Arch. Environ. Contam. Toxicol. 53, 66–76 (2007)
DOI: 10.1007/s00244-006-0121-4

ARCHIVES OF
Environmental
Contamination
and Toxicology

Biomarkers Study for Sediment Quality Assessment in Spanish Ports Using the Crab *Carcinus maenas* and the Clam *Ruditapes philippinarum*

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Received: 20 June 2006 / Accepted: 21 January 2007

Abstract. Intermolt crab *Carcinus maenas* and clam *Ruditapes philippinarum* were used to determine the toxicity of sediments collected in four Spanish ports (Cádiz, Huelva, Pasajes, and Bilbao) under laboratory conditions during 28 days. Sediment samples were analyzed to determine chemical concentration of metals (As, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb, and Zn), polyaromatic hydrocarbons, polychlorinated biphenyls, grain size distribution, and organic matter content. Different biomarkers of exposure of early biological stress were determined after 28-day exposure in crabs and clams, in the hepatopancreas and in the digestive gland, respectively: metallothionein, ethoxyresorufin O-deethylase (EROD), glutathione peroxidase (GPX), glutathione S-transferase (GST), and glutathione reductase activities (GR). The battery of biomarkers tested resulted in showing and linking the bioavailability of various contaminants and sediment characteristics to the toxicity of the different sediments. Significant induction of MTs was observed when organisms were exposed to metal-contaminated sediments (port of Huelva), and induction of EROD and GPX activities after exposure to sediments containing organic compounds (port of Bilbao and Pasajes). Higher induction was shown in biomarkers tested in crabs; nevertheless, only interspecies significant differences were observed in the induction of GR and GST activities. The present work confirms the necessity of using species with different ecological lifestyles for sediment toxicity assessment and validates the use of this set of biomarkers as a potential tool in sediment toxicity assessment.

Recently, the biomarkers approach has been incorporated into several pollution-monitoring programs in Europe and North America. Likewise, different methods for biological effects measurement have been evaluated in a series of practical workshops organized by the International Council for the Exploration of the Sea (ICES) and the Intergovernmental Oceanographic Commission (IOC), such as those in the North Sea (WHO Program). The United Nations Environment Program has founded a biomonitoring program in the Mediterranean Sea including a variety of biomarkers (Suter 1993). Recently, biomarkers have also been included in the Joint Monitoring Program of the Protection of the Marine Environment of the North-East Atlantic (OSPAR) convention of which Portugal and Spain are members. Nevertheless, the biomarkers approach has not been included in the guidelines for the management and monitoring of dredging and disposal activities. The current guidelines for control of these activities are based on several approaches that take into account chemical measurements, analysis of benthic communities, and exposure experiments. Very few approaches have already been studied about the inclusion of biomarkers for dredged material assessment in new guidelines.

Ecotoxicity studies based on biomarkers allow the determination of the impact of environmental stressors and facilitate following the evolution of the ecosystem towards degradation or restoration (Vasseur and Cossu-Leguile 2003). Indeed, in addition to their use as simple indices of exposure and effects to specific pollutants, biomarkers can provide insight into ecosystem health. Biomarkers are used to characterize both the ''exposure'' to specific classes of chemicals and different ''effects'' experienced by organisms (OSPAR 1995). An important advantage of biomarkers in assessing the impact of dredged materials is their inherent capacity to detect early biological effects within the organism and to monitor the temporal progression (or regression) of the disturbance of various levels of biological organization (Van der Oost *et al*. 2003).

The use of biomarkers for the evaluation of the toxic potential of sediments follows the identification of appropriate (*i.e.*, sentinel) species for the environmental compartment under study. Both the fresh and seawater protocols recommend that three species, representing different phyla (when possible), be tested for water-column effects and that three different ''life history strategies,'' or perhaps more appropriately, three ecological lifestyles (filter feeding, deposit feeding, burrowing) be used to assess sediment effects (Rodríguez-Ortega

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et al. 2003). In this sense, mollusks and crustaceans, particularly bivalves and crabs, respectively, have assumed a significant role in assessing levels of contaminants worldwide. Various studies have already been developed with these groups of species to assess dredged material through the use of different biomarkers (*e.g.*, de Lafontaine *et al*. 2000; Fossi *et al*. 1998; Regoli *et al*. 2002).

In the present study, a battery of biomarkers of exposure of early biological effects or defense (Gagné and Blaise 2004) to metals and to organic trace pollutants (Van der Oost *et al*. 2003) were utilized for the characterization of dredged material from four Spanish ports in the female crab *Carcinus maenas* and the clam *Ruditapes philippinarum*. The biomarkers used were as follows: metallothioneins (MTs), proteins for detoxification of metal contamination; ethoxyresorufin O-deethylase (EROD), phase I detoxification enzyme implicated in mono-oxygenation reactions of dioxins and polyaromatic hydrocarbons (PAHs); glutathione-S-transferase (GST) phase II detoxification enzyme but also implicated in oxidative stress events; glutathione peroxidase (GPX) and glutathione reductase (GR), antioxidant enzymes. This battery of biomarkers encompasses the biochemical responses to exposure to metals and organic compounds, and reveals environmental bioavailability of these compounds and the possible adverse effect on the organisms. The toxic potential of dredged sediments was determined under strict laboratory conditions to permit spatial comparison of sediment quality by controlling various abiotic and biotic factors. The battery of biomarkers, which encompasses the biochemical responses to exposure to metals and organic compounds, was tested and the potential toxicity of dredged material from Spanish ports was analyzed together with sediment chemical characterization. Differences between responses of the two bioindicator species utilized were also discussed.

Materials and Methods

Identification of Study Sites for Sediment Collection

The four Spanish ports chosen for the assessment of sediment toxicity were those described in Figure 1: the Port of Cádiz (SW, Spain): the port of Cádiz has been widely studied (DelValls *et al*. 1998), and it is characterized by the absence of significant contamination; the sampling sites corresponding to this port were Ca1 (negative toxicity control), Ca2, Ca3, and Ca4. The Port of Huelva (SW, Spain): it is characterized by heavy metal contamination resulting from mining and industrial operations; the sampling sites corresponding to this port were Hu1, Hu2, and Hu3. The Port of Bilbao (NNE, Spain): this port is characterized by high shipping activity; it is predominantly associated with organic contamination, especially by hydrocarbons; the sampling sites corresponding to this port were Bi1, Bi2, and Bi3. The Port of Pasajes (NNE, Spain): this port is also characterized by high shipping activity, resulting in organic contamination as in the Port of Bilbao; the sampling sites corresponding to this port were Pa1, Pa2, and Pa3. In the present study, Aznalcóllar mining spill mud, collected after the Aznalcóllar mining spill (April 1998, SW Spain) and characterized by high concentrations of metals (Gómez-Parra *et al*. 1998), was used as positive toxicity control for intersediment comparison, in a proportion of 30% dry weight of toxic mud. This positive toxicity control has been widely characterized and utilized in different toxicity tests performed by Riba *et al*. (2004a, 2004b).

Selection of Organisms

Two different species with different ecological lifestyles were used: the crab *C. maenas* (deposit feeder) and the clam *R. philippinarum* (filter feeder). These species are widely studied and have already been used in the biomonitoring of contaminated sites. They have been widely and successfully used as indicator organisms for monitoring polluted environments. Biochemical changes (biomarkers: metallothioneins and enzymatic activities) measured in these organisms have resulted in being sensitive to contaminants in water and sediment (Hoarau *et al*. 2004; Orbea *et al*. 2002; Astley *et al*. 1999).

