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Sublethal responses in caged organisms exposed to sediments affected by oil spills

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ABSTRACT

This study was performed to determine sublethal responses of two invertebrate species by using field deployments in areas affected by oil spills, which are acute in the Galician Coast (NNW, Spain) and chronic in the Bay of Algeciras (SSW, Spain). The organisms employed were the crab *Carcinus maenas* and the clam *Ruditapes philippinarum*, and during 28 days the animals were exposed to contaminated sediments in cages under field conditions. Different biomarkers of exposure were determined after a 28-day period exposure: ethoxyresorufin *O*-deethylase (EROD), phase I detoxification enzyme, glutathione-*S*-transferase (GST) phase II detoxification enzyme but also implicated in oxidative stress events, glutathione peroxidase (GPX) and glutathione reductase (GR), both antioxidant enzymes. In addition, histopathological effects in target tissues of the deployed organisms were evaluated. Biomarker measurements were linked with the concentration of chemicals in the sediments in order to elucidate the type, source and bioavailability of contaminants that produce adverse effects in the bioindicator species. Results obtained in this study have shown how the application of the selected battery of biomarkers under field bioassays allows for the identification of alternative sources of stress that are not observable in laboratory experiments.

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1. Introduction

Measurements of an organisms' response to a pollutant at the biochemical or physiological level can detect more quickly and specifically the presence of toxic compounds, The presence of toxic compounds can be detected more quickly and specifically by measuring an organism's response to a pollutant at the biochemical or physiological levels, allowing for earlier identification of change, before deleterious effects reach higher organization levels (Montserrat et al., 2003). Over the past decade, biomarkers have been used increasingly as diagnostic tools to investigate sublethal effects of toxic exposure and to elucidate the various modes of action of xenobiotics (De Coen et al., 2000).

The application of biomarkers under field conditions has been proposed by many authors in order to assess chronic responses in aquatic populations exposed under environmental realistic conditions (Suter, 1993; Depledge and Fossi, 1994; De Coen et al., 2006; Martín-Díaz et al., 2007). Field studies pose far greater difficulties due to the complex and fluctuating nature of the environment, and interactions among organisms within ecological communities. These studies address the integrated impact of anthropogenic and environmental stressors. Data collected in field studies may be more difficult to interpret than data from controlled laboratory experiments (Astley et al., 1999).

It has also been demonstrated that in situ assays are useful to determine the toxicity of sediments using different approaches including caging animals (Martín-Díaz, 2004). Sediment toxicity bioassays carried out in the laboratory are performed under strictly controlled parameters and thus do not reflect the variability in exposure that may occur in natural systems. This gives rise to uncertainty concerning the extrapolation of laboratory-based test results compared to natural environments in sediment risk assessment (Sibley et al., 1999). Environmental conditions can alter toxicity through complex exposure dynamics and these in situ-based designs are potentially more environmentally realistic than laboratory settings (Crane et al., 2007).

A biomarker approach was used with caged organisms exposed under field conditions to contaminants related to oil spills. The objectives were as follows: (1) to test the feasibility of a suite of biomarkers to assess the quality of the oil-contaminated sediment, (2) to identify the contaminants bound to sediments which produce the sublethal effects in the exposed organisms, and (3) to

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determine the differences between the biological responses associated to a deployment and the sediments that were recently or formerly contaminated by oil.

A battery of biomarkers of exposure of early biological effects was used in order to assess sediment toxicity of two coastal areas affected by oil spills: the Galician Coast, acutely impacted by the sinking of the tanker Prestige (2002) and the Bay of Algeciras chronically affected by several spills. Two invertebrate species with different feeding habits were selected to carry out the assessment: the crab *Carcinus maenas* and the clam *Ruditapes philippinarum*. The suite of biomarkers employed included: ethoxyresorufin *O*-deethylase (EROD), phase I detoxification enzyme implicated in monooxygenation reactions of dioxins and PAHs; glutathione-*S*-transferase (GST) phase II detoxification enzyme, implicated in oxidative stress events; glutathione peroxidase (GPX) and glutathione reductase (GR), antioxidant enzymes (Martín-Díaz et al., 2007). Histopathological alterations in target tissues were also evaluated because these are responsive and sensitive to a wide range of contaminants and have been developed and recommended as biomarkers for monitoring the effects of pollution (Au, 2004).

