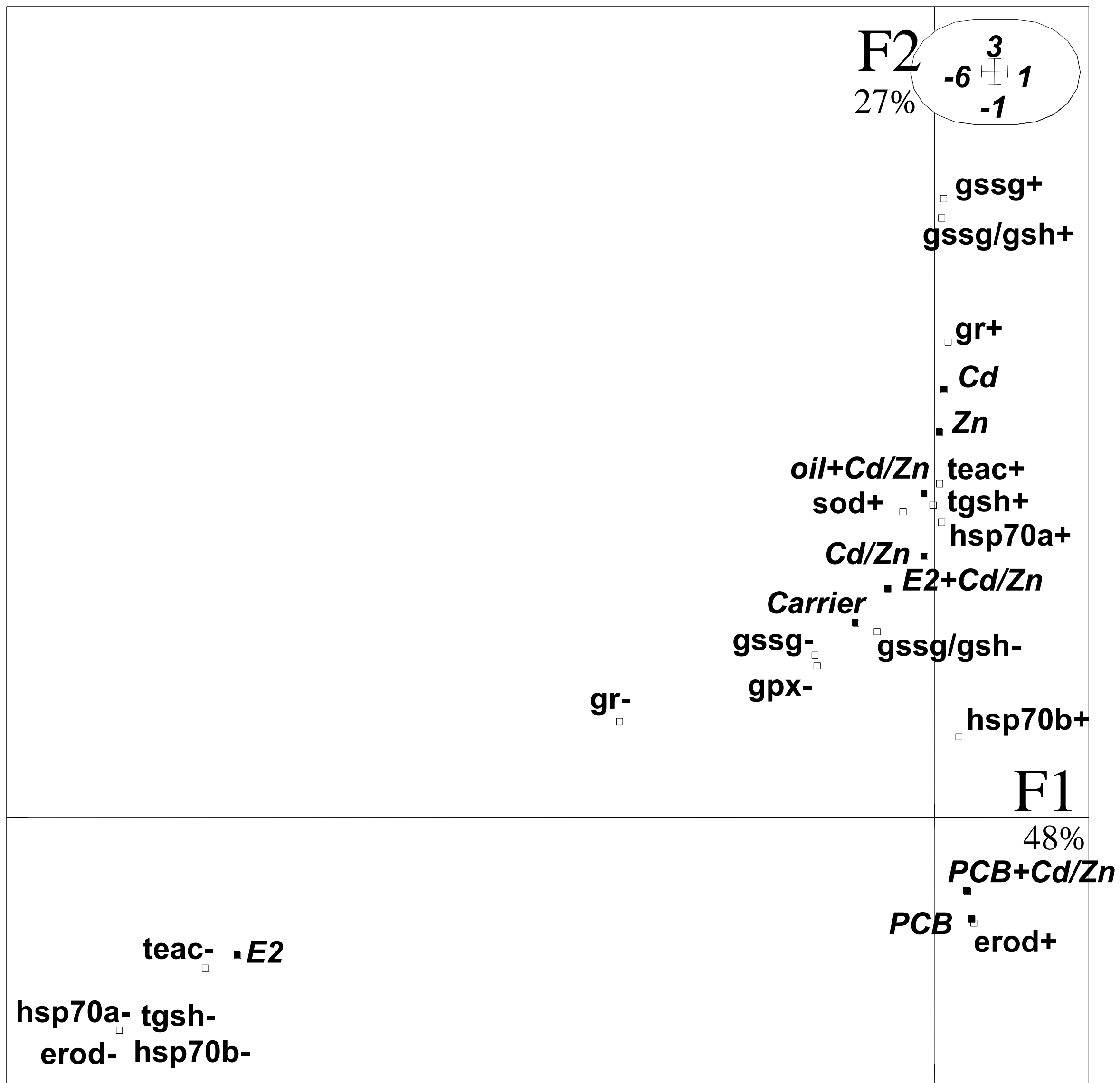
Figure 4. Representation of the treatments (italic, filled squares) and variables (empty squares) on the first factorial plane of a correspondence factor analysis. The variables were centred to the control value and duplicated using their absolute value as an induction variable (var+) and an inhibition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


Carrier control
PCB77
Oil +
Cd/Zn
PCB77
+Cd/Zn
purified HSP70
HSP70a
HSP70b
HSP60
β-actin



gr
F2
16 %
gssg
0.86
-1.6
1.5
-0.14
gssg/tgsh
gpx
sod
tgsh
teac
F1
63 %
hsp70a
hsp70b
erod


Cd
Zn
3.1
-5.3
0
-2
F2
16 %
PCB
Cd/Zn
F1
63 %
C
oil+Cd/Zn
Carrier
E2+Cd/Zn
E2
PCB+Cd/Zn


F2
27%
3
-6
1
-1
gssg+
gssg/gsh+
gr+
Cd
Zn
oil+Cd/Zn
teac+
sod+
tgsh+
hsp70a+
Cd/Zn
E2+Cd/Zn
Carrier
gssg-
gssg/gsh-
gpx-
gr-
hsp70b+
F1
48%
PCB+Cd/Zn
PCB
erod+
teac-
E2
hsp70a-
tgsh-
erod-
hsp70b-