In the present experiment, individuals of the intermolt female *C. maenas* and *R. philippinarum*, of standardized size (Martín-Díaz *et al*. 2004, 2005) were purchased from an aquaculture farm. The farm was located in a clean site of the coast. Crabs and clams were acclimatized before assay development for 2 weeks in the same conditions the bioassay was performed.

Sample Collection

Surface sediment samples (5–10 cm) were collected at five different Spanish Ports with a 0.025 m^2 Van Veen grab. Samples were brought to the laboratory and subsampled for physical–chemical characterization. Then, sediment samples were sieved through a 0.5-mm mesh into a tank in order to remove any associated macrofauna and larger sediment particles. They were kept at 4°C in dark until use in exposure experiments.

Exposure Experiment

Individuals (n = 10) were exposed to the different sediments for 28 days (long-term toxicity test). Each sediment samples were tested per duplicate in 20-L glass aquaria. The tests were carried out in whole sediment using a 1:4 v/v sediment:water relation containing a layer of 6-cm sediment and with constant aeration. The temperature (15°C ± 1°C), pH (7.8–8.2), salinity (33.8 ± 0.3), and dissolved oxygen (>5 $mg{\cdot}L^{-1}$, 60% saturation) were measured and controlled every day. Clams and crabs were respectively fed an algae preparation and frozen mussels every 3 days, respectively, always prior to water change.

Chemical Analysis of Sediments

Analyses of sediment (chemical and physical) were performed per replicate according to Spanish recommendations for dredged material before starting the experiment. The dry weight fraction was measured using the weight loss at 105°C. In order to perform the rest of the analyses, sediments were dried at 40°C during 24 h. Grain size distribution followed UNE 103 101. Metal content in the different sediments was determined following methods reported by Riba *et al*. (2002a, 2002b); polychlorinated biphenyl (PCB) (congeners #28, 52, 101, 118, 138, 153, and 180) and PAH content was analyzed according to U.S. EPA SW-846 method 8270/8082. All the analytical procedures were checked using reference material (MESS-1 NRC and CRM 277 BCR, for heavy metals and NCR-CNRC HS-1 for organic compounds) and allowed agreement with certified values higher than 90%.

Biochemical Analysis

Intermolt female crabs *C. maenas* and clams *R. philippinarum* were sampled on day 28 for biochemical analysis. After dissection, hepatopancreas and digestive gland from crabs and clams, respectively,

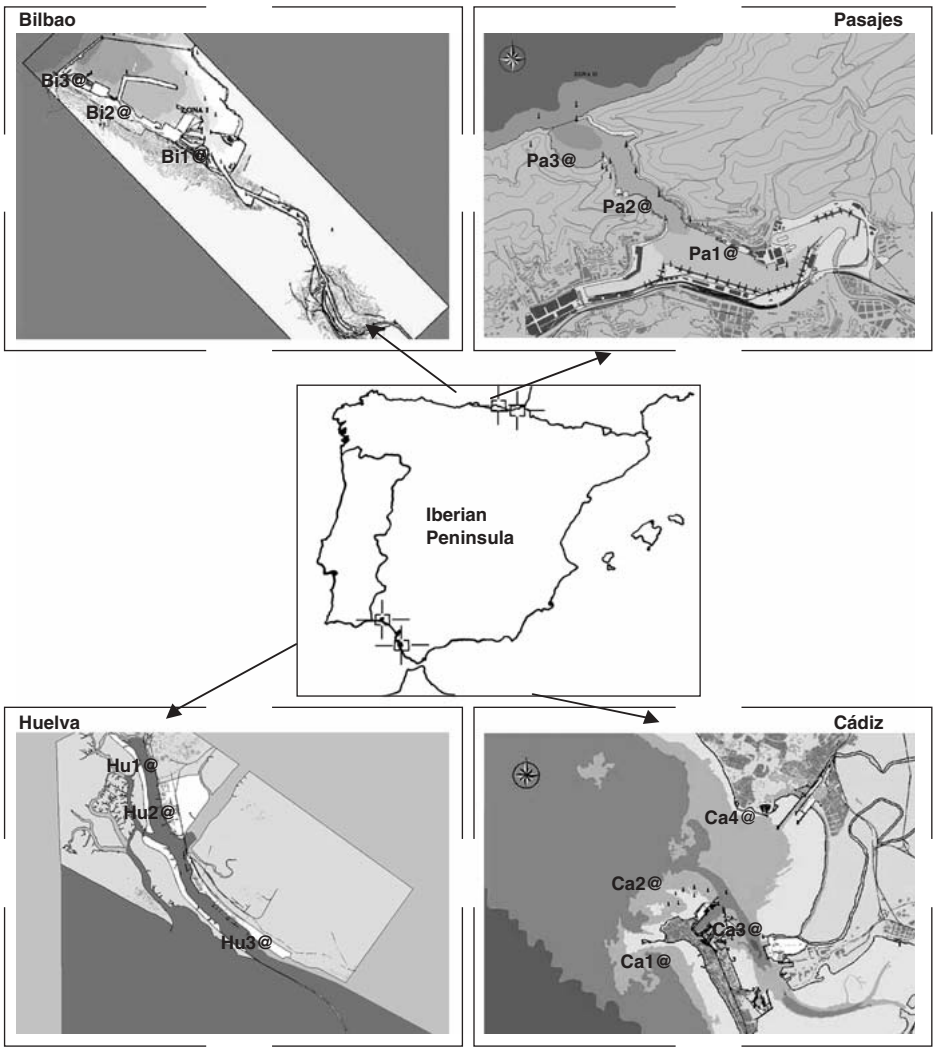

Fig. 1. Map showing the sites selected in this study and the localization of the stations in the Iberian Peninsula

were kept at –80°C prior to homogenization. The samples were homogenized following the procedure developed by de la Lafontaine *et al*. (2000). Once samples were homogenized, those for enzymatic activity determination were centrifuged at 10,000*g* for 30 min, and the supernatant was extracted for the determination of enzyme activity and total protein content. Samples obtained to determine metallothioneins content were centrifuged at 28 000g for 40 min. The supernatant was utilized for total protein determination according the dye-binding principle (Bradford 1976).

Biomarker Determinations

Metallothionein (MT) concentration in tissues were determined by Anodic Stripping Voltammetry (ASV) (Olafson and Olsson 1987). Briefly, 0.1 mL of the supernant (homogenate 28,000*g* for 40 min) was added to 0.9 mL of NaCl (0.9%), heated to 95°C for 4 min, then centrifuged at 10,000*g* for 15 min at 4°C. The supernatant was stored at –80°C prior to MT concentration determinations using purified rabbit metallothionein (Sigma-Aldrich). MT concentrations were expressed as μg MT mg^{-1} total protein. Mixed function oxidize activity was measured using the adapted EROD assay initially adapted for fingerling rainbow trout (Gagné and Blaise 1993). Briefly, 50 μL of supernatant (homogenate 10,000*g* for 30 min) were added to 10 μM 7-ethoxyresorufin and 10 μM reduced NADPH in 100 mM KH_2PO_4 buffer (pH 7.4). The reaction was started by the addition of NADPH, allowed to proceed for 60 min at 30°C, and stopped by the addition of 100 μL of 0.1 M NaOH. The formation of 7-hydroxyresorufin was determined fluorometrically using 520 nm (excitation) and 590 nm (emission) filters. 7-Hydroxyresorufin in the samples was achieved through a standard calibration curve developed with concentrations of 7-hydroxyresorufin. Results were expressed as (pmol min^{-1} mg^{-1} total protein). The procedure utilized for the determination of glutathione S-transferase activity was adapted from the method of McFarland *et al*. (1999). The activity was analyzed using 42 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 1 mM glutathione (GSH) as substrates and measured spectrophotometrically at 340 nm every 30 seconds for 3 min. Results were expressed as (nmol min^{-1} mg^{-1} total protein). The methodology used for the determination of glutathione peroxidase activity was also that adapted from McFarland *et al*. (1999). Briefly, GPX activities were measured spectrophotometrically at 340 nm every 2 min for 10 min, using as substrate 1 mM cumene hydroperoxide. Results were expressed as (nmol min^{-1} mg^{-1} total protein). GSH reductase activity was measured using the GR assay (McFarland *et al*., 1999). This activity was determined using 10 mM oxidized GSH as substrate and determined spectrophotometrically at 340 nm every 2 min during 10 min. Results were expressed as (nmol min^{-1} mg^{-1} total protein).

