Bioavailability of heavy metals bound to estuarine sediments as a function of pH and salinity values

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ABSTRACT

The role of two key-variables such as pH and salinity in chemical speciation and bioavailability of heavy metals originated by mining activities bound to sediments was evaluated under estuarine conditions. Two sediment samples collected in two estuaries in southern Spain (Ría of Huelva and Guadalquivir estuary) together with dilution of toxic mud from the Aznalcóllar mining spill (April, 1998) were used to determine their chemical speciation and bioavailability at different pH (6.5, 7.5, 8.5) and salinity (10, 20, 35) values using the estuarine clam *Ruditapes philippinarum*. The chemical speciation was established by means of measurements of the mobilization of heavy metals from sediments to waters and determining in it pH, salinity, alkalinity and heavy metal concentration. The geochemical model MINTEQA2 was used to establish the thermodynamic species in the assays. To assess the bioavailability of the heavy metals the concentration of metallothioneins in the clam tissues and the mortality of this organism was measured at different pH and salinity values. The influence of both salinity and pH was detected in the chemical behavior of metals and in their associated biological responses established by metallothioneins and the percentage of mortality. At low values of both variables (pH=6.5 and S=10), the biological effects were highest, and it was related to the free ion Zn^{2+}.

Keywords: sediment quality, Gulf of Cádiz, estuaries, metallothionein, *Ruditapes philippinarum*.

INTRODUCTION

Sediment contamination in estuaries and its biological effects need to be properly and fully assessed. Recent papers recommend conducting specific studies to determine the effects of pH and salinity on bioavailability of contaminants bound to estuarine sediments (Chapman and Wang, 2001). Trace metals are among the most common contaminants bound to estuarine sediments. The bioavailability of these metals to aquatic organisms is dependent on the physical and chemical forms of the metal (Luoma and Bryan, 1983; Vercauteren and Blust, 1996).

Many metal species show little or no bioavailability, *e.g*. metals adsorbed on particulate matter, carbonate complexes and metal complexes with strong chelating agents (EDTA) although still some debate about it (Allen and Hansen, 1996). Some authors have proposed that the bioavailability of a dissolved metal is related to its free ion activity (*e.g*., Morel, 1983). The first step in the uptake of metals by an organism comprises the transport of the cation through the plasmatic membrane. For many dissolved metals, entry into mollusks involves passive diffusion, perhaps facilitated by carrier molecules, and passage along a gradient maintained by intracellular ligands of increasing binding strength (Langston *et al*., 1998). This lipidic membrane is a barrier to the simple diffusion of ions due to its hydrophobic nature, and this transport is usually assumed to be mediated by transmembrane proteins with complex-

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ing groups that bind the metals (Simkiss and Taylor, 1995). Metallothioneins (MTs) are low-molecular-weight, cysteine-rich metal-binding proteins which have been shown to occur in most zoological taxa (Langston *et al*., 1998). It is generally considered that these proteins play a primary role in the homeostasis of essential metals such as Cu and Zn. However, non-essential metals, particularly Cd, are also able to induce MTs synthesis and according to their respective affinities for this ligand (Hg>Ag>Cu>Cd>Zn), to displace essential metals from pre-existing metallothioneins (Stillman *et al*., 1999).

The Free Ion Activity Model (FIAM) assumes, first, the diffusion of the cation from the bulk solution to the membrane surface and that the kinetic of the reaction between the ligand and the metal are rapid and non-limiting compared with the transport process through the membrane; and secondly, that the carrier is in excess and there is thus no saturation (Campbell, 1995). Taking into account these assumptions and the chemical reactions between the metal ion and the ligands present both in the solution and in the carrier protein, it can be demonstrated that the uptake is related only to the free ion activity of the metal. Nevertheless, changes in the composition and conditions of the medium may modify the structure of the plasmatic membrane and the number of available ligand groups present in the carrier proteins, hence affecting their chemical speciation and their bioavailability (Van Ginneken *et al*., 1999).

Because estuaries provide an interface between fresh and salt waters, they have strong gradients in many physical and chemical variables, including salinity, pH, dissolved oxygen, temperature, nutrients and amount and composition of particles. Unlike freshwaters, where pH is the controlling factor, in estuaries salinity is the controlling factor for the partitioning of contaminants between sediments and overlying or interstitial waters and both are the key-variables to control the bioavailability, and the toxicity of heavy metals bound to sediments.

The main objective of this paper is to assess the influence of pH and salinity values on the chemical speciation and bioavailability of heavy metals (Zn, Pb, Cd, Cu) bound to estuarine sediments using a ‘true estuarine species’ such as *Ruditapes philippinarum* simulating the typical values recorded for pH and salinity in most of the estuaries (pH ranged between 6 and 8 and salinity ranged between 10 and 35). Besides, this paper is a continuation of previously reported results (Riba *et al*., 2002a) about differences in toxicity using the same specie of clams and related to the total concentration of heavy metals in sediments and waters. In this sense, this paper addresses the influence of the chemical speciation of metals on the toxicity of *Ruditapes philippinarum* using a complementary and sublethal biological response such as metallothionein (MT).

