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The use of a metallothionein-like-proteins (MTLP) kinetic approach for metal bioavailability monitoring in dredged material

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Abstract

Ecotoxicological effects associated with contaminants present in dredged material from Spain were determined using a marine biotest based on the determination of metallothionein-like-protein concentrations (MTLPs) in the shore crab *Carcinus maenas*.

Intermoult female *C. maenas* were exposed in the laboratory to sediments from three Spanish ports, Ría de Huelva (SW, Spain), La Coruña (NW, Spain) and Bahía de Cádiz (SW, Spain) per replicate during 21 days. Hepatopancreas samples from crabs were taken for metallothioneins analysis on days 0, 7 and 21. Furthermore, chemical analysis was performed in the stations to determine the degree and nature of sediment contamination (Cr, Ni, Cu, Zn, Cd, Pb, Hg, As, PAHs and PCBs). A significant increase ($p<0.05$) in metallothionein concentration was observed over time in individuals exposed to sediment from the port Ría de Huelva characterized by high concentrations of metals. A toxicokinetic approach is proposed in this study related to the use of this biomarker in *C. maenas* to evaluate bioavailability associated with metals present in dredged material. As a first step, this toxicokinetic approach might reveal as a sensitive tool for evaluating bioavailability of contaminants present in dredged material.

Keywords: Biomarkers; *Carcinus maenas*; Metals; Sediment toxicity

1. Introduction

Recently the biomarker approach has been incorporated into several pollution monitoring programs in Europe and the USA. Likewise, different methods for biological effect 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, 1993). The United Nations Environment Programme has founded a biomonitoring programme in the Mediterranean Sea including a variety of biomarkers (Suter, 1993). Recently biomarkers have also been included in the Joint Monitoring Programme of the OSPAR convention where 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 yet. The current guidelines for the control of these activities are based on the several approaches which take into account chemical measurements, analysis of benthic communities and toxicity tests (Acosta and Lodeiros, 2004). Very few have already been studied about the utility of the use of biomarkers. Most of the regulation agencies comment the validation of them for this propose and their effectiveness in the new guidelines. Nevertheless, there are still some aspects that need to be defined before recommending them widely such as the complete understanding of the kinetic of these biological indicators of toxicity.

The use of biomarkers in the evaluation of the toxicity of dredged material is under continuous development and improvements are needed. However, significant contribution for the use of biomarkers could provide in the different programs, which involve characterization of dredged material and sediment quality (Martín-Díaz et al., 2004).

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The biomarker based in the measurement of metallothionein concentration in biological tissues, represents a detoxification function for structures, which have been reversible impaired by inappropriate metal binding. The role of metallothioneins is sequestering metals is well established, while their induction by exposure to a wide variety of metals (e.g.: Cd, Cu, Zn, Hg, Ni, Bi and Ag) is associated with their protective function (Stegeman et al., 1992; Rodríguez de la Rua et al., 2005). Therefore, they are early warning tools for the assessment of sediments contaminated by metals. At the present work, the potential use of the induction of metallothionein-like-proteins synthesis in the female crab *Carcinus maenas* as a biomarker of exposure to contaminated dredged material is studied. To reach this aim, and fully understand the biological function of this biomarker, a kinetic approach is proposed.

2. Materials and methods

2.1. Selection of organisms

Intermoult female *C. maenas* carapace width of 50–55 mm were purchased from an aquaculture farm (SW, Spain) placed in an uncontaminated site. To exclude variability, all animals used in this study were of the same size, length and moult stage and were collected on the same day from a single location. Only female *C. maenas* on the intermoult stage were used for the development of this assay in order to avoid variability of responses due to gender. Crabs were acclimatized before assay for 1 month.