Table 1. Summarized results of grain size, organic matter (%), and the concentration of chemicals, 10 metals and two kinds of organic compounds polycyclic aromatic hydrocarbon [PAHs] and polychlorinated biphenyl [PCBs] analyzed in different sediments collected in four Spanish Ports

Site	%Graves	%Sand	%Fines	%OM	As	Cd	Cr	Cu	%Fe	Hg	Mn	Ni	Pb	Zn	PCB	PAH
CA1	0.19	99.771	0.042	1.07	3.421	0.923	0.101	6.681	43.865	0.052	85.871	0.062	2.279	21.265	0.01	0.01
CA2	0.051	40.423	59.528	13.75	30.768	1.324	14.937	202.802	26.502	1.976	201.602	20.144	86.902	378.252	0.11	0.11
CA3	0.304	17.803	81.903	20.30	16.607	1.226	8.427	46.756	19.627	0.277	294.402	16.902	17.612	135.502	0.01	0.01
CA4	0.031	0.382	99.586	24.33	7.813	1.247	14.221	32.064	23.001	0.045	406.503	21.246	5.144	65.673	0.01	0.01
HU1	0.071	9.708	90.223	20.27	839.950	4.349	32.892	1938.502	65.747	2.378	383.304	34.568	383.101	2458.002	0.01	0.01
HU2	0.187	56.006	90.213	10.64	532.276	2.504	24.101	149.704	57.126	1.987	303.601	7.101	384.703	1857.003	0.01	0.01
HU3	0.025	16.128	43.946	6.30	272.778	1.318	8.132	772.504	41.248	1.203	354.446	128.552	217.604	1176.00	0.01	0.01
BI1	2.389	20.276	77.327	14.81	67.258	2.002	18.272	102.603	32.203	0.744	109.048	26.389	68.256	476.103	0.11	0.00
BI2	38.123	14.481	47.402	15.07	104.490	2.003	23.113	204.104	42.004	1.432	396.603	32.001	147.503	777.504	0.23	66.77
BI3	0.186	6.224	93.587	16.73	21.707	0.041	3.481	23.027	16.979	0.178	191.352	15.718	285.902	122.352	0.01	13.90
PA1	0.843	28.865	70.286	14.43	39.133	0.682	26.734	158.103	33.404	1.069	140.053	33.489	40.704	1085.002	0.61	0.01
PA2	3.666	5.078	91.237	18.47	28.758	0.704	23.4172	167.102	31.803	1.289	180.001	28.476	293.704	763.004	0.74	1.06
PA3	1.82	38.53	59.651	19.81	23.779	0.0337	18.614	162.504	22.001	1.358	152.499	19.615	246.003	576.004	0.24	0.26
TM	0.20	7.80	92.003	1.10	1234.004	13.681	25.302	643.703	13.603	2.014	230.003	42.504	2615.001	6635.002	0.09	0.04

Chemical concentrations are expressed as $mg \cdot kg^{-1}$.
OM organic matter

Statistical Analysis

Different biomarkers responses were analyzed by using SPSS/PC+ statistical package. Significant differences between individuals exposed to control sediment and individuals exposed to contaminated ones were determined using a one-way analysis of variance followed by a multiple comparison of Dunett's tests. The significance level was set at $p < 0.05$.

Factorial analysis was also performed in order to determine the major responses in respect to sediment contaminants levels. A MAA (factor analysis using the Principal Component Analysis (PCA) extraction procedure) was applied to the original set of variables by using the STATISTICA/PC+ statistical package. The factor analysis was performed on the correlation matrix; *i.e.*, the variables were autoscaled (standardized) so as to be treated with equal importance (DelValls and Chapman 1998). All analyses were performed using the PCA option of the FACTOR procedure, followed by the basic set-up for factor analysis procedure (P4M) from the BMDP statistical software package (Frane *et al*. 1985).

Correlation between chemical concentrations in sediments and interspecies biomarker responses was undertaken using a Pearson correlation analysis by using SPSS/PC+ statistical package. The level of significance was set at $p < 0.05$.

Results

Chemical Concentration in Sediment

Table 1 shows the metal (As, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Zn) and organic content (PCBs and PAHs) in the different sediments collected in the four ports located in the coast of Spain. It also shows the summarized results of conventional sediment parameters such as organic matter and grain size percentages (% fines, % sand, % graves).

The chemical characterization of the sediment samples indicated that sediments contained mixtures of contaminants (metals, PCBs, and PAHs). Furthermore, the summarized results of sediment conventional parameters (Table 1) such as organic matter and grain size percentages were found to be variable, depending on the sediment and area of concern. Sediments from the port of Huelva were characterized by high concentrations of metals as As, Cu, Pb, Zn, Ni, and Cd. Nevertheless, sediments from the ports of Pasajes and Bilbao were mostly characterized by contamination due to PCBs and PAHs, although contamination by metals as Cr, Mn, Ni, Pb, and Hg was also found. Sediments from the port of Cadiz were not found to be highly contaminated, and they were validated as a good negative toxicity control for the development of the present experiment, especially sediments from the site Ca1.

Metallothionein (MT) Concentration

MT concentrations determined in *R. philippinarum* digestive gland and female *C. maenas* hepatopancreas exposed to toxic mud from four Spanish ports during 28 days are shown in Figure 2. In clams, MT concentrations increased significantly, after the 28-day exposure period, in Hu1 (3.12-fold control) ($p < 0.05$), toxic mud (TM) (3.04-fold control) ($p < 0.05$), Hu2 (2.83-fold control) ($p < 0.05$), Ca4 (2.81-fold control) ($p < 0.05$), Pa1 (2.76-fold control) ($p < 0.05$), Ca3 (1.86-fold control) ($p < 0.05$), Bi3 (1.82-fold control) ($p < 0.05$), and Bi1 (1.36-fold control) ($p < 0.05$).

As for concentrations determined in clams, in crabs a significant increase was also shown over time in crabs exposed to Hu2 (12.58-fold control) ($p < 0.05$), Hu3 (4.43-fold control) ($p < 0.05$), Hu1 (3.82-fold control) ($p < 0.05$), Ca4 (2.63-fold control) ($p < 0.05$), TM (2.40-fold control) ($p < 0.05$), Bi3 (2.25-fold control) ($p < 0.05$), Ca3 (2.06-fold control) ($p < 0.05$) and Pa1 (2.03-fold control) ($p < 0.05$).

