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Heavy metal bioavailability and effects: II. Histopathology–bioaccumulation relationships caused by mining activities in the Gulf of Cádiz (SW, Spain)

I. Riba [a,b], J. Blasco [b], N. Jiménez-Tenorio [a], M.L. González de Canales [c], T. Ángel DelValls [a,*]

[a] *Dpto. de Química Física, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, Polígono río San Pedro s/n, 11510 Puerto Real, Cádiz, Spain*

[b] *Instituto de Ciencias Marinas de Andalucía (CSIC), Campus Río San Pedro, s/n, 11510 Puerto Real, Cádiz, Spain*

[c] *Dpto. de Biología, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, Polígono río San Pedro s/n, 11510 Puerto Real, Cádiz, Spain*

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Abstract

The relationship between bioaccumulation of heavy metals (Zn, Cd, Pb and Cu) and histological lesions in different tissues of organisms is assessed in three different areas located in the southwest of Spain in the Gulf of Cádiz (Ría of Huelva, Guadalquivir estuary and Bay of Cádiz) affected and non-affected by mining activities. Data included in these relationships were obtained along the years 2000 and 2001 to address the impact of the Aznalcóllar mining spill on the Guadalquivir estuary. The bioaccumulation and the histological lesions measured in this seasonal study in the Gudalquivir estuary were linked to derive tissue quality guidelines (TQGs) by means of a multivariate analysis approach (MAA). Sediments collected in the same areas of study were used to expose organisms during the survey carried out in autumn 2001 and to address the relationship between bioaccumulation and histological lesions under laboratory conditions and related to chemicals bound to sediments. Lesions show that the organisms collected in the ría of Huelva and exposed to their sediments were severe, intermediate in the Guadalquivir estuary and absent in the Bay of Cádiz. Results show that the Guadalquivir estuary trends to recover its initial status quo previous to the mining spill. The link between chemical concentration and the lesions measured in the same tissues using MAA permits to derive tissue quality guidelines for two organisms, oysters (*Crassostrea angulata*) and clams (*Scrobicularia plana*) collected in the Guadalquivir estuary and associated with the heavy metals from the mining spill (Zn and Cd). The TQG values expressed as concentrations (mg kg^{-1}—dry weight) not associated with biological effects are for oysters, Zn, 8603, Cd, 3.42; and for clams Zn, 800, Cd, 2.6.

Keywords: Aznalcóllar mining spill; Tissue quality guidelines; Multivariate analysis; Estuaries; Doñana

1. Introduction

The relationship between concentration of contaminants in tissues and toxic effects measured in organisms

* Corresponding author. Tel.: +34-956-016044/6159; fax: +34-956-016040.
E-mail address: angel.valls@uca.es (T. Ángel DelValls).

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is receiving increased attention during the last decades (Chapman, 1997). These relationships have as a final aim to derive tissue quality guidelines (TQGs) defined as the concentrations of the chemicals measured in the different tissues that are associated or not with the biological effect measured, in a parallelism with the sediment quality guidelines (SQGs) widely used around the world (Riba et al., 2003a). To identify possible toxic agent(s), requires body burden data collected from the same organisms exhibiting toxicity in a weight of evidence evaluation. This toxicity should involve only sublethal measurements (endpoints), otherwise (lethal) the organism is not alive and the bioaccumulation data are not significant for this propose (Chapman, 1997). Coupling tissue residue level with sublethal toxicity responses such as histopathological diseases allows clear identification of possible causative agent(s) and could permit to predict effects of chronic and low-level exposures, especially when surveys are carried out both under field and laboratory conditions and then compared.

The environmental behaviour of heavy metals exhibits direct toxicity, although some of them can be regulated in the organism tissues to greater or lesser degrees. For instance, essential metals such as Cu and/or Zn can produce toxicity both by deficiency or excess of them in the tissues. Most of previous studies establish that metal Bioaccumulation and/or Bioconcentration factors, BAFs—uptake from water and diet—and BCFs—uptake from water only—respectively, are not precise or, even in some case, reliable. It is based on that they are difficult to measure properly and are highly variable (Environment Canada, 1994). However, metals are bioaccumulated through highly specific physiological uptake mechanisms which are generally not conductive to biomagnification and depend on the chemical form of the metal and the properties of the surrounding medium (especially salinity and pH in estuaries—Riba et al., 2003b).

All these complexity pattern that can eclipse the relationship between concentration of metals and toxic effects can be partly neglected if a comparison using the same organism and the same tissues under both field and laboratory conditions is conducted to relate concentration of metals and histological diseases. Using this approach the question Is bioaccumulation–toxicity relationship useful for predicting anthropogenic impacts?, can be addressed.

The main objective of this work is to determine the sublethal effects provoked by an acute event of contamination in the Guadalquivir estuary associated with a mining spill comparing the histopathological lesions to those measured in areas chronically affected by mining activities (ría of Huelva), and in areas with absence of contamination (Bay of Cádiz), both under field and laboratory conditions. Also, an objective of this study is to derive tissue quality guidelines (TQGs) by linking the set of data of metal residues reported by Riba et al. (this issue) and the sublethal effects described in this work. A multivariate analysis approach (MAA) as previously described by DelValls and Chapman (1998) to derive sediment quality guidelines (SQGs) is performed on chemical residues and histopathological lesions measured in the same tissues to derive tissue quality guidelines (TQGs) in the Guadalquivir estuary.