METHODOLOGY

Sampling and experimental design

To establish the mobilization of heavy metals bound to sediments and to determine their chemical speciation in water and their bioavailability to clams we have used different nature sediment samples: different percentages of toxic mud collected near a pyrite-zinc mine were diluted using a clean sediment located in the Bay of Cádiz (0.3 and 7.9% dry weight of toxic mud), the procedures of dilution were previously reported by Riba *et al.* (2002b); two environmental stations collected in estuaries from the Gulf of Cádiz affected by different heavy metal contamination, Ría de Huelva (H) with a chronic origin of contamination and Guadalquivir estuary (GR) impacted by an acute event of contamination (Aznalcóllar mining spill, April, 1998) (Riba *et al*., 2002c). Figure 1 shows the location of the sites selected to collect the sediment samples. The sediment samples were collected using a 0.025 m^2 Van Veen grab and transferred to a cooler where it was homogenized until no textural differences were detected.

These sediment samples were used to expose individuals of the clam *Ruditapes philippinarum* as described below.

Two sediment bioassays were developed using the clam *Ruditapes philippinarum*. One based on different values of salinity (10, 20, 35) and the other one based on the use of different values of pH (6.5, 7.5, 8.5). The overlaying water was adjusted at the desired salinity and pH values as described by Riba *et al*. (in press). The test using the clam *Ruditapes philippinarum* was carried out in whole sediment. The sediment toxicity tests were developed using 15 L vessels containing 2 L of sediment overlaid with filtered seawater adjusted previously at the different salinities and pH and using a 1:4 v/v sediment:water relation. The pH, salinity, temperature (20°C) and the concentration of dissolved oxygen (>5 mg L^{-1}, 60% saturation) were measured and controlled every day.

Forty clams per tank previously acclimated to the different salinity and pH values (15 days) were used during 10 days at the assay conditions. Two different endpoints were selected to assess the toxic effects associated with the mobilization of metals (Zn, Pb, Cd, Cu): the percentage of mortality and the metallothionein levels in the soft body of the survival individuals at the end of the experiment.

Chemical analysis

Specimens of *Ruditapes philippinarum* of each tank were dissected out, and the soft body (pools of ten clams per replicate of each station) were taken and stored at –80°C until their processing. Samples were homogenized in a Potter–Elverhjem at a ratio 3-5 w/v in ice cold buffer 100 mM Tris-HCl/Base pH 8.1 (at

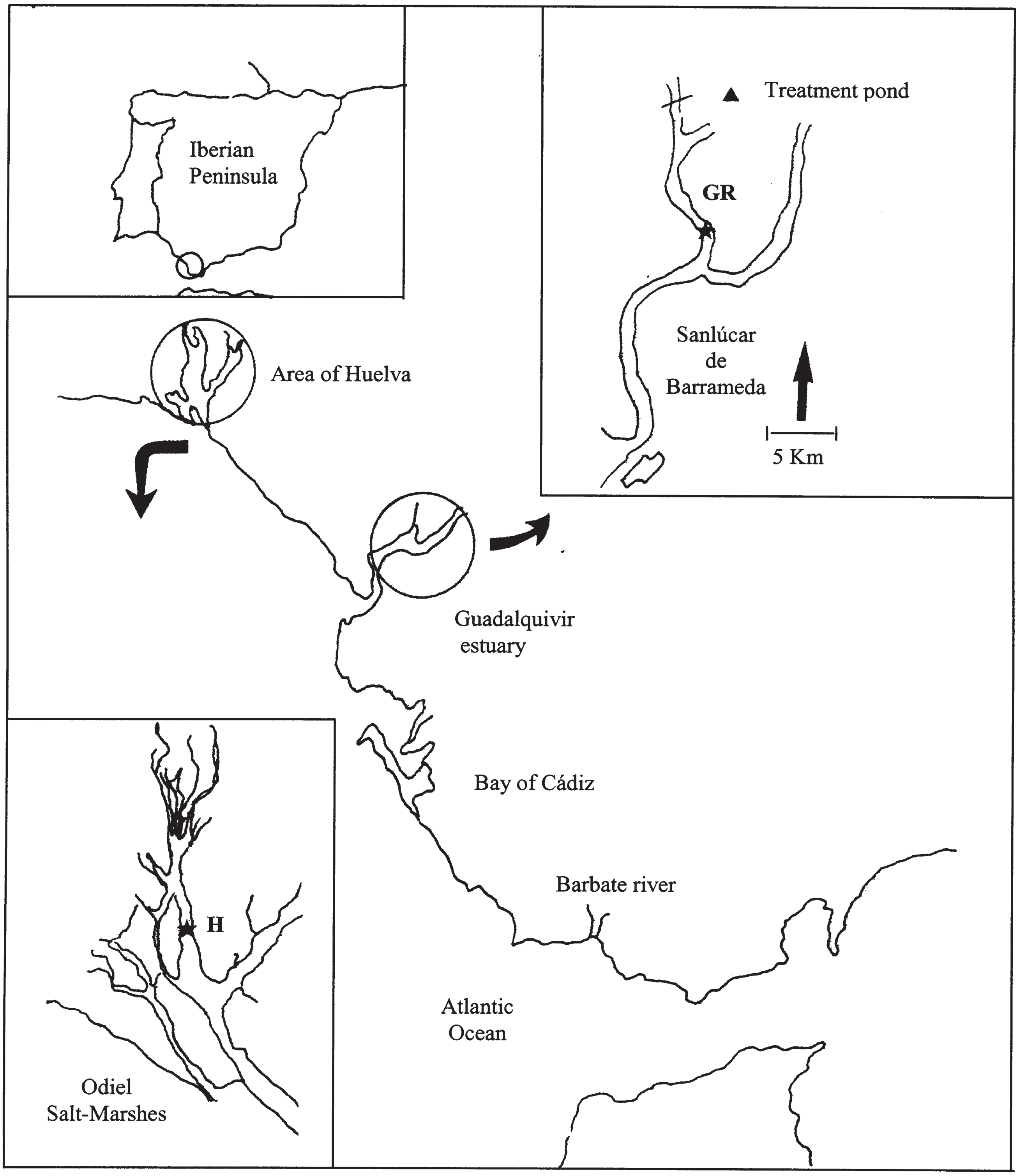

Figure 1 *Map of the studied area in the south of Spain showing the location of the environmental samples selected in the estuarine ecosystem, Ría de Huelva (H) and Guadalquivir estuary (GR).*

