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Interactive effects of benzo(*a*)pyrene and cadmium and effects of di(2-ethylhexyl) phthalate on antioxidant and peroxisomal enzymes and peroxisomal volume density in the digestive gland of mussel *Mytilus galloprovincialis* Lmk

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Exposure of marine animals to certain organic and metal pollutants is thought to enhance reactive oxygen species (ROS) production with concomitant alterations of antioxidant defence mechanisms. Some of these organic pollutants cause peroxisome proliferation, a process resulting also in possible enhanced production of ROS. The aim of this study was to investigate the effects of two organic xenobiotics, benzo(*a*)pyrene (B(*a*)P) and di(2-ethylhexyl)phthalate (DEHP), as well as the effects of cadmium (Cd), on antioxidant and peroxisomal enzymes and on peroxisomal volume density in the digestive gland of mussel, *Mytilus galloprovincialis* Lmk., experimentally exposed for 21 days. Special attention was paid to the interactive effects of organic and metal compounds by exposing one group of mussels to a mixture of B(*a*)P and Cd. Exposure of mussels to Cd caused a decrease in superoxide dismutase (SOD) activity, in Mn-SOD protein levels and in volume density of peroxisomes. B(*a*)P exposure significantly increased catalase and glutathione peroxidase (GPX) and inhibited Mn-SOD after 21 days of exposure. B(*a*)P also caused a slight increase in acyl-CoA oxidase (AOX) activity and peroxisomal volume density after 21 days of exposure. Cd tended to inhibit changes provoked by B(*a*)P, indicating that responses to organic xenobiotics can be modulated by concomitant exposure to metal contaminants. Exposure to DEHP increased catalase and AOX and inhibited SOD activity and Mn-SOD protein levels. In conclusion, peroxisome proliferation, measured as an increase of the peroxisomal enzymes catalase and AOX (up to 1.53-fold for AOX), is a specific response to organic contaminants such as B(*a*)P and DEHP, whereas Cd does not cause peroxisome proliferation. Thus, peroxisome proliferation may be a specific biomarker of organic pollutants in mussels. Both organic and metal pollutants inhibited SOD activity and protein levels (up to 0.21-fold for Mn-SOD protein levels), the latter offering potential as general marker of pollution.

Keywords: Benzo(*a*)pyrene, cadmium, interactive effects, di(2-ethylhexyl)phthalate (DEHP), antioxidant enzymes, peroxisome proliferation.

Introduction

The toxicity of organic xenobiotics such as polycyclic aromatic hydrocarbons (PAH) to aquatic organisms is likely partially mediated by the production of reactive oxygen species (ROS). These ROS are produced normally as by-products of the cellular metabolism but ROS production can be enhanced by xenobiotic metabolism (Di Giulio 1991, Livingstone *et al.* 1994, Lackner 1998). It is well

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established that the metabolism of the model PAH benzo(*a*)pyrene (B(*a*)P) and in general aromatic hydrocarbon quinones by mussel digestive gland microsomes involves the formation of ROS (García-Martínez and Livingstone 1995, Sjölin and Livingstone 1997, Mitchelmore *et al.* 1998) with the concomitant risk of oxidative stress processes that can damage DNA and other cellular macromolecules (Mitchelmore *et al.* 1998). The activity of antioxidant enzymes, among other mechanisms, plays a key role in preventing cellular damage to vital cellular components.

On the other hand, some organic pollutants are known to cause peroxisome proliferation in sensitive species, a process which also results in enhanced production of ROS (Lake 1993). Several compounds with dissimilar structure are known as peroxisome proliferators in mussels and fish, including the phthlate ester plasticizer dioctyl phthalate (also named di(2-ethylhexyl)phthalate, DEHP) and the PAH B(*a*)P (Cancio *et al.* 1998, Au *et al.* 1999, Cancio and Cajaraville 2000). Peroxisome-proliferating xenobiotics cause increased volume and numbers of peroxisomes and induction of certain peroxisomal enzymes, especially those of the fatty acid β-oxidation pathway such as acyl-CoA oxidase (AOX), which produces H_2O_2 as a by-product (Lake 1993). Besides enzymes producing ROS, peroxisomes contain the antioxidant enzymes catalase, Cu,Zn-superoxide dismutase (Cu,Zn-SOD) and glutathione peroxidase (GPX) (Dhaunsi *et al.* 1992, Singh *et al.* 1994, Orbea *et al.* 2000). The administration of peroxisome proliferators usually causes only slight increases in the H_2O_2-degrading catalase activity and even inhibition of GPX and SOD activities (reviewed by Lake 1993, Cancio and Cajaraville 2000). This imbalance between the induction of H_2O_2-producing enzymes and H_2O_2-degrading enzymes causes oxidative stress and may contribute to development of liver tumours in rodents (Lake 1993, Rao and Reddy 1996, Cancio and Cajaraville 2000). Thus, peroxisome proliferation has been proposed as a useful biological marker for identifying the potential carcinogenicity of these non-mutagenic genotoxic chemicals (Reddy *et al.* 1980, Reddy and Lalwani 1983).

Specificity of the peroxisome proliferation response is not known since only a reduced number of organic toxicants have been tested in aquatic organisms for their peroxisome proliferating capability (Cancio and Cajaraville 2000). Specifically, the possible effects of metal pollutants on peroxisome proliferation are not known. In addition, interactive effects of different contaminants on peroxisome proliferation have never been studied to our knowledge. Similarly, there are no studies reporting the interactive effects of different contaminants on antioxidant enzymes in aquatic organisms. This is an extremely important issue since environmental pollution is usually due to complex mixtures of contaminants.

In the present study, mussels *Mytilus galloprovincialis* were experimentally exposed for 21 days to B(*a*)P, Cd, a mixture of B(*a*)P and Cd and to DEHP. Activities of the antioxidant and peroxisomal enzymes catalase (EC 1.11.1.6), SOD (mainly cytosolic Cu,Zn-SOD, EC 1.15.1.1), GPX (EC 1.11.1.9) and AOX (EC 1.3.99.3) were assayed spectrophotometrically, while the protein levels of the two SOD forms, Cu,Zn-SOD and Mn-SOD, were studied using immunoblots. The volume density of peroxisomes was determined by catalase histochemistry coupled to stereology. The aim of the study was to investigate the effects of the toxicants tested on antioxidant and peroxisomal enzymes and on peroxisomal volume density, paying special attention to interactive effects between B(*a*)P and Cd.