2.2. Sediment sample collection

Surface sediment samples (5–10 cm) were collected at three Spanish ports: Ría de Huelva (SW, Spain), La Coruña (NW, Spain) and Bahía de Cádiz (SW, Spain) with a 0.025 m^2 Van Veen grab (Fig. 1). Samples were transported to the laboratory and subsampled for physical–chemical and toxicological characterization. Then sediment samples were sieved through a 0.5 μm mesh into a tank

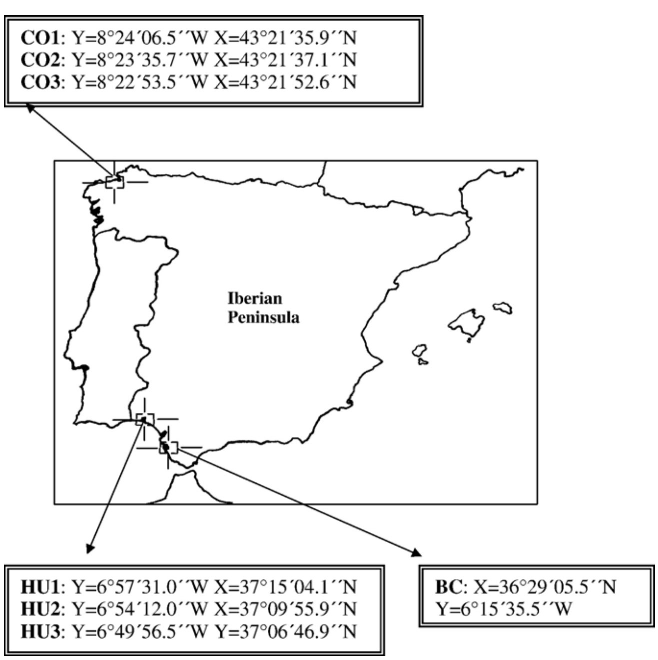

Fig. 1. Map of the studied areas and location of sampling sites (BC#: Port of Cadiz; HU#: Port of Ría de Huelva; CO#: Port of La Coruña).

in order to remove any associated macrofauna and larger sediment particle. Samples were kept at 4 °C in dark until they were used in toxicity testing. Sediments were stored at 4 °C no more than 2 weeks prior to the toxicity test.

2.3. Toxicity tests

Intermoult female *C. maenas* were exposed to the sediments from the ports of Ría de Huelva (SW, Spain) (HU1, HU2, HU3), La Coruña (NW, Spain) (CO1, CO2, CO3) and the negative toxicity control Bahía de Cádiz (SW, Spain) (BC) (Fig. 1) during 21 days. Each sediment was tested per replicate 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 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. Crabs were fed frozen mussels, always prior to water change (filtered seawater).

2.4. Chemical analysis of sediments

Analysis of sediment was performed according to Spanish recommendations for dredged material (CEDEX, 1994). Metal content in the different sediments was determined in microwave following methods reported by Riba et al. (2002), PCBs (congeners #28, 52. 101, 118, 138, 153 and 180) and PAHs content were 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 allow agreement with certified values higher than 90%.

2.5. Biomarker determination

On days 0, 7 and 21 individuals were sampled, dissected to obtain the hepatopancreas tissues in order to determine metallothionein-like-protein concentrations. In the present assay, the hepatopancreas tissues for the determination of this biomarker were chosen. This tissue plays a very important role in metal metabolism and it is considered as a long-term storage tissue reflecting persisting contamination (Duquesne and Coll, 1995). After dissection, hepatopancreas from crabs were kept at −80 °C prior to homogenization. The samples were homogenized in Tris-acetate buffer (25 mM, pH 7.6, containing 250 mM sucrose and 10 mM dithiothreitol 1:10 weight:volume, using a Teflon pestle and keeping the sample on ice at 2–4 °C following the procedure developed by de Lafontaine et al. (2000). Aliquots of this homogenate were sampled for total protein (Bradford, 1976) and MTLPs determination.

