Multi-response biomarker approach in the crab *Carcinus aestuarii* experimentally exposed to benzo(*a*)pyrene, polychlorobiphenyls and methyl-mercury

M. C. Fossi, C. Savelli, S. Casini, E. Franchi, N. Mattei and I. Corsi

The aim of this study was to test a multi-response biomarker approach for evaluating toxicological risk due to some of the main contaminants in the Mediterranean (benzo(*a*)pyrene, polychlorobiphenyls and methyl-mercury), using the Mediterranean crab *Carcinus aestuarii* as bioindicator organism. Forty crabs were injected with different doses of these contaminants. Several molecular, biochemical and genotoxic biomarkers were tested in different tissues and biological materials. The main conclusions were: (1) hepatopancreas, gills, haemolymph and excreta seem to be useful for biomarker studies in this species; (2) several biochemical, molecular and genotoxic biomarkers were found suitable for testing in these tissues; (3) several biomarkers were found suitable for evaluating chemical stress due to different Mediterranean contaminants.

Keywords: *Multi-response biomarker approach, invertebrates,* Carcinus aestuarii*, benzo(a)pyrene, polychlorobiphenyls, methyl-mercury.*

Introduction

The idea of measuring certain biological parameters as indicators of the health status of an organism dates back to the origins of medicine. With present-day molecular, biochemical, cytological and physiological techniques, biomarkers are now used in all fields of medicine, from oncogenesis to occupational medicine. The idea of using biomarkers in ecotoxicology is first found in the pioneering studies of Bayne *et al.* (1976) and Payne (1977) in marine environments. In the last 20 years biomarkers have been used in a vast range of environmental situations. In fact, the concept of biomarkers for evaluating risk in marine, terrestrial and fresh water environments has captured the attention of control agencies and is currently being assessed by several research commissions. This interest is confirmed by the increasing number of specialist manuals (McCarthy and Shugart 1990, Hugget *et al.* 1992, Peakall 1992, Fossi and Leonzio 1993, Peakall and Shugart 1993).

Various definitions of biomarkers have been proposed in the last decade, but here we propose the following definitions proposed by Depledge (1993) who defined the 'ecotoxicological biomarker' as a 'biochemical, cellular, physiological, or behavioural variation that can be measured in tissue or body fluid samples or at the level of whole organisms (either individuals or populations) that provides evidence of exposure and/or effects of one or more chemical pollutants (and/or radiations)'.

In most ecotoxicological studies, a single biomarker is not able to give enough information on the health status of the organism, and there is a need for the application of a number of biomarkers that can give information at different levels of biological organization. This suite of different biomarkers (*multi-response biomarker approach*) is in fact capable of indicating rapid responses to toxicant exposure and providing an early warning of long-term ecological effects. This simultaneous use of biomarkers to help to determine where an organism lies on the 'health status curve' illustrates an important principle, namely, that a suite of biomarkers offers greater possibilities for the detection of effects than do single biomarkers alone (Depledge 1993).

The historical development of the biomarker approach is closely linked to medicine and vertebrate biology (NCR 1987). In fact, most papers on the use of biomarkers in environmental hazard assessment have focused on the use of terrestrial, marine and freshwater vertebrates. However, biomarker measurements are equally feasible in invertebrate samples. There are several reasons why invertebrates are preferable in this ecotoxicological area. For example, invertebrates constitute 95% of all animal species (Barnes 1968). They are major components of all ecosystems, and invertebrate populations are often numerous, so that samples can be taken for analysis without significantly affecting population dynamics. In ecotoxicology studies invertebrates have an advantage over fish in that they remain in a confined area (Depledge and Fossi 1994). Increasing knowledge of the biochemistry of invertebrates (Livingstone 1991, 1992a, Stegeman *et al.* 1992) now permits reasonable interpretation of biomarker responses in terms of ecological risk assessment.

Most studies on biomarkers in invertebrates, especially crustaceans, have focused on mixed function oxygenase (MFO) (Burns 1976, Payne 1977, Singer and Lee 1977, James *et al.* 1979, Singer *et al.* 1980, Lee 1981, Lee *et al.* 1981, O'Hara *et al.* 1982, Bihari *et al.* 1984, Quattrochi and Lee 1984a, b, Batel *et al.* 1988, James 1989), because the MFO system is the main system detoxifying lipophilic compounds in all animals. Although MFO capacity is lower in marine invertebrates than in vertebrates, it has been shown to play a significant role in the metabolization of xenobiotics in the former (Lee *et al.* 1981, Livingstone 1981).

A few studies have evaluated other biomarkers in invertebrates, particularly the genotoxic effects of contaminants (Mortimer and Hughes 1991), interactions with esterase enzymes (Day and Scott 1990, Reddy and Venugopal 1990, Bocquené and Galgani 1991, Maheswari and Selvarajan 1991), changes in porphyrin metabolism (Brock 1992, Chamberland *et al.* 1994) and micronuclei frequencies. Here we also examine these aspects.

In the present study we tested a multi-trial biomarker approach for evaluating toxicologica

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contaminants in the Mediterranean (benzo(*a*)pyrene, (B(*a*)P), polychlorobiphenyls (PCBs) and methyl-mercury (MeHg)) using an invertebrate, the crab *Carcinus aestuarii* Nardo, 1847 (Crustacea: Decapoda) as bioindicator organism. B(*a*)P, a carcinogenic compound in mammals, is widespread in aquatic environments and accumulates more readily in invertebrates than in fish (Stegman 1981, Van der Oost *et al.* 1991) due to the lower rate of biotransformation of the former (Livingstone *et al.* 1992b). PCBs are toxic to many marine invertebrates and vertebrates (Duke *et al.* 1970, Nimmo *et al.* 1971) and are metabolized very slowly or not at all. The higher concentrations are found in organisms at the top of the food chain (Livingstone *et al.* 1992b). MeHg is a product of biotransformation of inorganic mercury and it is a toxic and easily bioconcentrated compound that potentially is biomagnified through the marine food-chains. The Mediterranean, a semi-enclosed sea, is a zone containing some of the richest natural reserves of mercury. Contamination from both anthropogenic and natural sources could generate pollution problems in the whole Mediterranean basin (Bacci 1989).