2. Materials and methods

2.1. Description of sites

The study was performed in two areas of the Spanish Coast: the Galician Coast (NW Spain) was chosen because it was affected by the Prestige oil spill in 2002, one of the most serious ecological catastrophes that occurred in the Iberian Peninsula, affecting more than 1000 km of coastline. The selected sites were located in the Cies Island in the included the mouths of the Palmones and Guadarranque Rivers in the Bay of Algeciras (S Spain). This location was selected because it is a highly industrialized area where heavy petrochemical activity has led to several accidental oil spills. A reference site was selected in a clean area in the Bay of Cádiz (S Spain) (Riba et al., 2004). The 10 selected study sites are shown in Fig. 1: A, B, C located in Cies; D, E, F in Corme-Laxe; GR3, GR4 and P1 in the Bay of Algeciras; and the reference site CA in the Bay of Cádiz. These areas are widely characterized by different ecotoxicological studies (DelValls et al., 1998, Riba et al., 2004; Martín-Díaz et al., 2005).

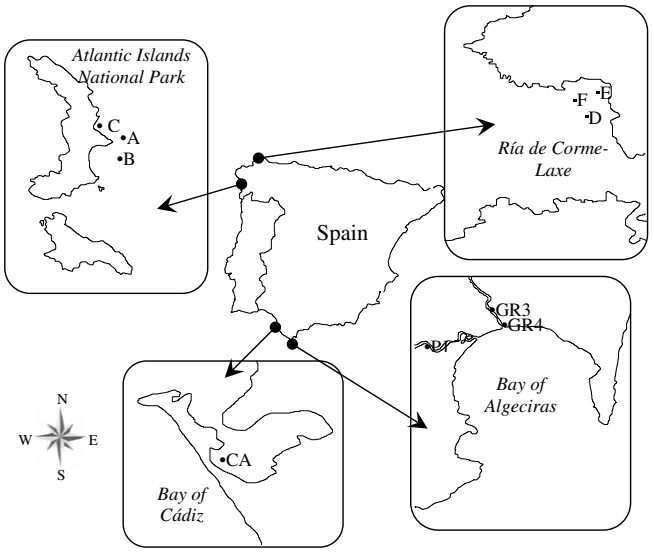

Fig. 1. Map of the coastal area of Galicia showing the locations of the sampling stations. A, B and C refer to the stations located in the Cies Island in the Atlantic Island National Park and D, E and F to those in the Bay of Corme-Laxe. The stations located in the Bay of Algeciras are GR3, GR4 and P1. The station CA located in the Bay of Cadiz corresponds to the sediment used as reference.

2.2. Sampling and deployment

The clam *R. philippinarum* (35–45 mm shell length) was obtained from an aquaculture farm whereas the crab *C. maenas* (carapace width (47–57 mm) was caught in a clean site located in the Bay of Cádiz (SW, Spain) (Riba et al., 2003). These invertebrate species were chosen for the study due to their commercial importance and ecological relevance. Both invertebrates have different feeding habits and previous toxicological studies have been successfully carried out with them under laboratory conditions (Martín-Díaz et al., 2007; Morales-Caselles et al., in press). The organisms were transferred to the laboratory and kept in tanks with continuous water replacement under controlled conditions until the beginning of the experiment. The test animals were carefully transported to the study sites and placed in cages made with plastic mesh (50 cm × 25 cm × 15 cm; approximately 0.5 cm mesh size) divided in two different compartments, one for crabs (n = 20) and one for clams (n = 40). The cages were positioned during low tide and were wedged into the sediment. The exposure lasted 28 days and the crabs were fed once a week with a mixed diet consisting of mussels or fish. Sediment samples from the study sites were collected and transported to the laboratory where they were kept in dark conditions at 4 °C prior to chemical analysis. In addition, non-exposed organisms were kept in the acclimatising tanks and used as controls for the chemical analysis and histopathological determinations.

2.3. Biochemical analysis

Deployed crabs and clams were collected and dissected after 28 days of exposure. The hepatopancreas (in crabs) and digestive gland (in clams) were extracted and kept at 80 °C prior to homogenization. The samples were homogenized with Tris–acetate buffer following the procedure developed by Lafontaine et al. (2000). Samples were centrifuged at 10,000*g* for 30 min, and the supernatant was used for determination of biomarkers and total protein content described by Bradford (1976). The phase II metabolizing Glutathione-*S*-transferase (GST) activity was determined by monitoring the rate of conjugation of glutathione (GSH) to 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm; the oxidation of 1 mM NADPH by Glutathione Reductase (GR) in the presence of 10 mM oxidized glutathione was monitored at 340 nm; the method to measure the antioxidant enzyme Glutathione Peroxidase (GPX) was adapted from McFarland et al. (1999) similar to GST and GR. Mixed function oxygenase activity, which is the first mode of detoxification of many organic pollutants, was measured using the EROD assay (Gagnè and Blaise, 1993). Biomarker results were normalized with the protein content.