2.6. Metallothionein-like-protein concentration (MTLP)

The supernatant (0.1 mL) was added to 0.9 mL of NaCl (0.9%), heated to 95 °C for 4 min, and centrifuged at 10,000 *g* for 15 min at 4 °C. Supernatant was stored at − 80 °C prior to MTLPs determination. Metallothioneins were analysed by the differential pulse polarographic assay based on — SH group quantification (Olafson and Olsson, 1987) have tested its specificity. Purified rabbit metallothionein (Sigma–Aldrich) was used as standard. MTLP concentrations were expressed as μg MTLP/mg total protein. Polarographic determination in heat-denatured cytosol is an analytical procedure based on several characteristics of metallothioneins, but it does not allow the assertion that the studied molecule is a true metallothionein since purification and sequencing are carried out. Thus, the terminology of metallothionein-like-protein (MTLP) is preferable.

2.7. Kinetic and statistical approach

A study of the kinetic of MTLPs concentration in *C. maenas* hepatopancreas was performed in order to determine the reaction of this protein as well as the rate of MTLPs increase. This kinetic study, could allow the establishment of kinetic considerations, related to the effects due to the exposure of the female crabs to metals and organic compounds in the sediment.

The increase of MTLP concentrations in hepatopancreas from days 0 to 21 of individuals exposed to contaminated sediments (HU1, HU2, HU3, CO1, CO2, CO3) and negative toxicity control (BC) were described by a Lineal kinetic approach. Metallothionein-like-protein concentrations were expressed as μg of protein per mg total proteins. A toxicokinetic approach was fitted with MTLPs results as follows:

$$\mathrm{Ln[MTLP]} - \mathrm{Ln}C_0 = K_t \text{ or } \mathrm{Ln[MTLP]} = \mathrm{Ln}C_0 + K_t \quad \text{(a)}$$

Where, C_0, is the concentration of MTLP in the hepatopancreas at $t=0$; [MTLP], is the concentration of metallothionein-like-proteins in the hepatopancreas at time t minus the metallothionein concentration at the initial time (0); K is constant rate of increase (MTLP concentration per day); and t is the time expressed in days. The data obtained of MTLP concentrations from crabs exposed to dredged material, as well as the constant rate of increase, K obtained in the Lineal Kinetic equation, were compared to those determined in control specimens by use of one-way ANOVA followed by a multiple comparison of Dunnett's tests.

A multivariate analysis approach (MAA) was also performed in order to determine the impact of the contamination on the different treatments studied. A MAA (Factor Analysis using the Principal Component Analysis (PCA) extraction procedure) was applied to the original set of variables. The Factor analysis was performed on the correlation matrix; i.e., the variables were auto-scaled (standardized) so as to be treated with equal importance (DelValls and Chapman, 1998). All analysis 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).

3. Results

3.1. Chemical characterization

Determination of metal and PCBs, are expressed as ratios (RAL1 and RAL2) between the concentrations determined in sediment samples and Action Levels applied in Spain for dredging material management actions (Table 1).

These ratios provide information about the potential biological effect of the dredging material. Risk (potential biological effect) associated to each concentration according to Action Level 1 is expressed as:

$$\mathrm{RAL1}(\chi)y = \frac{\mathrm{Cm}-\mathrm{AL1}}{\mathrm{AL1}} * 100$$

and Risk associated to each concentration according to Action Level 2:

$$\mathrm{RAL2}(\chi)y = \frac{\mathrm{Cm}-\mathrm{AL2}}{\mathrm{AL2}} * 100$$

Where RAL(X)y is the risk associated to compound X in the station y, Cm is the measured concentration of X, and AL1, AL2 is the Action Level 1, 2 respectively applied for characterization of dredged material

Table 1
Provisional Action Levels used for the dredged material classification of the Spanish ports

Contaminant	Action Level 1	Action Level 2
Hg	0.6	3.0
Cd	1.0	5.0
Pb	120	600
Cu	100	400
Zn	500	3000
Cr	200	1000
As	80	200
Ni	100	400
∑7PCBs*	0.03	0.1

(CEDEX, 1994) (* Some of the fellows IUPAC number 28, 52, 101, 118, 138, 153 and 180). These concentrations are expressed in mg·kg^{-1} (dry weight) and are related to the fine fraction of the sediment (diameter < 63 mm).