Several biochemical, molecular and cytogenetic biomarkers are examined in this study; they included: mixed function oxygenase (MFO), B esterases, porphyrins, DNA-based assays (micronuclei, alkaline unwinding assay) and some general condition indices. The main objectives of this study were: (1) to identify the tissues and biological materials useful for biomarker studies in *C. aestuarii*; (2) to evaluate various biochemical, molecular and genotoxic biomarkers in different tissues; (3) to identify the most suitable biomarkers for evaluating chemical stress due to the main Mediterranean contaminants: benzo(*a*)pyrene, polychlorobiphenyls and methyl-mercury.

MATERIALS AND METHODS

Treatment of the animals

Forty specimens of the marine crab, *C. aestuarii*, were collected in Orbetello lagoon (Grosseto, Tuscany, Italy) in the summer of 1994 and brought to the laboratory in containers kept at low temperature. The crabs, all male (average diameter of carapace 6 cm), were then divided into groups of five and placed in glass tanks with 10 litres of constantly oxygenated artificial marine water (37‰ salinity). They were kept at a temperature of 20 °C with a light period from 6 am to 8 pm. The crabs were left to acclimatize for 1 week. During this period they were fed every 3 days with mussels. The water was changed every 3 days.

After 1 week the groups were allocated different treatments: control (C), 50 µl DMSO (dimethyl sulphoxide) (CD), 1 µg benzo(*a*)pyrene (BPL), 10 µg benzo(*a*)pyrene (BPH), 10 µg polychlorobiphenyls (PCBL), 100 µg polychlorobiphenyls (PCBH), 1 µg methyl-mercury (HGL) and 10 µg methyl-mercury (HGH).

All the compounds were diluted in DMSO. The dose for each crab was contained in 50 µl of DMSO vehicle which was injected. After 3 days the crabs were sacrificed by pinching their nervous system very quickly. Before sacrificing the crabs, haemolymph was drawn and stored at –80 °C. Weight, carapace diameter and any limbs missing were noted for each specimen. Hepatopancreas and gills were removed and stored at –80 °C. We collected the faeces (pooled) of the group from every tank and stored at –80 °C as well. Biochemical, metabolic and genotoxic biomarkers were evaluated in these biological materials, as detailed in Figure 1.

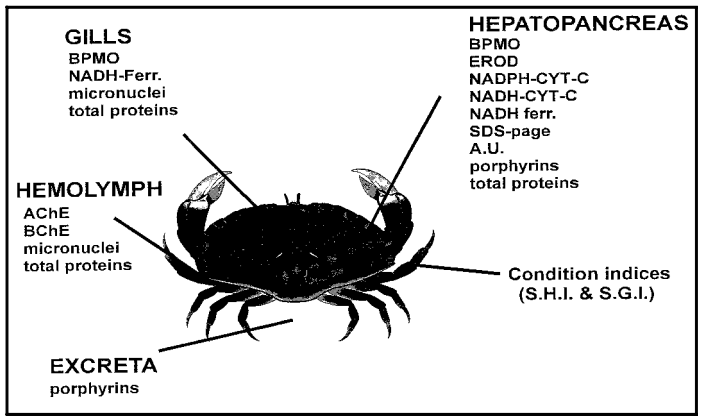


Figure 1. Biochemical, metabolic and genotoxic biomarkers detected in different biological materials of *Carcinus aestuarii* experimentally treated with benzo(*a*)pyrene, polychlorobiphenyls and methyl-mercury. BPMO, benzopyrene mono-oxygenase activity, EROD, ethoxyresorufin-*O*-deethylase activity; NADPH-CYT-C, NADPH cytochrome C reductase activity; NADH-CYT-C, NADH cytochrome C reductase activity; NADH-Ferr., NADH ferricyanide reductase activity; SDS-page, SDS–polyacrylamide gel electrophoresis; A.U., alkaline unwinding assay; AChE, acetylcholinesterase activity; BChE, butyrylcholinesterase activity; S.H.I., somatic hepatopancreas index; S.G.I., somatic gill index.

Biochemical assays

Microsomal preparation

One to two grams of tissue (hepatopancreas or gills) were weighed and homogenized in 10 mM Tris, 0.5 M sucrose, 0.15 M KCl, 1 mM EDTA, 1 mM DTT and 400 µM PMSF buffer (pH 7.6) in 1:4 (w/v) proportion. The homogenization was carried out with 10 passages in a Wheaton potter homogenizer. Gills were cut with scissors before homogenizing them for 1 min in a Turrax blender and eight passages in a Wheaton potter homogenizer. The homogenates were also sonicated for 30 s, and centrifuged at 9000 × *g* for 20 min at 4 °C. The supernatant centrifuged again at 100 000 × *g* for 60 min at 4 °C. A 0.5 ml aliquot of resuspension buffer (10 mM Tris, 20% glycerol, pH 7.6) was added to the pellet and the suspension stored at –20 °C for about half an hour and then at –80 °C until analysis. Before analysis, the microsomes were resuspended (five passages in a potter homogenizer) in 2.6 ml resuspension buffer per g of tissue. Gill microsomes were also sonicated for 30 s.

Microsomal enzyme activities

Mixed function oxygenase activity was evaluated in the microsomal fraction of hepatopancreas and gills by assaying: ethoxyresorufin-*O*-deethylase (EROD) and benzo(*a*)pyrene monooxygenase (BPMO) activities. In the same fraction were also detected the reductase enzyme activities (NADPH cytochrome C reductase, NADH cytochrome C reductase and NADH ferricyanide reductase). All the tests were carried out at 30 °C.

Ethoxyresorufin-O-deethylase (EROD) activity. EROD activity was measured by the method of Lubet *et al.* (1985), using 300 µl of resuspended microsomes and 5 µl of ethoxyresorufin in the incubation mixture. The incubation time was 15 min. The activity was expressed as pmol mg^{-1} prot min^{-1}.

Benzo(a)pyrene monooxygenase (BPMO) activity. BPMO activity was measured by the method of Kurelec *et al.* (1977), using 40

enzymes and incubating the reaction mixture for 1 h. The activity was expressed as FU mg^{-1} prot h^{-1}.

Reductase enzyme activities. NADPH cytochrome C reductase, NADH cytochrome C reductase and NADH ferricyanide reductase were assayed by the method of Livingstone and Farrar (1984).

