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Toxicokinetic approach for the assessment of endocrine disruption effects of contaminated dredged material using female *Carcinus maenas*

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Abstract Ecotoxicological effects associated with contaminants present in dredged material from three Spanish ports, Ría of Huelva (SW, Spain), La Coruña (NW, Spain) and Bay of Cádiz (SW, Spain) were determined using a marine biotest based on endocrine disruption effects (vitellogenesis process alteration). Intermoult female *Carcinus maenas* were exposed in the laboratory to sediments from the Spanish ports per replicate during 21 days. Crab haemolymph samples were taken for vitellogenin/vitellin (VTG) analysis on days 0, 7, 14 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 induction ($P < 0.05$) in vitellogenin/vitellin concentration was observed over time in individuals exposed to sediment samples containing significant concentrations of PAHs, PCBs, Hg, Pb and Zn. The toxicokinetic approach, proposed in this study related to the use of this biomarker in *C. maenas*, proved to be a powerful and sensitive tool to evaluate toxicity effects associated with contaminants present in dredged material. Moreover the integration of the results obtained through multivariate analysis approach (MAA) allowed the identification contaminants bound to sediments associated with adverse effects, validating the use of this marine biotest in a regulatory framework.

Keywords Vitellogenin · Biomarkers · Sediment contamination · Sediment toxicity · Sublethal endpoints

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Introduction

The disposal and relocation of dredged material and sediments in estuaries and marine ecosystems mostly occurs due to maintenance dredging operations on coastal harbours and waterways. Depending on their origin, the sediments and dredged material may be highly contaminated by various chemicals. There is an overwhelming amount of evidence that demonstrates that chemicals in sediments are responsible for toxicological and adverse effects such as histopathological lesions (Martín-Díaz et al. 2008) and biochemical alterations (Martín-Díaz et al. 2007). In order to avoid these adverse effects, the disposal of dredged material is controlled in areas that belong to the OSPAR and Helsinki Conventions. As described by Nendza (2002), these Conventions recommend marine sediment biotest methods which include established and less widely used techniques for acute toxicity, long-term toxicity, bioaccumulation, endocrine effects, toxic effects on reproduction, carcinogenicity and mutagenicity; covering laboratory tests, mesocosm studies, biomarkers and

field tests. Nevertheless, not all countries signed to these Conventions have adopted these recommended marine sediment biotest methods as part of their sediment assessment regulatory framework. This is the case for Spanish legislation with respect to characterization and management of dredged material, where bioassays are not required to supplement chemical analysis of areas of concern. The information obtained from physical–chemical characterization of the dredged material alone is not usually sufficient to carry out a suitable assessment of its toxicity on the biological community. Bioassays are therefore required as an additional tool (PIANC in press). The potential advantages of the inclusion of marine sediment biotests methods have been listed by several authors and are mainly based on the possibility of providing information not only on the contaminants present in the sediment but also with regard to their bioavailability and toxicity. In this sense, Rodríguez-Obeso et al. (2007) described different marine sediment biotest methods, mainly based in the study of short-term effects, for the characterization and management of dredged material in Spanish ports. Additionally, Martín-Díaz et al. (2004a) proposed distinct long-term toxicity tests, in the laboratory and in the field, applying a sensitive set of biomarkers as a suitable and responsive tool, applied using a range of bioindicator species (crustaceans and bivalves) to characterize dredged material toxicity.