2.4. Biomarker of effect: histopathology

Gills and digestive gland tissues of the organisms were fixed in phosphate buffered 10% formaldehyde (pH 7.2) for histopathology determination. After dehydration in graded concentrations of ethanol, the samples were embedded in paraffin wax. Histological sections of 6–8 μm thickness were stained with Haematoxylin–Eosin and Haematoxylin–VOF [15]. Sections were reviewed by light microscopy Leitz Laborlux S and photographed (Sony DKC-CM30).

2.5. Chemical analysis

The analyses of PAHs and PCBs bound to sediments were carried out according to USEPA SW-846 Method 827C78082 (USEPA,

1994). Briefly dried samples were Soxhlet extracted with *n*-hexane for 18 h, and the extracts were isolated by column chromatography on Florisile alumino-silica. PCBs and PAHs were eluted and their fractions were dried in a rotating evaporator and re-dissolved in isooctane. Aromatic fractions were analyzed on a Hewlett–Packard (HP) 5890 Series II gas chromatographer coupled with an HP 5970 mass spectrometer. PAHs were analyzed by GC–MS using selected ion monitoring (SIM). Analysis of PCBs as AROCLOR 1242 and AROCLOR 1260 was performed using the same instrument with an electron capture detector (GC/ECD). For both set of organic chemicals, PAHs and AROCLOR, the analytical procedure corresponded to the certified values of more than 90%.

Trace metals were analyzed as described by Casado-Martínez et al. (2006). Briefly, 2.5 g of sediments (<0.065 mm) were placed in Teflon containers and were digested in microwave (400 W, 15 min, twice) with HNO_3 2N. The extracts were purified by passing through a C-18 column and metals analyses were performed by anodic voltamperimetry (-Zn, Cd, Pb, Ni, Co and Cu-Metrohm Application Bulletin No. 147; – V-Metrohm Application Note No. V-81). The cold vapour technique was used for Hg and was quantified using atomic absorption spectrometry. The analytical procedures were checked using reference material (MESS-1 NRC and CRM 277 BCR) and showed a recovery greater than 90% of the certified concentration.

2.6. Statistical analysis

The induction of biomarkers of response was analyzed using the ANOVA and Tukey tests in order to determine significant differences ($p < 0.05$; $p < 0.01$) among the results obtained for the reference (CA) site and the other sampling sites, using the statistical package SPSS 11.5. No transformation of the data was necessary and homogeneity was verified. Multivariate analysis was carried out in an attempt to link contamination with adverse biological measurements. The principal component analysis (PCA) was used as the extraction procedure to derive a reduced number of new variables (factors) as linear combinations of the original variables (STATISTICA 6.0).

3. Results and discussion

3.1. Concentration of chemicals in the sediments

Results of the concentration of chemicals in the studied sediments are shown in Table 1. The highest concentration of PAHs was found in the sediments from GR3 (2961 mg kg^{-1} dry sediment) located in the Bay of Algeciras, followed by sediments from the station F (820 mg kg^{-1} dry sediment) located in Corme-Laxe, and GR4 (802 mg kg^{-1} dry sediment) and P1 (641 mg kg^{-1} dry sediment) in the Bay of Algeciras. These could be considered slightly contaminated by PAHs and adverse effects could be frequent according to MacDonald et al. (1996), and in the case of GR3 the concentration of this contaminant also exceeds the guideline proposed by the Dutch agencies (Tweede Kamer, vergaderjaar, 1994–1995). On the other hand, sediments from the Cies Island present the lowest concentrations of PAHs, whereas these chemicals were not detected in the sampling site located in the Bay of Cadiz. No special pattern was detected regarding to the concentration of metals in the different study sites; GR3 and F exceed some international guidelines defined for the metal Zn: GR3 (MacDonald et al., 1996) and F (MacDonald et al., 1996; DelValls and Chapman, 1998; NOAA, 1999). According to MacDonald et al. (1996), sediments from station D exceed the guideline proposed for Pb. Sites A and C from Cies and D, F from Corme-Laxe surpass the proposed guideline described by MacDonald et al. (1996) for Cu, whereas GR3 exceeds various guidelines proposed for this metal (Tweede Kamer, vergaderjaar, 1994–1995; MacDonald et al., 1996; NOAA, 1999; Riba et al., 2004). GR3 also exceeds the guidelines for Ni proposed by different international agencies and authors (Tweede Kamer, vergaderjaar, 1994–1995; MacDonald et al., 1996; NOAA, 1999).