2. Material and methods

2.1. Approach

A detailed description of the surveys, samples and conditions of the bioassays is reported by Riba et al. (see Table 1 and Fig. 1) in this issue. Briefly, two different surveys were used in this study both under field and laboratory conditions. Two different species (estuarine clam *Ruditapes phillipinarum*, 10 days, and juveniles of *Solea senegalensis*, 30 days) were exposed to whole sediments collected in the field. Field surveys were carried out using different species that includes oysters (*Crassostrea angulata*), clams (*Scrobicularia plana*) and fish (*Liza ramada*). Field surveys in the Guadalquivir estuary were conducted during different seasons from years 2000 to 2001. The histological lesions measured in the species collected in the last survey (autumn 2001) were compared to those obtained in the field and in sediment-exposed organisms from different areas of the Gulf of Cádiz (contaminated, ría of Huelva, and uncontaminated, Bay of Cádiz) to establish the potential recovery of the estuary after the spill.

2.2. Histological analysis

Organisms from the field and the toxicity tests were analyzed to determine the histopathological lesions in different tissues. In Table 1 showed in the previous paper describing the bioaccumulation survey (Riba et al., this issue) are described the different tissues and lesions analyzed for each survey either at field and laboratory surveys.

All the organisms (collected in the field and laboratory-exposed) were anaesthetized with 0.05% ethyl-4-aminobenzoate (benzocaine) during 5–10 min; then weight, length and externally examined. All the tissues from all the organisms were obtained by dissection and then fixed in phosphate buffered 10% formaldehyde fixative 24 h and included in paraffin. The histological sections were stained with Haematoxylin–Eosin and Haematoxylin/VOF (Gutierrez, 1967). Sections were reviewed by light microscopy and photographed (Olympus CH20). Damage to the tissues of organisms was semi-quantified by detecting the frequency of the lesions in each detected alteration. The samples of tissues analyzed by scanning electronic microscopy were fixed in glutaraldehide buffered 1% using sodium cacodilated, 0.1 M (pH 7.2–7.4) during 4 h. Then, it was post-fixing by means of osmium tetraoxide buffered 2% in sodium cacodilated 0.2 M, for 2 h. After dehydratation of samples using growing acetone the critic point is derived and finally the samples are covered by gold.

2.3. Data calculation and statistical analysis

General index of lesions were derived from the frequency of the histological lesions measured for each organism and tissue. In the field surveys, general index of lesions were derived for oysters, OIGG, gills, OIGE, external organs, OIGD, digestive, and OIGR, gonads, outside of the Guadalquivir estuary. The general index of lesions in the Guadalquivir estuary for fish were, FIGG, gills, FIGL, liver, and in the Bay of Cádiz and in the ría of Huelva were, FIGG, gills, FIGL, liver, FIGK, kidney. These index for clams were, CIGG, gills, CIGE, external organs, CIGD, digestive, and CIGR, gonads, in the Guadalquivir estuary and the same tissues, except external and gonads for the other two estuaries located in the Gulf of Cádiz. In the bioassays conducted in the laboratory, general indexes of lesion (CLID, CLIG, CLIGU) were calculated for each tissue as an average value of the clam damage semi-quantified as previously reported by DelValls et al. (1998). In a similar manner, general indexes of lesion were calculated for each tissue in the juveniles of the fish and for gills (FLIG), gut (FLIGU) and liver (FLIL) as an average value of the fish damage.

The general indexes were derived as an average value of the frequency of the lesions measured in each tissue. It is based on the use of six individuals of each species and during every survey both under laboratory and field conditions. To derive the final value we represent the number of organisms that show prevalence in any of the detected lesions using the next expressions and associated number of individuals: − (0 individuals), +/− (1 individual), + (2 individuals), ++/+ (3 individuals), ++ (4 individuals), +++/++ (5 individuals) and finally the maximum is associated with the presence of a disease in the total number of individuals, +++ (6 individuals). For instance, if we take into account a determined tissue disease (lamellar fusion in gills) for 3 of the 6 analyzed animals, we can derive a semi-quantitative value of ++/+ (1.5) for this station. To derive the final FIGG (general index of lesions in gills for fish) value an arithmetic average of the semi-quantitative values obtained for each of the detected lesions in gills is calculated.

The concentration of heavy metals and the general index of lesions in the tissues of the different organisms were analyzed by factor analysis, using principal components analysis (PCA) as the extraction procedure, which is a multivariate statistical technique (MAA) to explore variable (chemical concentration, metal concentrations in tissues and toxicity data, general index of lesions) distributions, for the different field surveys and the toxicity bioassays. The objective of PCA is to derive a reduced number of new variables as linear combinations of the original variables. This provides a description of the structure of the data with the minimum loss of information. The PCA was performed on the correlation matrix; i.e., the variables were centered (mean = 0) and scaled (standard deviation = 1), to be treated with equal importance. All analyses were performed using the PCA option of the FACTOR procedure, followed by the basic setup for factor analysis procedure (P4M) from the BMDP statistical software package (Frane et al., 1985). The MAA is applied to analyze bioaccumulation–toxicity relationship in the studied sites, in the Guadalquivir estuary along the seasonal survey using three different organisms together with the bioassay results obtained for the two stations located in this estuary (GL and GR). The samples (cases) included in the analysis were located in the Guadalquivir estuary (stations GL and GR), the data of oyster and fish were used as representative of the GL and GR stations, respectively. The cases studied in this MAA were defined by these two stations for each of the six seasonal periods (summer, autumn and winter 2000 and spring, summer and autumn 2001, see Table 1, Riba et al., this issue) and that defined by the bioassay conducted in the laboratory ($n = 28$, cases). The variables used in the MAA were those obtained from the bioaccumulation of four metals, Zn, Cd, Pb, Cu (Riba et al., this issue) and from the different general index of lesion obtained from each organism and described in this study, using those for gills, digestive and an average of the rest of organs named here as external.