Efforts to determine sediment toxicity, using sensitive and ecological relevant biomarkers in sentinel species have focused on the evaluation of the effect of certain compounds in the endocrine system. Vitellogenesis inhibiting hormone (VIH) and methyl farnesoate, for example which play and important role in regulating molting, metamorphosis and vitellogenesis in crustaceans, have been used as biomarkers (Oberdörster and Cheek 2001). Several authors have remarked that there have been very few measures of endocrine disruption involving sediment exposure, bioavailability, or dietary uptake by organisms associated with sediment (Depledge and Billinghurst 2003) able to result in endocrine disruption in benthic species. One of the most frequently used sublethal endpoints in the detection of the effects mediated by estrogenic substances is the measurement of the induction of hormone-dependent protein synthesis. These are proteins which are synthesized under the control of specific hormones. Inhibition or stimulation of vitellogenin/vitellin (VTG) levels in haemolymph, a protein implicated in reproduction and synthesized under hormone system regulation, is able to provide a useful indicator of direct repercussions on the reproductive capacity in female crabs *Carcinus maenas* (Martín-Díaz et al. 2004b). The induction of the synthesis of VTG due to a range of organic compounds and metal exposure in many Phyla is well documented (Nenzda 2002). Nevertheless, its potential use as a biomarker in the evaluation of toxicity in the environment is at an experimental stage, especially in marine crustaceans and bivalves. Marine sediment biotest methods which evaluate endocrine effects are mainly based on the determination of vitellogenin (VTG) synthesis in juvenile male fish. Nevertheless, synthesis of VTG has not been observed in male crustaceans following exposure to a range of contaminants. Despite crustaceans being one of the most ubiquitous groups of invertebrates, inhabiting all types of aquatic habitats and hence representing a suitable dredged material toxicity analysis bioindicator species, no recommended sediment biotest based on VTG synthesis in these species has been included by the OSPAR and Helsinki Conventions parties. Nevertheless, the study of the VTG synthesis (over time) in female *C. maenas* (Martín-Díaz et al. 2005), after exposure to environmental metal concentrations under laboratory conditions, has been shown to be a suitable biomarker in assessing endocrine effects.

In the present work, the potential use of the induction of vitellogenin/vitellin synthesis in the female crab *Carcinus maenas* as a biomarker of exposure to endocrine disrupter chemicals bound to sediments is studied. In order to meet this objective, and taking into consideration that VTG is naturally synthesized in female crabs, with its concentration changing over time, two important points have been taken into account to reduce the variability of this biomarker: (a) the use of a control test in parallel with individuals exposed to the same physicochemical conditions and to a characterized negative toxicity control sediment and b) the performance of a kinetic approach of the biomarker responses.

Material and methods

Sampling and preparation of sediments

Sediments from the port of Cádiz (BC1) (negative toxicity control), widely studied (DelValls et al. 1998) and characterized by the absence of significant contamination; Ría de Huelva (Hu1, Hu2, Hu3) and La Coruña (Co1, Co2, Co3) were collected with a 0.025 m^2 Van Veen grab and transferred to the cooler. The contents of the cooler were homogenized with a Teflon spoon until no color or textural differences could be detected. 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 they were used in toxicity testing, no more than two weeks after sediment tests.

Chemical analysis

Sediment characterization was performed according to Spanish guidelines for dredged material (CEDEX 1994). Dredged material subsamples were chemically analyzed. Dried sediments were gently homogenized after being dried at 60°C and the distinct chemicals determined. The heavy metal concentrations of As, Cd, Cr, Cu, Hg, Ni, Pb and Zn were determined through atomic absorption spectrophotometry and graphite furnace atomic absorption spectrophotometry depending on the heavy metal concentration in each station. Results are expressed as mg g^{-1} dry sediment. Mercury was determined by cold vapor technique and Arsenic by hydride generation. PAHs and PCBs were analyzed using a gas chromatography equipped with electron capture (Riba et al. 2002). 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) registering agreement with certified values in excess of 90%.

Sediment bioassay

Mature intermoult female *Carcinus maenas* ($n = 8$) were exposed to sediments from the different Spanish ports during 21 days per duplicate. Ovarian maturation in crustaceans includes a color change and an enlargement in size of the ovaries as the oocytes proliferate and increase in diameter during yolk incorporation (Charniaux-Cotton and Payen 1988). In order to determine the ovarian maturation stage, a sample of ovarian tissue was examined under a light microscope to estimate the oocyte diameter via ocular measurements. Only organisms in ovarian stage VI were used for this experiment. Sediments were tested per replicate in 20 l glass aquaria. The tests were carried out in whole sediment using a 1:4 v/v sediment water (filtered sea water) relation, containing a layer of 6 cm and with constant aeration. The temperature (15 ± 1°C), pH (7.8–8.2), salinity (33.8 p.s.u. ± 0.3) and dissolved oxygen (>5 mg l^{-1}, 60% saturation) were measured and controlled daily. Individuals used for the present study were intermoult female *Carcinus maenas*, having undergone a period of acclimatization of a month. Five individuals were placed in each tank and fed every 3 days, prior to water change (filtered seawater).