4°C) with 1 mM DTT. The homogenate was centrifuged at 50.000 g at 4°C for 120 min. The supernatant was heat-treated at 95°C for 4 minutes transfer immediately to a cool on ice. The samples were centrifuged at 10,000g for 15 min at 4°C. The supernatant was employed to quantify metallothionein levels using differential pulse polarography, according to the procedure described by Olafson and Olsson (1991). In the absence of a clam MT Standard, quantification of MT concentration in the cytosol of the soft body was based on rabbit liver MT, (MT-I), levels of MT are expressed as microgram per gram of protein. Total protein concentrations were measured by the Bradford method (1976) using BSA (bovine serum albumin) as reference standard material.

Water samples were taken of both, pH and salinity bioassays to determine the speciation of the metals in solution as a result of competition between different metal complexes, metal chelates and free metals ions and to relate this processes to metal bioavailability.

The total concentration of heavy metals (Cd, Pb, Zn, Cu) was carried out in water samples at different salinity and pH values using a differential pulse anodic stripping voltametry (DPASV). Measurements were taken with static drop mercury electrode (SMDE), using the Metrohm 693 processor as reported by Ponce *et al*. (2000). Briefly, the water samples were filtered through nuclepore 0.4µm polycarbonate filters (Millipore, HTTP047) and acidified using HNO_3 (Merck, Suprapur) to pH < 2, then samples were digested using UV radiation (6 h) at 85ºC (Metrohm, 705UV) and finally analyzed by DPASV. The heavy metal speciation in solution was determined at the different conditions using the geochemical equilibrium model MINTEQA2 (Allison *et al*., 1991). The modeling was based on chemical analysis of the next parameters in the water solutions: metals, temperature, salinity, pH, alkalinity and estimated concentrations of majority elements (SO_4^{2-}, Cl^-, K^+, Na^{2+}, Mg^{2+}, Br^-, F^-) from the two different bioassays and for each station.

The salinity was determined by means of an induction salinometer (Beckman RS-10). The total alkalinity and the inorganic carbon speciation in the samples was determined using a potentiometric analyzer (Metrohm 670) with glass combination electrodes (Metrohm, ref. 6.0210.100) as described by Forja *et al*. (in press). Briefly, the water quantities analyzed were 100 ± 0.001 g. The analyses were performed at constant temperature in a water jacketed open cell and they were cross-checked using an alkalinity reference material provided by Dr Andrew Dickson at Scripps Institution of Oceanography (Batch #33). The results for six samples gave an average value of 2010.6±2.9 µM for total inorganic carbon, while the certified value was 2009.85 ± 0.85 µM. This accuracy and precision is good enough for the inorganic carbon calculation in the used systems. The titration was carried out with 0.2M HCl. The inorganic carbon speciation has been calculated with the pH (NBS scale) and the total alkalinity values and using the dissociation constant reported by Mehrbarch *et al*. (1973).

The total concentration of SO_4^{2-}, Cl^-, K^+, Na^{2+}, Mg^{2+}, Br^-, F^- has been estimated by the salinity values and considering the stoichiometry of the major solute components and based on the assumption that the relative composition of sea water is constant (Millero and Sohn, 1992).

Data treatment

The biological adverse responses (percentage of mortality and the induction of the metallothioneins) from the replicates of the tests and the controls were compared using ANOVA and Scheffe's *F* tests to identify significant differences in sensitivity between mediums and controls ($P < 0.05$).

To link the set of data obtained, the original variables from chemical concentrations (total and free ion), percentage of mortality and induction of MTs were analyzed by factor analysis, using principal components (PC) as the extraction procedure, which is a multivariate statistical technique to explore variable (chemical concentration $n = 8$; toxicity data, $n = 2$) distributions. The factor analysis was performed on the correlation matrix, *i.e*., the variables were autoscaled (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).

RESULTS AND DISCUSSION

Mobilization of Zn, Cd, Cu and Pb

To determine the mobilization of the heavy metals from the sediment to water a partitioning coefficient β defined as the ratio of a heavy metal concentration in the water to that measured in the sediment was calculated and shown as a function of pH and salinity values in Figures 2 and 3 respectively. The units of β are kg L^{-1}, although for convenience it is assumed that a volume of 1 L sea water is equivalent to a mass of 1kg of sediment, rendering β dimensionless. To facilitate the interpretation of these results the β values have been shown in the Figures 2 and 3 as multiplied by a factor of 1000. Low values of this parameter are associated with a low mobilization of a determined metal for a station and vice versa. During the pH assay (Figure 2) the highest values of β (Cd, 96.6; Pb, 0.85; Cu, 20.3; Zn, 25.4) are calculated at the lowest pH value (6.5) and measured in the highest dilution of toxic mud (7.9%). It informs that the heavy metals bound to the toxic mud are more easily mobilized from sediments to water than those from the environmental samples. A similar pattern was determined for all the metals increasing their β with the decrease of the pH values. The acidification of the samples tends to liberate those metals less strongly associated with sediments to the overlaying water and potentially with a higher bioavailability.

