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THE USE OF A KINETIC BIOMARKER APPROACH FOR IN SITU MONITORING OF LITTORAL
SEDIMENTS USING THE CRAB *Carcinus maenas*

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Abstract

Caged, transplanted, intermoult, female shore crabs (*C. Maenas*) were exposed to sediments from the Port of Cadiz (SW, Spain); the Port of Huelva (SW, Spain), the Port of Pasajes (NE, Spain) and the Port of Bilbao (NW, Spain) for a period of 28 days.

Organisms were sampled on days 0, 7, 14, 21 and 28 allowing examination of different biomarkers to exposure to metals and organic compounds; Metallothioneins (MTs), Ethoxyresorufin O-deethylase (EROD), Glutathione-S-transferase (GST) and Glutathione peroxidase (GPX). Sediment samples were also analyzed to determine chemical concentration of metals (As, Cd, Cr, Cu, Hg, Mn, Ni, Pb and Zn), PAHs and PCBs.

Metals such as As, Cu and Zn associated with mining activities (Port of Huelva), and contaminants such as Ni, Pb, Hg and PCBs, associated with oil spills and industrial activities (Ports of Cadiz, Pasajes and Bilbao), were found to interfere in detoxification and/or anti-stress oxidative defenses in *C. maenas*. Positive, significant ($p < 0.05$) induction of MTs concentration was associated with increasing sediment As, Cu, Zn and Hg concentrations. EROD activity was associated with PCBs, GST activity with Hg and GPX activity with As, Cu, Ni, Pb and Zn. The use of the parameter AR, which involves the kinetic patterns of the battery of biomarkers tested, was found to be a powerful and sensitive tool for evaluating the bioavailability and adverse effects of contaminants bound to sediments, enabling polluted and non polluted sites to be easily distinguished.

Keywords: Metallothionein; Enzymatic activity; Oxidative stress; *Carcinus maenas*; Ecotoxicology; Dredged material; Invertebrates.

46 1. Introduction

47 Contaminated sediments are a risk to aquatic life, human health, and wildlife throughout the world.
48 Overwhelming evidence demonstrates that chemicals in sediments are responsible for toxicological
49 and adverse ecological effects (Andersen et al., 1998). Frequently, the chemicals causing these
50 effects are present in the sediment as mixtures of organic, metal, and other types of compounds.
51 The adverse environmental effects associated with contaminated sediments in ports and estuaries
52 have resulted in a number of international treaties and protocols for their environmental
53 management. The OSPAR and Helsinki Conventions (North Sea, North-East Atlantic, Baltic Sea)
54 propose strict guidelines to control the disposal of contaminated sediments. In recent years,
55 remediation and management of contaminated sediments is becoming increasingly important, which
56 has led to an increasing economical and technological burden on management agencies. Thus,
57 there is a clear need for the development of science based tools for the identification of sediments
58 that are impaired, allowing agencies to set priorities and make effective management decisions for
59 dealing with contaminated sediments.

60 The most widely applied methods for the assessment of sediment toxicity are acute and chronic
61 sediment bioassays. Although a wide range of acute toxicity tests are already recommended,
62 standardized and included in sediment assessment regulatory frameworks, the development and
63 application of chronic toxicity tests including the measurement of sub lethal responses (biomarkers),
64 although widely supported, is still under investigation. The use of biomarkers in the evaluation of the
65 toxicity of dredged material is under continuous development and improvements are still desirable.

66 An important advantage of biomarkers in assessing the impact of dredged materials is their inherent
67 capability to detect the early occurrence of various stress conditions within the organism and monitor
68 the temporal progression (or regression) of the disturbance at various levels of biological
69 organization (Regoli et al., 2002). When assessing the exposure to a mixture of contaminants in
70 dredged materials within the aquatic environment, biotransformation and antioxidant enzymes as

well as metal-binding proteins such as metallothioneins (MTs) play an important role in identifying contamination by organic and metal pollutants respectively. However, ecotoxicological measurements obtained in laboratory studies can be difficult to translate into accurate predictions of toxicological effects in the natural environment. Since both overestimation and underestimation of effects may occur, laboratory observations must always be validated with field research. The transplanting and caging of bioindicator organisms provides a combination of the experimental control of laboratory bioassays and the environmental realism of field monitoring (Salazar and Salazar, 1997). Thus, the combination of this methodology with biomarker measurements of suitable indicator species may provide a strong tool for the monitoring of contaminated sediments, allowing the determination of the total external load that is biologically available in the "real world" situation (van der Oost et al., 2003).