SDS–PAGE of microsomal proteins

The microsomal fraction of hepatopancreas and gills was denatured with Laemmli buffer in 1:4 (w/v) proportion and the isoforms of Cyt-P450 analysed by the method of Laemmli (1970) using SDS–PAGE, Phase System apparatus and 12.5% PhastGel Homogeneous. The amount of microsomal proteins in each sample was approximately 7.5 μg. Molecular weight was evaluated using bio-rad SDS–PAGE standards (High), containing proteins with molecular weights ranging from 40 000 to 200 000 Da. Densitometric analysis of the gels was performed using Gel Image Software by Pharmacia. Molecular weight (MW) and absorbance unit (AU) are reported for the proteins in the range between 45 and 60 kDa, and 60 and 97 kDa.

B esterase activities

Haemolymph was centrifuged at 8000 × *g* for 10 min and the pellet containing impurities from sampling, eliminated. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities were determined by the method of Ellman *et al.* (1961) and Westslake *et al.* (1981), using 10–20 μl of centrifuged haemolymph as enzyme source (30 °C). AChE and BChE activities were expressed in μmol min^{-1} ml^{-1}.

Protein concentrations

The concentrations of microsomal proteins were calculated using the bio-rad Protein Assay and expressed in mg ml^{-1}. Haemolymph was sonicated for 15 min and diluted 1:500 in resuspension buffer. The protein concentration was then calculated using the bio-rad Protein Assay and expressed in mg ml^{-1}.

Porphyrin concentrations

Porphyrin concentrations were determined in hepatopancreas and excreta. In hepatopancreas, about 300 mg of tissue was homogenized 1:10 with distilled water using a Turrax homogenizer; 0.2 ml of homogenate was then transferred to glass tubes and spiked with 1.6 ml of 50:50 methanol/perchloric acid mixture. After a vortex-mixing, the samples were kept in the dark for 10 min and then centrifuged for 5 min at a low speed. The porphyrin extract in the upper layer was then used for spectrofluorimetric quantitative determination (Grandchamp *et al.* 1980). The method of Lockwood *et al.* (1985) was used for the extraction of porphyrins from the pools of excreta: 1 ml of 5N HCl was added to a graduated centrifuge tube containing an accurately weighed excreta sample (100 mg, dry weight) and vortex-mixed. Diethyl ether (3 ml) was added and thoroughly mixed to give an emulsion, followed by water (3 ml) and further mixing. The mixture was then centrifuged at 700 × *g* for 10 min. The lower layer of aqueous acid containing the porphyrins was then taken for fluorimetric determination.

Quantitative determination of porphyrins was performed by the method of Grandchamp *et al.* (1980). This fluorimetric procedure is used to determine the percentages and concentrations of uroporphyrin, coproporphyrin and protoporphyrin in a mixture of porphyrins in the nanomolar range. The procedure is based on the three different excitation/emission wavelength couples of each porphyrin (uroporphyrin: 405–595 nm; coproporphyrin: 400–595 nm; protoporphyrin: 410–605 nm). By recording the fluorescence emission of the mixture at the three different couples of values it is possible to obtain three linear functions representing the concentrations of each porphyrin in the mixture, expressed as pmol g^{-1} biological material extracted.

Genotoxic assays

Alkaline unwinding assay

The alkaline unwinding test involves the binding of Hoechst 33258 fluorescent dye. The intensity of fluorescence is different for double and single strand DNA. Three different values of fluorescence are measured to obtain the rate of unwinding under alkaline and heat treatment. The number of breaks is directly proportional to the speed of unwinding. The three fluorescence values are that of the intact double strand (dsDNA) (maximum fluorescence), an intermediate value after alkaline treatment (auDNA) and that of single strand DNA (ssDNA) (minimum fluorescence). From these three values it is possible to determine the value of *F*, the residual DNA still in double strand form after alkaline treatment (Cesarone *et al.* 1979, Birnboim and Jevcak 1981, Daniel *et al.* 1985, Morris and Shertzer 1985, Shugart 1988):

$$F = \frac{fau\mathrm{DNA} - fss\mathrm{DNA}}{fds\mathrm{DNA} - fss\mathrm{DNA}} \quad (1)$$

A high *F* value indicates that the DNA unwound slowly and therefore has few breaks; a low *F* value means that the DNA unwound fast and therefore has many breaks. A measure of the number of breaks, in fluorescence units, is obtained by comparing the *F* values of a control organism with those of the stressed animal:

$$n = \frac{\ln F_e}{\ln F_o} - 1 \quad (2)$$

where F_e and F_o are the *F* values of exposed animals and controls, respectively.

Micronuclei

Micronuclei were determined by the method of Scarpato *et al.* (1990; modified). A small piece of gill was lacerated and digested with a solution of dispase in Hank's Balanced Salt Solution for 20 min then filtered with a plankton net to obtain a cell suspension. The pellet was centrifuged at 1000 rpm for 10 min and fixed in Carnoy's solution (acetic acid:methanol 3:1) for 20 min. After another centrifugation at 1000 rpm for 10 min, the cells were dropped onto clean glass slides and stained with 5% Giemsa/distilled water for at least 20 min, rinsed in distilled water, air dried and mounted in Eukitt. All slides were coded and analysed blindly. Gill cells were examined using a microscope with an oil-immersion objective at a magnification of 1000. Only cells with intact cell and nuclear membranes were scored. Two thousand gill cells per crab were scored and the results expressed as number of micronucleated cells per 1000 gill cells (MN‰).

Condition indices

The somatic hepatopancreas index (SHI) and the somatic gill index (SGI) were calculated for every sample [(weight of hepatopancreas or gills/crab weight) × 100].

Statistical analysis

All the data were processed by parametric statistical analysis using Statistica software (Microsoft) to obtain mean, standard deviation, standard error, correlation matrix and variance.

Results

The main results of this experiment are discussed separately for each of the biological materials analysed: hepatopancreas (Figures 2, 3 and 4), gills (Figure 5), haemolymph (Figure 6) and excreta (Figure 7).