Haemolymph samples (200 µl) were collected on days 0, 7, 14, 21 in the female crabs and introduced in liquid nitrogen, prior to storage at −80°C in order to determine vitelloegenin/vitellin concentration.

In order to perform a useful kinetic toxicity test based on VTG as a biomarker in the female crab *Carcinus maenas*, using field samples and taking into account the temporal VTG concentration variability in the female crabs, the following factors were controlled: (i) female shore crabs at the intermoult period and at the same ovarian stage were used, and (ii) a negative toxicity control was run in parallel.

Biomarker determination

Vitellogenin/vitellin (VTG) was measured in the haemolymph of intermoult female *Carcinus maenas* using an indirect enzyme-linked immunosorbent assay (ELISA) adapted from Pateraki and Stratakis (1997). VTG concentration was identified using a polyclonal antibody raised in rabbits against purified *Carcinus maenas* VTG and made monospecific by the addition of male haemolymph (Tuberty 1998).

The sample of interest (100 µl) and a serial dilution of vitellin (standard curve with values 0, 2, 10, 20, 50, 75 and 100 ng · 100 μl^{-1}) in dilution buffer were allowed to bind to the well of the microtitre plate, either overnight at 4°C or at room temperature for 2 h (all samples were run in triplicate). Excess protein in the well was removed with washing (PBS-T). Any sites on the wall of the plate well not covered by protein were then blocked using a general "bland" protein; in this case dried milk powder was reconstituted. This stopped antibody binding direct to the polystyrene wall and giving false positives. After further washing the primary antibody was added to the wells and incubated for 1 h at 37°C. If vitellogenin/vitellin was present in the sample this primary antibody would bind with it. After 1 h the plate was again washed out removing antibody. Then the secondary antibody was added. This second antibody was a goat anti-rabbit monoclonal preparation which will bind with any antibody produced in a rabbit. This antibody was commercially supplied by Sigma. The plate was incubated for a further hour at room temperature, and washed out to remove out secondary antibody. Following this, ABTS (2,2′-Azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt was allowed to react at room temperature in the dark for 10–30 min. The color change was quantified by an automated plate reader at 405 nm and the VTG standards were fit to a linear regression. The standard curve was linear over the range of standards.

No non-specificity of the antibody to male haemolymph was found. Vitellogenin/vitellin concentrations were expressed as µg of protein per ml of haemolymph.

Kinetic and statistical approach

As various authors have pointed out (Bouskill et al. 2006), a continuous series of biomarker responses over time rather than a single "snap-shot" to rationalize naturally synthesized biomarker versus other effects of toxic contaminants in the environment should be performed.

The increase of haemolymph VTG protein concentration from days 0 to 21 of individuals exposed to contaminated sediments (BC1, Hu1, Hu2, Hu3, Co1, Co2 and Co3) could be described by a Lineal kinetic approach. Vitellogenin/vitellin concentrations were expressed as μg of protein per ml of haemolymph. A toxicokinetic approach was fitted with VTG results:

$$\mathrm{Ln[VTG]} - \mathrm{Ln}\ C_0 = \mathrm{Kt} \quad \text{or} \quad \mathrm{Ln[VTG]} = \mathrm{Ln}\ C_0 + \mathrm{Kt} \tag{a}$$

where, C_0, is the concentration of VTG in the haemolymph at $T = 0$; [VTG], is the concentration of vitellogenin/vitellin in the haemolymph at time t minus the vitellogenin/vitellin concentration on $t = 0$; K is constant rate of increase (vitellogenin/vitellin concentration per day); and t is the time (days).

