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Sediment quality in the Guadalquivir estuary: sublethal effects associated with the Aznalcóllar mining spill

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Abstract

As a complementary assessment of the impact of the Aznalcóllar mining spill on the Guadalquivir estuary two different sediment toxicity tests using fish (*Solea senegalensis*) and clams (*Scrobicularia plana*) were performed. The histopathological alterations by recording lesions at 15 and 30 days in fish to the gills, liver, gut and kidney and at 14 days in clams to the gills and gut were used to determine the adverse effects associated with the contaminants bound to sediments. The lesions measured at different tissues in both organisms show that the enrichment of heavy metals from the mining spill stressed some areas in the ecosystem of the estuary. The comparison of these effects with those lethal effects detected in the same samples using a multivariate analysis approach permits to identify the adverse effects associated with the accidental spill on the estuary. The incidence of the impact, located in specific areas of the estuary show an acute effect related to the spill.

Keywords: Histopathological lesions; Sediment toxicity; Heavy metals; Multivariate analysis; Contamination; Gulf of Cádiz

1. Introduction

To measure the stress associated with contaminants in the ecosystem, toxicological methods have been developed to monitor effects of sediment-associated pollutants on benthic organisms, populations, and communities (Luoma and Ho, 1992). The toxicity tests that involve measurements of histological and cellular damages have shown a more sensitive response than survival for a selected organism to determine the adverse effects associated with chemicals bound to sediments. These sublethal responses have been found to be a powerful tool to evaluate sediment toxicity effects (DelValls et al., 1998).

As part of the monitoring of the impact caused by the Aznalcóllar mining spill (April 1998), the lethal effects associated with contaminants bound to sediments from the Guadalquivir estuary using individuals of amphipods and clams has been described by Riba et al. (2003). These toxicity tests have been complemented exposing to the same samples individuals of the fish *Solea senegalensis* and the clam *Scrobicularia plana* to determine the histopathological alterations in different tissues associated with the heavy metals bound to the toxic mud from the Aznalcóllar accident.

Histopathological measurements in fish and clams offer a number of practical advantages over other endpoints used in environmental risk assessment performed under laboratory or field conditions (Handy et al., 2002). The main advantage to be applied to our study is the high sensitivity of this kind of biomarker to sublethal levels of contamination that usually are found in the environmental samples. The comparison of the sublethal responses measured in the fish and clams, more sensitive than the lethal measurements, should permit us to clearly identify the real extension of the spill associated with the impact on the sediments located in the Guadalquivir estuary and furthermore the identification of the areas of concern located in the estuary and defined by the mining spill.

The multivariate analysis approach (MAA) has been shown as an useful tool to link sediment chemistry and biological effects with different proposes (e.g., DelValls and Chapman, 1998; DelValls et al., 2002). The MAA

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has been used to combines sediment chemistry, lethal effects on amphipods and clams, and sublethal responses in fish and clams measured in environmental samples collected in the estuary (GL2, GL6, GR2, GR4) and dilutions of toxic mud from the mine with clean sediment (0.3%, 1.8%, 7.9%, 20% and 32% dry sediment) to identify the areas of concern detected in the Guadalquivir estuary after the Aznalcóllar mining spill.

The objectives of this study were, (a) establish the sublethal toxicity associated with the concentrations of heavy metals in environmental samples and originated by the accidental spill by comparing the effects to those measured in dilutions of toxic mud, (b) determine the relationship between the lethal and sublethal toxicity in both kind of samples, toxic mud dilution and environmental samples, and (c) identify the areas of concern in the estuary impacted by the Aznalcóllar mining spill.

2. Material and methods

2.1. Approach

The sediment samples used in this study have been previously described related to their collection and treatment by Riba et al. (2003). These sediments were used to determine the histopathological effects associated with contaminants bound to them using different tissues, gills, liver, gut and kidney on the fish *S. senegalensis* and, gills and gut on the clam *S. plana*.

Clean sediment collected in the areas of the Bay of Cádiz (DelValls et al., 1998) and toxic mud collected 40–45 km far from the tailing spill point the day after the accident, were used to prepare the dilutions. These sediments were dried at room temperature not higher than 40 °C and then the different dilutions were set up as described by Riba et al. (2003). The dilutions are referred as percentage of toxic mud sediment and there were used 0.3%, 1.8%, 7.9%, 20% and 32% dry weight.

Sediment chemical concentration selected in this study and the analyses carried out in the sediment samples were performed as described by Riba et al. (2003).

2.2. Toxicity tests

Both sediment toxicity tests, using juveniles of the fish *S. senegalensis* (one month, exposure time) and the clam *S. plana* (14 days, exposure time) were developed in whole sediment using a 1:4 v/v sediment–water relation. About 3 l of previously wet filtered sediment aliquots (environmental samples) per replicate were placed on the toxic beakers (aquarium of about 20 l of total capacity) and filled with clean sea water (12 l). Toxic mud dilutions and negative controls were set up as described by Riba et al. (2003) and in same proportions than environmental samples.