Ethoxyresorufin O-deethylase (EROD) Activity

EROD activity determined in female crab *C. maenas* and the clam *R. philippinarum* after exposure period are found in Figure 2. In exposed clams, significant induction was observed in comparison with control individuals exposed to Pa2 (1.64-fold control) ($p < 0.05$), Pa3 (1.51-fold control) ($p < 0.05$), Pa1 (1.46-fold control) ($p < 0.05$), Bi1 (1.44-fold control) ($p <$

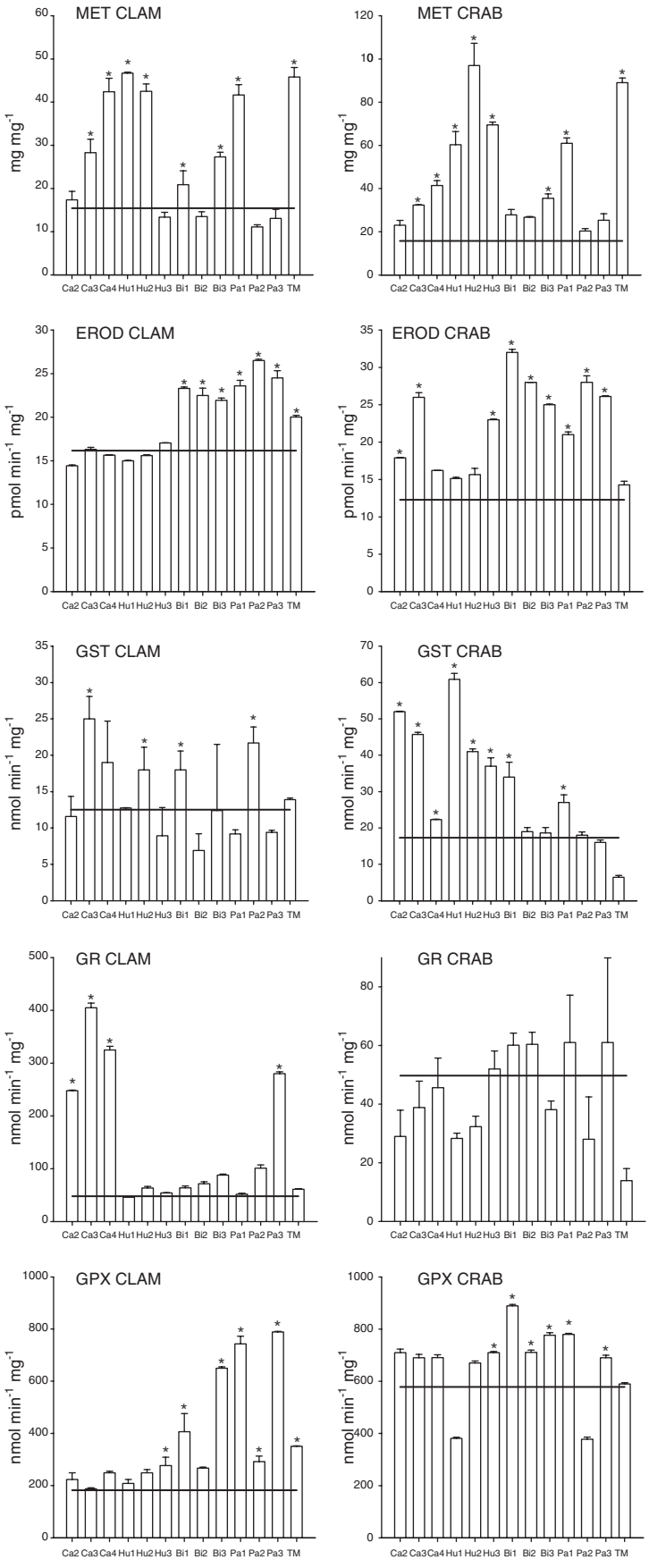

Fig. 2. Representation of metallothionein (MT) concentration, ethoxyresorufin O-deethylase (EROD), glutathione-S-transferase (GST), glutathione reductase (GR), and glutathione peroxidase (GPX) activities determined in digestive gland of *Ruditapes philippinarum* and hepatopancreas of *Carcinus maenas* after a 28-day exposure period to sediments from the port of Cadiz (Ca1, Ca2, Ca3, Ca4), the port of Huelva (Hu1, Hu2, Hu3), the port of Pasajes (Pa1, Pa2, Pa3), and the port of Bilbao (Bi1, Bi2, Bi3). The line represents the average value for each biomarker and species that correspond to the negative toxicity control (Ca1). Bars indicate the biomarker responses (mean values ± standard deviation). Asterisks (*) represent significant induction ($p < 0.05$) compared with control treatment

0.05), Bi2 (1.39-fold control) ($p < 0.05$), Bi3 (1.35-fold control) ($p < 0.05$) and TM (1.23-fold control) ($p < 0.05$).

Significant induction was also observed in crabs exposed to Pa3 (1.97-fold control) ($p < 0.05$), Pa2 (1.86-fold control) ($p < 0.05$), Ca3 (1.77-fold control) ($p < 0.05$), Hu3 (1.72-fold control) ($p < 0.05$), Bi2 (1.71-fold control) ($p < 0.05$), Bi1 (1.67-fold control) ($p < 0.05$), Bi3 (1.42-fold control) ($p < 0.05$), Pa1 (1.4-fold control) ($p < 0.05$) and Ca2 (1.36-fold control) ($p < 0.05$).

Glutathione S-transferase (GST) Activity

Enzymatic activity of glutathione S-transferase determined in female crab *C. maenas* and in the clam *R. philippinarum* are shown in Figure 2. For the exposed clams, significant induction was determined in Ca3 (2-fold control) ($p < 0.05$), Pa2 (1.74-fold control) ($p < 0.05$), Hu2 (1.44-fold control) ($p < 0.05$) and Bi1 (1.44-fold control) ($p < 0.05$).

Significant differences with control crabs were measured in Hu1 (3.52-fold control) ($p < 0.05$), Ca2 (3-fold control) ($p < 0.05$), Hu2 (2.37-fold control) ($p < 0.05$), Ca3 (2.19-fold control) ($p < 0.05$), Hu3 (2.14-fold control) ($p < 0.05$), Bi1 (1.97-fold control) ($p < 0.05$), Ca4 (1.58-fold control) ($p < 0.05$), and Pa1 (1.56-fold control) ($p < 0.05$).

Glutathione Reductase (GR) Activity

Glutathione reductase activities determined in female crab *C. maenas* and in the clam *R. philippinarum* over time are described in Figure 2. Significant differences compared with control were determined in clams exposed to Ca3 (8.45-fold control) ($p < 0.05$), Ca4 (6.79-fold control) ($p < 0.05$), Pa3 (5.84-fold control) ($p < 0.05$), and Ca2 (5.18-fold control) ($p < 0.05$). On the other hand, no significant differences were observed in this activity induction between control and toxic mud-exposed female crabs.

Glutathione Peroxidase (GPX) Activity

GPX activity measured in the clam *R. philippinarum* and the female crab *C. maenas* over time are expressed in Figure 2. Significant induction was also shown in comparison with control in clams exposed to Pa3 (4.34-fold control) ($p < 0.05$), Pa1 (4.09-fold control) ($p < 0.05$), Bi3 (3.58-fold control) ($p < 0.05$), Bi1 (2.22-fold control) ($p < 0.05$), TM (1.92-fold control) ($p < 0.05$), Pa2 (1.60-fold control) ($p < 0.05$), Hu3 (91.51-fold control) ($p < 0.05$) and Bi2 (1.47-fold control) ($p < 0.05$). Crabs exposed to different dredged material, showed significant differences with control in those exposed to sediment from Bi1 (1.54-fold control) ($p < 0.05$), Pa1 (1.35-fold control) ($p < 0.05$), Bi3 (1.34-fold control) ($p < 0.05$), Bi2 (1.23-fold control) ($p < 0.05$), Hu3 (1.23-fold control) ($p < 0.05$), Ca2 (1.22-fold control) ($p < 0.05$), Ca4 (1.20-fold control) ($p < 0.05$), Pa3 (1.19-fold control) ($p < 0.05$), Ca3 (1.19-fold control) ($p < 0.05$), and Hu2 (1.15-fold control) ($p < 0.05$).