The resulting sorted rotated factor loadings are coefficients correlating the original variables and the principal factors in this analysis. The variables are reordered so the rotated factor loadings for each factor are clustered together. In the present study, we selected to interpret a variable or group of variables as those associated with a particular factor where loadings were $\geqslant 0.25$, corresponding to an associated explained variance over 65%. This approximates Comreys' (1973) cut-off of 0.55 for a *good* association between an original variable and a factor, and also takes into account discontinuities in the magnitudes of loadings approximating the original variables.

Adequate quality assurance/quality control (QA/QC) measures were followed in all aspects of the study, from field sampling through to laboratory and data entry as per Blasco et al. (1999) and DelValls et al. (2001).

3. Results and discussion

3.1. Field surveys

3.1.1. Histopathology in the Guadalquivir estuary

The frequency of general indexes of lesions was derived for more than 150 individuals of the oyster *C. angulata* and more than 650 individuals of the clam *S. plana* collected in the two stations located in the Guadalquivir river (GL and GR) from June 2000 to autumn 2001. Also the lesions measured in individuals (more than 50) of the fish *L. ramada* collected in the confluence of the rivers Guadalquivir and Guadiamar and around station GR are shown in this analysis.

Typical lesions measured in tissues of *S. plana* from different areas of the Guadalquivir estuary are shown in Fig. 1. Highlighting for *C. angulata* infiltrations leucocites at level of gills, palpes, mantel, digestive glands, intestine, ovary and testicle, as well as alterations in epithelial and ciliar structures of gills and intestine. In *S. plana* an increase of mucous secretion was observed at level of gills as well as infiltration leucocytes in this structure and in the mantel. At level of the digestive apparatus it has been detected an apparent ciliar loss and epithelial decamation; without showing significant alterations in the gonads.

The average values and the standard deviation of the general index of lesions for the oyster *C. angulata* and for the fish *L. ramada* along the different seasonal period of study are shown in Fig. 2. These values were derived as an arithmetic average from the semi-quantitative lesions described and highlighted in the paragraph above and the procedure described in the methods section. As previously described by Riba et al. (this issue) the selection of these organisms in the areas located outside of the estuary (*C. angulata*) and in the confluence of rivers Guadiamar and Guadalquivir (*L. ramada*) was to address differences associated with the impact produced by the Aznalcóllar mining spill on the Guadalquivir estuary (Riba et al., 2002). No differences are detected between the trends of the general indexes of lesions measured in both organisms, except that *C. angulata* shows higher values of indexes of lesions during the last months in 2000 and the first months in 2001, whereas *L. ramada* shows a decreasing in their indexes of lesions from 2000 to the end of the surveys. These indexes decrease with the time of collection being the highest values of lesions measured during surveys carried out in summer 2000 and the lowest values measured in the last survey carried out in autumn 2001. For *L. ramada* and in general, lesions measured in gills were higher than those measured in liver, especially during the first months of monitoring (2000).

In Fig. 3 are shown the general indexes of lesions along the seasonal period of study for the clam *S. plana* in the two stations located in the Guadalquivir river (GL and GR). As in the case of the oyster and the fish none differences were detected in the trends of the indexes of lesions between the two studied stations showing in both cases a decrease of the lesions with the time, being highest those lesions measured during the first month of monitoring in summer 2000 and lowest the lesions measured during the last month of monitoring in autumn 2001.

In summary, from the results obtained during the seasonal monitoring of the histopathological lesions measured in the Guadalquivir estuary, for all the organisms, all the tissues and in all the samples studied it was shown a decrease of the damage with time that can be related to the decrease of the acute enrichment of heavy metals originated by the acute mining spill as recently reported by different authors in the area (Riba et al., 2003a, 2004a). Thus, it could confirm that the heavy metals that affected the estuary during the first months of the impact in 1998 produced some lesions in the organisms living in the estuary that after the cessation of the spill are recovering their original structure in the tissues of their populations. Furthermore, these results confirm the decrease in the sediment toxicity using the amphipod *Ampelisca brevicornis* with the time (from 1998 to 2001) that informed about the potential recovery of the environmental quality in the estuary as it has been reported by other studies in the area (Riba et al., 2004a).

3.1.2. Histopathology in the other areas

In Table 1 are shown the summarized results of the general index of lesions measured in the fish *S. senegalensis* and the clams *S. plana* in the other two areas selected in this study, the Bay of Cádiz (BC) and the ría of Huelva (H2 and H3) (Fig. 1, Riba et al., this issue). Results associated with the station H1 in the ría of Huelva were not available because organisms were not present in this area. For the studied areas the same kind of lesions was measured although the incidences of them, and then the frequency and the general index of lesion were different among the studied sites. In general, the severity of the lesions was highest in the ría of Huelva, intermediate in the Guadalquivir estuary and low or not detected in the Bay of Cádiz. The results obtained during the field survey along the time in the Guadalquivir estuary show that the lesions measured in the organisms collected during the summer of 2000 were more close to those measured in the ría of Huelva, whereas the lesions measured in autumn 2001 were more similar to the lesions measured in the clean area of the Bay of Cádiz, although still higher than in this area.