Fish individuals were obtained from the aquaculture facilities at the Faculty of Marine and Environmental Sciences previously to the test. Clams were obtained in an aquaculture industrial farm and acclimated to test conditions during one month. A baseline group of 10 randomly chosen individuals of both kinds of organisms were weighed to provide data for feeding calculations and later comparisons. Previously to the animals exposure to sediments and at the end of the acclimation period, the specimen size varied for juveniles of the fish from 14.21 ± 1.07 total length (cm) and 3.25 ± 1.50 weight averaged (g). For clams the size was 3.38 ± 0.93 total diameter length (cm). At the beginning of the experiments, another baseline group of 10 randomly chosen individuals for both organisms were measured, weighed, anaesthetized, and processed for pathological responses to be used as initial cellular control. Fish and clams were placed in the toxicity test and control beakers by replicate (6 fish and 10 clams per 20-l glass aquarium). Fish were fed with artificial food (Trow, Gemma, 0.9–1.6 mm) 2–3 times per day. Clams were fed by a mixture of micro algae (*Tetraselmis chuii*, *Isocrhysis galbana*, *Chaetoceros gracilis*) once per day. Aeration was provided to maintain adequate oxygen concentrations. Water replacement was performed every two or three days for fish and every day for the clam bioassay using a peristaltic pump. The physicochemical parameters, pH (7.5–8.5), Temperature (20 °C), Oxygen (>5 $mg\,l^{-1}$) and salinity (34) were recorded and controlled when necessary to maintain quality control during both sediment toxicity tests. Photoperiod during both tests was natural and under controlled conditions. The concentration of total ammonia in the interstitial water was monitored at the beginning and at the end of the bioassay being lower than 20 $mg\,l^{-1}$ (pH ranged between 7.5 and 7.9) in all the sediments and controls. These concentrations are considered with absence of influence in the toxic results produced by chemicals.

2.3. Data calculation and statistical analysis

When the water was renewed, the survival rate for all tanks was determined. For fish, pieces of selected tissues to assess the histopathological lesions were taken at 15 and 30 days of exposure time. For clams, the tissues were obtained at the end of the bioassay (14 days). Fish and clams were removed from the tanks and samples were collected. Fish were anaesthetized with 0.05% ethyl-4-aminobenzoate (benzocaine) during 5–10 min; then weight, length and externally examined. For both organisms, tissues 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 Hematoxilin–Eosin and Hematoxilin/

VOF (Gutierrez, 1967). Sections were reviewed by light microscopy and photographed (Olympus CH20). Damage to the tissues of both organisms was semi-quantified by detecting the frequency of the lesions in each detected alteration. 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.

Histopathological alterations were semi-quantitatively evaluated by ranking the severity of lesions (grades, 0 (−), 0.5 (+/−), 1 (+), 2 (++), and 3 (+++)) as described in detail by previous studies carried out in the area (DelValls et al., 1998). A general index of damages for each analyzed tissue (fish: index of gill damages –FGDIx-, index of liver damages –FLDIx-, index of gut damages –FUDIx-; clams: index of gill damages –CGDIx-, index of gut damages –CUDIx-) was established to permit comparison of toxic responses between treatments and organisms. An arithmetic average value was obtained from the original semi-quantitative assessment of the lesions for each tissue and organisms. For the sediment toxicity test using juveniles of the fish *S. senegalensis* these indexes of damages were determined at 15 and 30 days of exposure time.

To link the set of data obtained, the original variables from chemical concentrations, lethal responses in *Ampelisca brevicornis* and *S. plana* (Riba et al., 2003), and sublethal responses (*S. plana and S. senegalensis*), were analyzed by factor analysis, using PC as the extraction procedure, which is a multivariate statistical technique to explore variable (chemical concentration $n = 11$; toxicity data, $n = 12$) distributions. The factor analysis was performed on the correlation matrix, i.e., the variables were autoescaled (standardized) so as to be treated with equal importance (DelValls and Chapman, 1998). 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).

For this analysis, chemical data included were heavy metal concentration (Zn, Fe, Cd, Pb, Cu and Mn, expressed as $mg\,kg^{-1}$, except for Fe as percentage), Organic compound concentrations (PAHs and PCBs expressed as Aroclor standards 1242 and 1246). Geochemical matrix characteristics were covered by the organic carbon concentration (OC) and by grain size distributions. The toxicity data sets included the lethal responses of individuals of the amphipod *A. brevicornis* (percentage of mortality) and of the clams *S. plana* (TL50, this parameter was transformed to show an increases with biological damage deriving its inverse $TL50^{-1}$ and expressed as $hours^{-1}$). All these data are reported by Riba et al. (2003). Besides, the sublethal responses summarized as general indexes of damages (fish: index of gill damages –FGDIx-, index of liver damages –FLDIx-, index of gut damages –FUDIx-; clams: index of gill damages –CGDIx-, index of gut damages –CUDIx-, all are rendering as dimensionless) were used in the Multivariate analysis approach.