The results obtained will help to assess whether these parameters can be used as specific markers of pollution in estuarine environments.

Materials and methods

Animals and experimental procedure

Mussels, *Mytilus galloprovincialis* Lmk. of 3–4 cm length were collected in the estuary of Plentzia (Bay of Biscay, 43°24′N, 2°56′W) in April 1998. Animals were immediately transferred to the laboratory and placed in an aquarium at constant temperature of 14°C with natural seawater (dissolved oxygen 6.62 mg l^{-1}, pH 7.9–8.0, conductivity 18.6 mS cm^{-1}) from a relatively clean site (Gorliz, Bay of Biscay, 43°24'N, 2°56'W), previously passed through UV light and filtered through glass-wool and active charcoal. Animals were fed daily after the second day in the laboratory with a commercial food mixture for filtering marine invertebrates (Marine Invertebrate Diet, Carolina Ltd). Seawater was changed everyday and kept constantly aerated.

After a 7-day acclimatization, mussels were dosed everyday after changing seawater for 21 days with two organic xenobiotics previously dissolved in dimethyl sulphoxide (DMSO), the model aromatic hydrocarbon benzo(*a*)pyrene (B(*a*)P, Fluka Chem.-Biochem., Spain) and the most widely used plasticizer di(2-ethylhexyl)phthalate (DEHP, Aldrich-Chemie, Steinheim, Germany) both at a concentration of 500 μg l^{-1}, and with the heavy metal cadmium (as $CdCl_2$) at a concentration of 80 μg l^{-1}. A fourth group was dosed with a mixture of B(*a*)P (500 μg l^{-1}) and Cd (80 μg l^{-1}). The concentration of the organic compounds although higher than environmental levels was selected on the basis of the results obtained in Cancio *et al.* (1998) in which mussels exposed to 500 μg l^{-1} B(*a*)P and mussels injected with 60 μg DEHP showed induction of peroxisomal enzymes, but not mussels exposed to 100 μg l^{-1} DEHP. Thus, the water-borne exposure concentration of DEHP was increased in this study to 500 μg l^{-1}. Wofford *et al.* (1981) used the same dose of phthalates in oysters and other aquatic organisms. The concentration of Cd used here has been demonstrated to be sublethal and to induce biomarker responses in mussels (Etxeberria *et al.* 1994). Additionally, to the four treated groups, two controls were run under the same conditions, one group was left untreated (water control) and the other control group was exposed to DMSO (DMSO control) in the same concentration (33 μl l^{-1}) used in the B(*a*)P, B(*a*)P + Cd and DEHP groups.

The first sampling was performed just after collecting animals in the field in order to monitor natural conditions. A second sampling was carried out after the acclimatization period (day 0 of experiment), and the following samplings were made after 1, 7 and 21 days of xenobiotic exposure.

Measurement of antioxidant and peroxisomal enzyme activities

The activities of the antioxidant enzymes catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPX) were determined as described by Livingstone *et al.* (1990) in 10–20 animals per experimental group pooled in six subgroups. Each pool of digestive glands was homogenized in chilled 10 mM Tris-HCl buffer at pH 7.6, containing 0.15 M KCl and 0.5 M sucrose using an homogenizer held in an ice-water cooled bath. After a centrifugation at 500 *g* during 15 min, a small amount of the supernatants was aliquoted and frozen for subsequent measurement of palmitoyl-CoA oxidase (AOX) activity and immunochemical analyses. Mitochondrial and cytosolic fractions were obtained after centrifugation at 12 000 *g* (45 min) and 100 000 *g* (90 min) respectively at 4°C.

Enzyme activities were assayed at 25°C. Catalase activity was determined in the mitochondrial and cytosolic fractions by measuring the consumption of H_2O_2 at 240 nm (extinction coefficient 40 M^{-1} cm^{-1}). Total catalase activity was calculated as the sum of the activity of the two fractions. SOD activity was determined in the cytosolic fraction as the degree of inhibition of cytochrome c reduction by superoxide anion generated by the xanthine oxidase/hypoxanthine reaction monitored at 550 nm. The activity is given in SOD units (1 SOD unit = 50% inhibition of the xanthine oxidase reaction). GPX activity was measured in the cytosolic fraction by the NADPH consumption monitored at 340 nm (extinction coefficient 6.2 mM^{-1} cm^{-1}) during the formation of reduced glutathione by a commercial glutathione reductase using H_2O_2 as substrate.

Peroxisomal AOX activity was measured as described by Small *et al.* (1985) in 500 *g* supernatants. AOX assay is based on the H_2O_2-dependent oxidation of dichlorofluorescein catalysed by an exogenous peroxidase using palmitoyl-CoA as substrate. Total protein of all fractions was measured according to Bradford (1976) using bovine serum albumin as standard.

Catalase histochemistry and stereological analysis

For morphometric analyses of peroxisomes, the digestive glands of 10 animals per group were dissected out, cryoprotected in phosphate buffer 0.1 M + 10% sucrose, embedded in Cryo-M-Bed, frozen in liquid N_2 and stored at −40°C until staining. The histochemical demonstration of catalase activity was performed using the diaminobenzidine method according to Cajaraville *et al.* (1997). Briefly, 8-μm

thickness cryostat sections were fixed in 4% formaldehyde in phosphate buffer 0.1 M at pH 7.4, containing 6% sucrose and 2.5% NaCl for 5 h at 4°C and rinsed in the same buffer without fixative. The incubation was carried out in a medium containing 0.2% 3,3′-diaminobenzidine, 0.01 M imidazole and 0.3% H_2O_2 as substrate in 0.01 M Teorell–Stenhagen buffer, pH 10.4, for 40 min at 42°C in a shaking water bath and darkness. After rinsing in Teorell–Stenhagen buffer, sections were dehydrated in a graded series of ethanol and mounted with DPX.