In the salinity bioassay (Figure 3) the highest values of β were measured in different stations and depending on the analyzed metal. In general, the lowest values of β were obtained in waters exposed to sediment collected in environmental stations (C, H and GR). The

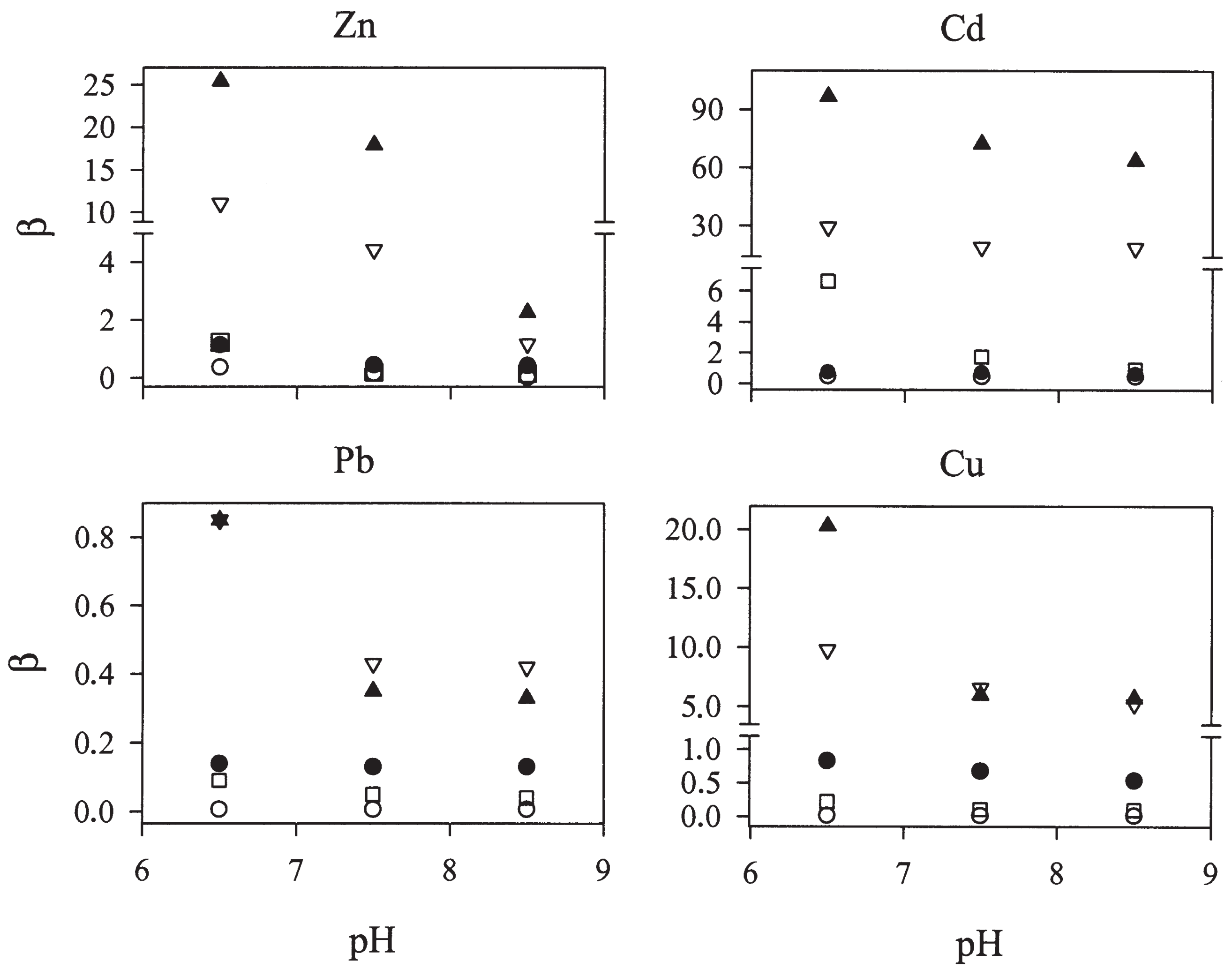

Figure 2 *β (10^3) values defined as the ratio of the total concentration of a determined metal in water divided by the total concentration in sediment of all the stations, Bay of Cádiz (●), toxic mud 0.3% (▽), toxic mud 7.9% (▲), Ría of Huelva (○) and Guadalquivir estuary (□), at different pH values used in the bioassay. (β are dimensionless).*

mobility of heavy metals from the sediment to the water when the salinity varies will depend on the relative importance of the two counteractive processes (Chapman and Wang, 2000): (1) desorption due to increase complexation with sea water anions (Cl^- and SO_4^{2-}) and/or increasing competition for particle sorption sites with sea water cations (Na^+, K^+, Ca^{2+}, Mg^{2+}); (2) coagulation, precipitation and flocculation. In this sense, βs for metals may decrease, increase or be constant depending on the nature of the sediments and the estuarine conditions. The values of β for Cd decrease for all stations when salinity decrease so this metal tends to be more efficiently trapped by low-salinity sediments and its concentration in water is higher at high salinity than at low salinity values. For this metal the highest value (198) was derived at the highest salinity value (35) in the highest toxic mud dilution (7.9). The mobilization for Pb, Cu and Zn are similar to Cd in control sediments (β, Figure 3) and in sediments from the Ria of Huelva, being different in the dilutions of toxic mud and in the sediments from the Guadalquivir estuary (GR), in which βs increase when salinity decrease. In this sense, metals from those sediments are more easily mobilized to the water than in the other stations.