Still, studies that use biotransformation and antioxidant enzymes as tools to monitor polluted sites should be performed carefully. Various authors have pointed out the serious risk of misinterpretation associated with using a single "snap-shot" (i.e., a single measurement at the end of exposure) to rationalize oxidizing versus other effects of toxic contaminants in the environment (Bouskill et al., 2006). Thus, a study of a continuous series of biomarker responses over time rather than a single measurement after concrete exposure time should be performed in order to overcome the problem mentioned above.

The aims of this study were to determine the measured biomarker responses within *C. maenas* over a 28-day exposure period to contaminated sediments. This protocol was chosen to: 1) fill the lack that exists from measuring a single time point,, 2) reveal temporal variation in oxidative injury related to increasing exposure time, and 3) allow the distinction and classification of sites with different magnitudes (and types) of sediment toxicity. To do this caged, female crabs were exposed *in situ* to sediments with different levels of metals and organic compounds from a number ports within Spain. The responses of a suite of biomarkers, including metallothioneins (MTs), mixed function oxidase

(EROD), glutathione-S-transferase (GST), and glutathione peroxidase (GPX): antioxidant enzyme were determined during a 28-day exposure period and through a kinetic approach.

2. Materials And Methods

2.1. Study site

Eight sampling sites were chosen (Figure 1) for the assessment of sediment toxicity in Spanish ports. Ca2 and Ca3 (negative toxicity control) were situated in the Port of Cádiz (SW, Spain), which has been studied in some depth (DelValls et al., 1998; Riba et al., 2004a,b) and is characterized by an absence of significant contamination. Hu2 and Hu3 were situated in the Port of Huelva (SW, Spain) and are characterized by extensive heavy metal contamination related to historic mining activity. Bi2 and Bi3 were situated in the Port of Bilbao (NNE, Spain), which is characterized by significant shipping activity with sediment contamination predominantly associated with organic contamination, especially hydrocarbons. Pa2 and Pa3 were situated in the Port of Pasajes (NNE, Spain), which is also characterized by significant shipping activity and by contamination associated with organic compounds similar to the Port of Bilbao.

2.2. General approach

2.2.1. Chemical characterization of the sediment

Analysis of sediment was performed before starting the experiment according to Spanish recommendations for dredged material (CEDEX, 1994). 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 over 24h. Metal content in the different sediments was determined following the methods reported by Riba et al. (2004a,b); PCBs (congeners #28, 52, 101, 118, 138, 153 and 180) and PAHs 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).

122

123 2.2.2. *In situ* toxicity test

Intermoult, female, *C. maenas* of a standardized size (Martin-Diaz et al., 2004) were enclosed in rectangular cages (50 x 25 x 10 cm) made of plastic net. During the experiment 24 crabs were placed in each cage and 2 cages were placed per site. Crabs were fed frozen mussels. No condition indexes were noted along the experiment. Low mortality was observed in individuals exposed to control sediments which indicated that crabs conditions were feasible for field caging experiments.

The cages were anchored to the sediment at each site by a scuba-diver within each of the four Spanish ports: Port of Cadiz (SW, Spain) (Ca2, Ca3-control sediment), Port of Huelva (SW, Spain) (Hu2, Hu3), Port of Pasajes (NE, Spain) (Pa2, Pa3) and Port of Bilbao (NW, Spain) (Bi3, Bi2). Cages were exposed to sediments for 28 days. On days 0, 7, 14, 21 and 28, cages were collected and 10 individuals sampled. The temperature ($15^{\circ}\text{C} \pm 1^{\circ}\text{C}$), pH (7.8-8.2), salinity (33.8 ± 0.3) and dissolved oxygen ($>5 \text{ mg} \cdot \text{L}^{-1}$, 60% saturation) were measured.