The data obtained of vitellogenin/vitellin concentrations from females 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 the SPSS/PC+ statistical package. Significant differences between individuals exposed to control sediment and individuals exposed to contaminated ones were determined using a one-way ANOVA followed by a multiple comparison of Dunetts tests. The significance level was set at $P < 0.05$.

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 et al. 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).

Results

Chemical characterization

Results obtained from the chemical characterization are described in Table 1. Determination of metals 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.

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:

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

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

$$\mathrm{RAL1}(\chi)\mathrm{y} = \frac{\mathrm{Cm} - \mathrm{AL2}}{\mathrm{AL2}} * 100$$

where RAL(χ)y is the risk associated to compound X in the station y, Cm is the measured concentration of X, AL1, AL2 is the action level 1, 2 respectively applied for characterization of dredged material from Spanish ports. Determinations of PAHs are expressed as mg kg^{-1} due to absence of Action Level in Spanish recommendations.

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. In Table 2 are shown the Action Levels that correspond to each compound.

The highest values obtained of most heavy metals are found in dredged material from the Ría de Huelva port, with the exception of Hg whose highest values were at Co1. Conversely, the port of La Coruña was characterized as a PCB and PAH contaminated port (Fig 1).

Biomarker response

Summarized results of VTG concentration in the haemolymph of female *Carcinus maenas* exposed to control (Ca1) and contaminated sediments (Hu1, Hu2, Hu3, Co1, Co2 and Co3), over time is shown in Fig. 2. In this figure, the experimental VTG concentration fitted to a Lineal Kinetic Equation can be observed. In all treatments, an increase in VTG concentration over the 21 days of exposure was observed.

The fitted results correlate well with the experimental data as well as data from predictions derived from expression [a] (Fig. 2). A good approximation of VTG induction to a Lineal Kinetic model for most of the treatments is confirmed. Nevertheless, for Co1 and Co3, there appears to be an initial rise to day 7, then a levelling off or even small reduction to day 21. Concerning the constant rate of VTG increase (K), differences were observed between control and contaminated sediments exposed individuals. Thus, the highest K was determined in individuals exposed to sediment from Hu2 ($K = 0.126$) ($P < 0.05$), followed by those exposed to sediment from Co1 ($K = 0.125$) ($P < 0.05$), Hu3 ($K = 0.115$) ($P < 0.05$), Co3 ($K = 0.095$), Hu1 ($K = 0.074$), and Co2 ($K = 0.020$). The lowest value was found for the control treatment BC1 ($K = 0.011$).

For a better understanding of the relationship between chemical concentration in sediment and the kinetic response

Table 1 Chemical characterization for each sample

	BC1	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; H#: Port of Ría de Huelva; CO#: Port of La Coruña. N.D.: not detected. Positive RALs are bold characters

Table 2 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
Σ7PCBs[a]	0.03	0.1

[a] 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)