The stereological analysis of peroxisome structure was carried out essentially as described by Cajaraville *et al.* (1997). A lattice with 168 test points (multipurpose test system P168) was superimposed onto the preparations with the aid of a *camera lucida* attached to the light microscope, and the fraction of test points falling onto peroxisomes was determined. The digestive epithelium was considered as the reference space and test points falling on any other structure or tissue were not taken into account. Counts were made with the 100× objective at a total magnification of 1250× on five randomly selected microscopical fields per animal. In addition, the diameters of 90 peroxisomes per animal were measured using measuring paper superimposed onto the preparations. Approximate values of 0.5, 1.0 or 1.5 mm were assigned to each peroxisome, 1 mm in the measuring paper being equivalent to 0.73 μm in the tissue section. The volume density of peroxisomes (V_{vp}) was calculated as the ratio of peroxisomal volume (V_p) to digestive epithelium volume (V_c) with the formula:

$$V_{vp} = V_p/V_c = k(x/m)$$

where $k = (2/(3t))(\sum Y_i^3/\sum Y_i^2)$, t is the section thickness, x is the number of points falling on peroxisomes, m is the total number of points falling on the digestive epithelium and Y_i is the peroxisomal diameter.

SDS-PAGE and Western blotting

Polyclonal antibodies against rat Cu,Zn-SOD and Mn-SOD were kindly provided by Professor J. D. Crapo (Durham, NC, USA). These antibodies had previously been shown to cross-react with the mussel protein (Orbea *et al.* 2000). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots were performed as described previously (Orbea *et al.* 1999a). Four samples per experimental group were prepared to a final protein concentration of 2 μg μl^{-1} and 5 μl per lane were loaded in the gels. Two gels were always run simultaneously containing all the samples of one experimental day and processed under the same conditions. Additionally, the samples of the control groups of the three experimental days were loaded and run together to compare results obtained for the three samplings. After electrophoresis, proteins were transferred onto nitrocellulose membranes and these were blocked for 1 h at room temperature in 5% non-fatty dried milk. Incubations with the diluted specific antibodies against Cu,Zn-SOD and Mn-SOD (1:2000) were performed overnight at 4°C. After washing, membranes were incubated with the diluted (1:10 000) secondary antibody bound to peroxidase for 1 h at room temperature. Peroxidase activity was visualized with an ECL kit.

The average optical density of the immunoreactive bands corresponding to the monomeric form of Cu,Zn-SOD and Mn-SOD (Orbea *et al.* 2000) were quantified using the analysis software '1-D Main' (American Applied Biotechnology, Fullerton, CA, USA).

Statistical analysis

Statistical analyses were performed with the aid of the SPSS/PC+ statistical package (SPSS, Inc., Microsoft Co., Redmond, WA, USA). One-way analyses of variance (ANOVAs) were performed to study the effects of keeping animals under laboratory conditions on the activity of antioxidant and peroxisomal enzymes, on protein levels of SODs and on peroxisomal volume density. To analyse the effects of the different treatments, data were subdivided into three groups depending on the treatment type. Animals exposed to Cd were compared with the water control group and animals exposed to DEHP to the DMSO control group. Statistical differences between pairs of means for each experimental day were analysed by the Student's t-test. Animals treated with B(a)P and B(a)P + Cd were compared with the DMSO control group and analysed by one-way ANOVAs. Significant differences between means were studied using the Duncan's test for multiple range comparison between pairs of means. Significance level was established at $p < 0.05$ for all cases.

Results

Antioxidant and peroxisomal enzyme activities

The effect of keeping mussels in laboratory conditions during 4 weeks was assessed by means of one-way ANOVAs in the water-control group. Significant effects were detected for SOD, GPX and AOX but not for catalase (table 1). A

Table 1. Results recorded in the water control group in the whole experimental period for all parameters studied.

	Field	Day 0	Day 1	Day 7	Day 21
CAT ($n = 6$)	0.65 ($\pm$0.03)	0.61 ($\pm$0.02)	0.76 ($\pm$0.06)	0.55 ($\pm$0.07)	0.59 ($\pm$0.041)
SOD (*) ($n = 6$)	18.84 ($\pm$2.09)	16.20 ($\pm$1.31)	17.90 ($\pm$1.80)	36.07 ($\pm$3.62)	22.56 ($\pm$2.50)
GPX (*) ($n = 6$)	9.82 ($\pm$1.23)	10.22 ($\pm$0.71)	12.74 ($\pm$2.01)	15.26 ($\pm$1.68)	18.18 ($\pm$2.97)
AOX (*) ($n = 6$)	0.93 ($\pm$0.05)	0.61 ($\pm$0.06)	1.07 ($\pm$0.07)	0.50 ($\pm$0.04)	0.42 ($\pm$0.04)
$V_{vp} \times 10^4$(*) ($n = 10$)	1.95 (+0.24, −0.22)	1.83 (+0.30, −0.25)	1.76 (+0.45, −0.36)	1.02 (+0.14, −0.12)	2.65 (+0.18, −0.17)
Cu,Zn-SOD ($n = 4$)			90.04 ($\pm$3.79)	103.4 ($\pm$12.5)	113.8 ($\pm$10.7)
Mn-SOD (*) ($n = 4$)			71.76 ($\pm$9.45)	31.51 ($\pm$5.32)	90.35 ($\pm$3.62)

Standard errors (SE) are in parentheses.

Note that for V_{vp}, superior and inferior SE are provided, since due to the logarithmical transformation of data previous to statistical analyses, intervals of SE are asymmetric.

* Statistical significant differences ($p < 0.05$) according to one-way ANOVA analyses.

CAT, catalase (mmol min^{-1} mg protein^{-1}); SOD, superoxide dismutase (SOD Units mg protein^{-1}); GPX, glutathione peroxidase (nmol min^{-1} mg protein^{-1}); AOX, acyl-CoA oxidase (mUnit mg protein^{-1}); V_{vp}, volume density of peroxisomes (μm^3 μm^{-3}) $\times 10^4$.

slight increase in catalase activity was detected after 8 days of collecting animals in the field (day 1 of exposure) but catalase activity returned to initial values at the end of the experiment (table 1). GPX activity increased progressively during the experimental period (figure 1C). In the case of SOD activity, a significant increase was observed after 2 weeks in the laboratory, the activity decreasing again after 4 weeks (figure 1B). AOX activity decreased significantly along the experimental period although the highest value was reached after 8 days of sampling (day 1 of experiment) (figure 1D).

Cd exerted no significant effect on catalase, GPX and AOX activities but affected significantly SOD, lower values being recorded in the Cd group than in the water-control group after 7 days of exposure (figure 1).

In general, no significant differences were observed between the DMSO-control and the water-control groups in the four enzyme activities studied, with the exception of catalase and GPX activities after 21 days of exposure, when animals of the DMSO-control group showed significantly lower activities than those of the water-control group (data not shown).