A qualitative relationship could be established comparing the lesions measured in the Guadalquivir estuary compared to the different areas and the set of data from chemical residues reported by Riba et al. (this issue). The concentration of metals from the mining spill may have impacted on the organisms in the estuary producing

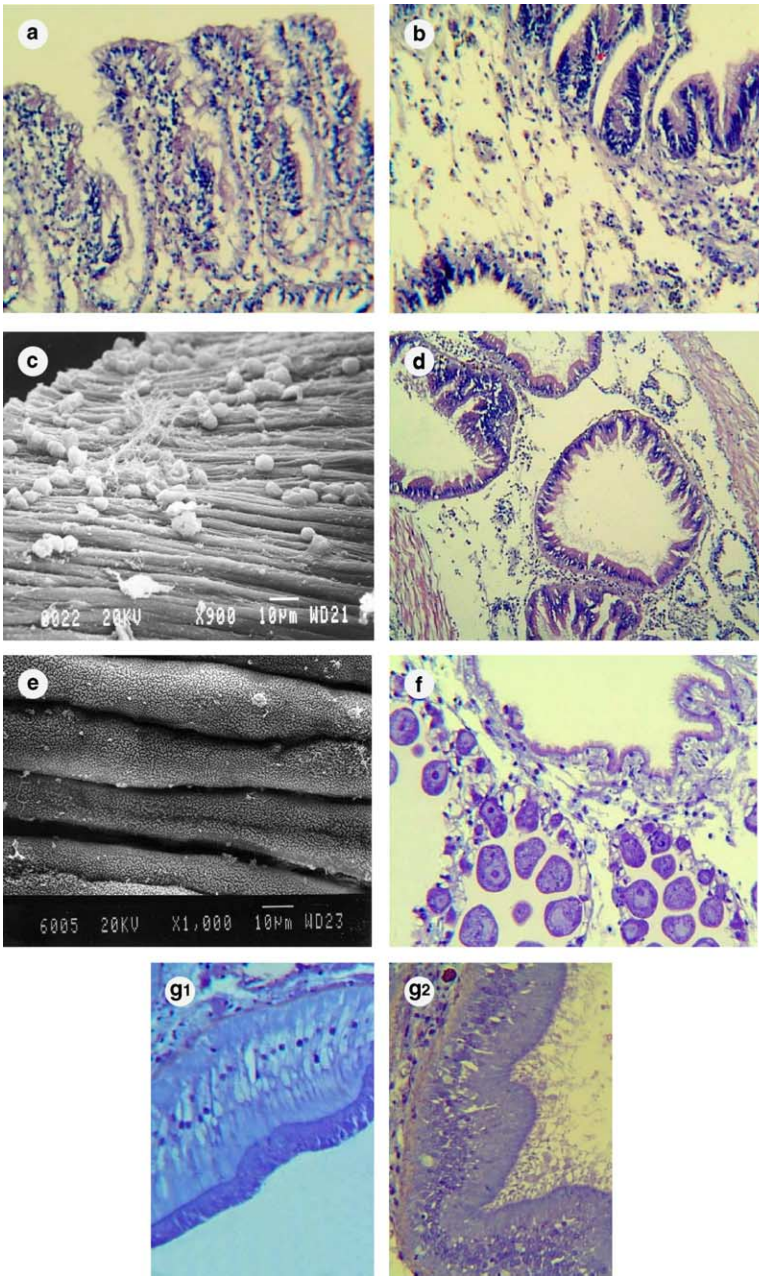

Fig. 1. Example of histological sections used to semi-quantify lesions (Table 1) associated with contaminant bound to sediments used in the *Scrobicularia plana* bioassay. (a) Gill with leucocitary infiltrations (H/E ×10). (b) Gill with necrosis and epithelial decamation (H/E ×20). (c) Scanning electronic microscopy of gills with mucosa secretion (MEB ×900). (d) Digestive gland (H/E ×4). (e) Siphon (MEB ×1000). (f) Female gonads (A.T. ×10). (g) 1. Controls of intestinal epithelium (A.T ×10). 2. Ciliate loss and desorganization in intestinal epithelium (A.T. ×10).

some lesions measured during the surveys carried out in year 2000. The decrease in the concentrations of the metals from 1998 to 2000 and 2001 (Riba et al., this issue) originated by the accident, specially Zn could be related to the decrease of the lesions in all the areas of the estuary from 2000 to 2001, and that started after the cessation of the spill (González de Canales et al., 2001). However, the concentration of other metals such as Cu does not decrease with the time so the lesions may not be related to the enrichment of these metals that are produced by other sources different than the mining spill. These correlations should be confirmed by further