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 described per ASTM (1991a,b) and for the MAA for DelValls et al. (2002).

3. Results and discussion

3.1. Chemical analysis

Heavy metal concentrations measured in the toxic mud dilution are summarized in Table 1. The error coefficient (ε), measured to compare the theoretical concentration with the measured concentration in the laboratory is similar to the error calculated in Riba et al. (2003). It demonstrates the efficiency of the analytical method to set up the different dilutions of toxic mud. The rest of the chemical concentrations and the conventional parameters of the sediments used in this study are shown by Riba et al. (2003, see Table 1).

3.2. Toxicity tests

No mortality of individuals of the fish *S. senegalensis* was detected during one month of exposure to either toxic mud dilutions or samples collected in the Guadalquvir estuary. The mortality results obtained using the clam *S. plana* were in the same range that those reported by Riba et al. (2003). In both sediment toxicity tests several sublethal damages were measured. Summarized results of the semi-quantitative evaluation of the frequency of the lesions measured are shown in Tables 2 and 3 for the fish tissues and in Table 4 for the clam tissues. An example of the diseases detected in different tissues of the fish and the clams exposed to toxic mud dilutions and environmental sediment samples are shown in Figs. 1 and 2 respectively. These kinds of alterations showed in the photographs were used to derive the values shown in Tables 2–4.

Fish exposure to chemical concentrations bound to the toxic mud dilution produced several damages that were in general related to a linear accumulation in the main target organs used in the analysis: liver, gills, gut

Table 1
Theoretical (C_{Th}) and measured (C_m) concentrations of heavy metals (Zn, Fe, Cd, Pb, Cu and Mn expressed as mg kg^{-1}, except Fe as % dry weight) for each of the toxic mud dilution tests used in the sediment toxicity test using *S. senegalensis* and *S. plana*

		0.3%	1.8%	7.9%	20%	32%
[Zn]	C_m	99.6	450	1700	4189	7024
	C_{Th}	106	430	1746	4335	6946
	ε	6.31	4.70	2.65	3.39	1.13
[Fe]	C_m	0.56	1.22	3.92	8.01	12.7
	C_{Th}	0.58	1.20	3.71	8.65	13.6
	ε	3.44	1.67	5.66	7.40	6.99
[Cd]	C_m	0.18	1.11	2.45	9.61	15.3
	C_{Th}	0.20	0.90	3.60	9.20	14.7
	ε	10.0	23.3	31.9	4.46	4.56
[Pb]	C_m	87.3	223	650	1562	2235
	C_{Th}	95.3	212	688	1632	2568
	ε	8.36	5.15	5.61	4.30	13.0
[Cu]	C_m	14.0	40.0	191	368	600
	C_{Th}	15.5	45.9	169	412	657
	ε	9.92	12.9	12.6	10.7	6.74
[Mn]	C_m	155	159	166.33	220	200
	C_{Th}	163	167	180	206	232
	ε	5.00	4.65	7.58	6.69	14.0

The relative error (ε) is calculated as described in the text and expressed as percentage.

and kidney. It can be observed different histomorphological alterations previously reported in these kinds of organisms when affected by heavy metal contamination (González de Canales et al., 1996) such as hyperplasia, hypertrophia, necrotic cells among others (Fig. 1). The effects measured as microscopic lesions increase with the exposure time. The damages identified in all the tissues analyzed are present almost always after 30 days of exposure to the toxic mud dilutions. In general, it is observed that lesions measured in the organs extracted in *S. senegalensis* increase with the toxic mud dilution and with the time of exposure (Table 2). The lesions detected in the tissues from fish exposed to sediments collected in the estuary (Table 3) are lower than those measured in toxic mud dilutions higher than 0.3%, except for the station GR2. Fish exposed to sediments of this station showed several damages at 15 and 30 days and mainly associated with liver and gills. These damages measured in tissues increase with the time of exposure as detected in the toxic mud dilutions treatments.

Clam exposure to toxic mud dilution provoked sublethal responses in those treatments equal or lower of 7.9% of toxic mud. Dilutions higher than 7.9% provoked mortality of 100% after the first 96 h and no specimens were available for the histological study. Toxic mud dilutions produced sublethal effects increasing with the amount of toxic mud used, being highest at 7.9% for the two tissues used in the analysis. At this dilution all the damages in all the tissues showed the highest frequency. The tissues obtained from individuals exposed to environmental sediment samples shown lower frequency of the damages than those measured in the toxic mud dilutions, being in the same range than those obtained at dilution 0.3%, except for sediments obtained in the station GR2 that presented damages ranged between those identified in tissues exposed to toxic mud dilution of 0.3% and 1.8%. In general, the lesions measured in gill tissues were more severe than those identified in gut tissues and increase with the toxic mud dilution used in the tests (Table 4 and Fig. 2).