Exposure to B(*a*)P provoked significant changes in catalase activity. After 7 days of exposure, catalase decreased in the B(*a*)P group with respect to the DMSO-control, but after 21 days catalase as well as GPX showed significantly higher activities in B(*a*)P-exposed mussels than in DMSO controls (figure 2A, C). In the case of SOD, mussels exposed to B(*a*)P presented significantly lower activity of SOD than the DMSO-control group after 7 days of exposure (figure 2B). With regard to the peroxisomal AOX activity, exposure to B(*a*)P caused a significant inhibitory effect after 1 day when compared with the DMSO-control group, but a slightly higher activity than in DMSO controls was recorded after 7 and 21 days of exposure (figure 2D).

The treatment with B(*a*)P + Cd caused inhibition of the significant decrease of catalase activity recorded in animals exposed only to B(*a*)P after 7 days (figure 2A). An inhibitory effect was also observed for GPX after 21 days of treatment with B(*a*)P + Cd (figure 2C). At day 7 of treatment with B(*a*)P + Cd, a significant

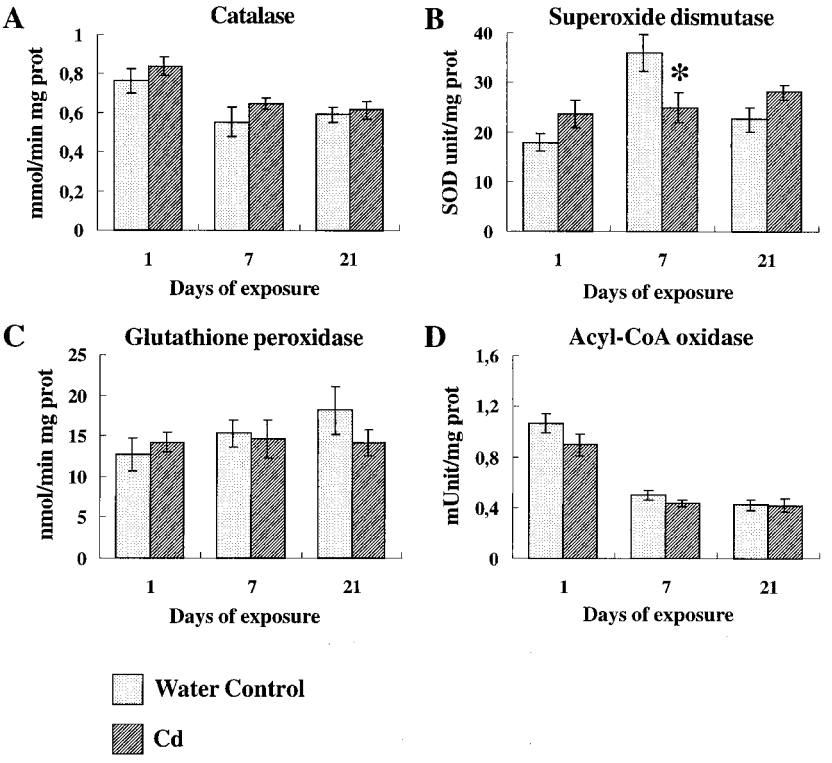

Figure 1. Activities of antioxidant and peroxisomal enzymes in the control and Cd-treated mussel. (A) Catalase; (B) superoxide dismutase; (C) glutathione peroxidase; (D) acyl-CoA oxidase. Animals were sacrificed after 1, 7 and 21 days of treatment. Vertical segments show SE. Significant differences between pairs of means according to Student's *t*-test are indicated by an asterisk (*) $p < 0.05$, $n = 6$).

inhibition of SOD activity occurred when compared with the DMSO-control group although not as marked as that caused by B(*a*)P alone (figure 2B). In all the cases SOD activity was higher in animals exposed to B(*a*)P + Cd than in those treated only with B(*a*)P. In the case of AOX, treatment with B(*a*)P + Cd for 1 day inhibited the significant decrease provoked by exposure to B(*a*)P alone. After 21 days of treatment, the B(*a*)P + Cd group showed significantly higher AOX values than animals from the DMSO-control group (figure 2D).

In mussels exposed to DEHP, catalase and GPX activities were higher than in the DMSO-control group after 21 days of experiment (significant for catalase) (figure 3A, C). SOD activity decreased significantly after 7 days of treatment with DEHP compared with the DMSO-control (figure 3B). AOX activity showed higher values in DEHP-treated mussels than in the DMSO-control group along the whole experimental period, these differences being significant after 21 days of exposure (figure 3D).

Peroxisomal volume density

No differences were found in V_{vp} between animals dissected just after collection in the field and mussels kept in the laboratory for 1 week (table 1). After 7 days