3.2. Biomarkers of exposure

Mean values of the biomarkers of exposure determined in crabs and clams obtained after the 28-day exposure are summarized in Fig. 2. In general, organisms deployed in the Corme-Laxe area (D, E, and F) present the highest induction of the biomarkers of exposure. Significant differences ($p < 0.01$) in GPX activities in crabs were observed in sites D, E and F (Corme-Laxe) and the reference station CA, whereas clams exposed in site D also presented significant differences in relation to the reference station. Differences were significant for the phase II enzyme GST measured in crabs from D, F and the reference site CA. The GR antioxidant enzyme activity for both, crabs and clams resulted significantly different from the reference site CA for the three study sites located in Corme-Laxe (D, E and F). EROD activity, which accounts for the enzymatic activity occurring in phase I of detoxification, was significantly different among the crabs collected in locations B (AINP), D, E and F (Corme-Laxe), and clams exposed to sediments from D (Corme-Laxe) and GR3, GR4, P1 (Bay of Algeciras).

Table 1
Total PAHs, PCBs and metal concentration (Zn, Cd, Pb, Ni, Co and V)-mg kg^{-1} dry sediment- measured in the sediments from Galicia: Atlantic Islands National Park (A, B, C), Corme-Laxe (D, E, F); the Bay of Algeciras (GR3, GR4 and P1) and the Bay of Cadiz (CA) used as the reference station

	PAHs	PCBs	Zn	Cd	Pb	Cu	Ni	Co	V
CA	n.d.	n.d.	21.3	0.92	2.28	6.98	0.06	3.40	80.0
A	257	n.d.	76.2	n.d.	26.6	18.9[d]	12.0	0.52	n.d.
B	370	6.52	43.4	n.d.	9.13	n.d.	6.88	n.d.	n.d.
C	239	4.76	37.5	n.d.	6.54	31.6[d]	5.02	0.87	n.d.
D	537	2.60	65.7	n.d.	44[d]	22.1[d]	9.39	1.21	13.4
E	558	4.29	31.8	n.d.	4.25	n.d.	5.61	0.37	2.34
F	820[d]	2.28	243[a,b,d]	n.d.	14.3	19.1[d]	7.03	0.67	5.94
GR3	2961[d,e]	22.0	138[d]	0.17	21.6	5.01	74.7[a,d,e]	12.8	26.1
GR4	802[d]	1.75	35.3	0.10	6.21	3.67	13.1	5.59	n.d.
P1	641[d]	0.84	56.7	0.12	12.3	75.2[a,c,d,e]	13.3	n.d.	6.84

n.d: not detected.
[a] Concentration that exceeds the ERL (effects range-low) defined by NOAA (1999).
[b] Value that exceeds the sediment quality guideline suggested by DelValls and Chapman (1998).
[c] Concentration which surpass the guideline described by Riba et al. (2004).
[d] Value that exceds the guideline proposed by MacDonald et al. (1996).
[e] Concentration that surpass the guidelines defined by Dutch agencies, Tweede Kamer, vergaderjaar (1994–1995).