Table 2
Chemical characterization for each sample

	BC	HU1	HU2	HU3	CO1	CO2	CO3
Hg							
RAL1	−96	**286.7**	**480**	**145**	**968.3**	−22	−10.5
RAL2	−99.2	−23	**16**	−51	11.4	−8.4	−8.2
Cd							
RAL1	−96.8	**176**	**582**	−3.8	−3.8	−48.7	−74.9
RAL2	−99.4	−64.8	**16.4**	−80.8	−80.8	−89.7	−95
Pb							
RAL1	−96.2	**253**	**260.7**	**124.8**	**116.3**	−31.4	−54.9
RAL2	−99.2	−29.4	−27.9	−55	−56.7	−86.3	−91
Cu							
RAL1	−98.7	**1912**	**2338**	**878.8**	**109**	−46.9	−64.7
RAL2	−99.7	**403**	**509.5**	**144.7**	−47.7	−86.7	−91.2
As							
RAL1	−97.7	**329.1**	**558.6**	**165.9**	**65.7**	−71.9	−83
RAL2	−99	**71.6**	**75.6**	**6.3**	**8.3**	−88.7	−93.2
Cr							
RAL1	−98.3	−67.6	−63.2	−78.2	−85.7	−84.3	−83.3
RAL2	−99.7	−93.5	−92.6	−95.6	−97.1	−96.9	−96.7
Ni							
RAL1	−98.3	−66.9	−62.8	−78.6	−80.1	−80	−80.8
RAL2	−99.6	−91.7	−90.7	−94.6	−95	−95	−95.2
Zn							
RAL1	−98.7	**410.2**	**439**	**162**	**2.6**	−61.7	−73
RAL2	−99.8	−15	−10.2	−56.3	−82.9	−93.6	−95.5
PCBs							
RAL1	−98.8	−88.7	−66.7	−94	**748**	**96**	**34.7**
RAL2	−99.6	−96.6	−90	−98.2	**154.4**	−41.2	−59.6
Total PAHs (mg kg^{-1})	N.D.	< 0.12	1.29	< 0.13	7.38	7.07	3.2

(BC#: Port of Cadiz; HU#: Port of Ría de Huelva; CO#: Port of La Coruña). N.D.: not detected. Positive RALs are bold.

from Spanish ports. Determinations of PAHs are expressed as mg kg^{-1} due to absence of Action Level in Spanish recommendations.

Results obtained from the chemical characterization are described in Table 2s. RAL1 and RAL2 values equal or bigger than 0 are consider having high potential biological effect according to the corresponding Action Levels. On the other hand, RAL1 and RAL2 under 0 are not considered to have any associated risk according to the corresponding Action Levels.

The highest values obtained for most of the heavy metal determined are found in dredged material belonging to the port of Ría de Huelva, except for Hg whose values were significantly important in CO1. On the other hand, the port of La Coruña could be characterized as PCBs and PAHs contaminated port in its sediments.

3.2. Biomarker response

Summarized results of MTLP concentrations in the hepatopancreas of female *C. maenas* exposed to contaminated sediments (HU1, HU2, HU3, CO1, CO2, CO3 and BC), over time are shown in Fig. 2. It could be observed the experimental MTLP concentration fitted to a Lineal Kinetic Equation. In all the treatments, it was observed an increase in MTLP concentration along the 21 days of exposure.