In general, high values of β are calculated for heavy metal concentrations measured at low salinity (10) and low pH (6.5) tested in the bioassay, except for Cd in the salinity bioassay. The bioavailability of these metals could be higher at those low values than at higher values of salinity and pH.

Chemical speciation

Computer speciation modeling allows insights into all aspects of a system dealing with the effects of numerous metals and ligands present and is able to optimize the effects of concentration and other environmental variables such as pH and salinity (Davidge *et al.*, 2001). The environmental effect of the heavy metals is strongly

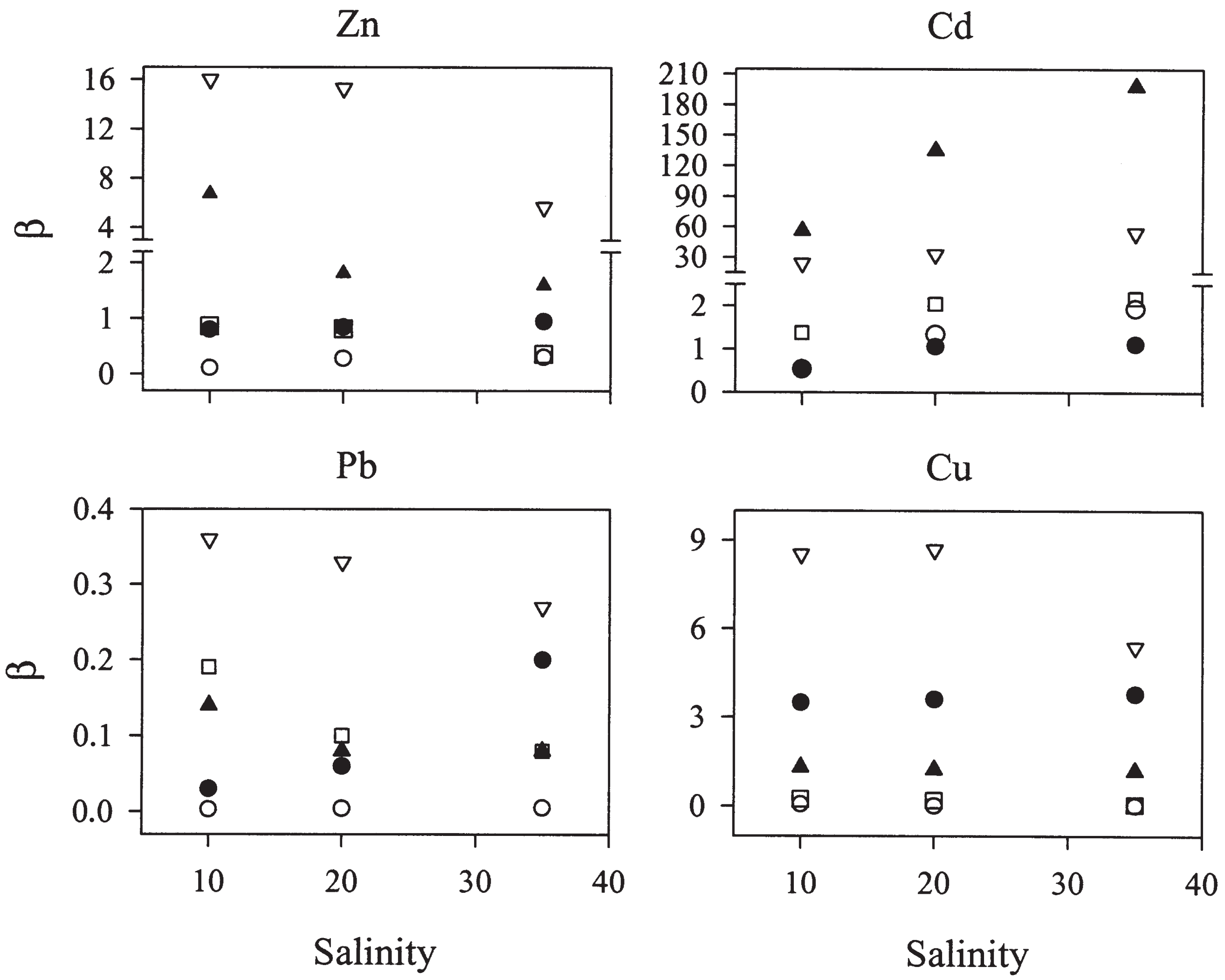

Figure 3 *β (10^3) values defined as the ratio of the total concentration of a determined metal in water divided by the total concentration in sediment of all the stations, Bay of Cádiz (●), toxic mud 0.3% (▽), toxic mud 7.9% (▲), Ría of Huelva (○) and Guadalquivir estuary (□),at different salinity values used in the bioassay. (β are dimensionless).*

dependent on the chemical speciation since it will affect the bioavailability and, hence, the mortality provoked by the metals on the clams used in the bioassays.