These lesions measured in both kind of organisms have been compared to the histomorphological diseases in organisms collected in the estuary during three years of monitoring from 1999 to 2001 (Riba, 2003). The lesions detected in environmental stations and those from the lower dilutions of toxic material were similar to those measured in organisms collected in the area of the estuary defined by the confluence of the rivers Guadiamar and Guadalquivir and in the vicinity of station GR2 (see Fig. 1, Riba et al., 2003). The repercussions of these lesions can be associated with a sequel produced by the spill on the estuary and that could become persistent in the estuary during the next years.

Table 2
Summarized semi-quantitative results of lesions detected in microscopic abnormalities of individuals of the fish *S. senegalensis* exposed to control sediments and toxic mud dilution (0.3%, 1.8%, 7.9%, 20% and 32%) and during 15 and 30 days of exposure period

Organ	Parameter	Exposure time (days)	Control	0.3%	1.8%	7.9%	20%	32%
Liver	Lipid-like vacuoles	15	–	++	++	++	+++	+++
		30	–	+	++	+++	+++	+++
	Hepatocellular anisocytosis	15	–	++	++	++	+++	+++
		30	–	+	++	+++	+++	+++
	Hyperemic capillaries	15	–	+/–	+	+	+	+++
		30	+/–	+/–	+	+	++	+++
	Foci of cellular alteration	15	–	–	–	–	++	+++
		30	+/–	–	++	+	+	+++
	Hepatocellular shrinkage	15	–	–	+/–	+/–	++	+++
		30	+/–	–	+/–	+	+++	+++
Gills	Clavate lamellae	15	–	–	+/–	+	+	+++
		30	–	–	+	+	++	+++
	Shortening of secondary	15	–	+/–	+	+	++	++
	lamellae	30	–	+	+	++	+++	+++
	Epithelial lifting	15	–	–	–	–	+/–	+/–
		30	–	–	–	–	+	+++
	Hyperplasia	15	–	+/–	+	+	+	+++
		30	+/–		+	++	+++	+++
	Deformation of secondary	15	–	+	+	+	++	++
	lamellae	30	–	+	++	+++	+++	+++
	Vascular congestion	15	–	–	+	+	+	+++
		30	–	+	+	++	+	+++
Gut	Increase of lipid content in	15	+/–	+	++	+++	+++	+++
	enterocytes	30	–	+/–	+	++	+	+++
	Hyperplasia	15	–	+/–	+	+	++	+++
		30	+/–	+	+	+/–	+++	+++
Kidney	Tubular epithelial necrosis	15	–	+/–	+	+	+	+++
		30	+/–	+/–	+	++	+	+++
	Lost of hematopoietic tissue	15	–	+/–	+	+	++	++
		30	–	+/–	+	++	+++	+++

Incidence of lesions: (–) absent, (+/–) sometimes, (+) frequent, (++) very frequent, (+++) always present.

To confirm these results and to summarize them in the multivariate analysis approach (MAA) a general index of damages for each tissue and for both organisms were calculated as an arithmetic average value and shown in Table 5. These values confirm that the diseases measured in fish were higher in liver and gills than in the other two tissues and highest for the highest toxic mud dilution. In general, the environmental stations shown general indexes of damages lower than those measured in the toxic mud dilutions and similar to those measured in the lowest dilution of toxic mud (0.3%), except GR2 which obtained indexes ranged between those calculated for toxic mud dilutions 0.3% and 1.8%, and being similar to this last dilution. For clams the results were similar than those obtained using fish although the values calculated for both tissues (gills and gut) are much higher in clams than in fish. This difference in the range of the damage between organisms using gills and especially gut tissues could be associated with the mechanism used for each animal to get food. The clams fed through the filtration of the overlain water in which could be particles of contaminated suspended sediments. The heavy metals bound to these particles could be available in the gut of the clam whereas the fish fed ate balls of artificial food and it is more difficult that the contaminants access to the gut of the fish than in the case of clams.

Evaluations of histology of gills, liver, gut and kidney revealed clear differences between the negative control of toxicity and the GR2 station and the dilutions of toxic mud higher than 0.3%.