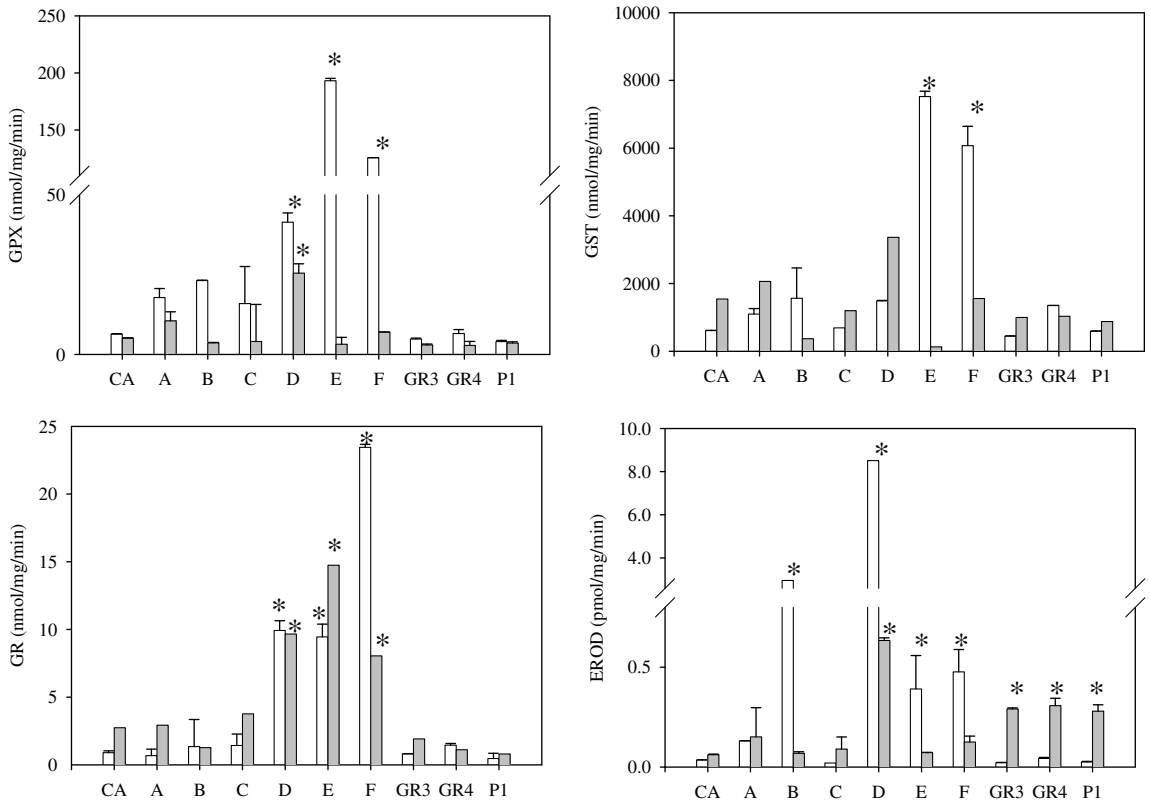

Fig. 2. General health biomarkers for both invertebrate species, the clam *Ruditappes philippinarum* and the crab *Carcinus maenas*: glutathione peroxidase activity GPX (nmol/min/mg prot), glutathione transferase GST activity (nmol/min/mg prot), glutathione reductase GR activity (nmol/min/mg prot) and EROD activity (pmol/mg/min). Asterisks indicate significant differences with the reference treatment CA ($^{*}p < 0.05$; $^{**}p < 0.01$).

Crabs were fed weekly in order to avoid biological responses caused by starvation. Additional feeding could have reduced the exposure to contaminants. Without feeding, however, and after 28 days of deployment, the crabs would have diminished their food intake because of their limited access to natural food resources and different symptoms related to stress and weakness would have appeared.

3.3. Biomarkers of effect

The relationship between pollutants and pathologies in target tissues has been previously reported (Ortiz-Delgado et al., 2007). Histopathology results showed no alterations in the organisms from the negative control (Figs. 3 and 4). In general, damage to crab and clam tissues was lower than detected in laboratory deployments (Morales-Caselles, 2007). Most of the organisms analyzed showed several histological unspecific lesions related to symptoms of general stress, including loss of digestive epithelial cells, rupture of gill epithelium, respiratory lamellar fusion, as well as haemocitic infiltration or loss of connective tissue of the gills and hepatopancreas. Most of these effects can also be observed in different marine invertebrate or vertebrate species exposed to different inorganic or organic contaminants, parasitic or infectious diseases, nutritional stress, or physico-chemical disorders (Rodríguez de la Rua et al., 2005; Ortiz-Delgado et al., 2007). Organisms exposed to sediments from the Bay of Algeciras were the most affected followed by clams and crabs exposed to sediments from Corme-Laxe, and finally organisms from the Cies site showed alterations due to general environmental stress. The lesions observed in clams that had been exposed to sediments from Algeciras during 28 days included: desquamation of digestive epithelium, occlusion of the digestive ducts, haemocitic infiltrations and weak alterations or loss of the supporting digestive connective tissue (hepatopancreas), ciliar alterations, loss of support connective tissue, and hypertrophy or fusion of lamellae (gills). Crabs deployed in site GR3 presented disrupted pillar cells, epithelial changes, desquamation in gills and vacuoles in hepathopancreas of caged organisms. The presence of parasites in some of the crabs studied makes it more difficult to determine the cause of the damages. In this sense, clams related better to pollutants than crabs. Organisms from the reference site did not present alterations in target tissues.

3.4. Linking chemicals and biomarkers

As it has been shown above, in the current study the highest activities of biomarkers of exposure where observed in those individuals deployed "in situ" in the Bay of Corme-Laxe. Studies carried out with the same organisms and similar sediments under laboratory conditions (Morales-Caselles et al., in press) showed higher biomarker responses in organisms exposed to sediments from the Bay of Algeciras, mainly due to the concentration of PAHs in the sediments. Occasionally, in situ exposures showed greater toxicity than laboratory exposures to sediments from the same sites (Burton et al., 2005).

In order to elucidate the source and type of contaminant that is causing stress in the organisms, a multivariate analysis was performed to link biomarkers of exposure with the chemicals bound to sediments. Three new factors were defined to describe the 17 original variables by explaining a 75% of the total variance (Table 2). The main Factor (29.9%) links the phase I detoxification activity determined by EROD in clams and crabs and the GST and GPX activity in clams to the concentration of Pb in the sediment. This factor has a positive loading mainly in site D from Corme-Laxe and followed by site A in Cies (Fig. 5). The low score in site A