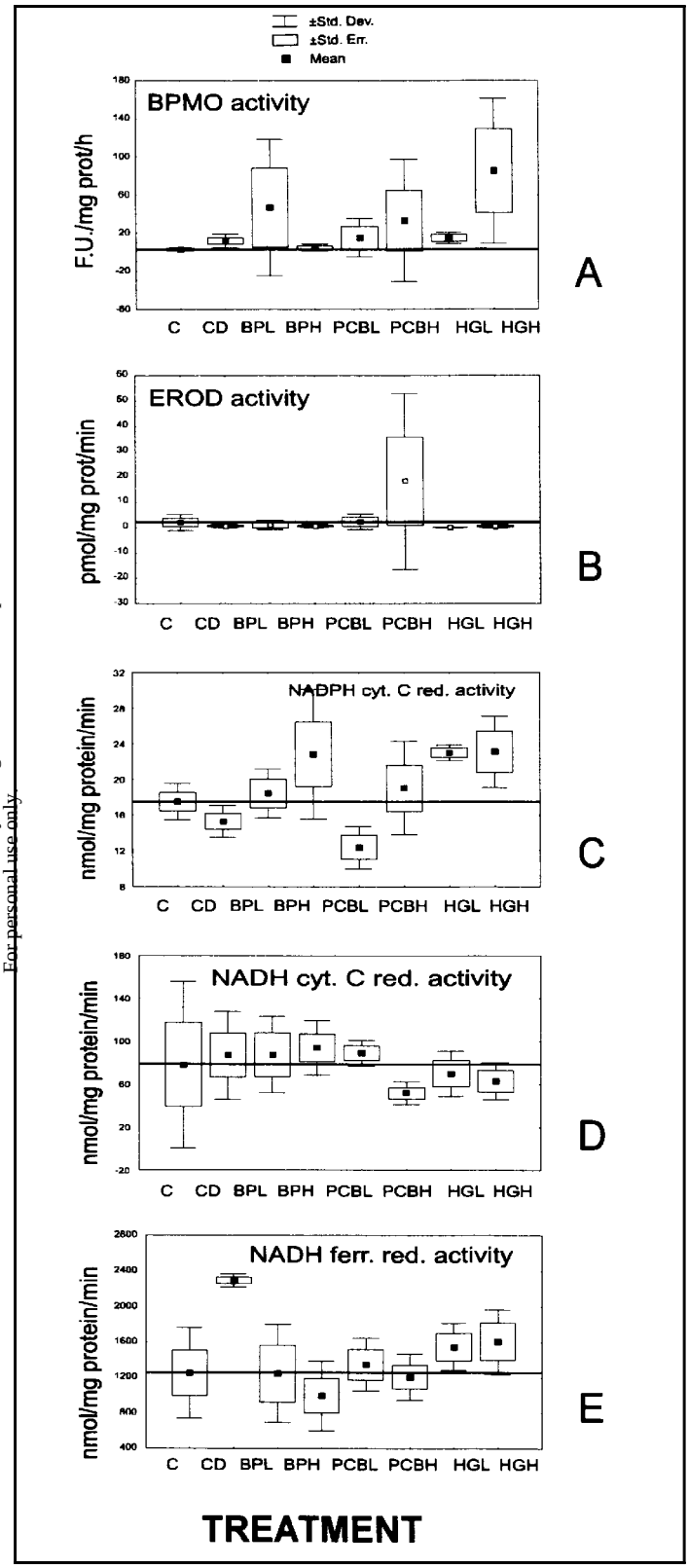


Figure 2. Biomarkers: benzopyrene monooxygenase activity (A), ethoxyresorufin-*O*-deethylase activity (B), NADPH cytochrome C reductase activity (C), NADH cytochrome C reductase activity (D), NADH ferricyanide reductase activity (E), in hepatopancreas of *Carcinus aestuarii* experimentally treated with benzo(*a*)pyrene, polychlorobiphenyls and methyl-mercury. C, control ($n = 4$); CD, control + 50 µl DMSO ($n = 2$); BPL, 1 µg benzo(*a*)pyrene ($n = 3$); BPH, 10 µg benzo(*a*)pyrene ($n = 4$); PCBL, 1 µg polychlorobiphenyls ($n = 4$); PCBH, 10 µg polychlorobiphenyls ($n = 4$); HGL, 1 µg methyl-mercury ($n = 3$); HGH, 10 µg methyl-mercury ($n = 3$).

Hepatopancreas

BPMO activity in this tissue (Figure 2(A)) was highly enhanced in the BPL, PCBH and HGH groups. EROD activity (Figure 2(B)) was induced only in the group treated with PCBs. NADPH cytochrome C reductase (Figure 2(C)) was induced in the BPH and HGH groups, whereas NADH ferricyanide reductase and the NADH cytochrome C reductase (Figure 2(D and E)) did not differ statistically between groups. Molecular weight determination by SDS–PAGE of the microsomal fraction (Figure 3) showed induction of proteins in the area between 66 and 97 kDa in the CD (AU 0.02) and BPL (AU 0.02 and 0.03) groups, and in the area between 66 and 97 kDa in CD (AU 0.03), BPL (AU 0.04 and 0.12), BPH (AU 0.01 and 0.06), PCBL (AU 0.04 and 0.13), PCBH (AU 0.02 and 0.09), HGL (AU 0.06 and 0.23) and HGH (AU 0.05 and 0.26) groups. DNA damage (Figure 4(A)) assayed by alkaline unwinding was statistically significant ($p < 0.05$) in all treated groups with respect to controls. All compounds assayed produced a similar number of breaks, while DMSO decreased DNA breaks. These results present some difficulties in the interpretation related to the concomitant presence of positive and negative effects related to the different experimental treatments. Total porphyrin concentrations (Figure 4(C)) decreased in all treatment groups, particularly in those treated with methyl-mercury, while uroporphyrin concentrations decreased in the BPH, PCBH and HGL groups ($p < 0.05$). Total protein concentrations increased in the BPL, PCBL and HGL groups but the difference was not statistically significant with respect to controls (Figure 4(D)).