The application of PCA to the original 23 variables indicates that they can be grouped in four new factors. These new variables explain more than 93% of the total variance in the original data set. In the present study, we selected to interpret a group of variables as those associated with a particular component where loading were 0.35 or higher (Table 6), corresponding to an associated explained variance of more than 30%. This approximates Comreys (1973) cut-off of 0.6 for a good association between an original variable and a component and also takes into account discontinuities in the magnitudes of the loading of the original variables. The loading following varimax rotation for the four components are

Table 3
Summarized semi-quantitative results of lesions detected in microscopic abnormalities of individuals of the fish *S. senegalensis* exposed to environmental stations (GL2, GL6, GR2 and GR4) and during 15 and 30 days of exposure period

Organ	Parameter	Exposure time (days)	GL2	GL6	GR2	GR4
Liver	Lipid-like vacuoles	15	+	+	++	+
		30	+/–	+	++	+
	Hepatocellular anisocytosis	15	+	+	++	+
		30	+/–	+/–	++	+
	Hyperemic capillaries	15	–	–	+/–	+/–
		30	+/–	–	+	+/–
	Foci of cellular alteration	15	+/–	–	–	–
		30	–	+/–	+	+/–
	Hepatocellular shrinkage	15	–	–	+/–	+/–
		30	+/–	+/–	–	+/–
Gills	Clavate lamellae	15	–	–	+/–	–
		30	–	–	+	+/–
	Shortening of secondary lamellae	15	–	–	+/–	+/–
		30	–	–	+	+/–
	Epithelial lifting	15	–	+/–	–	–
		30	–	–	–	–
	Hyperplasia	15	+/–	+/–	–	+/–
		30	+	+	+	+
	Deformation of secondary	15	+/–	+/–	+	+
	lamellae	30	+/–	+/–	+	+/–
	Vascular congestion	15	–	–	+/–	–
		30	–	+/–	+/–	–
Gut	Increase of lipid content in en-	15	+/–	+/–	+	+
	terocytes	30	–	–	+	+/–
	Hyperplasia	15	–	–	+/–	–
		30	+/–	+/–	+	+/–
Kidney	Tubular epithelial necrosis	15	–	–	+/–	+/–
		30	–	–	+/–	+/–
	Lost of hematopoietic tissue	15	–	–	+/–	–
		30	+	+/–	+	+/–

Incidence of lesions: (–) absent, (+/–) sometimes, (+) frequent, (++) very frequent, (+++) always present.

Table 4
Summarized semi-quantitative results of lesions detected in microscopic abnormalities of individual of the clam *S. plana* exposed to toxic mud dilutions (0.3%, 1.8% and 7.9%) and to environmental stations (GL2, GL6, GR2 and GR4) during 14 days of exposure period

	Control	0.3%	1.8%	7.9%	GL2	GL6	GR2	GR4
Gills								
Hemocitary infiltration	–	+	++	+++	+/–	+/–	+	+
Fusion of lamellae	+/–	+	+++	+++	+/–	+	++	+
Lost of cell	–	+/–	+	++	–	+/–	+	+/–
Hypertrophya hyperplasia	–	+	+	+++	+/–	+/–	+	+/–
Necrosis	–	+/–	+	++	–	+/–	+/–	+/–
Gut								
Increase of lipid content in enterocytes	–	+/–	++	+++	–	–	++	+/–
Hyperplasia	–	+/–	++	++	–	+/–	+	+

given in Table 6. Each factor is described according to the dominant group of variables and based on their scores for each of the cases studied and that are shown in Fig. 3.

Factor 1. Toxicity associated with the heavy metals from the toxic mud originated by the accidental spill. This component, accounting for 66.54% of the total variance, has high positive loading on the heavy metals Zn, Cu, Pb, Cd and Fe and all the toxic responses, both lethal and sublethal, except the FLDIx obtained after 15 days of fish exposure. This new variable can be defined as the toxic responses of all the organisms to heavy metals from the Aznalcóllar mining spill bound to sediments. The factor score is only positive in the toxic mud