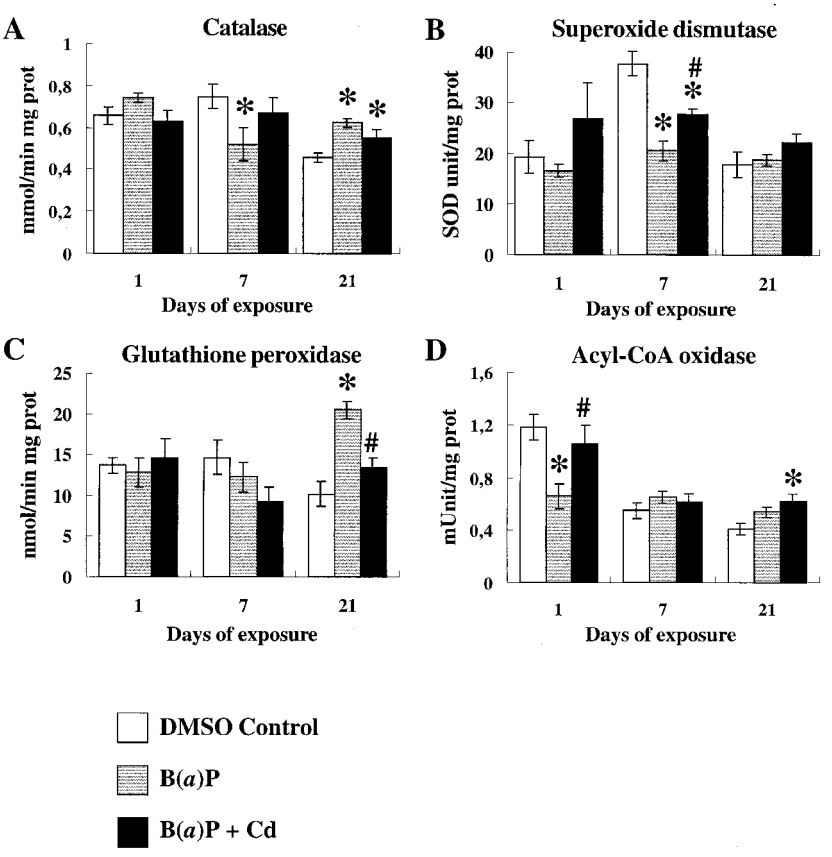

Figure 2. Activities of antioxidant and peroxisomal enzymes in mussels treated with DMSO (DMSO control group), B(*a*)P and B(*a*)P + Cd. (A) Catalase; (B) superoxide dismutase; (C) glutathione peroxidase; (D) acyl-CoA oxidase. Animals were sacrificed after 1, 7 and 21 days of treatment. Vertical segments show SE. Significant differences between pairs of means with respect to the DMSO control group are indicated by an asterisk (*). Significant differences with respect to the B(*a*)P-treated group are indicated by hash (#). Statistical signification is based on the multiple range test of Duncan ($p < 0.05, n = 6$).

of experiment the means of this parameter decreased but values higher than initial ones were reached after 21 days (figure 4A).

After 1 and 21 days of exposure to Cd, animals showed significantly lower V_{vp} when compared with the water-control group (figure 4A).

Mussels exposed to DMSO showed similar effects to those described for Cd treated mussels when compared with water-controls. Thus, a significant decrease in V_{vp} after 1 and 21 days of treatment was observed. In animals exposed to B(*a*)P a slight non-significant increase in V_{vp} was observed after 21 days of exposure when compared with the DMSO-control group (figure 4B). In the B(*a*)P + Cd group the trend to increase V_{vp} provoked by B(*a*)P at day 21 was inhibited (figure 4B).

Animals exposed to DEHP showed an increase in V_{vp} when compared with the DMSO-control group at day 1 of experiment but afterwards they showed lower values than controls (figure 4C).

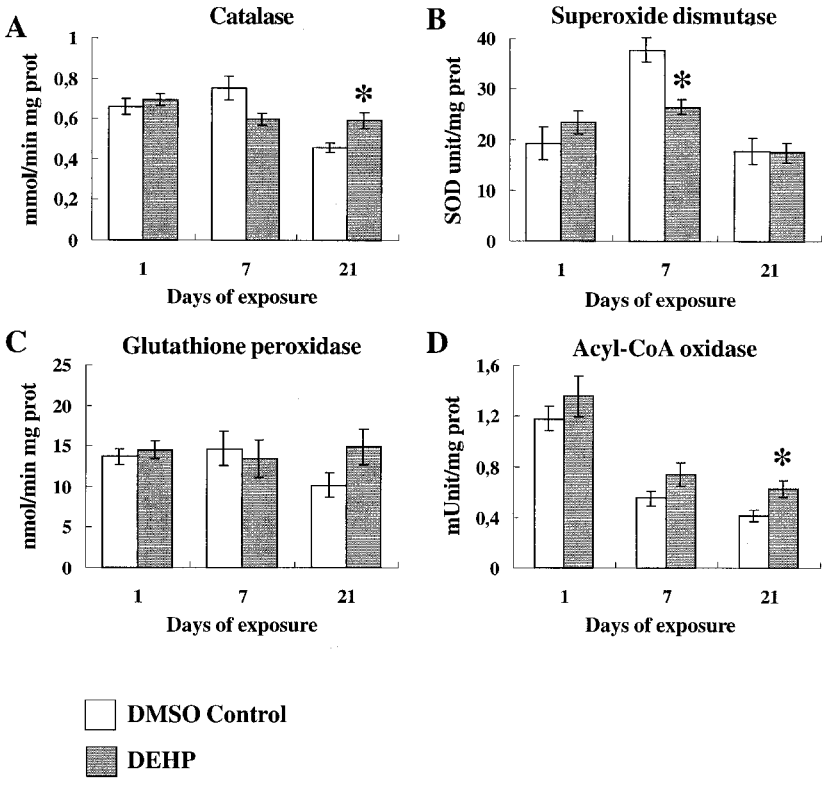

Figure 3. Activities of antioxidant and peroxisomal enzymes in mussels treated with DMSO (DMSO control group) and DEHP. (A) Catalase; (B) superoxide dismutase; (C) glutathione peroxidase; (D) acyl-CoA oxidase. Representation of results and statistical significance as in figure 1, $n = 6$.

Protein levels of Cu,Zn-SOD and Mn-SOD forms

A representative immunoblot of SDS-polyacrylamide gels of digestive gland 500*g* supernatants incubated with the antibody against Cu,Zn-SOD is shown in figure 5. Blots of this kind were used to quantify the protein levels of Cu,Zn-SOD and Mn-SOD along the experiment. Keeping mussels in the laboratory had significant effects over the Mn-SOD protein levels (figure 6B and table 1), which presented its lowest value after 7 days of experiment. Cu,Zn-SOD was not significantly altered along the experimental period in controls (figure 6A).

Treatment with Cd did not affect significantly the protein levels of Cu,Zn-SOD (figure 6A) but after 1 and 21 days caused a significant decrease in levels of Mn-SOD compared with controls (figure 6B).

Exposure to DMSO alone provoked a reduction in levels of Cu,Zn-SOD after 21 days. Mn-SOD showed lower values than the water controls during the whole experiment, these differences being significant at days 1 and 21 (*cf.* figures 6 and 7). The B(*a*)P treated group presented similar levels of Cu,Zn-SOD than the DMSO-control group (figure 7A). Mn-SOD levels showed a trend to decrease along the experiment in the B(*a*)P group, values being significantly lower than in the DMSO control after 21 days (figure 7B).