The output of the computer modeling, using the water-ligand model MINTEQA2 are shown in the Figure 4 related to the four heavy metals at different pH and for the control (C), toxic mud dilutions (0.3% and 7.9%) and the two environmental sediment samples (H and GR). The chemical species that associates the mobilized heavy metal are for cadmium: Cd^{2+}, $\Sigma CdOH$, $\Sigma CdCl$, $\Sigma CdSO_4$, $\Sigma CdCO_3$; for the heavy metal copper: Cu^{2+}, $\Sigma CuOH$, $\Sigma CuCl$, $\Sigma CuSO_4$, $\Sigma CuCO_3$, $Cu_2(OH)_2^{2+}$, for the heavy metal Zn: Zn^{2+}, $\Sigma ZnOH$, $\Sigma ZnCl$, $\Sigma ZnSO_4$, $\Sigma ZnCO_3$, ZnOHCl and for the heavy metal lead: Pb^{2+}, $\Sigma PbOH$, $\Sigma PbCl$, $\Sigma PbSO_4$, $\Sigma PbCO_3$. All the chemical species affected by the symbol "Σ" are referred to the sum of all the associations of the metal with the charges available for each ligand.

In general, acidification of the assay provokes an increase of the free ion for all the metals at all the station, except for Cd and Pb in sediments collected in the Guadalquivir estuary. Furthermore, the increase of the free ion specie is specially detected in Zn and Cu in the highest dilution of toxic mud, being the predominant chemical specie for Zn. A difference between the speciation of Zn and Cu is related to the trend in the percentage of free ion with the increase of the toxic mud amount in the dilutions. The free ion formation of Zn increases with the amount of toxic mud diluted, whereas for Cu it decreases. In this sense, the chemical speciation of copper is more related to the mobilization of Cu from sediments in the Bay of Cádiz than that determined by the toxic mud. For the heavy metal Cd and Pb the chemical specie predominant in all the samples was ΣCdCl and ΣPbCl.

Summarized results of the output of the computer

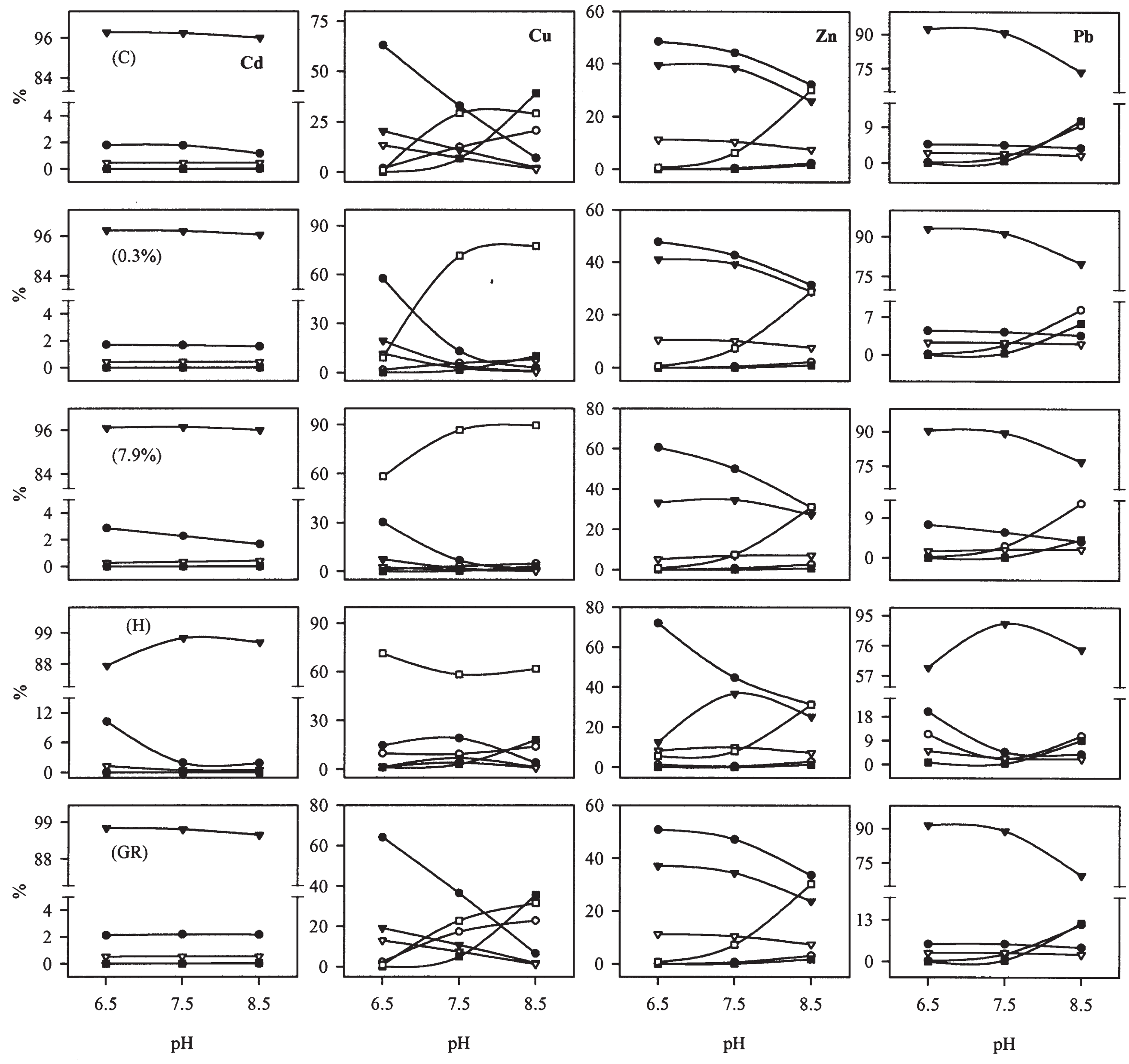

***Figure 4**. Diagrams of chemical speciation of heavy metals Cd, Cu, Zn and Pb bound to sediments studied, Bay of Cádiz (C), toxic mud dilution (0.3% and 7.9%), Ría of Huelva (H) and Guadalquivir estuary (GR) as a function of pH values. Chemical species (● Free metal, ○ ΣMOH, ▼ ΣMCl; ▽ ΣMSO_4; ■ ΣMCO_3; □ $Cu(OH)_2^{2+}$ and ZnOHCl) are expressed as percentage of the total dissolved concentration of the metals mobilized from the sediments assayed.*