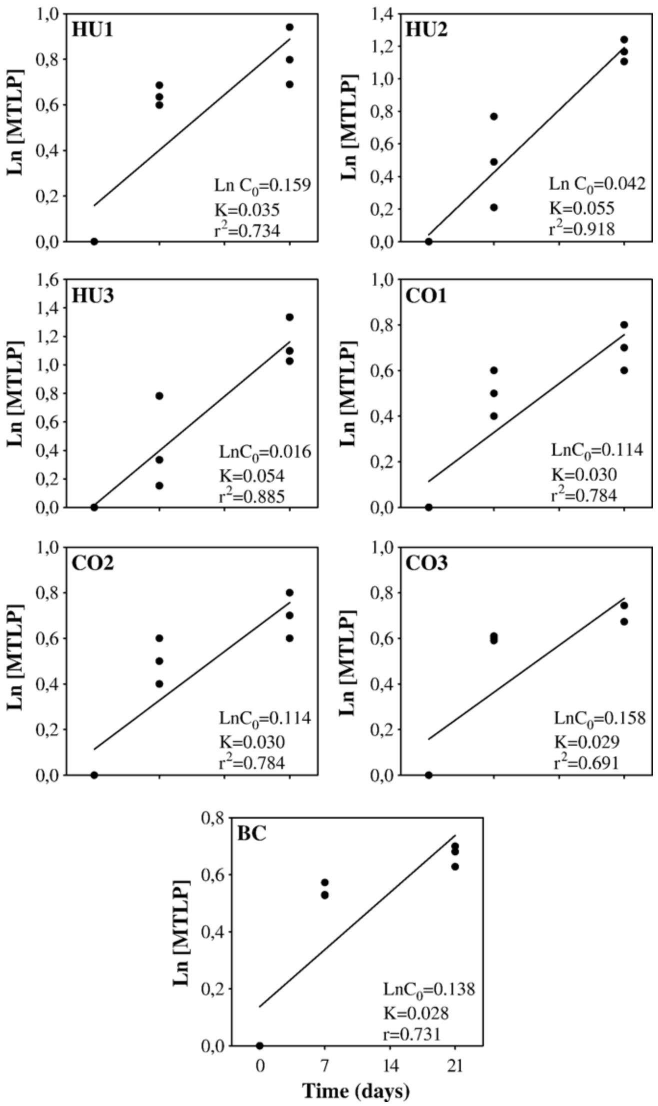

Fig. 2. Ln of metallothionein-like-protein (MTLP) ($\mu g \cdot mg^{-1}$) concentration in hepatopancreas of *C. maenas* over time. In the graph are shown Lineal Kinetic Equation fitted parameters from the approximation of the experimental MTLP data ($[MTLP]_t - C_0$).

The fitted results show a good correlation between the experimental data and the predicted by the expression [*a*] (Fig. 2). It confirms a good approximation of MTLP induction to a Lineal Kinetic model. The best approximation to a Lineal Kinetic model was determined in individuals exposed to sediment from HU2 (r^2=0.918), followed by that determined in sediment from HU3 (r^2=0.885), CO1 (r^2=0.784), HU1 (r^2=0.734), BC (r^2=0.731) and CO3 (r^2=0.691) (Fig. 2).

Concerning to the constant rate of MTLP increase (K), differences were observed between control and contaminated sediment exposed individuals. Thus, the highest K was determined in individuals exposed to sediment from HU2 (K=0.055) (p<0.01), followed by those exposed to sediment from HU3 (K=0.054) (p<0.01), HU1 (K=0.035) (p<0.01), CO1 (K=0.030), CO2 (K=0.030), CO3 (K=0.029) and BC (K=0.029) (Fig. 2).

For a better understanding of the relationship between chemical concentration in sediment and the kinetic response of the MTLP concentration in the crabs exposed (K=rate of increase of MTLP concentration) potential correlation among these variables was performed. A multivariate analysis approach (MAA) was applied to the chemical concentration in the sediments from the different ports to link with the biological adverse effect variable, the rate of increase of the concentration of MTLP (K) in female crabs exposed to sediments from three Spanish Ports in the laboratory. The MAA was performed using the set of data obtained for the 6 cases defined by the different sampling sites (HU1, HU2, HU3, CO1, CO2 and CO3) and 11 variables (As, Cd, Cr, Cu, Hg, Ni, Pb, Zn, ∑PCBs, ∑PAHs and K). In total we applied the MAA on 11 variables for 6 cases. The application of MAA indicated that the original variables could be described by three variables or factors (Table 3). These factors explained more than 94.28% of the original data set. The criteria selected to interpret a variable associated with a particular factor was a loading of 0.3 or higher; this approximates Comrey's (1973) cut-off of a 0.6 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 was described according to the dominant group of variables. Here are described the different components:

Factor 1. This component accounts for 72.31% of the total variance. This factor has positive loading on the concentration of As, Cd, Cr, Cu, Ni, Pb, and Zn, and K values. It shows a strong induction of MTLPs (K values) in individuals exposed to sediments containing these metals (Table 3).

Factor 2. This component, accounting for 13.42% of the total variance has positive loadings on the concentration of Hg, Pb and PAHs in the sediment. These positive loadings do not show any relationship with the induction of MTLPs (K values) in the hepatopancreas of *C. maenas*. Thus, it could be described as a factor informing about the no influence of these metals and organic content of sediments in the induction of MTLP synthesis (Table 3).

Factor 3. This component, accounting for 8.56% of the total variance shows a double description based in the difference between positive loadings (metals content associated with MTLP induction) and negative loadings (PCBs and PAHs content not associated with MTLP induction). This factor demonstrates (on its positive loadings) a prevalence of metal contamination (As, Cr, Cu, Pb and Zn) associated with the induction of MTLP (Table 3).

Table 3
Sorted rotated factor loadings (pattern) of 11 variables for the two principal factors resulting from the multivariate analysis of results obtained from *C. maenas*

Carcinus maenas	#1	#2	#3
%variance	72.307	13.418	8.558
K	0.585	–	0.507
As	0.911	–	0.404
Cd	0.947	–	–
Cr	0.909	–	0.392
Cu	0.879	–	0.455
Hg	–	0.991	–
Ni	0.944	–	–
Pb	0.715	0.453	0.504
Zn	0.834	–	0.507
PCBs	–	–	−0.861
PAHs	–	0.300	−0.903

Only loadings greater than 0.2 are shown in the table. Factors (#) are numbered consecutively from left to right in order of decreasing variance.

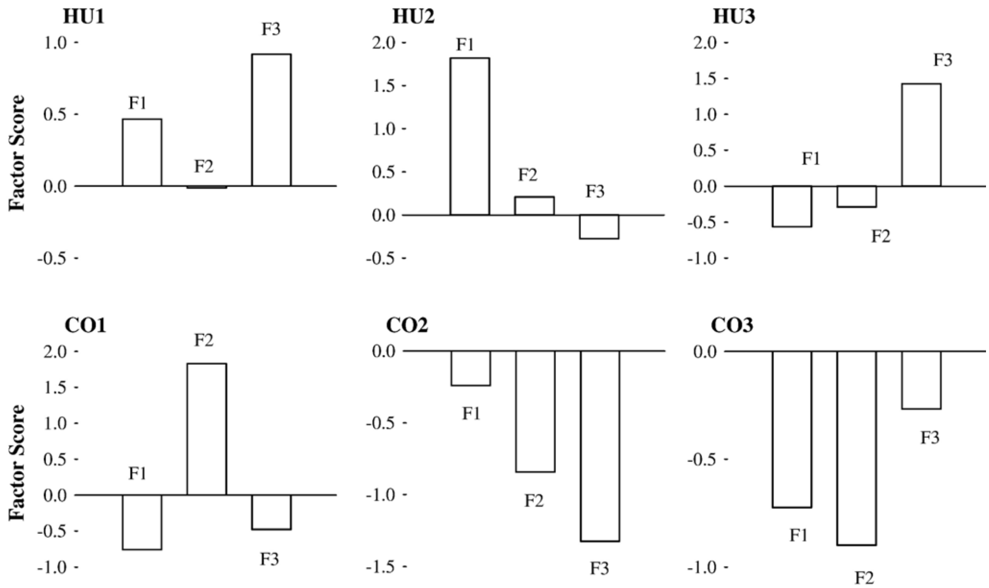

Fig. 3. Representation of Factor scores estimation for each of the 6 cases (HU1, HU2, HU3, CO1, CO2, CO3) evaluated using the crab *C. maenas* and after a multivariate analysis approach (MAA) that was applied to the chemical concentration in the sediments from the different ports to link with the biological adverse effect variable, the rate of increase of the concentration of MTLPs (*K*).