135

136 2.2.3. Sublethal responses determination

Ten, intermoult, female *C. maenas* were collected from within each port on days 0, 7, 14, 21 and 28. Crabs were dissected and their hepatopancreas were removed for biomarker determination. Hepatopancreas tissues were homogenized 1:4 in cold homogenization buffer: Hepes-NaOH (10 mM) buffer containing 125 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol, pH 7.4 at 4°C at a 1:5 weight:volume ratio (Lafontaine et al. 2000). After homogenization, samples were centrifuged (4°C , 10 000g, 30min) and the supernatant collected for biomarker determination (S9 fraction). Samples obtained to determine MTs content were centrifuged at 28 000 g for 40 min. The supernatant was utilized for total protein determination according the dye-binding principle (Bradford, 1976).

145 Metallothionein (MT) concentration in hepatopancreas tissues was determined by Anodic Stripping
 146 Voltammetry (ASV) (Olafson and Olsson 1987). In brief, 0.1 mL of the supernatant (homogenate 28
 147 000 g for 40 min) was added to 0.9 mL of NaCl (0.9 %), heated to 95 °C for 4 min, then centrifuged
 148 at 10 000 g for 15 min at 4°C. The supernatant was stored at –80 °C prior to MT concentration
 149 determinations using purified rabbit metallothionein (Sigma-Aldrich). MT concentrations were
 150 expressed as $\mu\text{g MT (mg total protein)}^{-1}$. Mixed function oxidize activity was measured using the
 151 adapted EROD assay initially adapted for fingerling rainbow trout (Gagné and Blaise, 1993). 50 μL
 152 of supernatant (homogenate 10 000 g for 30 min) were added to 10 μM 7-ethoxyresorufin and 10
 153 μM reduced NADPH in 100 mM KH_2PO_4 buffer (pH 7.4). The reaction was started by the addition of
 154 NADPH, allowed to proceed for 60 min at 30°C, and stopped by the addition of 100 μL of 0.1 M
 155 NaOH. The formation of 7-hydroxyresorufin was determined fluorometrically using 520 nm
 156 (excitation) and 590 nm (emission) filters. 7-hydroxyresorufin in the samples was achieved through a
 157 standard calibration curve developed with concentrations of 7-hydroxyresorufin. Results were
 158 expressed as $\text{pmol}\cdot\text{min}^{-1}\cdot(\text{mg total protein})^{-1}$. The procedure utilized for the determination of
 159 Glutathione S-transferase activity was adapted from the method of McFarland et al. (1999). The
 160 activity was analyzed using 42 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 1 mM GSH as
 161 substrates and measured spectrophotometrically at 340 nm every 30 seconds for 3 min. Results
 162 were expressed as $\text{nmol min}^{-1} (\text{mg total protein})^{-1}$. The methodology used for the determination of
 163 glutathione peroxidase activity was also adapted from McFarland et al. (1999). GPX activities were
 164 measured spectrophotometrically at 340 nm every 2 minutes for 10 minutes, using as substrate 1
 165 mM cumene hydroperoxide. Results were expressed as $\text{nmol min}^{-1} (\text{mg total protein})^{-1}$.

166

167 2.3. Data and statistical analysis

168 A continuous study of biomarker responses over time as opposed to a measurement of sublethal
 169 responses at a single time point was performed. Metallothioneins and enzymatic activity responses

170 were fitted to different kinetic models using SIGMA PLOT 8.0 software package. Linear regression
171 was used to determine the kinetic parameters of MTs at each site. GST 's, EROD's and GPX's
172 kinetic equations were obtained through hyperbolic regression analysis, where the different
173 enzymatic activities were fitted to the best hyperbola equation.

174 In order to summarize the temporal variation of the different biomarkers, the area (AR) under the
175 fitted kinetic curves was calculated using Mathematica 4.0 Differences between the AR of control and
176 contaminated sediments were analyzed using SPSS/PC+. Significant differences between biomarker
177 responses of crabs exposed to control and contaminated sediments were determined using one-way
178 ANOVA. Post-hoc examination of significant differences between sites was carried out using
179 Dunett's test. The significance level was set at $p < 0.01$.

180 Significant correlations between chemical concentrations in sediments and biomarker responses
181 (using AR parameters) were examined using a Pearson's correlation analysis using the SPSS/PC+
182 statistical package. The level of significance was set at $p < 0.01$.