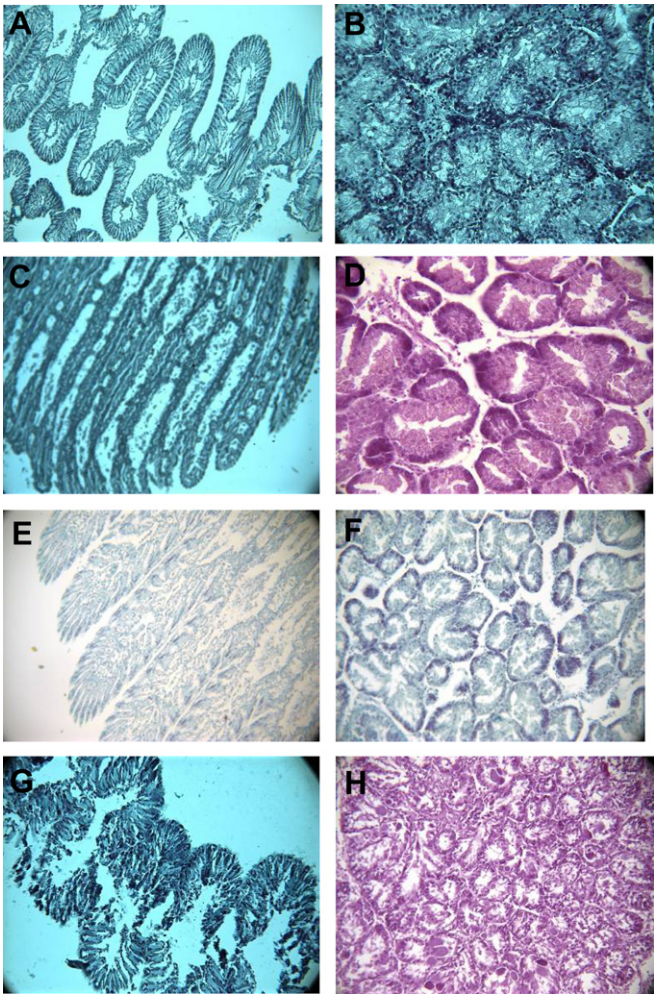

Fig. 3. Histological sections of gills and digestive gland of the clam *Ruditapes philippinarum* after 28-d exposure to the sediments. (A) Histological section of a control gill (day 0); (B) histological section of a control digestive gland (day 0); (C) histological section of gill from a clam exposed to sediments from AlNP; (D) histological section of gill from a clam exposed to sediments from Corme-Laxe; (E) histological section of digestive gland from a clam exposed to sediments from Corme-Laxe; (F) histological section of gill from a clam exposed to sediments from Algeciras; (G) histological section of digestive gland from a clam exposed to sediments from Algeciras.

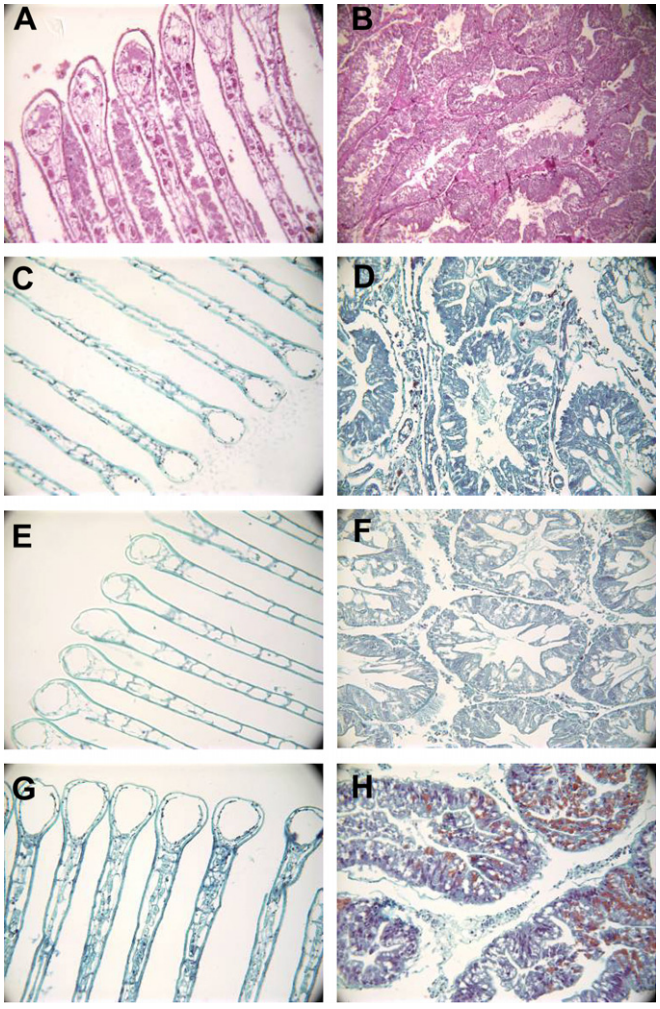

Fig. 4. Histological sections of gills and hepathopancreas of the crab *Carcinus maenas* after 28-d exposure to the sediments. (A) Histological section of a control gill (day 0); (B) histological section of a control hepathopancreas (day 0); (C) histological section of gill from a crab exposed to sediments from AlNP; (D) histological section of gill from a crab exposed to sediments from Corme-Laxe; (E) histological section of hepathopancreas from a crab exposed to sediments from Corme-Laxe; (F) histological section of gill from a crab exposed to sediments from Algeciras; (G) histological section of hepathopancreas from a crab exposed to sediments from Algeciras.