The values obtained in the analysis corresponding to factor 1 explains the induction of MTLP (constant rate of increase values) determined in individuals exposed to sediment from the port of Ría de Huelva (HU1, HU2) highly contaminated by As, Cd, Cr, Cu, Ni, Pb and Zn in sediment (Fig. 3). The factor 2 score (on its positive value) explains the absence of relationship between the presence of the metal Hg and Pb and the organic compound PAHs in the sediment and the induction of MTLP (constant rate of increase values) determined in individuals exposed to sediment from the port of La Coruña (CO1). The port of La Coruña is characterized by high concentrations of these compounds. In this sense, concerning to the metals Pb and Hg, they have widely been associated to the induction of metallothioneins (Stegeman et al., 1992). In this case, the absence of induction could be due to the high concentration of the organic compound PAHs, also present in the sediment from CO1. It has already been suggested by other authors that metallothioneins synthesis may be reduced in the presence of high levels of organic contaminants due to an increased demand for cysteine residues for Reduced Glutathione (GSH) synthesis which has important functions in detoxification of electrophilic compounds (Commandeur et al., 1995). Finally, the factor 3 score (on its positive value), explains the smooth induction of the constant rate of increase of MTLP in individuals exposed in the laboratory to sediments from the port of Ría de Huelva (Hu1 and Hu3), characterized by high concentrations of (As, Cr, Cu, Pb, and Zn) (Fig. 3). On the other hand, it shows (on its negative loadings) the presence of organic compounds, not associated with the induction of MTLP, and in this case, it describes the absence of sublethal effect provoked by PCBs and PAHs in the port of La Coruña (CO1, CO2, CO3) (Fig. 3).

4. Discussion

The results obtained from the comparison of the rate of increase of MTLPs (*K*), obtained from the lineal approximation of the concentration of MTLP induced in female crabs *C. maenas* over 21 days of exposure to contaminated sediments from the ports of Cádiz, Ría de Huelva, and La Coruña have demonstrated significant MTLP induction in organisms exposed to sediments from HU1 ($p<0.05$), HU2 ($p<0.01$) and HU3($p<0.01$) compared with the induction observed in individuals exposed to control sediments. Moreover, a direct relationship has been shown between the increase of *K* values and the exposure to As, Cd, Cr, Cu, Ni, Pb and Zn (HU1, HU2, HU3) and an indirect relationship between PCBs and PAHs exposure and metallothionein induction in the hepatopancreas of *C. maenas* (CO1, CO2, CO3).

As it was observed, MTLPs induction was associated to metal rich sediments. The levels of this biomarker have demonstrated to increase in a dose-response (George, 1989) or a time-response (Beyer et al., 1997) manner after administration of heavy metals (Narváez et al., 2005). The induction of this protein gene expression provided evidence that induction by metals is a direct response to increases in the intracellular metal concentration which is mediated through the action of metal binding regulatory factors (Thiele, 1992). The present study demonstrates the significant relationship between MTLPs induction over time and the concentration of metals in sediment in the laboratory. As a first approach, the use of this parameter (K=constant rate of increase of MTLPs), which integrates the kinetic characteristics of the different systems is shown, in certain circumstances, as a powerful and sensitive tool to evaluate bioavailability of metals in sediments. The feasibility of metallothioneins as biomarkers for metal exposure or metal-induced stress has been discussed by Stegeman et al. (1992). Given the extensive scientific information base and available methods for measuring changes in metallothionein synthesis and its metal composition (Van der Oost et al., 2003) and taking into account the results obtained from this first approach, these proteins might show a strong potential for use as biomarkers for ERA (Environmental Risk Assessment) of sediments highly contaminated by metals.