Gills

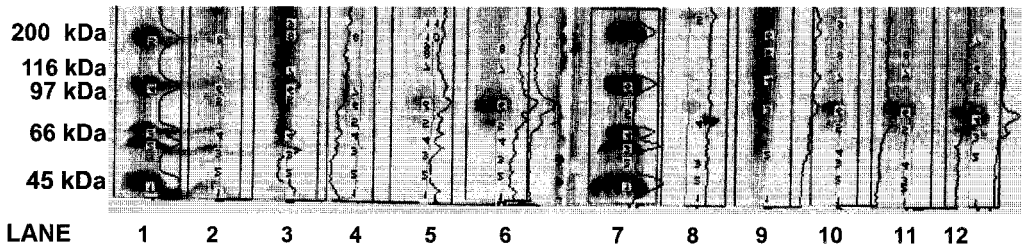
Gills (Figure 5) were found suitable for MFO assay. The experiment confirmed that BPMO and the reductase activities (Figure 5(A and B)) can be tested in this tissue. BPMO activity was induced in the BPL and HGH groups. One of the most interesting results was that of the analysis of the micronuclei. Frequencies of micronuclei increased in the BPL, BPH, PCBH, HGL and HGH groups and the increase was statistically significant ($p < 0.05$) in all treated groups with respect to controls (Figure 5(C)).

Haemolymph

It was possible to test AChE activity in haemolymph (Figure 6(A)). AChE activity was much lower in the BPH group. BChE activity (Figure 6(B)) did not show any statistically significant difference between treated groups and controls. Total protein concentration (Figure 6(C)) was higher in the PCBH, HGL and HGH groups.

Excreta

Excreta were found to be particularly suitable for the assay of porphyrins (Figure 7). All the contaminants tested and especially MeHg produced a drastic decrease in porphyrin concentrations with respect to the control group. Protoporphyrin concentrations decreased in the HGH group, and uroporphyrin concentrations decreased in the BPL, PCBH and HGL groups in a statistically significant manner ($p < 0.05$).



Lane	Group	Range 45-60 kDa		Range 60-97 kDa	
		M.W. kDa	Area A.U.	M.W. kDa	Area A.U.
2	C	54.8	0.0	70.4 65.3	0.01 0.11
3	CD	57.6	0.02	69.1	0.03
4	BPH			81.1 71.1	0.01 0.06
5	PCBH	54.2 48.5 45.0	0.01 0.0 0.0	81.1 70.4	0.02 0.09
6	HGH	54.8 45.7	0.01 0.01	82.3 70.4	0.05 0.26
8	C			78.8	0.08
9	CD	58.9 46.4	0.05 0.01	71.1	0.07
10	BPL	56.9 47.1	0.03 0.02	81.1 71.1	0.04 0.12
11	PCBL	54.8 46.4	0.01 0.01	82.3 71.8	0.04 0.13
12	HGL	52.8	0.01	88.0 75.0	0.06 0.23

Figure 3. SDS–PAGE of microsomal fraction of hepatopancreas of *Carcinus aestuarii* experimentally treated with benzo(*a*)pyrene, polychlorobiphenyls and methyl-mercury. The results of densitometric analysis are reported in the table. For the proteins in the range between 45 and 60 kDa and 60 and 97 kDa molecular weight (MW) and for absorbance unit (AU) are reported. C, control; CD, control + 50 μl DMSO; BPL, 1 μg benzo(*a*)pyrene; BPH, 10 μg benzo(*a*)pyrene; PCBL, 1 μg polychlorobiphenyls; PCBH, 10 μg polychlorobiphenyls; HGL, 1 μg methyl-mercury; HGH, 10 μg methyl-mercury.