183

184 3. Results

185 3.1. Chemical characterization

186 Summarized results of the chemical analysis are shown in Table 1. The chemical characterization of
187 the sediment samples indicates that the sediments contain a mixture of contaminants (metals, PCBs
188 and PAHs). Sediments from the port of Huelva were characterized by higher concentrations of metals
189 (As, Cu, Pb, Zn, Ni and Cd) compared with the reference site Ca3. Whereas, sediments from the
190 ports of Pasajes and Bilbao were mostly characterized by high concentrations of PCBs and PAHs,
191 although contamination by metals such as Cr, Mn, Ni, Pb and Hg was also found. Sediment from the
192 port of Cadiz was not highly contaminated (Ca3), although high concentrations of Hg were
193 determined in Ca2.

194

195 3.2. Specimen survival

196 During the 28-day, *in situ*, bioassay exposure to sediments from four Spanish ports, no sediment-
197 dependent mortalities were observed for intermoult female *C. maenas*. Survival in all treatments was
198 more than 90%.

199

200 3.3. Biomarker responses

201 Summarized results of biomarker responses determined in hepatopancreas tissues of *C. maenas*
202 exposed to contaminated sediments (Ca2, Ca3, Hu2, Hu3, Bi2, Bi3, Pa2, Pa3) are shown in Figure
203 2. In this figure experimental biomarker responses over time and their approximation to kinetic
204 models could be observed. Biomarker responses described different patterns over time for each
205 biomarker response measured in *C. maenas*. GST and GPX activities increased until days 14 and
206 21, respectively, after which they declined reaching basal levels by the end of the experiment.
207 EROD activity and MTs concentration increased during the 28-day exposure; with linear induction
208 observed for MTs and exponential induction for EROD activity. No significant induction of
209 detoxification and antioxidant parameters was observed in the control individuals (Ca3).

210 The equations to the different biomarkers, the correlation coefficient and the representation of the
211 areas (AR) calculated for each biomarker and sites are shown in Figure 3. The results show a good
212 correlation between the experimental data and those predicted by the results as described in Figure
213 2. It confirms a good approximation of biomarker induction (MTs, GPX, GST and EROD) to Linear
214 and Hyperbolic Kinetic models.

215 Significant induction of GST activity (compared to controls) were observed in crabs exposed to Ca2
216 (2.38% of control) ($p < 0.01$), Pa3 (1.70%) ($p < 0.01$), Bi2 (1.68%) ($p < 0.01$), Hu3 (1.45%), Hu2 (1.40%)
217 Bi3 (1.27%) and Pa2 (1.12%). Conversely, significant induction of GPX activity was also observed in
218 crabs exposed to Bi3 (16.75% of control) ($p < 0.01$), Pa3 (15.08%) ($p < 0.01$), Hu2 (14.32%) ($p < 0.01$),
219 Hu3 (14.07%) ($p < 0.01$), Bi2 (12.02%) ($p < 0.01$), Pa2 (10.85%) ($p < 0.01$) and Ca2 (9.38%) ($P < 0.01$).

220 Significant induction of EROD activity in crabs exposed to Pa3 (16.71.52% of control) ($p < 0.01$), Pa2
221 (1.47%) ($P < 0.01$), Bi2 (1.34%) ($p < 0.01$), Bi3 (1.19%) ($p < 0.05$) and Ca2 (1.12%) was also found. No
222 induction was observed in Hu2 and Hu3. Finally, significant MTs increase was observed in crabs
223 exposed to Hu3 (1.75% of control) ($p < 0.01$), Hu2 (1.66%) ($p < 0.01$), Ca2 (1.45%) ($p < 0.01$), Bi2
224 (1.28%), Pa3 (1.16%). No induction in Bi3 and Pa2 was detected.

225 Significant positive correlations were observed between contaminants bound to sediments and
226 biomarker responses (Table 2). A significant correlation between GST activity induction and Hg ($p <$
227 0.05) was observed. Induction of GPX activity was correlated with As ($p < 0.01$), Cu ($p < 0.01$), Pb
228 ($p < 0.01$), Zn ($p < 0.01$) and Ni ($p < 0.01$). Significant induction of EROD activity was only observed in
229 individuals exposed to Σ PCBs. Whereas, MT induction was associated with the metals As ($p < 0.05$),
230 Cd ($p < 0.05$), Cu ($p < 0.05$), Zn ($p < 0.05$) and Hg ($p < 0.01$).