Link Between Chemical Concentration and Effect

For a better understanding of the relationship between chemical concentration and the biomarkers determined in different tissues (hepatopancreas in crabs and digestive glands in clams), potential correlation between chemical concentration, MT concentrations, and enzymatic activities (EROD, GST, GPX, and GR) were examined for each species. Two multivariate analysis approaches (MAA) were applied to the chemical concentration in sediments from the different ports in association with adverse biological effect variables, the concentration of MTs and the enzymatic activities registered on day 28, in female crabs and in clams exposed to 14 sediment samples. The MAA was performed using the set of data obtained for the 14 cases defined by the different sampling sites (Ca1 (negative toxicity control), Ca2, Ca3, Ca4, Hu1, Hu2, Hu3, Bi1, Bi2, Bi3, Pa1, Pa2, Pa3, and TM (positive toxicity control). Five variables were utilized (MT, EROD, GST, GPX, and GR), which correspond with biomarkers determination after 28 days of exposure and 16 variables (%Graves, % Sand, %Fines, % Organic Matter, As, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Zn, PCBs, and PAHs) related to physical–chemical characterization of the sediment. In total, we applied the MAA on 21 variables for 14 cases.

After the MAA was undertaken, the original variables could be described by three new variables or factors (Table 2 and 3) for each species. The criteria selected to interpret a variable associated with a particular factor was a loading of 0.4 or higher; this approximates Comrey's (1973) cut-off of a 0.7 or higher for a good association between an original variable and a factor, and also takes into account discontinuities in the magnitudes of the loadings of the original variables. Each component is described according to the dominant group of variables. The different components for each species are described here.

Concerning the clam *R. philippinarum* exposed to different sediments (Table 2):

- The first principal factor, #1, accounts for 32% of the variance and combines the MT induction after 28 days of exposure with metal concentration in the sediment of different sites (As ($p < 0.01$), Cd ($p < 0.01$), Cr ($p < 0.01$), Cu ($p < 0.05$), Fe ($p < 0.01$), Mn ($p < 0.05$), Pb ($p < 0.01$), Zn ($p < 0.01$) and Hg). This factor can be defined as induction of MTs by exposure to metals.
- The second factor, #2, accounts for 16% of the variance and associates variables describing the induction of enzymatic activities GST, GR, and MTs; concentrations of the metals Cr and Mn; and the percentage of fines. The only significant relationship was observed between MTs induction and the metals Cr ($p < 0.01$) and Mn ($p < 0.05$).
- The third factor, #3, accounts for 15% of the variance and reveals the direct relationship of the induction of EROD and GPX, and the presence of PAHs and PCBs in the sediment. This relationship was significant between EROD and PCBs ($p < 0.01$); and GPX and PCBs ($p < 0.05$).

Table 2. Sorted rotated factor loadings (pattern) of 66 variables for the three principal factors resulting from the multivariate analysis of results obtained from *Ruditapes philippinarum*.

R. philippinarum	Factor 1	Factor 2	Factor 3
%Variance	31.70	15.59	15.08
GST28	—	0.55	—
GPX28	—	—	0.52
GR28	—	0.45	—
MT28	0.49	0.49	—
EROD28	—	—	0.83
%Graves	—	—	0.66
%Sand	—	−0.74	—
%Fines	—	0.90	—
%OM	—	0.79	—
As	0.96	—	—
Cd	0.89	—	—
Cr	0.60	0.46	0.40
Cu	0.63	—	—
Fe	0.97	—	—
Hg	0.72	—	—
Mn	—	0.41	—
Ni	—	—	—
Pb	0.85	—	—
Zn	0.96	—	—
PCBs	—	—	0.71
PAHs	—	—	0.62

The variables utilized were the biomarkers of exposure (metallothioneins [MT], ethoxyresorufin O-deethylase [EROD], glutathione peroxidase [GPX], glutathione reductase [GR], and glutathione-S-tranferase [GST] determined after a 28-day exposure period in the laboratory); metal content in the sediment; organic compounds content, sediment characteristics (grain size, organic matter [MO]. Only loadings >0.4 are shown in the table. Factors (#) are numbered consecutively from left to right in order of decreasing variance.

Table 3. Sorted rotated factor loadings (pattern) of 66 variables for the three principal factors resulting from the multivariate analysis of results obtained from *Carcinus maenas.*

Carcinus maenas	Factor 1	Factor 2	Factor 3
%Variance	31.59	17.81	15.81
GST28	—	0.56	—
GPX28	—	—	0.67
GR28	—	—	0.4
MT28	0.65	0.31	—
EROD28	—	—	0.73
%Graves	—	—	0.71
%Sand	—	—	—
%Fines	—	0.68	—
%OM	—	0.60	—
As	0.89	—	—
Cd	0.95	—	—
Cr	0.41	0.66	—
Cu	0.34	0.63	—
Fe	0.95	—	—
Hg	0.53	0.52	—
Mn	—	0.71	—
Ni	—	—	—
Pb	0.97	—	—
Zn	0.98	—	—
PCBs	—	—	0.68
PAHs	—	—	0.50

The variables utilized were the biomarkers of exposure (metallothioneins [MT], ethoxyresorufin O-deethylase [EROD], glutathione peroxidase [GPX], glutathione reductase [GR], and glutathione-S-tranferase [GST], determined after 28-day exposure period in the laboratory and); metal content in the sediment; organic compounds content, sediment characteristics (grain size, organic matter (MO). Only loadings greater than 0.4 are shown in the table. Factors (#) are numbered consecutively from left to right in order of decreasing variance.

Concerning the crab *C. maenas* exposed to different dredged material (Table 3):

- The first principal factor, #1, accounts for 32% of the variance and associates the MT induction on day 28 with metal concentrations of As ($p < 0.05$), Cd, Cr, Cu, Fe, Hg ($p < 0.05$), Pb, and Zn present in the different sediments. It is defined as a factor related to induction of MTs by sediment-bound metals.
- The second factor, #2, accounts for 18% of the variance and associates variables describing the induction of enzymatic activities GST and MTs on day 28 as a result of metal content of Cr, Cu, Hg, and Mn and % fines. This relationship was significant between GST, Cu ($p < 0.01$), Hg ($p < 0.05$), and Mn ($p < 0.05$); and for MTs and Mn ($p < 0.05$).
- The third factor, #3, accounts for 16% of the variance and reveals the direct relationship between the induction of GPX, EROD, and GR on day 28 and the concentration of PAHs and PCBs. The only significant relationship was achieved between EROD and PCBs ($p < 0.01$).

Interspecies Biomarker Response Correlation

Table 4A–E shows the correlation between species for each biomarker determined after a 28-day exposure period to the Spanish ports sediments. The biomarkers responses between species followed a similar trend; nevertheless, the only significant correlation between biomarker responses in clam and crabs was found for EROD ($p < 0.01$), MTs ($p < 0.01$), and GPX ($p < 0.05$).