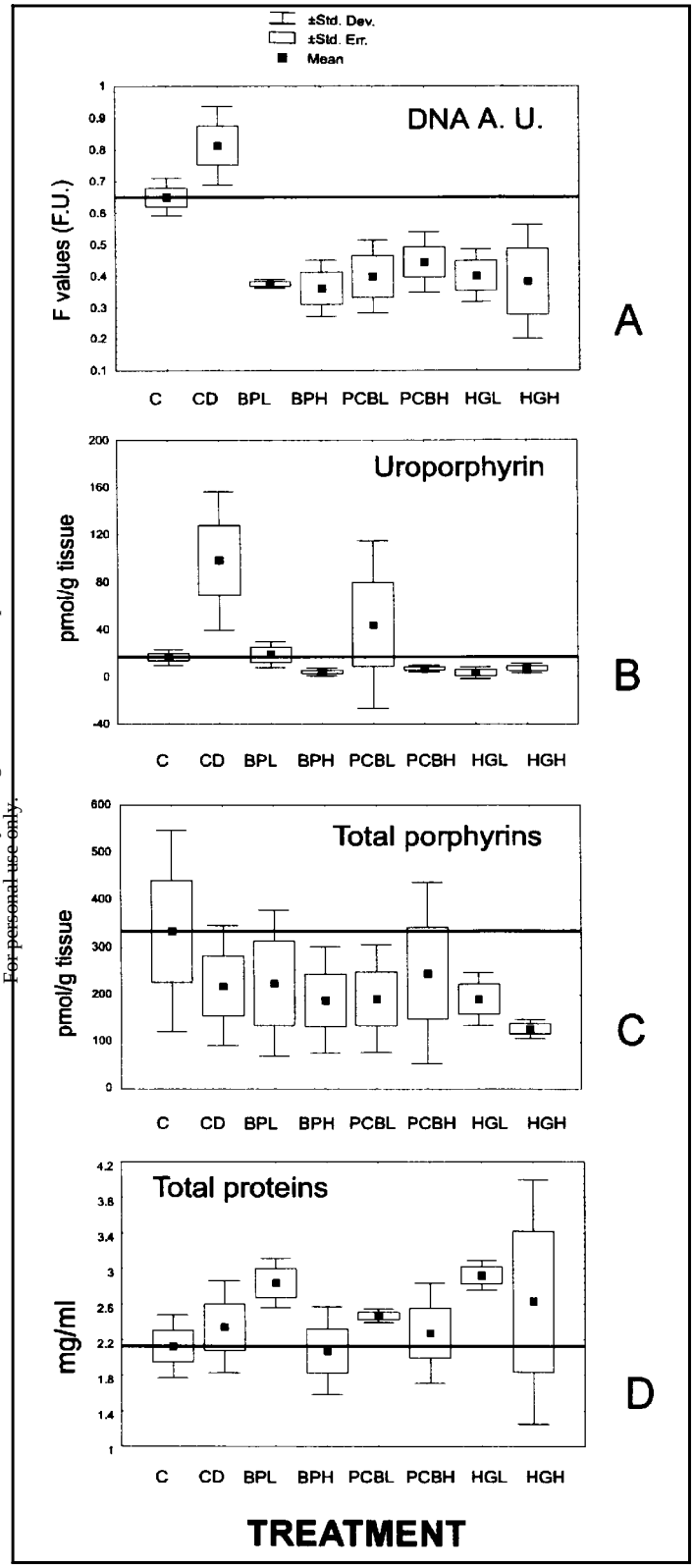
Discussion

Comparing the data of the present experiment with that of other authors in crustacea the following points emerge: the values of NADPH cytochrome C reductase were in the same range as the data in other species (James 1989); the results of the SDS–PAGE are similar to those of Quattrocchi and Lee (1984a,b) who identified the Cyt.P450 in crabs with the molecular weight of 49.5 and 54 kDa; our results are in line with those of Bihari *et al.* (1984) who reported similar or higher MFO activity in gills than in the hepatopancreas. Several biomarkers tested in this study, such as NADH cytochrome C, NADH-ferricyanide reductases, AChE and BChE activities, total protein content and total porphyrin content, do not show any statistical difference with respect to control with the conditions of treatment used in this experiment. Nevertheless the utility of these data consist in the ability to detect these biomarkers in this species.

Two main methodological points must be considered in the interpretation of the above results. The first is related to the method of administration of the contaminants. A criticism can be made on the potential effects of the vehicle in some biomarker responses and the lack of uniformity of treatment, possibly responsible for the absence of statistically significant differences with respect to controls. This observation confirms the data previously obtained by Calleja and Persoone (1993) who observed that the organic solvents such as DMSO can influence the acute toxicity of some lipophilic chemicals in invertebrates.

The second methodological problem is related to the presence of proteolytic activity in the hepatopancreas that may interfere with the detection of MFO activity. Inhibitors of monooxygenase activity affecting NADH cytochrome P-450 reductase are known to exist in the microsomal fraction of hepatopancreas (James *et al.* 1979). These inhibitors, that do not act when the tissue is intact, are released during homogenization of the hepatopancreas (James 1989). In order to solve this kind of problem, Lee *et al.* (1981) underline the importance of using reagents that stabilize MFO components, particularly cytochrome P-450 in microsomal preparations. MFO activity can be drastically reduced without antiprotease in the homogenizing buffer. Bihari *et al.* (1984) also suggest that phospholipase inhibitor in homogenizing buffer increases the enzyme activity from 2.5- to 14-fold in the crab *Maja crispata.*

The influence of both these interference factors, vehicle and proteolytic activity in hepatopancreas, can be taken into consideration in the interpretation of the results, and particularly in the high variability d



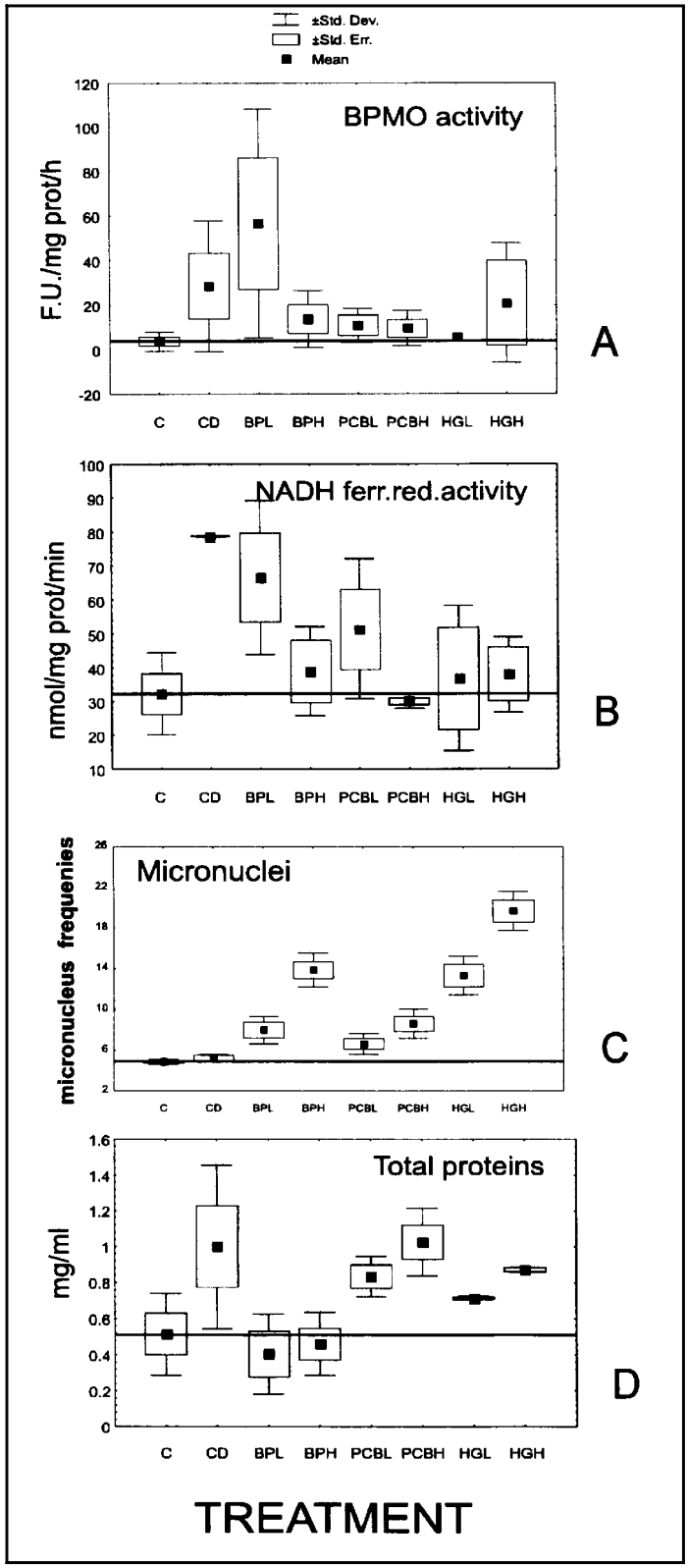


Figure 4. Biomarkers: DNA A.U. (alkaline unwinding assay) (A), uroporphyrin concentration (B), total porphyrin concentrations (C) and total protein concentrations (D) in hepatopancreas of *Carcinus aestuarii* experimentally treated with benzo(*a*)pyrene, polychlorobiphenyls and methyl-mercury. C, control ($n = 4$); CD, control + 50 μl DMSO ($n = 2$); 1 μg benzo(*a*)pyrene ($n = 3$); BPH, 10 μg benzo(*a*)pyrene ($n = 4$); PCBL, 1 μg polychlorobiphenyls ($n = 4$); PCBH, 10 μg polychlorobiphenyls ($n = 4$); HGL, 1 μg methyl-mercury ($n = 3$); HGH, 10 μg methyl-mercury ($n = 3$).