suggests that Pb bound to sediments produced some stress although the high prevalence of this factor in site D, which exceeds the sediment quality guideline proposed by MacDonald et al. (1996) for this contaminant, implies that there is a source of Pb that involves the activation of "early warning" biomarkers.

On the other hand, the fact that the biomarkers of exposure have a significant induction in the organisms exposed to sediments from the Bay of Corme-Laxe, which was not detected under laboratory exposure (Morales-Caselles et al., in press), could be related to the existence of a contaminant in the sea water that was not measured. In this case, a possible source could be the presence of caged mussels for aquaculture in the proximities that may cause an input of organic matter, therefore causing stress among the deployed organisms. The addition of organic matter to the surrounding water involves a decrease in levels of dissolved oxygen that is used in the oxidation processes. The oxygen content of water can be important in determining the nature and the rate of both chemical and biochemical transformations (Walker et al., 2006). On the other hand, there have been a number of studies documenting the fact that UV radiation can greatly increase the toxicity of PAHs in a broad phylogenetic spectrum of aquatic species (Anckley et al., 2003) including molluscs and crustaceans; in this case photo-induced PAH toxicity could be occurring under field conditions.

According to the second factor (23.8%), a relationship is observed between the organic contaminants PAHs and PCBs with metals Ni, Co and Zn. This factor, with negative loading, does not relate the association of these contaminants to the biomarkers and accounts for a contamination in sediments from sites GR3 and GR4 from the Bay of Algeciras and F in Corme-Laxe (Fig. 5). Biomarker responses were expected in organisms exposed to the contamination bound to sediments from Algeciras, as it was shown in laboratory studies (Morales-Caselles et al., in press). However, biomarkers of exposure were generally low, mainly in crabs, in comparison to sediments from Corme-Laxe. Previous studies with vertebrates and invertebrates have demonstrated an inhibition of the CYP1A1 system due to metal exposure (Faverney et al., 2000; Kutlu and Susuz, 2004); Faverney et al. (2000) observed that co-treatment with an organic compound and a heavy metal induced apparently opposite effects compared to those obtained in individual treatments with one or the other of these compounds. As

Table 2
Sorted rotated factor loadings of 17 variables for the three principal factors resulting from the multivariate analysis of results obtained from the biomarker responses in crabs and clams and the chemical analysis

	FACTOR 1 *29.9*	FACTOR 2 *23.8*	FACTOR 3 *21.1*
GPX-crab	–	–	0.93
GPX-clam	0.97	–	–
GR-crab	–	–	0.85
GR-clam	–	–	0.82
GST-crab	–	–	0.94
GST-clam	0.91	–	–
EROD-crab	0.90	–	–
EROD-clam	0.94	–	–
PAHs	–	−0.98	–
PCBs	–	−0.93	–
Zn	–	−0.46	0.51
Cd	–	–	–
Pb	0.93	–	–
Cu	–	–	–
Ni	–	−0.97	–
Co	–	−0.88	–
V	–	–	–