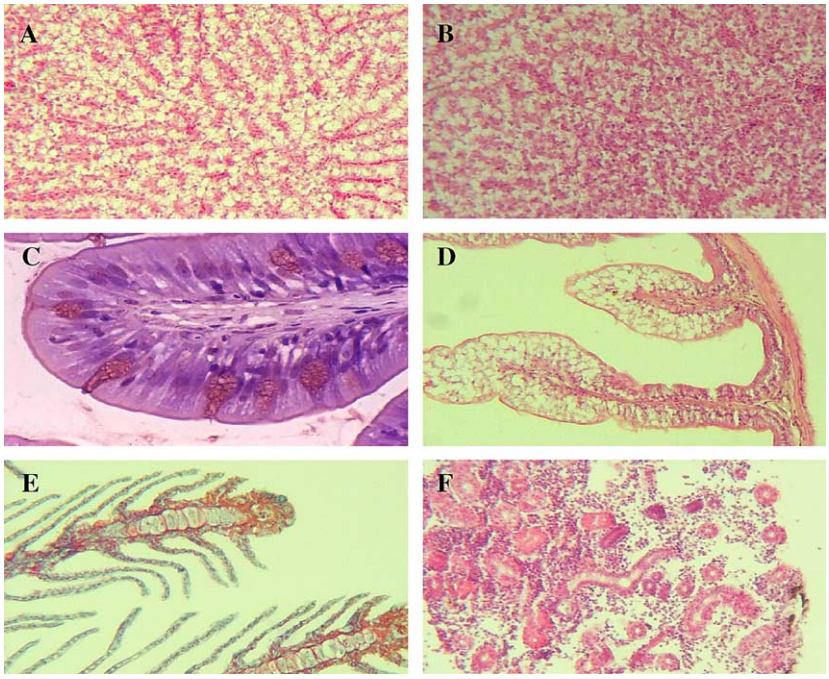

Fig. 1. Example of histological sections used to semi-quantify lesions (Tables 2 and 3) associated with contaminant bound to sediments used in the *S. senegalensis* sediment toxicity test. (A) Liver of untreated specimens, (B) Liver, toxic mud dilution of 20%, 15 days, (C) gut from control organisms, (D) gut, toxic mud dilution of 20%, 15 days, (E) gills, toxic mud dilution of 7.9%, 15 days, (F) kidney, toxic mud dilution of 20%, 30 days.

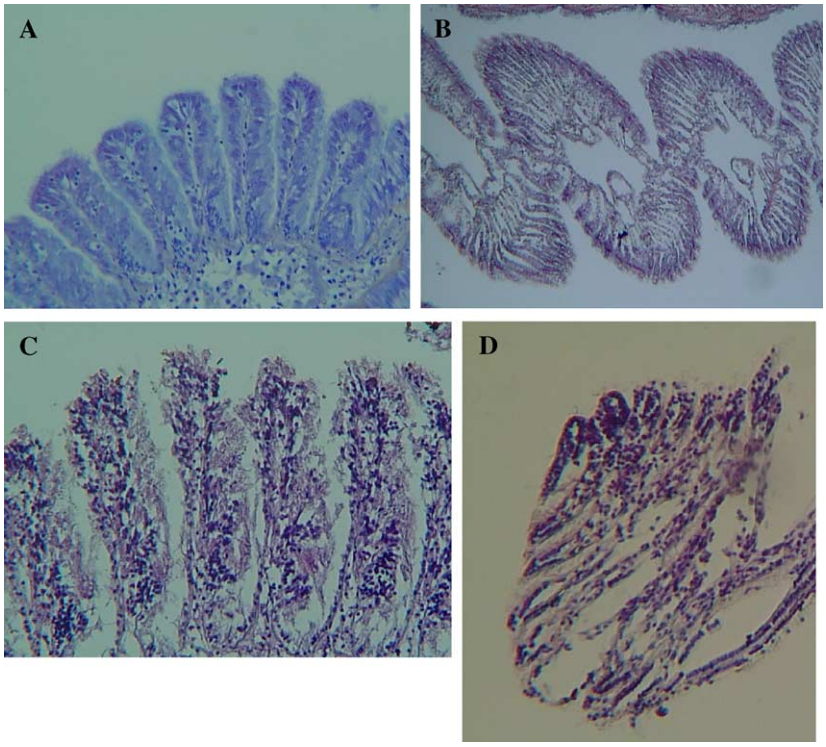

Fig. 2. Example of alterations caused by exposition of *S. plana* to sediments and controls and used to semi-quantify lesions (Table 4). (A) Clam gills exposed to 0.3% of dilution of toxic mud. (B) Clam gills exposed to sediments from environmental stations GR2. (C) Clam gills exposed to 1.8% of dilution of toxic mud. (D) Clam gills exposed to 7.9% of dilution of toxic mud.

Table 5
Summarized results of the general indexes of tissue damages (dimensionless) derived from the original lesions measured in clams (index of gill damages –CGDIx-, index of gut damages –CUDIx-) and fish (index of gill damages –FGDIx-, index of liver damages –FLDIx-, index of gut damages –FUDIx-, and index of kidney damage –FKDIx-) during their exposition to negative control of toxicity (C), environmental stations (GL2, GL6, GR2, GR4), and toxic mud dilutions (0.3%, 1.8%, 7.9%, 20% 32%)

Index	C	GL2	GL6	GR2	GR4	0.3	1.8	7.9	20	32
CGDIx	0.10	0.30	0.60	1.10	0.70	0.80	1.60	2.60	–	–
CUDIx	0.00	0.00	0.25	1.50	0.75	0.50	2.00	2.50	–	–
FGDIx-15d	0.00	0.17	0.25	0.42	0.33	0.33	0.75	0.83	1.25	2.25
FGDIx-30d	0.08	0.25	0.33	0.75	0.42	0.50	1.00	1.67	2.17	3.00
FLDIx-15d	0.00	0.40	0.40	1.00	0.60	0.90	1.10	1.10	2.20	3.00
FLDIx-30d	0.30	0.40	0.50	1.20	0.70	0.50	1.50	1.80	2.40	3.00
FUDIx-15d	0.25	0.25	0.25	0.75	0.50	0.75	1.50	2.00	2.50	3.00
FUDIx-30d	0.25	0.25	0.25	1.00	0.50	0.75	1.00	1.25	2.00	3.00
FKDIx-15d	0.00	0.00	0.00	0.50	0.25	0.50	1.00	1.00	1.50	2.50
FKDIx-30d	0.25	0.50	0.25	0.75	0.50	0.50	1.00	2.00	2.00	3.00

Clam lesions measured at toxic mud dilution of 20% or higher were not established due to the mortality of all the individuals exposed.