Simultaneous exposure to B(*a*)P + Cd caused a decrease in Cu,Zn-SOD after 1 and 7 days compared with animals exposed solely to DMSO or B(*a*)P (figure 7A),

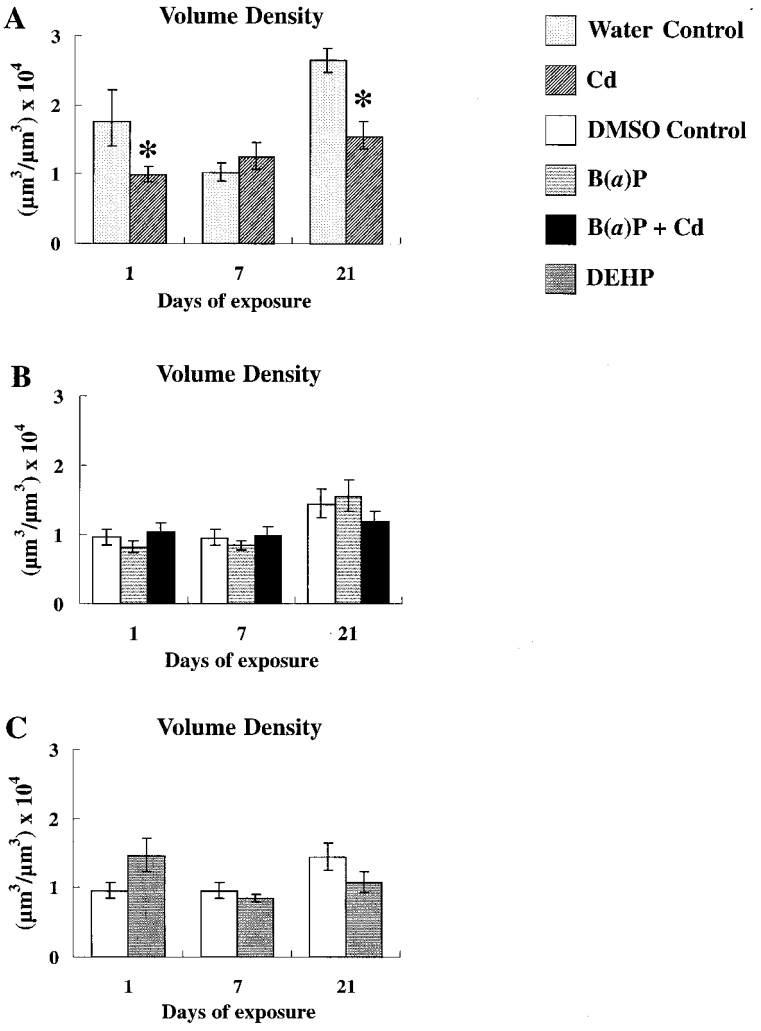

Figure 4. Peroxisomal volume density in (A) water control and Cd-treated mussels, (B) mussels treated with DMSO (DMSO control group), B(*a*)P and B(*a*)P + Cd, and (C) mussels treated with DMSO (DMSO control group) and DEHP. Representation of results and statistical significance as in figure 1 for A and C, and as in figure 2 for B, $n = 10$.

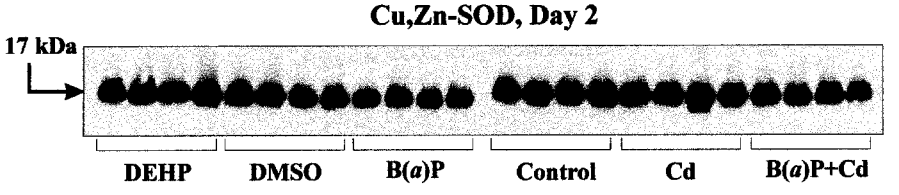

Figure 5. Representative immunoblot of SDS-polyacrilamide gels of digestive gland 500*g* supernatants of mussels exposed for 7 days to different treatments. Four different samples were loaded per experimental group. The immunoreactive band corresponding to Cu,Zn-superoxide dismutase was detected in all samples, with different intensities. The same protein concentration was loaded in all cases.

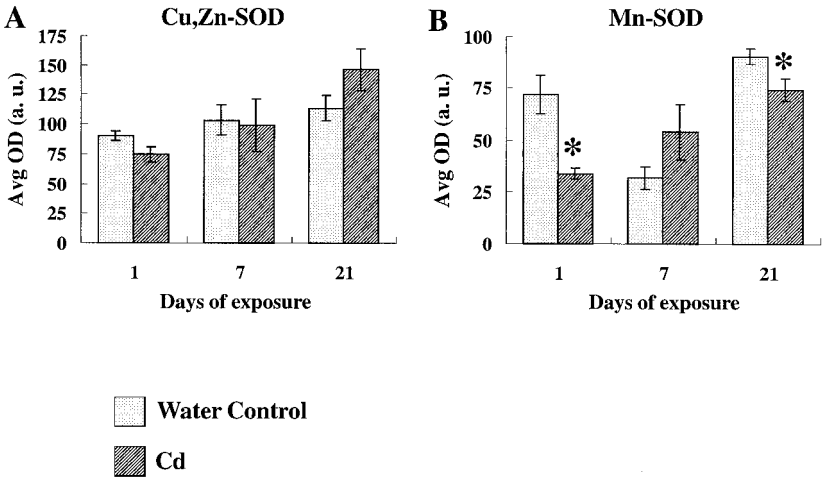

Figure 6. Protein levels of superoxide dismutase forms measured as the average optical density (in arbitrary units, a.u.) of immunoreactive bands in the control and Cd-treated mussels. (A) Cu,Zn-superoxide dismutase; (B) Mn-superoxide dismutase. Representation of results and statistical significance as in figure 1, $n = 4$.

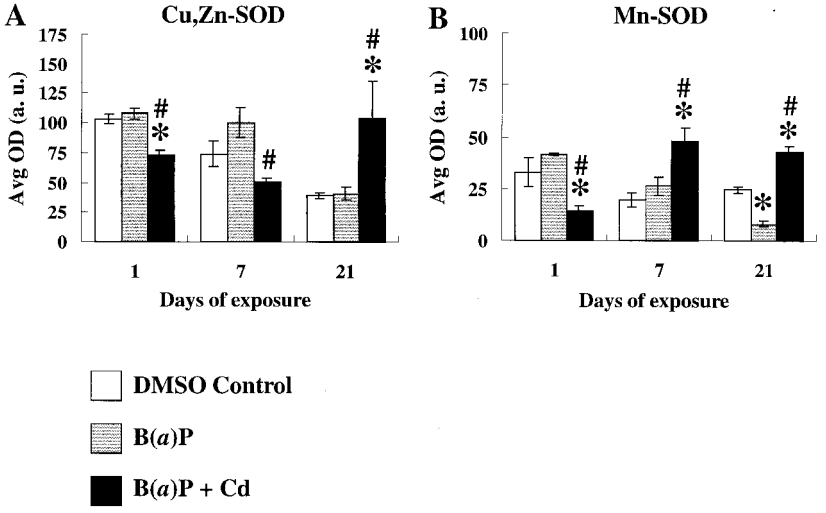

Figure 7. Protein levels of superoxide dismutase forms measured as the average optical density (in arbitrary units, a.u.) of immunoreactive bands in mussels treated with DMSO (DMSO control group), B(*a*)P and B(*a*)P + Cd. (A) Cu,Zn-superoxide dismutase; (B) Mn-superoxide dismutase. Representation of results and statistical significance as in figure 2, $n = 4$.

but at day 21, the B(*a*)P + Cd group shows significantly higher values of Cu,Zn-SOD than in DMSO or B(*a*)P-treated animals. With regard to Mn-SOD, after 1 day levels were significantly lower than in the DMSO-control and B(*a*)P groups. By days 7 and 21 however, the B(*a*)P + Cd group showed significantly higher values than the group treated with B(*a*)P alone or the DMSO control group (figure 7B).