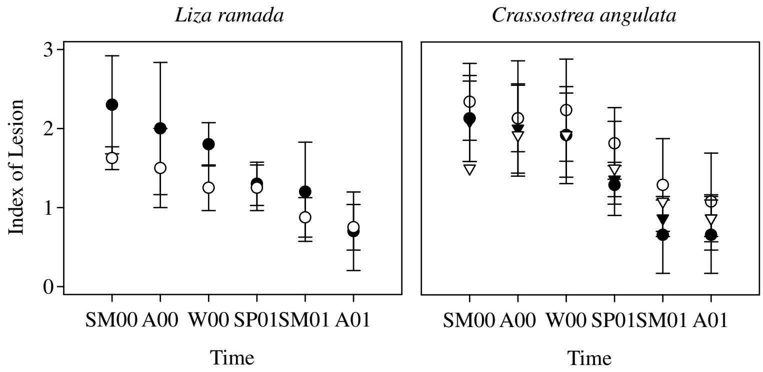

Fig. 2. General indexes of lesion obtained for *Liza ramada* gills (FIGG (●)) and liver (FIGL (○)) tissues; and for *Crassostrea angulata* gills (OIGG (●)), external organs (OIGE (◯)), digestive (OIGD (▼)) and gonad (OIGR (▽)) tissues. The organisms were collected in the Guadalquivir estuary along different seasons: summer (SM00), autumn (A00) and winter (W00) in the year 2000 and in spring (SP01), summer (SM01) and autumn (A01) during year 2001.

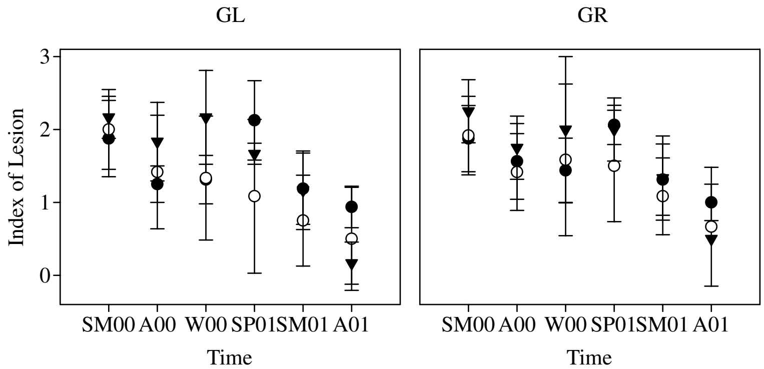

Fig. 3. General indexes of lesion measured in *Scrobicularia plana* for gills (CIGG (●)), external organs (CIGE (○)), digestive (CIGD (▼)) and gonads (CIGR (▽)), in the stations GL and GR located in the Guadalquivir river. The organisms were collected in the Guadalquivir estuary along different seasons: summer (SM00), autumn (A00) and winter (W00) in the year 2000 and in spring (SP01), summer (SM01) and autumn (A01) during year 2001.

studies and specifically using quantitative approaches different than the qualitative here described.

3.1.3. Bioassays

The histopathological lesions measured in exposed organisms to sediments collected during autumn 2001 in the stations GL and GR located in the Guadaquivir estuary show similar damages for the fish *S. senegalensis* and the clams *R. phillipinarum* than those measured in the field surveys. For the fish, gills show clavate lamellae, shortening of secondary lamellae, epithelial lifting, hyperplasia, deformation of secondary lamellae and vascular congestion; liver, lipid-like vacuoles, hepatocellular anisocytosis, hyperaemic capillaries, foci of cellular alteration and hepatocellular shrinkage; gut, increased of lipid content in enterocytes and hyperplasia and kidney, tubular epithelial necrosis and loss of hematopoietic tissue. For the clams it was detected in the gills, hemocitary infiltration, fusion of lamellae, lost of cells, hyperplasia and hypertrophia, and necrosis; in gut it was detected increasing of lipid content in enterocytes and hyperplasia.

In Fig. 4 are shown the general indexes of lesions in tissues of the clam *R. phillipinarum* and the fish *S. senegalensis* exposed to sediments collected in different estuaries of the Gulf of Cadiz and adapted from those reported by Riba et al. (2004b). The derivation of these general index of lesions was conducted as previously reported in the methods section. The highest general indexes of lesions for all the tissues were measured in both organisms exposed to sediments collected in the areas of the ría of Huelva, and mainly in stations H1 and H2. The lesions measured in the Guadalquivir estuary were low and similar to those measured in the

Table 1
Summarized semi-quantitative results of lesions detected in microscopic abnormalities of individuals of the fish *Solea senegalensis* and the clam *Scrobicularia plana* collected in the Bay of Cádiz and H2 and H3 from the area of Huelva

Tissues and organisms	Histopathology	Sample zones		
		H2	H3	Cádiz
Fish				
Gills	Lamellar fusion	+++	++	+
	Hyperplasia	+++	++	+
	Distention capillaries	+++	++/+	+
	Areas adematous	+++	+	+
	FIGG	3.00	1.63	1.00
Liver	Lipid-like vacuoles	+++	+	+
	Hyperplasia/hypertrophy	++	+	+
	Blood stasis	+++	+/++	+
	FIGD	2.67	1.17	1.00
Kidney	Tubular occlusion	+++	+	+
	Pycnosis	+++	+	+
	Tubular disintegration	++	++	+
	Loss of interstitial tissue	+++	+/++	+
	FIGK	2.75	1.38	1.00
Clams				
Gills	Necrosis	+++	+/++	+/–
	Hyperplasia	++/+++	+	–
	Hemocitary infiltration	++	+	–
	Fusion of lamellae	+++	++	+
	CIGG	2.63	1.38	0.38
Digestive	Hypertrophy	++	++	+
	Hemocitary infiltration	+++	+	–
	Enterocytes	++	+	–
	CIGD	2.33	1.33	0.33

The results are referred to the frequency of the lesions measured in a total number of 6 individuals in each survey: – (none), +/– (one), + (two), ++/+ (three), ++ (four), +++/++ (five) and +++ (six). Examples of some of the measured lesions are shown in Fig. 1.