Discussion

In the present study, different biomarkers were determined in the clam *R. philippinarum* and the female crab *C. maenas* in order to validate the use of this battery of biomarkers to assess exposure to contaminants in sediments collected from several Spanish ports. The biomarkers utilized in this work indicated exposure to pollutants through a warning response of defense that might have effect consequences and affect other levels of the biological community.

Among the exposure biomarkers tested, the MT concentration showed a significant ($p < 0.05$) induction in individuals

Table 4. Pearson correlation results of metallothionins concentrations determined at the 14 sites in the clam *Ruditapes philippinarum* and the crab *Carcinus maenas*

A) Metallothionein	Clam	Crab	As	Cd	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Zn	PCBs	PAHs
Clam	1	0.41**	0.61**	0.50**	0.46**	0.34*	0.53**	0.22	0.31*	−0.16	0.40**	0.53**	−0.26	−0.29
Crab	0.41**	1	0.36*	0.08	0.24	0.07	0.25	0.37*	0.34*	0.03	0.06	0.20	−0.30	−0.14
B) Ethoxyresorufin O-deethylase	Clam	Crab	As	Cd	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Zn	PCBs	PAHs
Clam	1	0.51	−0.23	−0.14	0.27	−0.25	−0.05	−0.05	−0.42**	−0.04	0.02	−0.04	0.74**	0.26
Crab	0.51	1	−0.47**	−0.44**	−0.07	−0.21	−0.34*	−0.10	0.02	0.27	−0.32*	−0.38	0.40**	0.29
C) Glutathion-S-transferase	Clam	Crab	As	Cd	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Zn	PCBs	PAHs
Clam	1	0.09	−0.06	−0.01	−0.07	−0.23	−0.07	−0.28	0.01	−0.33*	−0.04	−0.09	−0.02	−0.40**
Crab	0.09	1	0.07	−0.20	0.19	0.49**	−0.11	0.36*	0.36*	0.10	−0.39*	−0.19	−0.31*	−0.23
D) Glutathione peroxidase	Clam	Crab	As	Cd	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Zn	PCBs	PAHs
Clam	1	0.40**	−0.21	−0.19	0.07	−0.22	−0.12	−0.10	−0.47**	−0.05	−0.00	−0.06	0.38*	−0.05
Crab	0.40*	1	−0.40**	−0.24	−0.33*	−0.54**	−0.27	−0.38*	−0.23	0.01	−0.23	−0.30	−0.19	0.12
E) Glutathione reductase	Clam	Crab	As	Cd	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Zn	PCBs	PAHs
Clam	1	0.71	−0.37*	−0.25	−0.26	−0.31*	−0.34*	−0.37*	−0.18	−0.24	−0.24	−0.34	−0.15	−0.16
Crab	0.71	1	−0.60**	−0.57**	−0.19	−0.31*	−0.55**	−0.41**	−0.15	0.10	−0.58**	−0.56**	−0.13	0.29

Asterisks represent significant correlation (*$p < 0.05$, **$p < 0.01$) between metallothionein concentration, ethoxyresorufin O-deethylase, glutathione-S-transferase, glutathione peroxidase, glutathione reductase; and contaminants in each species and interspecies
PCBs polychlorinated biphenyls, PAHs polyaromatic hydrocarbons.

exposed to 30% toxic mud containing high concentrations of metals compared to control treatment. Likewise, a significant ($p < 0.05$) MT induction in individuals exposed to sediments characterized by metal contamination was observed, as those from the port of Huelva (Hu1, Hu2, Hu3). The role of MT-sequestering metals is well established, whereas their induction by exposure to a wide variety of metals (*e.g.*, Cd, Cu, Zn, Hg, Co, Ni, Bi, and Ag) is associated with an exposure protection function (Stegeman *et al*. 1992). Increased MT expression provides evidence that metals in sediments were bioavailable to both species. The shore crab *C. maenas* and the clam *R. philippinarum* have been demonstrated to be useful in MT concentration biomarker studies to detect the potential effects of metal contamination (Bebianno and Serafim 2003; Hamza-Chaffai *et al*. 2000). In this sense, factors 1 and 2, in both species, were related to MT induction due to metal contamination in the sediments of the port of Huelva. Nevertheless, induction of MTs was also observed in individuals exposed to sediments from the ports of Cadiz, Bilbao, and Pasajes, principally because of the presence of Cr and Mn in the different sediments. To date, some authors have published studies showing that a positive correlation between Cr and MT induction has been determined (Fulladosa *et al*. 2006), although no studies have been found where relationships between Mn exposure and MTs had been measured.

Concerning EROD, induction of this activity was observed in both species exposed to sediments containing high concentrations of PCBs and PAHs, although it was only significantly ($p < 0.05$) related to the presence of PCBs. These results are confirmed by Factor 3, which related, for each species, the induction of this biomarker with sediments from the ports of Pasajes and Bilbao, highly contaminated by organic chemicals. In the crab *C. maenas*, this biomarker induction was also determined in individuals exposed to sediments from Ca2 and Ca3, not associated with any of the chemicals determined in the sediment. EROD activity is involved in the first phase of metabolism, unmasking or adding reactive functional groups, which involve oxidation, reduction, or hydrolysis (Goeptar *et al*. 1995). Its induction is a clear signal of CYP1A1 and CYP1A2 enzyme activities, two of the many cytochrome isoforms known. Increases in EROD activities have been reported in many species of invertebrates, including different species of clams and crabs, after exposure to organic trace pollutants (de Lafontaine *et al*. 2000, Perez *et al*. 2004, Fossi *et al*. 2000). It is suggested that EROD activity may not only indicate chemical exposure, but may also precede effects at various levels of biological organization (Whyte *et al*. 2000).

GST activity was found to be significantly ($p < 0.05$) induced in the organisms exposed to sediments from the ports of Pasajes and Bilbao, and TM, preferentially by the presence of various metals (Cr, Cu, Hg, and Mn), in *C. maenas* and by Cr and Mn in the clam *R. philippinarum*. Consequently, Factor 2 in both species explained the sublethal effects determined in the individuals exposed to the sediments from the ports of Pasajes, Bilbao, and the positive toxicity control. GSTs are a family of enzymes that utilize GSH as a substrate in reactions that permit the biotransformation and disposal of a wide range of exogenous compounds (Contreras-Vergara *et al*. 2004). These compounds may be xenobiotics, drugs, or products of oxidative stress. In comparison to mammals and insects, marine invertebrate GSTs are less known, although reports about their induction after exposure to toxic chemicals are becoming available. This enzyme is a phase II type enzyme and catalyzes the synthetic conjugation reactions of the xenobiotic parent compounds and its metabolites, in order to facilitate the excretion of chemicals. An increase in hepatic GST activity has been reported in several studies after exposure to PAHs and PCBs (Van der Oost *et al*. 2003; Quinn *et al*. 2005). Nevertheless, induction of GST activity because of the presence of Cu and Cr in the sediments from Pasajes, Bilbao, and toxic mud has been shown. It is important to note that these metals belong to a group of metals that are redox-active

and can directly generate free radicals (Ercal *et al*. 2001), which could explain the induction of this biomarker after the exposure to these metals.

Many pollutants (or their metabolites) may elicit toxicity related to oxidative stress. Oxygen toxicity can be a potent oxidant capable of reacting with critical cellular macromolecules, possibly leading to DNA damage and cell death. Defense systems that tend to inhibit oxyradical formation include the antioxidant enzymes such as GR and GPX.