Table 6
Sorted rotated factor loading (pattern) of 23 variables on the six principal factors

% Variance	Factor 1	Factor 2	Factor 3	Factor 4
	66.54	12.41	10.19	4.75
Zn	0.912	–	–	0.378
Fe	0.651	−0.660	–	–
Cd	0.820	–	–	0.484
Pb	0.866	0.381	–	0.408
Cu	0.921	–	–	–
Mn	–	−0.937	–	–
PAHs	–	0.926	–	–
Aroclor 1242	–	–	–	–
Aroclor 1260	–	–	−0.849	–
O.C.	–	0.597	0.677	–
Grain size	–	–	0.963	–
Mortality	0.529	–	–	0.794
$TL50^{-1}$	0.547	–	–	0.671
CGDIx	0.691	–	–	0.694
CUDIx	0.560	–	–	0.764
FGDIx-15d	0.492	–	–	0.852
FGDIx-30d	0.702	–	–	0.689
FLDIx-15d	–	–	–	0.920
FLDIx-30d	0.585	–	–	0.755
FUDIx-15d	0.603	–	–	0.726
FUDIx-30d	0.442	–	–	0.777
FKDIx-15d	0.381	–	–	0.846
FKDIx-30d	0.801	–	–	0.568

The loading matrix has been rearranged so that the columns appear in decreases order of variance explained by factors. Only loading greater than 0.35 are shown in table. Factors (#) are numbered consecutively from left to right in order of decreasing variance explained.

dilution of 7.9% being negative in the rest of the dilutions, environmental stations and in the negative control. Note that the metal Fe is considered not toxic but representative of the geochemical matrix of the sediment studied. Its association in this factor is related to the origin of the toxic mud which was a pyrite mine reach in this metal.

Factor 2. Contaminant and organic concentrations not associated with toxic responses. This factor accounts for 12.41% of the total variance and groups the concentration of PAHs, the organic carbon concentration and the heavy metal Pb with positive loading and the concentration of Fe and Mn with negative loading. It could be related to the presence of PAHs and the trend of the behaviour of organic carbon concentration inverse to the geochemical matrix of sediments defined by Fe and Mn. It does not group any of the toxic responses measured in the different sediment toxicity tests. The factor score shows only positive values in control and in the toxic mud dilutions, decreasing when the amount of toxic mud increases.

Factor 3. Relationship between organic carbon and grain size. This factor group the organic carbon con-

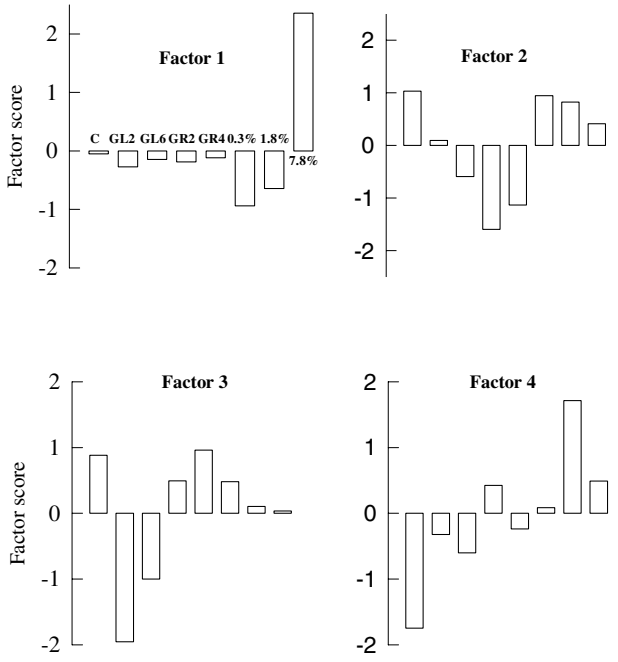

Fig. 3. Estimated factor scores for the four factors in each of the eight cases (one control, C, for environmental stations, GL2, GL6, GR2, GR4, and three dilutions of toxic mud, 0.3%, 1.8%, and 7.9%) to the centroid of all cases for the original data. The factor scores quantify the prevalence of every factor for each station and is used to establish the description of each factor.

centration, the grain size with positive loading and the Aroclor 1260 concentrations with negative loading, counts for 10.19% of the total variance. This factor can be related to the relationship between organic carbon and grain size in sediments, and not includes any of the toxic responses measured. It shows factor scores positive in the negative control, stations GR2 and GR4 and toxic mud dilutions (decreasing when the amount of toxic mud increases).