231 The balance between bioactivation on the one hand and detoxication on the other can be crucial for
232 the assessment of possible hazards due to the exposure to toxic substances (van der Oost et al.
233 1998). For this reason, in the present study the biotransformation index (BTI) (van der Oost et al.,
234 1998), which is the ratio between phase I and phase II enzyme activities was determined (Figure 4).
235 EROD:GST in caged female crabs are maximally induced after 14 in Pa2, and after 21 days of
236 exposure in Ca2 and Pa3 (Figure 4). As opposed to the EROD activity, the BTI values in crabs
237 which belong to the mentioned sites decrease after 21 days of exposure due to the increased phase
238 II activities (Figure 4).

239

240 4. Discussion

241 Comparisons between the temporal biomarker responses in female crabs (*C. maenas*) exposed to
242 sediments from four Spanish ports and a new parameter, which involves biomarker responses over
243 time, have been calculated. This new parameter, AR, allowed the comparison of temporal biomarker

244 responses in organisms exposed to control and contaminated sites, as well as contaminants bound
245 to sediment which have provoked adverse effects.

246

247 4.1. Metallothionein concentration (MT)

248 Significant induction ($p < 0.01$) of MTs after 28-day exposure to sediments contaminated with As, Cd,
249 Cu, Hg and Zn was found in Hu2, Hu3 and Ca2 ($p < 0.05$). *C. maenas* has previously been shown to
250 be a useful species in metallothionein biomarker testing studies, allowing the detection of the
251 potential effects of metal contamination *in situ* (Bebianno et al. 2003). High concentrations of metals
252 were observed in the port of Huelva, presumably due to the historic mining activity in the area. Ca2
253 was also highly contaminated with Hg. MTs constitute a family of low-molecular-weight, cysteine-rich
254 proteins functioning in the regulation of the essential metals Cu and Zn, and in the detoxication of
255 these and other, non essential, metals such as Cd and Hg (Roesijadi and Robinson, 1994). The
256 cellular interactions involving MTs are expected to follow two general lines, the first being the
257 interception and binding of metal ions that are initially taken up by the cell and the second being the
258 removal of metals from nonthionein ligands that include cellular targets of toxicity. The latter may
259 represent a detoxication function for structures, which have been reversibly impaired by
260 inappropriate metal binding. The role of MTs in sequestering metals is well established, while their
261 induction by exposure to a wide variety of metals (e.g. Cd, Cu, Zn, Hg, Co, Ni, Bi and Ag) is
262 associated with their induction by exposure protection function (Stegeman et al., 1992). Relatively
263 few studies in marine environments have reported the induction of MTs associated with As.
264 Nevertheless, induction of MTs due to As has been described in the freshwater invertebrate *D.*
265 *polymorpha* (Bauskill et al., 2006).

266

267 4.2. Ethoxyresorufin-O-deethylase activity (EROD)

EROD activity is involved in the first phase of metabolism, unmasking or adding reactive functional groups, which involve oxidation and reduction or hydrolysis (Goeptar et al., 1995). Induction is a clear sign of CYP1A1 enzyme activity, which is among the many known cytochrome isoforms. Increases in EROD activities have been reported in many species of invertebrates, including clam and crab species, following exposure to organic trace pollutants (Lafontaine et al., 2000). In the present study significant induction ($p < 0.01$) of EROD activity was measured in hepatopancreas homogenates of *C. maenas* exposed to PCBs ($p < 0.01$) bound to sediments from the port of Pasajes (Pa2, Pa3).

4.3. Glutathione-S-transferase activity (GST)

Chemical compounds, such as heavy metals, are biotransformed to conjugates of reduced glutathione (GSH). Conjugation with GSH is a very important detoxification pathway that is catalyzed by the enzyme GST. A significant increase in GST activity was observed in crabs exposed to sediments from Ca2 ($p < 0.05$) associated with the presence of Hg ($p < 0.01$). This finding indicates that Hg (at the concentrations found at site Ca2), induces GST activity in the hepatopancreas of *C. maenas* and may be used to elucidate the redox-activity generated by the oxidative stress produced by Hg (Elunlai et al. 2007).