GR is not always recognized as an antioxidant enzyme. It can nevertheless be included in this category because it makes glutathione disulfide reduction possible (GSS G) via a NADPH-dependent process. Therefore, it is at the basis of the regeneration of reduced GSH, which is necessary to the operation of GPXs and many other cell enzymes (Manduzio *et al*. 2003). GR enzyme is also involved in antioxidant defense in the same way as GPX. Significant ($p < 0.05$) induction of GR was only observed in clams exposed to sediments from the port of Cadiz and Pasajes. No significant induction was observed in female crabs. Results obtained in studies of this enzyme in different species are confounding. Activities have been reported in individuals exposed to PCBs and PAHs (Van der Oost *et al*. 2003). Although responses of GR to pollutants have apparently received little attention, this enzyme plays a fundamental role in the face of oxidative stress, maintaining the proper redox status of glutathione, which is important both as cofactor of several antioxidant enzymes and as an indirect scavenger of oxyradicals (Regoli *et al*. 2002).

Significant ($p < 0.05$) induction of GPX activities in comparison to control were registered in individuals exposed to sediments from the ports of Bilbao, Pasajes, and Cadiz, which contained high concentrations of PCBs ($p < 0.05$) and PAHs. In this sense, Factor 3 in crabs and clams revealed a significant relationship between induction of GPX activity on day 28 due to PCBs, explaining the results observed in Bilbao and Pasajes. GPX is involved in the inhibition of oxyradical formation in the presence of the redox-active compounds such as PCBs and PAHs. An increased GPX activity was observed in experiments with fish exposed to PCBs and PAHs (Van der Oost *et al*. 2003); nevertheless, more research is required in invertebrate species.

Chemical compounds, such as heavy metals, are biotransformed into conjugates of reduced glutathione (GSH). Conjugation with GSH is a very important route of detoxification of toxicants and it is catalyzed by the enzyme GST. After this biotransformation, toxicants are destroyed in the cytosolic and mitochondrial compartments by GPX in the presence of reduced glutathione (GSH) (Regoli *et al*. 2002). In this study, GR activity was related to these other biomarkers depending on the species. For the crab *C. maenas*, it was associated with GPX and the presence of organic compounds (Factor 3), and for the clam *R. philippinarum* with GST and metal content.

In the present study, interspecies responses to mixture of different contaminants in sediment from Spanish ports has been tested under controlled laboratory conditions in two species, the clam *R. philippinarum* and the crab *C. maenas*. For all the biomarkers tested, higher induction was observed in the crab than in the clam, except for GR activity. Nevertheless, all the biomarker responses, for both species, were significantly correlated ($p < 0.01$), except for GST and GR activities. Neither in clams nor in crabs was any significant correlation detected between GR induction and contaminant presence in the sediment, although in the MAA performed, an association of this biomarker was achieved with Cr and Mn in clams and PAHs and PCBs in crabs. No significant induction of GST activity was observed related to contaminants in clams; nevertheless, significant ($p < 0.01$) induction was observed of this enzymatic activity with Cu, Hg, and Mn content in sediments for crabs.

Therefore, taking into account these interspecies results, it could be summarized that *C. maenas* is a more sensitive species for sediment toxicity assessment. Nevertheless, different concepts should be discussed and taken into account before a conclusion of this magnitude is made:

a). These species have different feeding styles. The fact that the clams are filter feeders and the crabs deposit feeders could influence the bioavailability of sediment contaminants.
b). In the present assay, no contaminated food was provided to the different organisms, although it was not stated to what extent food could be contaminated when being introduced in the aquaria.
c). The possibility of the presence of different detoxification pathways concerning GR and GST activities in clams and crabs should be taken into consideration.

In order to chose one species for the evaluation of sediment or dredged material toxicity, the sensitivity of the species, life stage tested, its degree of phylogenetic and ecological relatedness to receptors at the disposal site, its preferences and tolerance to the particle size makeup of the test sediment, among other characteristics, should be taken into consideration. Consideration of these factors allows establishment of each test species as a surrogate for organisms living at the disposal site (Munns *et al*. 2002).

Finally, it is important to take into consideration that tests for assessing dredged material have been developed in the laboratory under strictly controlled parameters, so they do not reflect the variability in exposure that may occur in natural systems, and the results lack the ecological relevance that *in situ* assays could provide. Because effects may be both overestimated and underestimated, laboratory observations on biomarkers must always be validated with field research.

Conclusions

The battery of exposure biomarkers utilized in the present work could be interpreted as a sensitive early warning contamination signal in female *C. maenas* and the clam *R. philippinarum* exposed to several contaminated sediments. It could be concluded that:

a). This set of biomarkers tested resulted in showing and linking the bioavailability of the contaminants determined in the sediment with the toxicity of these compounds in the biomarkers tested. MT induction was related to the exposure to metals, EROD activity to PAHs and PCBs, and GST to the exposure to Cu, Cr, and Mn. GPX together with GR were induced to protect the organism from the oxidative stress provoked by the presence of PAHs, PCBs, and Cr, Mn, respectively. The only significant interspecies

differences were observed in the induction of GR and GST.

b). The authors consider the necessity of using at least two species with different ecological lifestyles, with different sensitivity to contaminants, in order to perform a sediment quality assessment study.

c). Sediment toxicity assessment studies in the laboratory should be validated with *in situ* studies.

Acknowledgments. The authors would like to thank the Port Authorities of Cádiz, Huelva, Pasajes, Bilbao, and AZTI Foundation. We would also like to thank the collaboration of the technician Pablo Vidal and the scuba diver Antonio Moreno. This research has been funded by a Grant of the Spanish Ministry for Education and Science (REN-2002-01699) and by a Grant from the Ministry of Development (BOE, 13-12-2002). We especially appreciate the careful English revision of the manuscript performed by Darcy Longpré (Environment Canada).

References

Astley KN, Meigh HC, Glegg GA, Braven J, Depledge MH (1999) Multi-variate analysis of biomarker responses in *Mytilus edulis* and *Carcinus maenas* from the Tees Estuary (UK). Mar Poll Bull 39:145–154

Bebianno MJ, Serafim MA (2003) Variation of metal and metallothionein concentrations in a natural population of *Ruditapes decussatus.*. Arch Environ Contam Toxicol 44:53–66

Bradford MB (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Comreys AL (1973) A first course in factor analysis Academic Press, New York

de Lafontaine Y, Gagné F, Blaise C, Costan G, Gagnon P, Chan HM (2000) Biomarkers in zebra mussels (*Dreissena polymorpha*) for the assessment and monitoring of water quality of the St. Lawrence River (Canada). Aquat Toxicol 50:51–70

Contreras-Vergara CA, Harris-Valle C, Sotelo-Mundo RR, Yepiz-Plascencia G (2004) Mu-class glutathione S-transferase from the marine shrimp *Litopenaeus Vannamei*: Molecular cloning and active site structural modeling. J Biochem Toxicol 18:245–252

Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR) (1995) Report of the workshop on biological effects monitoring techniques. Abeerden, Scotland, 2–6 October

DelValls TA, Chapman PM (1998) Site-specific sediment quality values for the Gulf of Cádiz (Spain) and San Francisco Bay (USA), using the sediment quality triad and multivariate analysis. Cienc Mar 24:3313–3336

Ercal N, Gurer-orhan H, Aykin-Burns N (2001) Toxic metals and oxidative stress part I: mechanisms involved in metal-induced oxidative damage. Curr Top Med Chem 1:529–539

Fossi MC, Savelli C, Casini S (1998) Mixed function oxidase induction in *Carcinus estuarii*. Field and experimental studies for the evaluation of toxicological risk due to Mediterranean contaminants. Comp Bioch Physiol 121C:321–331