The results shown in this study, point out the feasibility to use this biomarker for metal bioavailability monitoring in dredged material using different sediment samples collected in three ports located in Spain. The results validate the use of this biomarker of exposure to assess the sediment quality and the potential use of it in the monitoring programmes for disposal of dredged material and besides, for characterization of sediments before being dredged. In this sense, this biomarker is proposed to be used as part of a tiered testing approach for dredged material characterization when metals are chemicals of concern in the port sediments. Nevertheless, it should be taken into account that this biomarker and toxicokinetic approach, showed in this paper, are presented as an initial estimation, and more research in this direction should be performed.

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References

Acosta V, Lodeiros C. Heavy metals in the clam *Tivela mactroides* Born, 1778 (Bivalvia: Veneridae) from coastal localities with different degrees of contamination in Venezuela. Cienc Mar 2004;30:323–33.

Beyer J, Sandvik M, Skaare JU, Egaas E, Hylland K, WaagbØ R, et al. Time- and dose-dependent biomarker responses in flounder (*Platichthys flesus* L.) exposed to benzo[*a*]pyrene, 2,3.3′,4,4′,5-hexachlorobiphenyl (PCB-156) and cadmium. Biomarkers 1997;2:35–44.

Bradford MB. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.

CEDEX (Centro de Estudios y Experimentación de Obras Públicas). Recomendaciones para la gestión del material de dragado en los puertos Españoles. Madrid: Puertos del Estado; 1994.

Commandeur JNM, Stijntjes GJ, Vermeulen NPE. Enzymes and transport systems involved in the formation and disposition of glutathione S-conjugates. Role in bioactivation and detoxification mechanisms of xenobiotics. Pharm Rev 1995;47:271–330.

Comreys AL. A first course in factor analysis. New York, USA: Academic Press; 1973.

DelValls TA, Chapman PM. 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 1998;24:3313–36.

Duquesne SJ, Coll JC. Metal accumulation in the clam *Tridacna corcea* under natural and experimental conditions. Aquat Toxicol 1995;32:239–53.

Frane J, Jenrich R, Sampson P. Factor analysis. In: Dixon WJ, editor. BMDP Statistical Software. Berkeley, CA, USA: University of California Press; 1985. p. 480–500.

George SG. Cadmium effects on plaice liver xenobiotic and metal detoxification systems: dose response. Aquat Toxicol 1989;15:303–10.

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

Martín-Díaz ML, Blasco J, Sales D, DelValls TA. Biomarkers as tools to assess sediment quality. Laboratory and field surveys. Trac 2004;23:807–18.

Narváez N, Lodeiros C, Nusetti O, Lemus M, Maeda-Martinez AN. Uptake, depuration and effect of cadmium on the green mussel *Perna viridis* (L. 1758) (Mollusca: Bivalvia). Cienc Mar 2005;31:91–102.

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

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

Rodríguez de la Rua A, Arellano ML, González de Canales ML, Blasco J, Sarasquete C. Accumulation of copper and histopathological alterations in the oyster *Crassostrea angulata*. Cienc Mar 2005;31:455–66.

Stegeman JJ, Brouwer M, Richard TDG, Förlin L, Fowler BA, Sanders BM, et al. Molecular responses to environmental contamination: enzyme and protein systems as indicators of chemical exposure and effect. In: Huggett RJ, Kimerly RA, Mehrle Jr PM, Bergman HL, editors. Biomarkers: biochemical, physiological and histological markers of anthropogenic stress. Chelsea, MI, USA: Lewis Publishers; 1992. p. 235–335.

Suter GW. Ecological Risk Assessment. Boca Raton, FL, USA: Lewis Publishers; 1993. p. 538.

Thiele DJ. Metal-regulated transcription in eukaryotes. Nucl Acids Res 1992;20:1183–8.

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

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