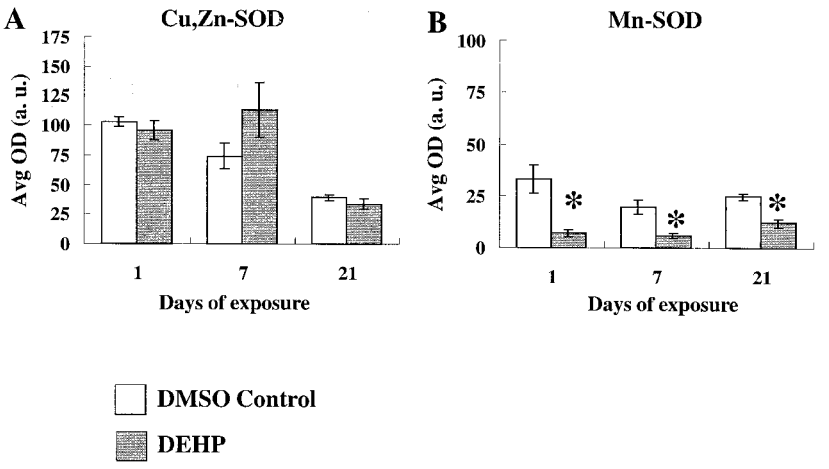

Figure 8. Protein levels of superoxide dismutase forms measured as the average optical density (in arbitrary units, a.u.) of immunoreactive bands in mussels treated with DMSO (DMSO control group) and DEHP. (A) Cu,Zn-superoxide dismutase; (B) Mn-superoxide dismutase. Representation of results and statistical significance as in figure 1, $n = 4$.

No differences were found between animals exposed to DEHP and the DMSO-control for Cu,Zn-SOD (figure 8A) whereas Mn-SOD values were significantly lower at the three sampling days in the DEHP treated group than in the DMSO control group (figure 8B).

Discussion

In this study, antioxidant and peroxisomal enzymes namely catalase, SOD, GPX and AOX, involved in the metabolism of ROS, as well as peroxisomal volume density, were analysed in digestive glands of mussels exposed to B(*a*)P, Cd, B(*a*)P + Cd and DEHP.

The experiment was performed during Spring months. Mussels were collected at the end of April and, after 1 week of acclimatization, the exposure experiment was run during May. Results obtained from control groups showed that enzyme activities and protein levels varied during the experimental period. This was especially true at day 7 when there was an increase in SOD activity, but a significant decrease in the protein levels of Mn-SOD together with a decrease in AOX activity, when compared with the first day of experiment. SOD activity and protein levels returned to initial values by the end of the experiment. Peroxisomal volume density also decreased by day 7 and returned to initial values at the end of the experiment. It is remarkable that both control groups (water-control and DMSO-control) showed the same temporal trends. Previous studies in mussels sampled at different seasons along the year have demonstrated that major changes in antioxidant and peroxisomal activities and peroxisomal structure occur in the Spring, when they show a maximum peak (Viarengo *et al.* 1991, Solé *et al.* 1995, Cancio *et al.* 1999, Orbea *et al.* 1999b). The time-related variations recorded in control groups could reflect those seasonal changes occurring in natural

conditions, whereas the treatment with xenobiotics induces an alteration of this natural trend.

In animals treated with Cd there was a significant inhibition of SOD activity after 7 days of exposure but this transient effect disappeared by day 21. Protein levels of Mn-SOD, but not Cu,Zn-SOD, was inhibited after 1 and 21 days of Cd exposure. In agreement, exposure of mouse to Cd provokes inhibition of catalase, GPX and SOD activities (Sugiyama 1994). Under our experimental conditions, Cd had no effects on the peroxisomal enzymes catalase and AOX, but it caused a significant decrease in peroxisomal volume density at days 1 and 21 of exposure. This toxic effect of Cd, and also those exerted on SOD, seem not to be mediated by ROS, as Cd (at a dose of $40\,\mu g\,l^{-1}$) does not produce lipid peroxidation or GSH depletion in mussels (Viarengo *et al.* 1990). The fact that Cd did not induce any of the antioxidant enzymes studied or the activity of the ROS-producing enzyme AOX gives further strength to this idea. Although it is known that in fish Cd impairs Zn homeostasis (Beyer *et al.* 1997), the protein levels of mussel Cu,Zn-SOD was not affected by exposure to Cd. The decrease of Mn-SOD protein levels in Cd-treated mussels suggests that Mn-SOD is more sensitive than Cu,Zn-SOD to this metal which could indicate an interaction of Cd with Mn homeostasis or a general damage to mitochondrial function.

In this study, DMSO was used as vehicle for organic contaminants and a control group exposed solely to DMSO was run in parallel. This group showed inhibition of catalase and GPX activities and SOD protein levels as well as a decrease in peroxisomal volume density after 21 days of treatment when compared with the water control group. We selected DMSO as vehicle because previous studies using acetone showed that the latter affects peroxisomal enzyme activities (Cancio *et al.* 1998). Since DMSO causes toxic and genotoxic effects at concentrations higher than $0.5\,ml\,l^{-1}$ (Gauthier 1989), only $0.033\,ml\,l^{-1}$ was used in this study. Doyotte *et al.* (1997) also used DMSO to expose freshwater mussels (*Unio tumidus*) to an organic toxicant and the DMSO control group showed similar values for catalase, SOD, GPX, glutathione reductase and GSH redox status than the water control group even when they used a higher concentration (0.05%) than that used in this work. From our results it becomes clear that, as observed previously for acetone, DMSO has also some effects on the studied parameters and a more suitable vehicle should be used in future studies.