Figure 5. Biomarkers: benzopyrene monooxygenase activity (A), NADH ferricyanide reductase activity (B), micronuclei (C) and total proteins (D) in gills of *Carcinus aestuarii* experimentally treated with benzo(*a*)pyrene, polychorobiphenyls and methyl-mercury C, control ($n = 4$); CD, control + 50 μl DMSO ($n = 2$); BPL, 1 μg benzo(*a*)pyrene ($n = 3$); BPH, 10 μg benzo(*a*)pyrene ($n = 4$); PCBL, 1 μg polychlorobiphenyls ($n = 4$); PCBH, 10 μg polychlorobiphenyls ($n = 4$); HGL, 1 μg methyl-mercury ($n = 3$); HGH, 10 μg methyl-mercury ($n = 3$).

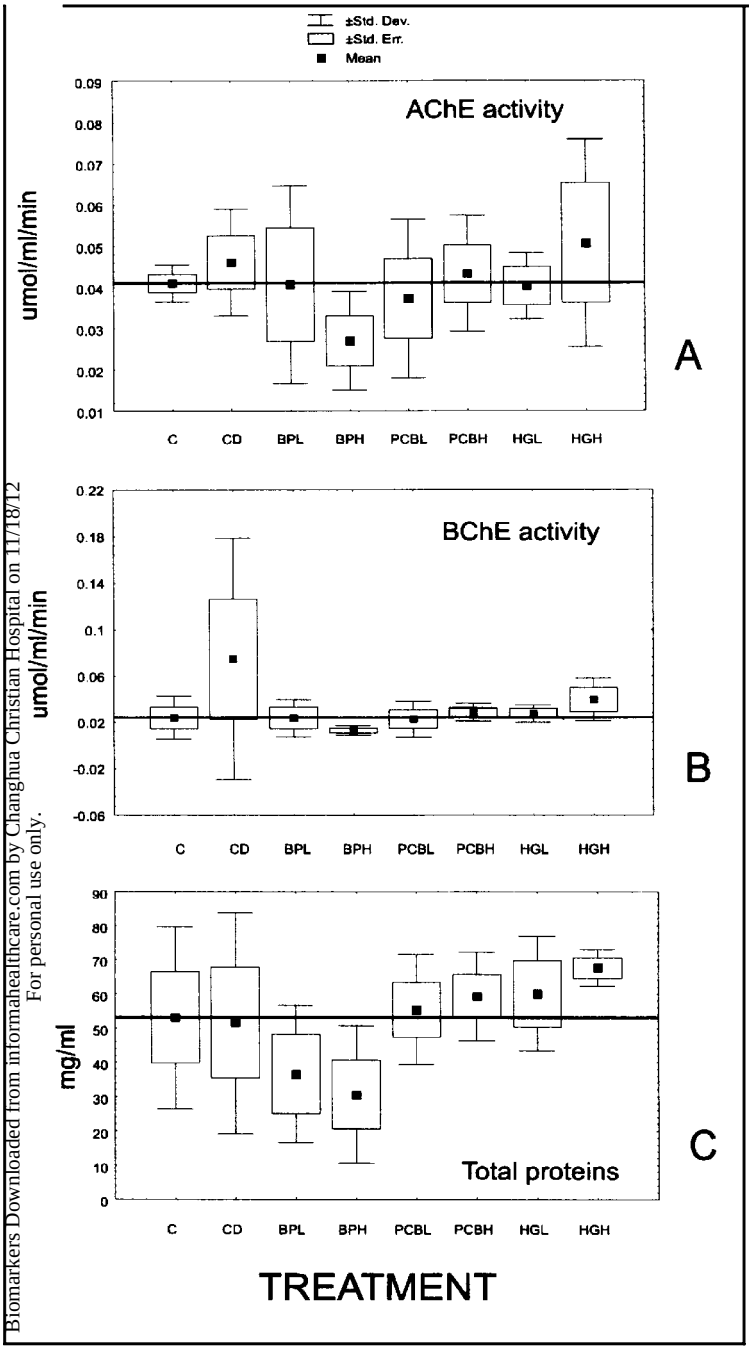


Figure 6. Biomarkers: acetylcholinesterase activity (A), butirylcholinesterase activity (B) and total protein concentrations (C) in haemolymph of *Carcinus aestuarii* experimentally treated with benzo(*a*)pyrene, polychlorobiphenyls and methyl-mercury. C, control ($n = 4$); CD, control + 50 μl DMSO ($n = 2$); BPL, 1 μg benzo(*a*)pyrene ($n = 3$); BPH, 10 μg benzo(*a*)pyrene ($n = 4$); PCBL, 1 μg polychlorobiphenyls ($n = 4$); PCBH, 10 μg polychlorobiphenyls ($n = 4$); HGL, 1 μg methyl-mercury ($n = 3$); HGH, 10 μg methyl-mercury ($n = 3$).