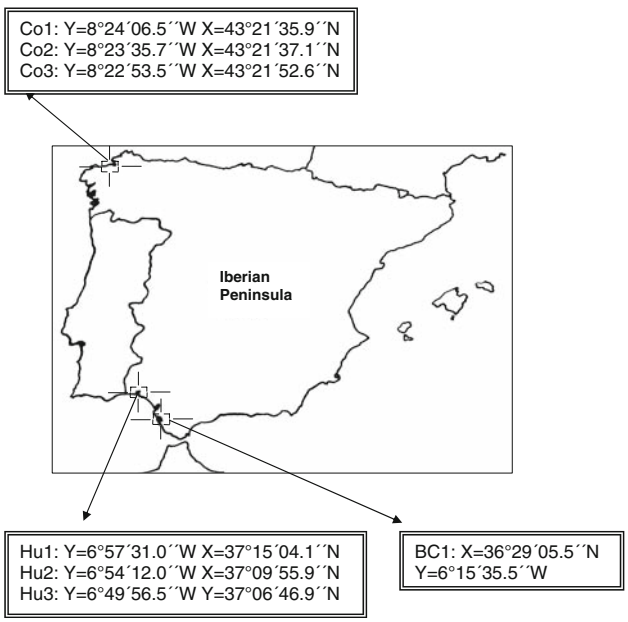

Fig. 1 Map of the studied areas and location of sampling points

of the VTG concentration in the crabs exposed (K = rate of increase of VTG concentration) potential correlation among these variables was analysed. 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 VTG (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, Co3) and 11 variables (As, Cd, Cr, Cu, Hg, Ni, Pb, Zn, ΣPCBs and ΣPAHs and K). In total the MAA was applied to 11 variables for 6 cases. The application of MAA indicated that the original variables were able to be described by three variables or factors