modeling are shown in Figure 5 related to the four heavy metals at different salinity values for the control (C), toxic mud dilutions (0.3% and 7.9%) and the two environmental sediment samples (H and GR). The chemical species that associates the mobilized heavy metal are for cadmium: Cd^{2+}, $\Sigma CdOH$, $\Sigma CdCl$, $\Sigma CdSO_4$, $\Sigma CdCO_3$; for the heavy metal copper: Cu^{2+}, $\Sigma CuOH$, $\Sigma CuCl$, $\Sigma CuSO_4$, $\Sigma CuCO_3$, $Cu_2(OH)_2^{2+}$, for the heavy metal Zn: Zn^{2+}, $\Sigma ZnOH$, $\Sigma ZnCl$, $\Sigma ZnSO_4$, $\Sigma ZnCO_3$, ZnOHCl and for the heavy metal lead: Pb^{2+}, $\Sigma PbOH$, $\Sigma PbCl$, $\Sigma PbSO_4$, $\Sigma PbCO_3$. All the chemical species affected by the symbol "Σ" are referred to the sum of all the associations of the metal with the charges available for each ligand.

In general, the concentration of the free ion for all the metals increases with the decrease of the salinity values, except for copper in all the station but the highest toxic mud dilution. In this dilution, the percentage of this specie still low and about the 18% of the total copper in solution at a value of 10 for salinity. The highest amount of the free ion is associated with the heavy metal Zn in the highest percentage of toxic mud