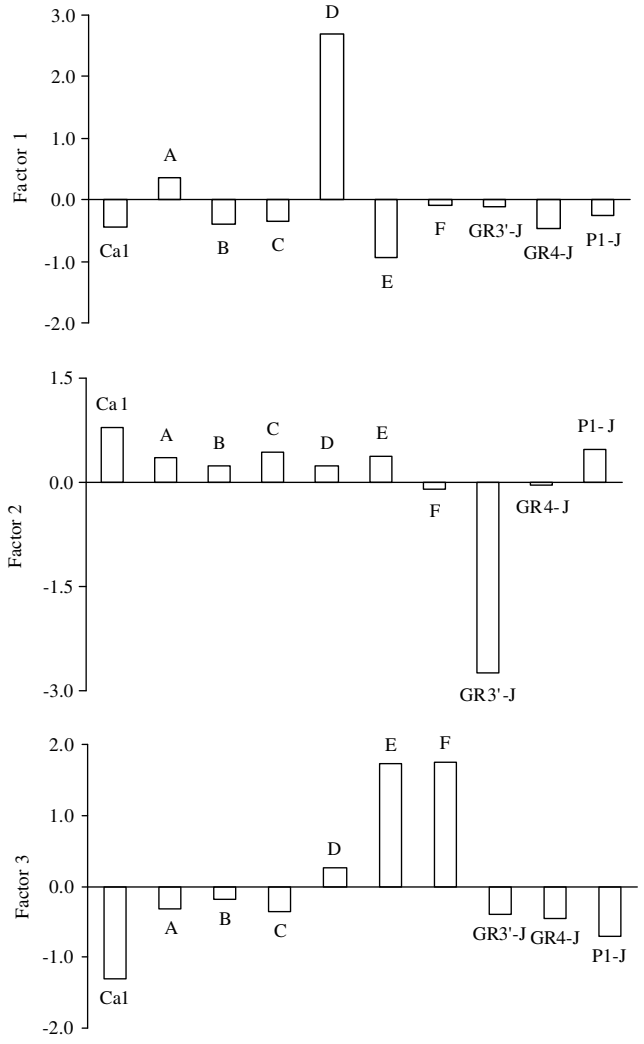

Fig. 5. Factor loadings for the three principal factors resulting from the multivariate analysis of results obtained from the chemical analysis and the suite of biomarkers.

mentioned above, sites from the Bay of Algeciras exceed SQGs defined for various metals and this could also explain the inhibition of EROD activity. The area of the deployment, the mouth of the Guadarranque River, is under the influence of natural tides and this could reduce the bioavailability of contaminants to the organisms. Sediments in intertidal zones along the seashore experience fluctuating oxygen levels in accordance with tidal movements (Walker et al., 2006). As the oxygen content declines, there will be a tendency for oxidative transformations to be replaced by reductive ones. Oxidations by the microsomal monooxygenase system depend upon the activation of hemoprotein molecular oxygen (O_2) after it has been bound to an associated hemoprotein, cytochrome P450. (Walker et al., 2006).

The third factor (21.1%) connects the concentration of Zn in sediments to the biomarkers of response related to antioxidant activity: GR induction in crabs and clams and GST and GPX activity in crabs. Sites D, E and F from the Bay of Corme-Laxe present the influence of this factor (Fig. 5), which indicates this area presents stress caused by the Zn pollution. Previous studies have determined that the Bay of Corme-Laxe is not contaminated and have attributed the presence of metals to basal levels (Cobelo-García et al., 2005). However, regarding site D where levels of Zn surpass several sediment quality guidelines (Table 1), an anthropogenic source of this metal is probably the cause of the stress in the organisms, mainly in crabs. Previous studies have shown sublethal responses in *C. maenas* exposed to sediments contaminated by Zn (Martín-Díaz et al., 2005).

In general, histopathological responses have shown moderate damage in the studied organisms deployed in Corme-Laxe, which suggests that the antioxidant and detoxification activities are successful. However, defence may involve a trade-off between production and survival: increased survival may be obtained only at a cost of reduced growth of reproduction (Walker et al., 2006).

4. Conclusions

This study has analyzed the sublethal responses in organisms deployed in sites affected by different spills in the areas of Galicia and the Gulf of Cádiz. In addition, biomarker results have been linked with chemicals bound to sediments in order to elucidate the cause, source and bioavailability of adverse affects after exposure. A set of biomarkers including antioxidant and detoxification activities has been evaluated in addition to histopathological damages in target tissues. Two invertebrate species were used, the clam *R. philippinarum* and the crab *C. maenas*, with difference feeding habits, in order to better assess the responses. The lowest sublethal responses were observed in organisms exposed to sediments from the Cíes Islands in the AINP, although the presence of some metals could have induced some stress in the deployed animals. This points to a recovery of the area four years after the Prestige oil spill although the presence of some metals is considered a potential risk. Organisms exposed in Corme-Laxe presented high levels of stress that were not observed in laboratory exposures (Morales-Caselles et al., in press), which points to the impact of sources of contaminants, not only hydrocarbons, such as the material from the aquaculture cages. In the case of the Bay of Algeciras, the toxic effects of contaminants were probably diminished by the removal of water caused by tidal fluctuation, although target tissues presented the most serious alterations of all the study sites.

Previous studies concur with the fact that the in situ, caged organism approach should be used together with other assessment methods such as laboratory toxicity testing (Burton et al., 2005) as a new and important line of evidence in ecological risk assessment (Baird et al., 2007; Crane et al., 2007).

This report has shown how field studies allow for the identification of alternative sources of stress that are not observable in laboratory experiments. Therefore, the authors consider that

bioassays should not be limited to experimental designs under laboratory conditions and propose that field deployments are an improvement over the uncontrolled circumstances of in situ surveys and the excessive control of laboratory tests.

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