Bay of Cádiz and considered without toxic effects, although some of the tissues for both organisms showed moderated damages measured in organisms exposed to sediments collected in the confluence of both rivers in the Guadalquivir estuary (GR). It confirms the trend in the decrease of lesions in tissues of organisms collected in the Guadalquivir estuary pointed out in the field survey carried out at different seasonal period of the years 2000 and 2001.

3.2. Deriving tissue quality guidelines in the Guadalquivir estuary

The set of data associated with the chemical concentrations of the metals Zn, Cd, Pb and Cu and the general indexes of lesions obtained in this area during the field surveys together with those results obtained in the toxicity tests carried out in the laboratory were linked by means of a MAA. The application of PCA to the chemical and toxicological data represents the original variables (metals and general indexes of lesions) by three new variables, or principal factors (Table 2). These factors explain 84.6% of the variance in the original data set. Negative values of sorted rotated factor loadings (negative salience) are as important as positive values (positive salience); however, in this analysis, the positive loadings are in general of larger magnitude than the negative loadings. The loadings following varimax rotation for the three factors are given in Table 2. Each factor is described according to the dominant group of variables. The first principal factor, #1 is predominant and accounts for 50.0% of the variance; this factor combines the chemical concentrations of the metals Zn and Cd with all the indexes of lesions (gills, IGG; digestive, IGD; and other tissues, IGE). It represents the histopathological lesions in the Guadalquivir estuary and associated with the concentrations of Zn and Cd in the organisms. The second factor, #2 accounts for 24.4% of the variance and combines the concentrations of Cu and Cd and with negative loadings the concentrations of

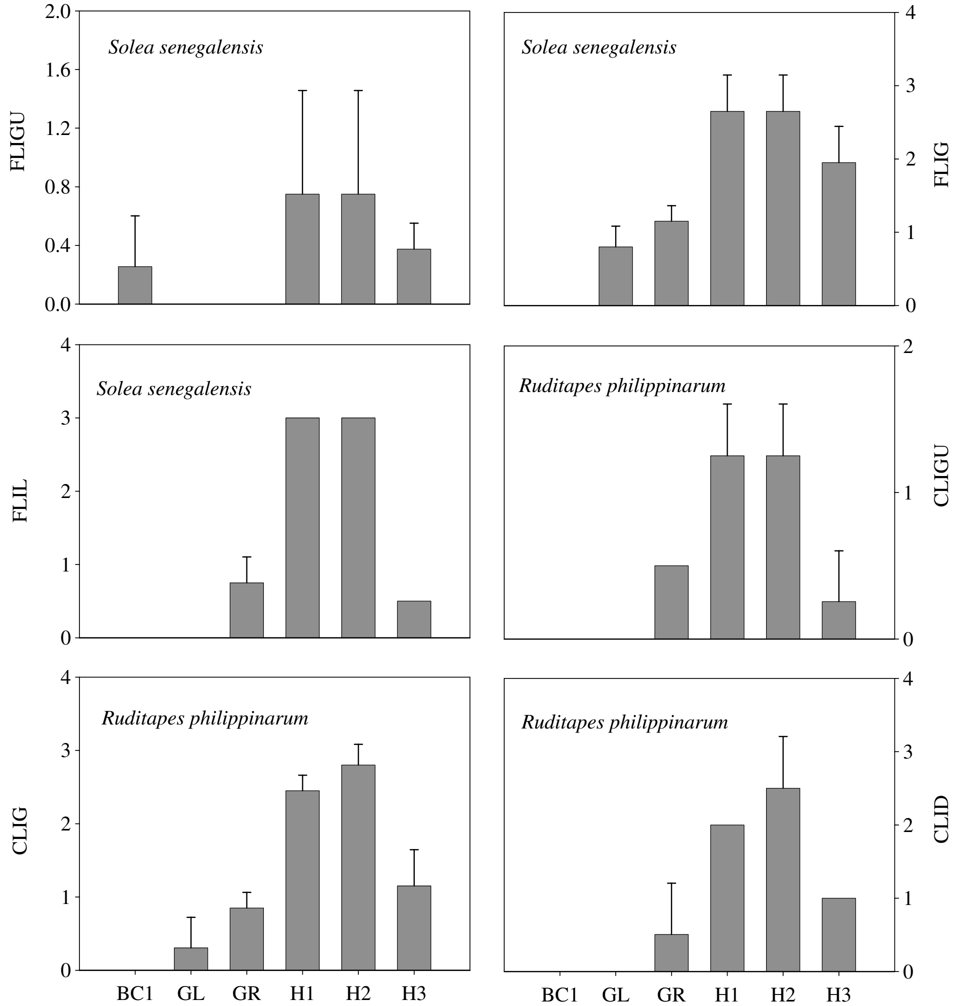

Fig. 4. General indexes of lesions measured in the fish *Solea senegalensis* for gut (FLIGU), gills (FLIG), and liver (FLIL) and in the clam *Ruditapes philippinarum* for gut (CLIGU), gills (CLIG), and digestive (CLID), exposed to sediments collected in autumn 2001 (A01) in the three studied areas: Bay of Cádiz (BC), Guadalquivir estuary (GL and GR) and the ría of Huelva (H1, H2 and H3).