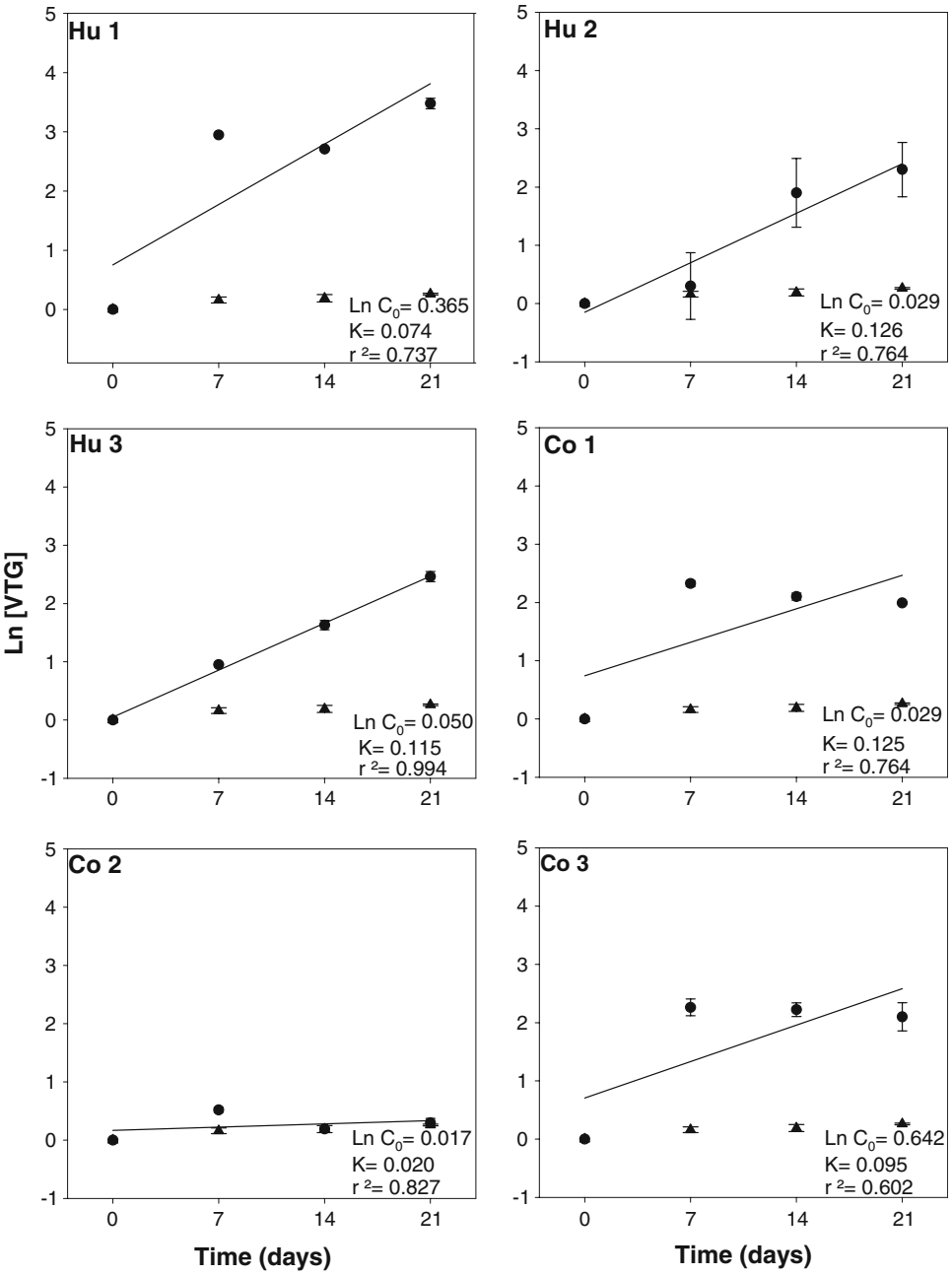

Fig. 2 Ln of the vitellogenin/vitellin (μg ml^{-1}) concentration in haemolymph of *Carcinus maenas* along the 21 days of exposure (0, 7, 14, 21) to contaminated (●) (Hu1, Hu2, Hu2, Co1, Co2, Co3) and control site (▲) (BC1). In the graph are shown Lineal Kinetic Equation fitted parameters from the approximation of the experimental VTG data ($[VTG]_t - C_0$)

(Table 3). These factors better described 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 Comreys (1973) cut-off of 0.4 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. The different components are as follows.

Factor 1 accounted for 64.13% of the total variance shows a double description based on the difference between positive loadings (metals content not associated with VTG induction) and negative loadings (PCBs and PAHs content with VTG induction). This factor demonstrates (its positive loadings) a prevalence of metal contamination (As, Cd, Cr, Cu, Ni, Pb, and Zn) not associated with the induction of VTG. In addition, it associates the induction of VTG over time with the concentration of organic chemicals in sediments (on its negative loadings) ΣPCBs and ΣPAHs.

Factor 2, which accounted for 19.96% of the total variance has positive loadings relating to the concentration of Pb and Zn and the constant rate of VTG increase. Under the bioassay conditions, it may be associated with an induction of VTG (K values) in individuals exposed to sediments containing Pb and Zn. Thus, it may be described

Table 3 Sorted rotated factor loadings (pattern) of 11 variables for the two principal factors resulting from the multivariate analysis of results obtained from *Carcinus maenas* only loadings greater than 0.4 are shown in the table

Carcinus maenas	#1	#2	#3
%Variance	64.13	19.96	13.11
K	−0.4	0.93	−0.5
As	0.97	–	–
Cd	0.89	–	–
Cr	0.99	–	–
Cu	0.98	–	–
Hg	–	–	−0.96
Ni	0.99	–	–
Pb	0.87	0.40	−0.60
Zn	0.96	0.42	–
PCBs	−0.63	–	–
PAHs	−0.46	–	−0.55

Factors (#) are numbered consecutively from left to right in order of decreasing variance

as a factor which provides information on the influence of metal content of sediments on the induction of VTG synthesis.

Factor 3 accounted for 13.11% of the total variance (a low loading with respect to this variance). This factor has negative loading in relation to the concentration of Cd, Pb, Hg, ΣPAHs and K values. It may display induction of VTG (K values) in individuals exposed to sediments containing Pb and Cd; and a strong induction of K values in organisms exposed to sediments containing Hg and ΣPAHs. In this sense, the negative prevalence of this factor may be related to an induction of VTG synthesis by the presence of metal and ΣPAHs in sediments.

The values obtained in the analysis corresponding to factor 1 (negative value), explains the induction of the constant rate of increase of VTG in individuals exposed in the laboratory to sediments from the port of La Coruña, characterized by high concentrations of PCBs and PAHs (Fig. 3). On the other hand, it shows (for positive loadings) the presence of metals not associated with the induction of VTG, in this case describing the absence of sublethal effects provoked by metal contamination (As, Cd, Cr, Cu, Ni, Pb, and Zn) in the port of Ría de Huelva (Hu1, Hu2) (Fig. 3). The factor 2 score (positive value) explains the induction of VTG (constant rate of increase values) determined in individuals exposed to sediment from the Ría de Huelva port (Hu3, Hu1) highly contaminated by Pb and Zn in sediment (Fig. 3). Finally, factor 3 score (negative value) is related to the induction of K values in the port of La Coruña, principally in Co1 (Fig. 3), characterized by high concentrations of Hg and PAHs (Table 1). It also relates the smooth effect of metals such as Cd and Pb in the induction of VTG in the Ría de Huelva port (Hu2).