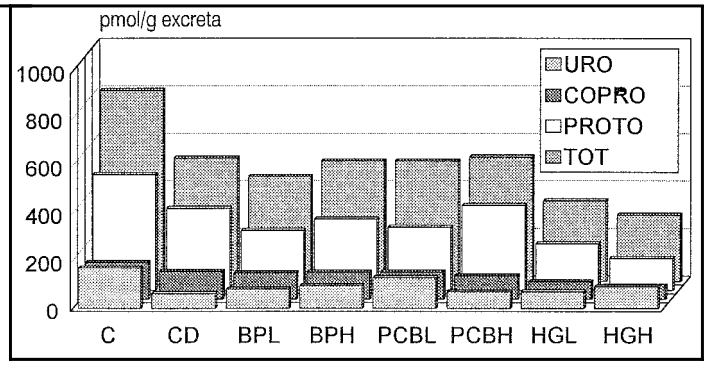


Figure 7. Biomarkers: coproporphyrin, uroporphyrin, protoporphyrin and total porphyrins in excreta of *Carcinus aestuarii* experimentally treated with benzo(*a*)pyrene, polychorobiphenyls and methyl-mercury (the samples of each treatment are pooled). C, control ($n = 4$); CD, control + 50 μl DMSO ($n = 2$); BPL, 1 μg benzo(*a*)pyrene ($n = 3$); BPH, 10 μg benzo(*a*)pyrene ($n = 4$); PCBL, 1 μg polychlorobiphenyls ($n = 4$); PCBH, 10 μg polychlorobiphenyls ($n = 4$); HGL, 1 μg methyl-mercury ($n = 3$); HGH, 10 μg methyl-mercury ($n = 3$).

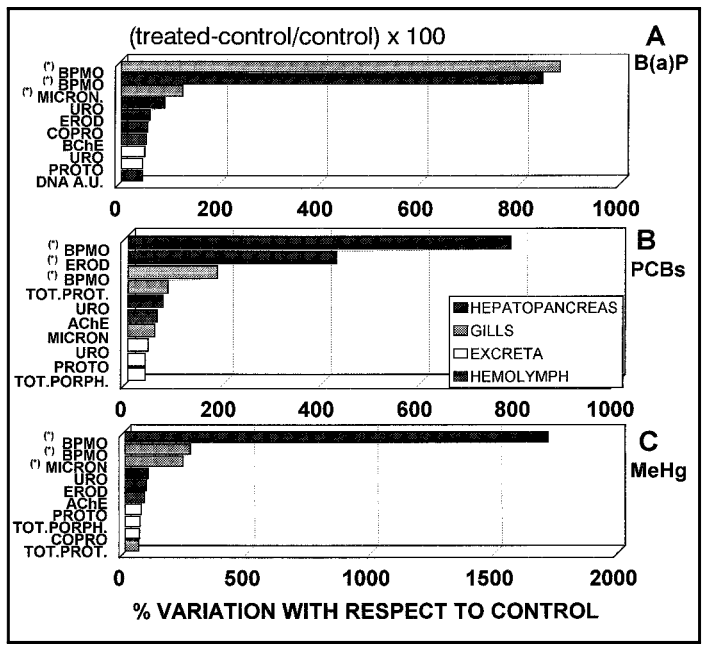


Figure 8. Identification of the most sensitive biomarkers in *Carcinus aestuarii* for the detection of B(*a*)P(A), PCBs and MeHg (C) contamination. (*) = More sensitive biomarkers that can be allowed to identify specific class of contaminants ('Discriminant' biomarkers). BPMO, benzopyrene monoxygenase activity; MICRON, micronuclei; URO, uroporphyrin; EROD, ethoxyresorufin-*O*-deethylase activity; COPRO, coproporphyrin; BChE, butyrylcholinesterase activity; PROTO, protoporphyrin; DNA A.U., alkaline unwinding assay; TOT.PROT., total proteins; AChE, acetylcholinesterase activity; TOT.PORPH; total porphyrins.

activity in the same group of treatment. The main question of this study was the identification of the most suitable biomarkers for evaluating chemical stress due to the three contaminants. The percentage variations of the biomarker responses with respect to controls, were calculated using the average values obtained in the two dose treatments for each contaminant, according to the formula: (treated–control/control) ×100 (Figure 8). In the case of B(*a*)P contamination the most sensitive biomarker was BPMO induction in gills followed by BPMO induction in hepatopancreas, porphyrins and induction of EROD activity in hepatopancreas (Figure 8(A)). In the case of PCBs, the highest response with respect to controls was again the induction of MFO activities, especially BPMO and EROD in hepatopancreas (Figure

treatment groups, the most reliable biomarkers for contamination were BPMO activity in hepatopancreas, followed by BPMO in gills and uroporphyrin in hepatopancreas (Figure 8(C)).

These results allow us to select the most sensitive biomarkers and the most suitable tissues for the identification of the different chemical insults. BPMO activity in hepatopancreas is a good biomarker of exposure of PCBs and B(*a*)P contamination in this species, and could be used, in field studies, to identify general stress due to lipophilic contaminants. Moreover the ability of methyl-mercury to induce MFO activity, as found in the present study, was previously reported by Leonzio *et al.* (1996) in birds experimentally exposed to this contaminant. More specific identification of the molecules responsible for the chemical stress can be done with a suite of 'discriminant' biomarkers. B(*a*)P contamination can be detected by the concomitant presence of induction of BPMO activity in gills and hepatopancreas, with an increase in micronucleus frequencies. PCB contamination seems to be mainly characterized by MFO induction (BPMO and EROD activities) in hepatopancreas and gills. 'Discriminant' biomarkers for MeHg contamination could be the concomitant presence of BPMO induction in hepatopancreas and an increase of micronucleus frequencies.

In the total of the four biological materials analysed in this experiment the most useful for the detection of the main biomarker responses are hepatopancreas and gills, while the haemolymph and excreta seem to be less suitable for these compounds.

Conclusion

The main conclusions of this first experiment in the Mediterranean species *Carcinus aestuarii* can be summarized as follows:

(1) Some tissues and biological materials seem to be useful for biomarker studies in this species: hepatopancreas, gills, haemolymph and excreta. The most suitable tissues in terms of sensitivity of biomarker responses are hepatopancreas and gills.

(2) A number of biochemical, molecular and genotoxic biomarkers were found to be detectable in several tissues. Biomarkers, such as BPMO in hepatopancreas and gills, EROD activity in hepatopancreas and esterase activity in haemolymph, are confirmed to be suitable biomarkers in crustaceans. A series of biomarkers very seldom measured in invertebrates, such as micronuclei in gills, DNA damage tested by alkaline unwinding assay in hepatopancreas and porphyrin concentrations in hepatopancreas and excreta, can also be used in this species.

(3) These results will enable the definition of a simple suite of biomarker tests for the three contaminants to be applied in field studies in the Mediterranean Sea.

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