Pb and not associated with lesions in the different organisms. It represents the bioaccumulation of the three metals without correlations with the biological diseases. The third factor, #3 accounts for 10.1% of the variance and it is a combination of metals Zn and Cd and two of the general indexes of lesions IGD and IGE. It is important to note that this factor account for highest loading of the variable Zn, being those associated with the variables Cd and the indexes of lesions lower than those measured in factors 2 and 1 respectively. It could be related to the decrease of bioaccumulation of the metals from the Aznalcóllar mining spill (Zn and Cd) and associated with the same pattern in decreasing the incidence of histopathological lesions measured in the organisms collected in the estuary.

In order to confirm these factor descriptions and to establish the site-specific values of tissue quality, here defined as tissue quality guidelines (TQGs) in the Guadalquivir estuary, we propose a representation of estimated factor scores from each case (organisms at different seasonal period of time from field survey and organisms exposed to sediments) to the centroid of all cases for the original data. These results are shown in Table 3 and Fig. 5. In general, factors 1 and 3 show positive prevalence during the first months of the impact of the mining spill in both stations located in the Guadalquivir estuary (GL and GR) and for all the organisms, although F3 was negative for all the stations in the fish *L. ramada*. Besides, correlations between bioaccumulation of Zn and Cd and lesions measured in the organisms showed in F1 and for the station GR located in the estuary were similar between fish and mollusks being higher in clams. On the other hand, these correlations were higher in GR than in GL using all the

Table 2
Sorted rotated factor loadings (pattern) of seven variables on the three principal factors results from the multivariate analysis in the study using organisms collected in the Guadalquivir estuary during six different periods of time along years 2000 and 2001

Factors	#1	#2	#3
% variance	50.0%	24.4%	10.1%
Zn	0.251	–	0.936
Cd	0.371	0.546	0.420
Pb	–	−0.837	–
Cu	–	0.882	–
IGG	0.961	–	–
IGD	0.904	–	0.319
IGE	0.783	–	0.501

The loading matrix has been rearranged so that the columns appear in decreasing order of variance explained by factors. Only loadings greater than 0.25 are shown in the table. Factors (#) are numbered consecutively from left to right in order of decreasing variance explained.

organisms and during the first months of the impact. The negative values of F1 and F3 in the cases represented by the results obtained in the sediment toxicity tests at the end of the period of monitoring (autumn 2001) shows not correlation between bioaccumulation of metals from sediments and lesions measured both in field and laboratory conditions in the Guadalquivir samples collected during autumn 2001. It confirms the process of recovery the initial environmental quality in the estuary that has been previously discussed in this work and pointed out by previous studies in the area (Riba et al., 2002).

The bioaccumulation of Cu represented by the prevalence of F2 in the organism studied shows that it was significant at oyster and fish organisms for all the periods and in the sediment bioassay conducted using benthic fish *S. senegalensis*. It again points out the presence of different sources of Cu in the area of the Guadalquivir estuary than the mining spill.

Table 3
Estimated factor scores from each 28 cases evaluated using the concentration of metals and the histopathological lesions in organism collected in the Guadalquivir estuary (CA, *C. angulata*; SP, *S. plana*; LR, *L. ramada*; SS, *S. senegalensis*; RP, *R. phillipinarum*) during different periods of time (SM, summer, A, autumn, W, winter, SP, spring), and using data from the toxicity tests (SS# and RP#) to the centroid of all cases for the original data

	Factors		
	#1	#2	#3
CASM00	1.197	1.685	0.432
CAA00	1.031	0.685	1.065
CAW00	0.750	0.820	1.756
CASP01	−0.290	0.779	1.385
CASM01	−1.470	0.639	1.362
CAA01	−1.733	0.782	2.094
SPGLSM00	1.165	−1.171	−0.320
SPGLA00	0.197	−1.203	0.121
SPGLW00	0.379	−1.154	0.178
SPGLSP01	0.780	−1.084	−0.292
SPGLSM01	−0.480	−1.314	0.026
SPGLA01	−1.212	−0.553	0.266
SPGRSM00	1.194	−0.965	−0.081
SPGRA00	0.426	−0.828	0.314
SPGRW00	0.508	−1.110	0.429
SPGRSP01	1.048	−0.936	0.007
SPGRSM01	−0.155	−0.894	0.485
SPGRA01	−0.979	−0.663	0.612
LRSM00	1.515	1.221	−0.759
LRA00	1.000	1.089	−1.215
LRW00	0.775	0.963	−0.488
LRSP01	0.133	0.871	−0.574
LRSM01	−0.244	1.235	−0.344
LRA01	−0.937	0.871	−0.124
SSGL	−1.226	0.866	−1.978
SSGR	−0.642	0.800	−1.849
RPGL	−1.820	−0.671	−1.195
RPGR	−0.907	−0.763	−1.314

The factor scores quantify the prevalence of every factor for each station and it is used to derive the tissue quality guidelines (TQGs) as described through the text and using Fig. 5.