Discussion

The results obtained from the comparison of the rate of increase of VTG (K), obtained from the lineal approximation of the concentration of VTG induced in female crabs *Carcinus maenas* over 21 days of exposure to contaminated sediments from the ports of Cádiz, Ría de Huelva, and La Coruña have demonstrated significant VTG induction in organisms exposed to sediments from Hu3, Hu2 and Co1 ($P < 0.05$) 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 exposure to PCBs and PAHs (Co1, Co2, Co3), exposure to Zn and Pb (Hu3 and Hu1) and exposure to Hg and PAHs (Co1) as well as Pb and Cd (Hu2). These results agree with the higher levels of

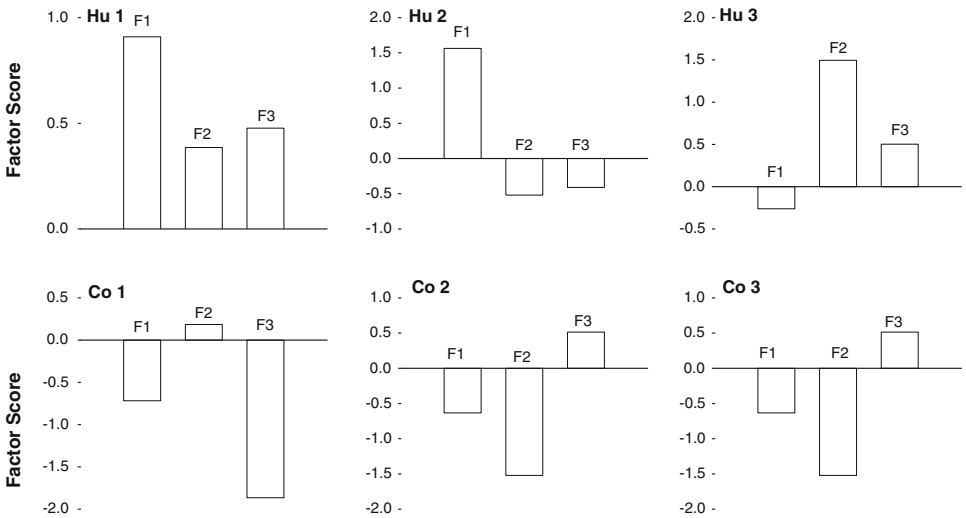

Fig. 3 Representation of Factor scores estimation for each of the 6 cases (Hu1, Hu2, Hu3, Co1, Co2, Co3) evaluated using the crab *Carcinus maenas*

vitellogenin, measured as organic labile phosphates, found in both haemolymph and gonad homogenates of the clam *Mya arenaria* and the mussels *Elliptio complanata*, related with chemical contamination of different sites (Blaise et al. 1999; Gagné et al. 2002; Blaise et al. 2003). These studies take into account the fact that this protein is synthesized during the intermoult period and that it is responsible for nutrient contributions and yolk development of the future egg. It may be affirmed that high VTG in females is therefore a warning of possible disruption to normal reproductive function (Kime et al. 1999). It has long been known that atretic eggs which do not mature normally can be reabsorbed and may result in high concentrations of VTG being carried through the haemolymph to the hepatopancreas for digestion (Lee and Walker 1995). Injury from chemical content exposure may have disrupted the normal development of the oocytes and induced this process of reuptake of yolk proteins leading to an increase in VTG concentration in haemolymph. Martín-Díaz et al. (2004, 2005, 2007) demonstrated the induction of vitellogenin/vitellin in *C. maenas* and *P. clarkii* after exposure to diluted environmental concentrations of Cd and Zn, as well as histopathological lesions in gonad (atretic oocytes). Naqvi and Howell (1993) corroborated these results when they found that individuals of *P. clarkii* exposed to cadmium had a lower eclosion percentage in new eggs, although the number of eggs was higher for those in the bioaccumulation of these metals. Vitellogenesis may also be affected by pollutants with known affinity for the estrogenic receptor, such as nonyphenol, bisphenol A, PCBs and PAHs (Van der Oost et al. 2003), this perhaps supporting findings of VTG induction due to PAHs and PCBs in the present work. Studies on the specific mechanisms by which the endocrine system are able to be disrupted are scarce, and this is a critical point to be addressed in order to fully understand the risk of known endocrine disrupting compounds (EDCs), as well as predicting the potential deleterious effects of pollutants whose disruptive effects on endocrine systems are not still evident (Rodríguez et al. 2006)