Animals exposed to B(*a*)P showed a significant increase of catalase and GPX activities after 21 days of treatment. B(*a*)P also caused a slight increase in AOX activity and peroxisomal volume density after 21 days of exposure. Possibly the rise of catalase, GPX and AOX activities and the increase in peroxisomal volume density could have been higher if DMSO would have not been used as a vehicle since this compound inhibits catalase and GPX activities, and causes a decrease in peroxisomal volume density. Nevertheless, the results indicate a moderate increment of peroxisome proliferation in mussels treated with B(*a*)P which agrees well with previous reports showing increased activities of catalase (Livingstone *et al.* 1990) and AOX (Cancio *et al.* 1998) in B(*a*)P-treated mussels. Peroxisome proliferation and increased catalase activity has also been reported in mussels exposed to complex mixtures of PAH such as the water accommodated fraction of crude and lubricant oils (Cajaraville *et al.* 1992, 1997). In the liver of the fish *Solea aurata* B(*a*)P also caused an increase in the number of peroxisomes (Au *et al.* 1999).

In addition to catalase, B(*a*)P treatment raised the activity of the other H_2O_2-metabolizing enzyme GPX at day 21 but inhibited SOD activity and protein levels of Mn-SOD after 7 and 21 days exposure, respectively. Several studies in mammals have reported that peroxisome proliferators generally inhibit the activity of SOD (Lake 1993). In aquatic organisms, Eertman *et al.* (1995) reported an increase in SOD activity in mussels *M. edulis* exposed to $0.5\,\mu g\,l^{-1}$ of B(*a*)P or fluoranthene for 2 or 4 weeks, but SOD activity decreased at concentrations of $6\,\mu g\,l^{-1}$ for both compounds. This inhibitory effect of PAH on enzyme activities could be due to a narcotic effect, as discussed by Eertman *et al.* (1995). On the other hand, fish SOD, especially the Cu,Zn-SOD form, is very sensitive to oxidative stress, since Cu,Zn-SOD is oxidatively modified and inactivated by ROS (Pedrajas *et al.* 1995).

Similar to the effects caused by B(*a*)P, in animals exposed to $500\,\mu g\,l^{-1}$ DEHP a significant augmentation of catalase, GPX and AOX activities is observed after 21 days of exposure whereas SOD activity and Mn-SOD protein levels are inhibited after 7 days, and after 1, 7 and 21 days of exposure, respectively. We have previously reported increased AOX activity in mussels injected with DEHP (60 μg/animal) but not in mussels exposed to DEHP at a lower concentration ($100\,\mu g\,l^{-1}$) of that used in the present study (Cancio *et al.* 1998). The capacity of DEHP and its metabolite MEHP (mono-2-(ethylhexyl) phthalate) to induce the peroxisomal β-oxidation has been demonstrated in several mammalian species (Lhugenot and Cornu 1993) especially rats and mice. In these experiments, catalase increment has also been demonstrated but to a lesser extent than AOX (Conway *et al.* 1989, Ganning *et al.* 1989, Cimini *et al.* 1994). This imbalance in the induction of H_2O_2-producing and -degrading peroxisomal enzyme activities can give rise to a situation of oxidative stress because the exceeding H_2O_2 can pass through the peroxisomal membrane as consequence of its high permeability and react with cell structures causing oxidative damage (Lake 1993, Reddy and Mannaerts 1994, Cancio and Cajaraville 2000). Oxidative damage to SOD and ROS-mediated down-regulation of Mn-SOD may explain the decrease in SOD activity and protein levels respectively, as observed in mussels treated with B(*a*)P or DEHP.

Although complex interactions among contaminants must occur in the aquatic environment, studies addressing this issue in mussels are rare (Moore *et al.* 1984). Thus, one of the aims of the present work was to study the possible interactive effects between organic and metallic contaminants in mussels exposed simultaneously to a mixture of B(*a*)P and Cd. Mussels exposed to B(*a*)P + Cd showed differences in peroxisomal and antioxidant enzyme activities as well as in peroxisomal volume density when compared with animals treated with B(*a*)P alone. In general, Cd appeared to inhibit the effects caused by B(*a*)P, i.e. Cd inhibits partially the increase of catalase and GPX activities as well as the inhibition of SOD activity caused by B(*a*)P. Moreover, Cd inhibits the increase in peroxisomal volume density observed in the B(*a*)P group at day 21. In agreement with the results reported in this study, in the plaice *Platichthys flesus* L. the administration of these two compounds affected biomarker responses differently from compounds administered alone, although the bioaccumulation pattern of the two chemicals given alone was not modified (Sandvick *et al.* 1997). Thus, pre-treatment with Cd provokes a partial inhibition of cytochrome P4501A (CYP1A) induction caused by B(*a*)P, and pre-treatment with B(*a*)P and PCB prior to Cd inhibited metallothio-

nein induction compared with Cd alone (Sandvick *et al.* 1997). In mussels it has been reported that exposure to a mixture of phenanthrene and Cu does not cause interactive effects on the accumulation and depuration of these compounds, but interactive effects occur in biomarker responses, i.e. latency of lysosomal hydrolases or activity of the microsomal respiratory chain (Moore *et al.* 1984).

In conclusion, peroxisome proliferation, measured as increased activity of the peroxisomal enzymes catalase and AOX, is a response to organic contaminants such as B(*a*)P and DEHP, whereas Cd does not cause peroxisome proliferation. Therefore, these enzyme activities, or other comparable measurements of peroxisome proliferation, could be used as biomarkers in pollution monitoring programs but always taking into consideration that responses of mussels to organic xenobiotics can be modulated by concomitant exposure to metal contaminants. Interestingly, the biomarker most widely used to monitor organic pollutants, i.e. induction of EROD activity and/or the induction of CYP1A protein, is very reliable in fish but not in molluscs (Canova *et al.* 1998) and thus peroxisome proliferation has been proposed as a possible alternative biomarker for organic pollutants in molluscs (Cajaraville *et al.* 2000). Nevertheless, further studies with other metals might be needed to assure the specificity of peroxisome proliferation for organic contaminants. On the other hand, all the organic and metal pollutants tested inhibit SOD activity and protein levels, possibly as a consequence of oxidative stress linked to overproduction of ROS in the case of organic pollutants. Thus, SOD inhibition offers potential as general marker of pollution.

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