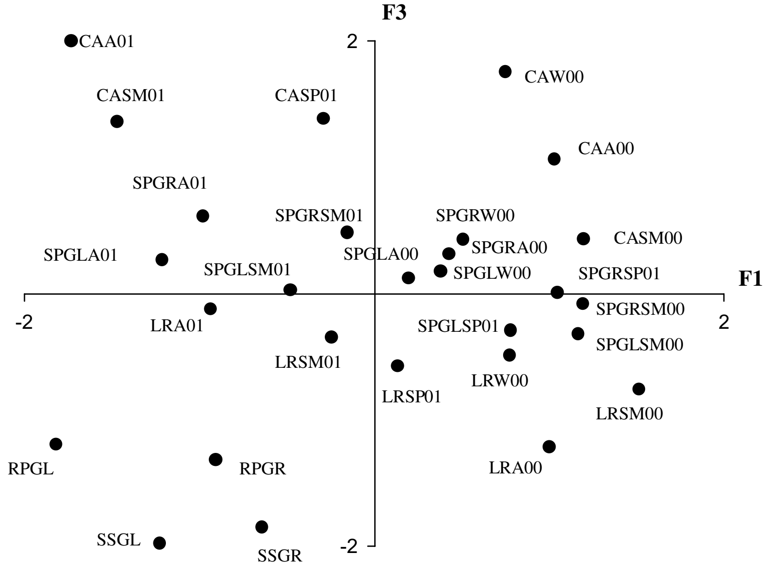

Fig. 5. Results of the MAA (PCA, estimated factor store) used for the case (seasonal surveys) for different organisms (*C. angulata*, CA#; *S. plana*, SP#; and *L. ramada*, LR#) distributions in the space defined by the factor couples associated with biological effect (F1 and F3). The explained variance for each factor is reflected in Table 2.

To derive TQGs the prevalence of factor scores associated with histological lesions F1 and F3 were used and are shown in Fig. 5. Based on the results of original variables loadings (Table 2) in both F1 and F3 that shows F1 with higher loadings of histological lesions than F3 and that F1 shows lower loadings of chemical concentrations Zn and Cd than in F3, we have considered that only those case (organisms) that showed positive prevalence of both factors then showed in the x, y-positive quadrant in Fig. 5, can be considered to derive TQGs. Thus, when the scores of both factors, the factor showing relationships between the metals and adverse effects, are $\leqslant 0$, the maximum concentrations of the metals at these organisms are the maximum chemical concentrations in the organisms not associated with adverse effects, "not hazardousness". In contrast, to establish the minimal concentrations above which hazardousness can be present, the minimum concentration of the metals at the organisms where the score of both factors >0 were selected and described here as "hazardousness". The minimal and major effect concentrations, as well as the intermediate ranges of concentrations representing an interval of uncertainty, are shown in Table 4.

To facilitate the understanding of the above-mentioned process to derive tissue quality guidelines (TQGs) we have described the calculation method for the case of Zn in *C. angulata* (Table 4). The metal is included in the factors #1 and #3 so correlated to biological effect (Table 2). Both factors are positive at organisms collected in the three first surveys carried out in the mouth of the estuary (GL). On the other hand, F1 is negative in

Table 4
Summary of tissue quality guidelines (TQGs) proposed for Zn and Cd, in this study derived using chemical residues (Riba et al., this issue) and histological lesions measured in the same tissues of different organisms: A) oysters, *Crassostrea angulata*; B) clams, *Scrobicularia plana*, collected in the Guadalquivir estuary by means of a multivariate analysis approach (MAA)

Chemical		Tissue quality guidelines (TQGs)		
		"Not hazardousness"	"Uncertainty interval"	"Hazardousness"
Zn	A)	<8603	8603–8865	>8865
	B)	<800	800–862	>862
Cd	A)	<3.42	3.42–5.33	>5.33
	B)	<2.60	2.60–2.62	>2.62

For *Liza ramada* none TQGs was possible to derive. All concentrations are expressed as $mg\,kg^{-1}$ dry weight.

the rest of the surveys although F3 is positive in the same period of time (probably due to the process of bioaccumulation of Zn but without a consequence in the lesions measured in this organism) then they are not considered for deriving TQGs as concentrations of bioaccumulated metal causing effects. The maximum concentration of Zn among these three cases in the year 2001 with negative prevalence in F1 is 8603 mg kg^{-1} (dry weight) measured in the oysters collected during autumn 2001. This is the ''not hazardousness'' concentration (Table 4). Similarly, to develop the guideline 'hazardousness' we find the minimum concentration of the metals among the stations with factors 1 and 3 positive: three first surveys (Table 3, Fig. 5), which for Zn is 8865 mg kg^{-1} (dry weight) at the organisms collected in autumn 2000. The uncertainty interval is the difference between these two concentrations.

4. Final remarks

This study presents the results of a combined chemical and biological assessment of environmental quality at different periods during the monitoring of the Aznalcóllar mining spill in the Guadalquir estuary compared to the values obtained in other areas. These conclusions obtained are summarized below,

(a) All the histological lesions measured in all the tissues and in all the stations studied in the Guadalquivir estuary decrease with time. The highest values of these lesions in the estuary were measured in the confluence of the Guadiamar and Guadalquivir rivers.
(b) The comparison between the bioaccumulation (cause identification) and the histological lesions (effect identification) measured in organisms collected either at field or laboratory-exposed permits characterize differences among areas affected by the mining spill, affected by chronic mine activities and without contamination.
(c) The correlations between the bioaccumulation of heavy metals Zn and Cd and histological lesions measured in organisms along the studies carried out in the Gudalquivir estuary during the years 2000 and 2001, establishes the basis to derive tissue quality guidelines (TQGs) for the oysters and clams as consequence of an acute mining spill.

This work is an attempt to use the relationship between metal residues and sublethal levels to derive site-specific values of tissue quality (TQGs). However, the values here derived should be used with caution and only associated with organisms used in the area of the Guadalquivir estuary.

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