Prompted by several reports of endocrine disruption in fish, amphibians and aquatic invertebrates, the establishment of robust ecotoxicity test methods is a high priority for the international scientific and regulatory community. Keeping in mind that ecological risk assessment fundamentally depends on field monitoring and laboratory testing, there is a need to ensure that new OECD test guidelines incorporate priority endpoints (e.g. vitellogenin and gonad histology) which are also used in environmental monitoring programmes (Hutchinson and Pickford 2002). In line with this, the use of the present toxicity test for assessing endocrine effects associated with dredged material contamination, would involve the incorporation of new action levels related to Hg, PCBs and PAHs that do not reflect Spanish guidelines for dredged material, being characterized by lower concentrations than those described in these guidelines and associated with adverse effects. Such criteria would classify the sites Hu3 (due to Hg), Co1, Co2 and Co3 (due to PCBs and PAHs) with an associated risk corresponding to Action Level 2.

With respect to the kinetic approach performed in the present study, results suggest that: (i) VTG concentration in the haemolymph of female *Carcinus maenas* during the intermoult period increases over time, being able to be fitted to a Kinetic Lineal Equation and (ii) differences can be observed through the comparison of the constant rate of increase of VTG (K) between individuals exposed to contaminated sediments and control, and between contaminants and their concentrations. Therefore, in the present research work a study of the kinetic of VTG concentration in *Carcinus maenas* haemolymph was performed in order to determine the reaction of this protein as well as the rate of VTG increase. This kinetic study may allow the establishment of kinetic criteria, related to effects on vitellogenesis processes in mature intermoult female crabs due to the exposure of the female crabs to metals and organic compounds in the sediment.

Most of ecotoxicological studies performed to date regarding VTG as indicator of endocrine effects, are based in the measurement of this protein in juvenile male fish, where this protein synthesis has been induced due to the exposure to PCBs and PAHs. The advantage of the approach developed in this study is the determination of the synthesis of VTG in invertebrates, specifically, the intermoult female *C. maenas*, this proving to be a suitable bioindicator species for sediment assessment given its natural synthesis of VTG during the intermoult period.

In summary, a kinetic approach has been performed in order to analyze the differences in VTG synthesis between control and contaminated sediment exposed crabs over time. This approach avoids the prior existing disadvantage related to the natural synthesis of VTG by *C. maenas* females, and has allowed the determination of differences in VTG synthesis between organisms exposed to polluted and non-polluted sites. The parameter K, described herein and calculated for VTG induction has made it possible to analyze and compare the bioavailability of contaminants bound to sediments. The induction of VTG as biomarker of endocrine disruption proved to be a sensitive biomarker, with the information it provides being of great importance in assessing the response of the ecological status of the ecosystem, without being specific to any single group of contaminants. Hence it is able to be induced due to metals or organic compounds. Nevertheless, the integration of the results through a multivariate analysis (chemical and biological measurements) permitted the identification of

groups of contaminants bound to sediments, their bioavailability and their associated adverse effects in the endocrine system of *C. maenas*. Results obtained validate the use of this marine sediment biotest, supported by a solid statistical approach, in a regulatory framework for the assessment of dredged material and sediment toxicity. Moreover, it potentially offers a more sensitive assessment than that proposed by Spanish guidelines for dredged material characterization and management, producing endocrine disruption in exposed biota.

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