4.4. Glutathione peroxidase activity (GPX)

Many pollutants (or their metabolites) may elicit toxicity reactions related to oxidative stress. Oxygen toxicity may act as a strong oxidant, capable of reacting with critical cellular macromolecules, possibly leading to DNA damage and cell death. Defense systems that inhibit oxyradical formation include the antioxidant enzymes such as glutathione peroxidase (GPX) (van der Oost et al., 2000). GPX activity measured was significantly increased after exposure to sediments from sites Ca2, Ca3, Hu2, Hu3, Bi2, Bi3, Pa2 and Pa3. Interestingly, the induction of this biomarker ranged from 9.38% to 16.75% over control. The induction of this biomarker was correlated with high concentrations of sediment-

bound metals, such as, As, Cu, Pb, Ni and Zn. Previous reports have also found that As (Bouskill et al., 2006), Cu (Sánchez et al., 2005), Ni (Tsangaris et al., 2006), Pb (Alves de Almeida et al., 2004) and Zn (Elumalai et al., 2007) have the capacity to promote oxidative stress and modulates enzymatic and non-enzymatic antioxidant expression within biological tissues.

4.5. Integrated time-dependant biomarkers responses

By using a battery of biomarkers to evaluate sediment toxicity over a 28-day period, we were able to analyze the time dependent trends in the biochemical responses of *C. maenas*. According to Bouskill et al. (2006), if the cellular effects of contaminants bound to sediment followed a simple linear etiology over time, initial EROD and MTs induction should protect cells from injury, followed by antioxidant enzymes induction, GST and GPX, to avoid oxidative stress and lipid peroxidation when MTs or EROD are overwhelmed. However, the biomarker responses observed in this study did not appear to follow this simple linear etiology. Determined biomarker responses vary over the 28-day exposure time due to differences in the contaminant levels for each site. Metallothioneins and EROD enzymatic activity were induced by As, Cd, Cu, Hg, Zn and PCBs, respectively. Since, for these biomarkers, the highest responses were found on day 28, their induction was slower than that of GPX and GST activities. This behavior suggests that after the beginning of the exposure, antioxidant enzymes such as GST and GPX were induced to avoid the oxidative stress caused by As, Hg, Cu, Ni, Pb and Zn, as found in previous studies. After the initial induction, a return of antioxidant enzymes to basal levels, suggests that MTs detoxification mechanisms were involved in the acclimation of *C. maenas* to contamination by As, Cd, Cu, Hg and Zn. In contrast, PCB exposure (at the concentration tested) did not produce significant oxidative stress.

The delay in the induction of MTs could be due to the sequestration of metals by MTs via the displacement of Zn, leading to a release of free Zn that, in turn, induces de novo MTs synthesis.

Indeed, in previous studies of invertebrate species (Correia et al., 2002; Rigwood et al., 1998) exposed to As, induction of MTs was not fast enough to prevent lipid peroxidation.

In the present study biomarker responses have been correlated with the contaminants measured in the sediments. Contaminants determined in the sediments tested are those recommended for dredged material characterization (CEDEX, 1994). Nevertheless, more studies should be performed in this direction since other contaminants not measured and synergetic and/or antagonistic effects have not been determined and could have affected the results obtained.

The BTI values determined in crabs at the sites Pa2, Pa3 and Ca2, indicate that the actual hazards of environmental contaminants slightly decrease in time after an initial increase. These results support those described by van der Oost et al. (1998) which were found in caged carps exposed to polluted sites.

5. Conclusions

In summary, by examining the temporal evolution of a suite of biomarkers in the test organism *C. maenas*, it was revealed that As, Cu and Zn associated with mining activities and Ni, Pb, Hg and PCBs, associated with oil spills and industrial activities, interfere in detoxification and/or anti-stress oxidative defenses under field conditions, suggesting that these contaminants are likely to cause adverse effects in wild populations of *C. maenas*.