Factor 4. Acute toxicity in environmental stations associated with the Aznalcóllar mining spill (Cd, Zn and Pb). This factor accounts for 4.74% of the total variance and groups the concentration of the heavy metals Cd, Pb and Zn together with all the toxic responses measured at both lethal and sublethal responses. It should be noted that some of the responses (mortality of amphipods among others) shows higher loading in this factor than in the factor 1 that was associated with sediment toxicity from the toxic mud dilutions. The definition of the acute toxicity is based on the positive scores of this factor that is measured only in one of the environmental stations (GR2) and in all the toxic mud dilutions although for the lowest (0.3%) it is about 0. Besides, the low variance explained by this factor confirms the results reported by previous studies in the area (DelValls et al., 1999; Gómez-Parra et al., 2000; Riba et al., 2002a,b,c) that although the heavy metals from

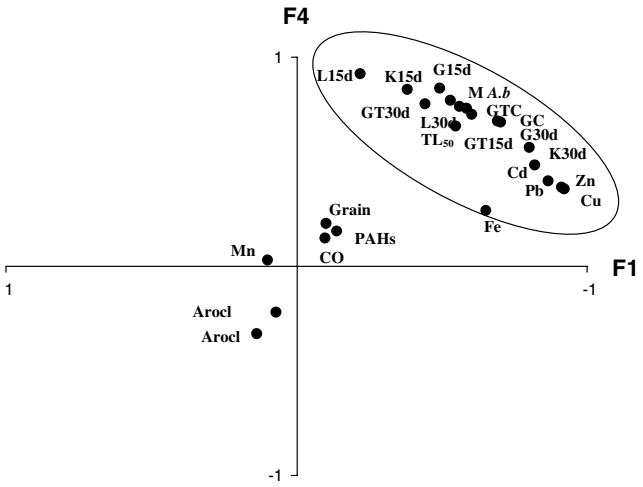

Fig. 4. Results of the multivariate analysis approach (MAA) used for distribution of the original variables in the space defined by factors correlating contamination and biological effects (F1 66.5% and F4 4.8%). Variables included in the ellipse are grouped to show the correlation between acute and chronic biological effects and contaminants.

the accidental spill impacted the estuary during the first days of the accident they affected the ecosystem acutely and in specific areas of the estuary.

In Fig. 4 is shown the ordination of the original variables in the space defined by factors 1 and 4 which grouped chemical concentrations and toxicity endpoints. The other two factors (2 and 3) were not shown in the figure because they not grouped original variables correlated with biological effects. In the figure it is shown that biological effects are correlated to heavy metal concentrations of Cd, Pb, Zn and Cu, including in this correlation the metal Fe which is associated with the geochemical origin of the toxic material (mine pyrite material) but not to the toxic effect. From this ordination it is also shown that the influence of the grain size, organic carbon and the contamination associated with PAHs and PCBs is not correlated with the biological effects measured in this study.

4. Conclusions

The difference in the frequency of the lesions for different tissues in fish compared to clams establishes that the heavy metals bound to sediments affected in a different way to the organisms used in the assays depending in their feeding habits. The clams get the food through the overlain water filtration and show higher frequency of lesions in both tissues (gills and gut) whereas the fish directly get in contact with overlain water through gills but these heavy metals have more difficulties to reach the gut than in the case of clams. In

this sense, the use of both organisms permit to identify the extension of the biological effects associated with the heavy metals bound to the sediments from the mining spill. The comparison of these lesions to those reported in the area in field collected organisms have shown that the impact of the spill although acute could provoke a sequel in the estuary that should be monitorized in the next years.

The comparison between the different toxic responses, lethal and sublethal and between the different organism responses determines an useful battery of sediment toxicity test to determines the effects associated with the mining spill. Both kinds of responses, mortality measured in the amphipod test and histopathological lesions in fish and clams have shown sensitivity enough to identify 'grey' zones of concern in the estuary. However, the easier performance (faster, cheaper, etc.) of the amphipod compared to the other two tests have determined its selection to the monitoring of the biological effects provoked by the accidental spill in the estuary during the last three years (Riba et al., 2003).

The estuary was impacted by the accidental spill showing an enrichment of the metals from the mine that produced biological effects in different organisms typically occurring in the area. Only one station of the studied site showed lethal toxicity to amphipods and sublethal toxicity to clams and fish considering the impact of the mining spill on the Guadalquivir estuary as acute. Further studies under an integrated point of view comparing the effects measured in this work and associated with the mining spill to the sediment quality in areas affected by chronic contamination originated by mining activities are recommended. These studies need to address the biological effects under field conditions to confirm the recovery in the estuarine environmental conditions previous to the spill. Besides, the comparison to other areas contaminated by mining activities but with a different kind of heavy metal impact, the area of Huelva is considered highly and chronically contaminated by heavy metals, could permit to compare the difference between a chronic and an acute impact originated by mining spills.

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