Fossi MC, Casini S, Savelli C, Franchi E, Mattei N, Sanchez-Hernandez JC, Corsi I, Bamber S, Depledge MH (2000) Biomarker responses at different levels of biological organization in crab (*Carcinus aestuarii*) experimentally exposed to benzo (a)pyrene. Chemosphere 40:861–874

Frane J, Jenrich R, Sampson P (1985) Factor analysis. In: Dixon WJ (ed) BMDP Statistical Software. University of California Press, Berkeley, California, pp 480–500

Fulladosa E, Deane E, Ng AHY, Woo NYS, Murat JC (2006) Stress proteins induced by exposure to sublethal levels of heavy metals in sea bream (*Sparus sarba*) blood cells. Toxicol In Vitro 20:96–100

Gagné F, Blaise C (1993) Hepatic metallothionein level and mixed function oxidase activity in fingerling rainbow trout (*Oncorhynchus mykiss*) after acute exposure to pulp and paper mill effluents. Wat Res 27:1669–1682

Gagné F, Blaise C (2004) Review of biomarkers and new techniques for in situ aquatic studies with bivalves. In: Thompson C, Wadhia K, Loibner AP, (eds) Environmental toxicity testing. Blackwell Publishers, UK, pp 207–228

Goeptar AR, Scheerens H, Vermeulen NPE (1995) Oxygen reductase and substrate reductase activity of cytochrome P450. Crit Rev Toxicol 25:25–65

Gómez-Parra A, Forja JM, Del Valls TA, Sáenz I, Riba I (2000) Early contamination by heavy metals of the Guadalquivir Estuary after the Aznalcóllar mining spill (SW Spain). Mar Poll Bull 40:1115–1123

Hamza-Chaffai A, Amiard JC, Pellerin J, Joux L, Berthet B (2000) The potential use of metallothionein in the clam Ruditapes decussatus as a biomarker of in situ metal exposure. Comp Biochem Physiol (C) 127:185–197

Hoarau P, Garello G, Gnassia-Barelli M, Roméo M, Girard JP (2004) Effect of three xenobiotic compounds on Glutathione S-Transferase in the clam *Ruditapes decussatuss*. Aquat Toxicol 68:87–94

Manduzio H, Monsinjon T, Rocher B, Leboulenger F, Galap C (2003) Characterization of an inducible isoform of the Cu/Zn superoxide dismutase in the blue mussel *Mytilus edulis*. Aquat Toxicol 64:73–83

Martín-Díaz ML, Bamber S, Casado-Martínez MC, Sales D, DelValls TA (2004) Toxicokinetic of heavy metals from a mining spill using *Carcinus maenas*. Mar Environ Res 58:833–837

Martín-Díaz ML, Blasco J, González de Canales ML, Sales D, DelValls TA (2005) Bioaccumulation and toxicokinetic of dissolved metals from the Guadalquivir estuary after the Aznalcóllar mining spill using *Ruditapes philippinarum*. Arch Environ Contam Toxicol 48:233–241

McFarland VA, Inouye SL, Lutz CH, Jarvis AS, Clarke JU, McCant DD (1999) Biomarkers of oxidative stress and genotoxicity in livers of field collected brown bullhead, Ameiurus nebulosus. Arch Environ Contam Toxicol 37:236–241

Munns WR Jr, Berry WJ, DeWitt TH (2002) Toxicity testing, risk assessment, and options for dredged material management. Mar Poll Bull 44:294–302

Olafson RW, Olsson PE (1987) Electrochemical detection of metallothionein, zinc and copper levels during an annual reproductive cycle in Rainbow trout (*Salmo gairdneri*). Fish Physiol Biochem 31:39–47

Orbea A, Ortiz-Zarragoitia M, Solé M, Porte C, Cajaraville MP (2002) Antioxidant enzymes and peroxisome proliferation in relation to contaminant body burdens of PAHs and PCBs in bivalve molusks, crabs and fish from Urdaibai and Plentzia estuaries (Bay of Biscay). Aquat Toxicol 58:75–98

Perez E, Blasco J, Sole M (2004) Biomarker responses to pollution in two invertebrate species: *Scrobicularia plana* and *Nereis diversicolor* from the Cádiz bay (SW Spain). Mar Environ Res 58:275–279

Quinn B, Gagné F, Weber JP, Blaise C (2005) Ecotoxicological effects of a semi-submerged municipal dump (Castle Harbour, Bermuda) on the Calico scallop *Argopecten gibbus*. Mar Poll Bull 51:534–544

Regoli F, Pellegrini D, Winston GW, Gorbi S, Giuliani S, Virno-Lamberti C, Bompadre S (2002) Application of biomarkers for assessing the biological impact of dredged material in the Mediterranean: the relationship between antioxidant responses and

susceptibility to oxidative stress in the red mullet (*Mullus barbatus*). Mar Poll Bull 44:912–922

Riba I, DelValls TA, Forja JM, Gómez-Parra A (2002a) Influence of the Aznalcóllar mining spill on the vertical distribution of heavy metals in sediments from the Guadalquivir estuary (SW, Spain). Mar Poll Bull 44:39–47

Riba I, Forja JM, DelValls TA, Guerra R, Iacondini A (2002b) Determining sediment toxicity associated with the Aznalcóllar mining spill (SW, Spain) using a bacterial bioassay. First International Conference on Remediation of Contaminated Sediments, Batelle Press

Riba I, Conradi M, Forja JM, DelValls TA (2004a) Sediment quality in the Guadalquivir estuary: lethal effects associated with the Aznalcóllar mining spill. Mar Poll Bull 48:144–152

Riba I, Casado-Martínez MC, DelValls TA, Forja JM, Gómez-Parra A (2004b) Sediment quality in the Atlantic coast of Spain. Environ Toxicol Chem 23:271–282

Rodríguez-Ortega MJ, Grøsvik BE, Rodríguez-Ariza A, Goksøyr A, López-Barea J (2003) Changes in protein expression profiles in bivalve molluscs (*Chamaelea gallina*) exposed to four model environmental pollutants. Proteomics 3:1535–1543

Stegeman JJ, Brouwer M, Richard TDG, Förlin L, Fowler BA, Sanders BM, Van Veld PA (1992) Molecular responses to environmental contamination: enzyme and protein systems as indicators of chemical exposure and effect. In: Huggett RJ, Kimerly RA, Mehrle PM Jr, Bergman HL (eds) Biomarkers: biochemical, physiological and histological markers of anthropogenic stress. Lewis Publishers, Chelsea, Michigan, pp 235–335

Suter GW (1993) Ecological risk assessment Lewis Publishers, Boca Raton, Florida, p 538

Thiele DJ (1992) Metal-regulated transcription in eukaryotes. Nucl Acids Res 20:1183–1188

Van der Oost R, Beyer J, Vermeulen NPE (2003) Fish bioaccumulation and biomarkers in environmental risk assessment. Environ Toxicol Pharmacol 13:57–149

Vasseur P, Cossu-Leguile C (2003) Biomarkers and community indices as complementary tools for environmental safety. Environ Int 28:711–717

Whyte JJ, Jung RE, Schmitt CJ, Tillit DE (2000) Ethoxyresorufin-O-deethylase (EROD) activity in fish as a biomarker of chemical exposure. Crit Rev Toxicol 30:347–570

WHO: International Programme on Chemical Safety (IPCS) (1993) Biomarkers and risk assessment: concepts and principles. Environmental Health Criteria 155, World Health Organization, Geneva