Different behaviors of the biomarker responses were observed related to the time-dependant induction of metallothioneins, phase I enzymes and phase II enzymes. It is of increasing demand that the study of biomarker responses over time and the search of a parameter that involves this temporal evolution be undertaken. By using the calculated parameter AR we were able to summarize the temporal progression of the ecotoxicological responses, allowing the comparison of toxic contaminant bioavailability between polluted and non-polluted sites. Oxidative injury has a

342 temporal progression, which must be considered when using biotransformation and antioxidant
343 enzymes as biomarkers of sediment toxicity. Thus, we recommend the use of biomarker assays that
344 specifically consider the temporal progression of ecotoxicological responses, as these will provide a
345 stronger basis for the identification of sediment toxicity, particularly when sediments contain a
346 mixture of contaminants.

347

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478 Figure captions:

479 Figure 1. Localization of sampling points in the Iberian Peninsula.

480

481 Figure 2. Representation of metallothioneins (MTs) concentration, Ethoxyresorufin O-deethylase
482 (EROD), Glutathione-S-transferase (GST), Glutathione Reductase (GR) and Glutathione Peroxidase
483 (GPX) activities determined in hepatopancreas of *Carcinus maenas* over a 28-day exposure period to
484 sediments from the port of Cadiz (Ca2, Ca3), the port of Huelva (Hu2, Hu3), the port of Pasajes (Pa2,
485 Pa3) and the port of Bilbao (Bi2, Bi3).

486

487 Figure 3. Representation of the area under the curve (AR) calculated for each biomarker and
488 sediment. Results of correlation coefficient, r^2 and fitted equation are also shown. Asterisks show
489 significant differences of the AR with control sediment (Ca3) (** $p < 0.01$).

490

491 Figure 4. Biotransformation index: EROD : GST in *C. maenas* hepatopancreas exposed over a 28-day
492 period to sediments from the ports of Cadiz (Ca2, Ca3), the port of Huelva (Hu2, Hu3), the port of
493 Pasajes (Pa2, Pa3) and the port of Bilbao (Bi2, Bi3).

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EROD : GST ratio

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73 Table 1. Summarized results of chemical concentrations of sediments from the Port of Cadiz (Ca2,
74 Ca3), Port of Huelva (Hu2, Hu3), Port of Bilbao (Bi2, Bi3) and Port of Pasajes (Pa2, Pa3). Chemical

SITE	As	Cd	Cr	Cu	Hg	Mn	Ni	Pb	Zn	Σ PCBs	Σ PAHs
CA2	30.77	1.32	14.94	202.80	1.98	201.60	20.14	86.90	378.25	0.11	0.11
CA3	16.61	1.23	8.43	46.76	0.28	294.40	16.90	17.61	135.50	0.01	0.01
HU2	532.27	2.50	24.10	149.70	1.99	303.60	7.10	384.70	1857.00	0.01	0.01
HU3	272.78	1.32	8.13	772.50	1.20	354.45	128.55	217.60	1176.00	0.01	0.01
BI2	104.49	2.00	23.11	204.10	1.43	396.60	32.00	147.50	777.50	0.23	66.77
BI3	21.71	0.04	3.48	23.03	0.18	191.35	15.72	285.90	122.35	0.01	13.90
PA2	28.76	0.70	23.42	167.10	1.29	180.00	28.48	293.70	763.00	0.74	1.06
PA3	23.78	0.04	18.61	162.50	1.36	152.60	19.61	246.00	576.00	0.24	0.26

85 s are expressed as mg·kg⁻¹.

86

87 Table 2. Significant positive Pearson's correlation between biomarkers responses and contaminants
 88 of interest. Data were pooled across sites and areas (AR) of the fitted kinetic approaches. Asterisks
 89 indicate significant relationships (* $p < 0.05$; ** $p < 0.01$).

Biomarker/ Contaminant	GST	GPX	EROD	MT
As	-	0.48**	-	0.40*
Cd	0.01	0.02	-	0.4*
Cr	0.01	0.00	0.04	0.02
Cu	-	0.46**	-	0.47*
Hg	0.4*	0.27	-	0.51**
Mn	-	0.15	-	0.25
Ni	-	0.42**	-	0.15
Pb	-	0.69**	0.25	0.23
Zn		0.52**	-	0.4*
Σ PCBs	-	0.00	0.73**	-
Σ PAHs	0.13	0.04	0.30	0.09