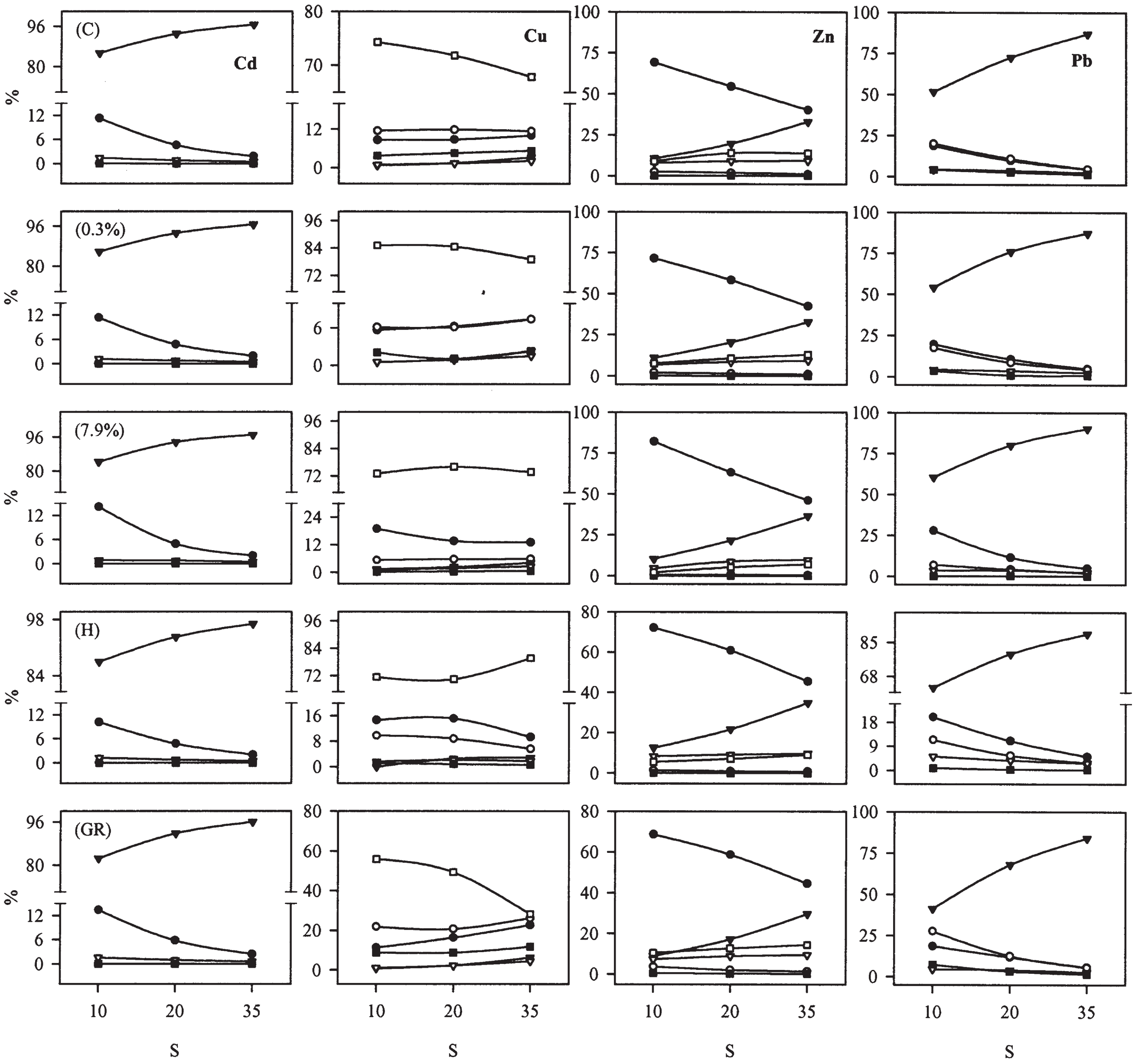

Figure 5 *Diagrams of chemical speciation of heavy metals Cd, Cu, Zn and Pb bound to sediments studied, Bay of Cádiz (C), toxic mud dilution (0.3% and 7.9%), Ría of Huelva (H) and Guadalquivir estuary (GR) as a function of salinity values. Chemical species (● Free metal; ○ ΣMOH; ▼ ΣMCl; ▽ ΣMSO_4; ■ ΣMCO_3; □ $Cu(OH)_2^{2+}$ and ZnOHCl) are expressed as percentage of the total dissolved concentration of the metals mobilized from the sediments assayed.*

diluted being higher than 80% of the total dissolved Zn. Besides, the concentration of the free ion of this metal increases with the toxic mud dilution. Concentrations of this free ion in control, lowest toxic mud dilution (0.3%), and the two environmental sediment samples are in a similar range and about 70% of the total concentration of dissolved Zn. A similar behavior is detected for the heavy metals Pb and Cd but as in the case of copper they are in a percentage lower than 20% appearing as free ions. The chemical species associated with these two metals are predominantly related to the species ΣCdCl and ΣPbCl.

Bioavailability of metals

The bioavailability of the mobilized heavy metals was determined by means of the induction of the metal-binding proteins (MTs) and by the percentage of the mortality in the two bioassays after ten days of exposure time.

The percentage of survival in the different sediment bioassays at different pH and salinity values is shown in Figure 6. The mortality at highest dilution of toxic mud was always significantly different compared to those measured in control at any pH and salinity values. Significant differences are associated with the toxic mud dilution of 0.3% for pH values of 6.5 and for salinity values of 10. For pH and salinity values higher than 6.5 and 10 respectively the mortality at this dilution was not significantly different to control.

In Figure 7 shows the summarized results of the concentration of MTs measured in the total body of the individuals exposed to control (C), toxic mud dilution (0.3% and 7.9%) and the two sediments from the estuaries (Ria of Huelva (H) and Guadalquivir (GR)). Significant differences in the induction of the MTs were measured among treatments and control and among pH and salinity assays. Results obtained in the pH bioassay show that the induction of MTs are significantly different at any pH treatment related to the individuals exposed to the highest toxic mud dilution. For this treatment the induction of MTs increases with the increase of the proton concentration, being highest at the lowest pH value assayed (6.5). The other toxic mud dilution (0.3%) shows only significantly different in the induction of MTs measured in clams compared to those measured in control associated with the lowest pH value (6.5). The environmental stations were not significantly different than the results obtained in the control at any pH assayed.

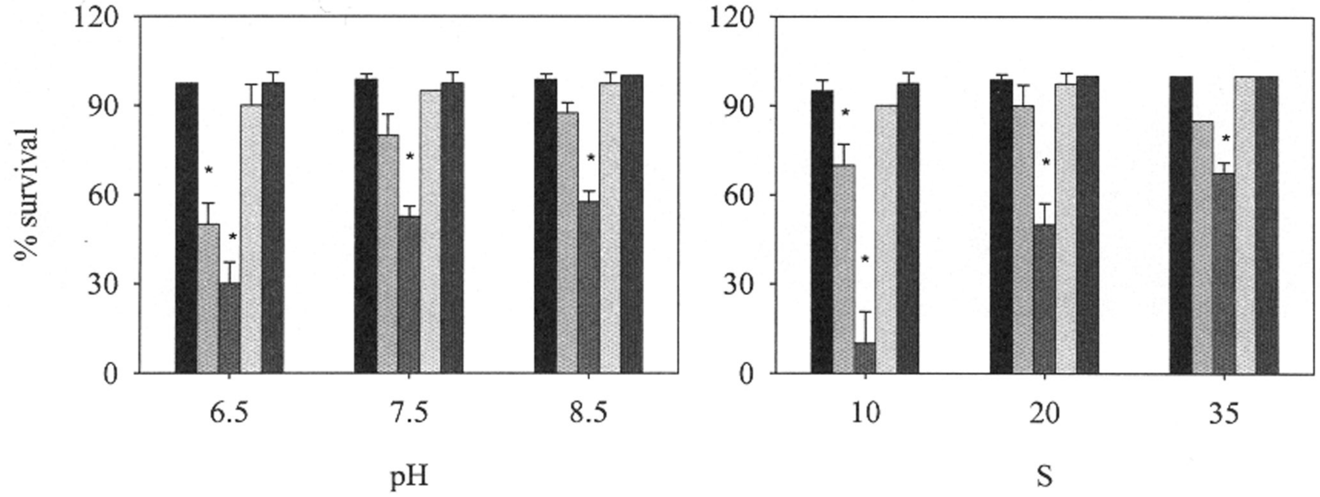

Figure 6 *Histogram of percentage of clam survival measured in the pH and in the salinity bioassays. Asterisks show those survival significantly different (*P*<0.05) to those measured in control. Stations ordered from left to right in each pH and salinity values were: Bay of Cádiz (C), toxic mud dilution (0.3%), toxic mud dilution (7.9%), Ría of Huelva (H) and Guadalquivir estuary (GR).*

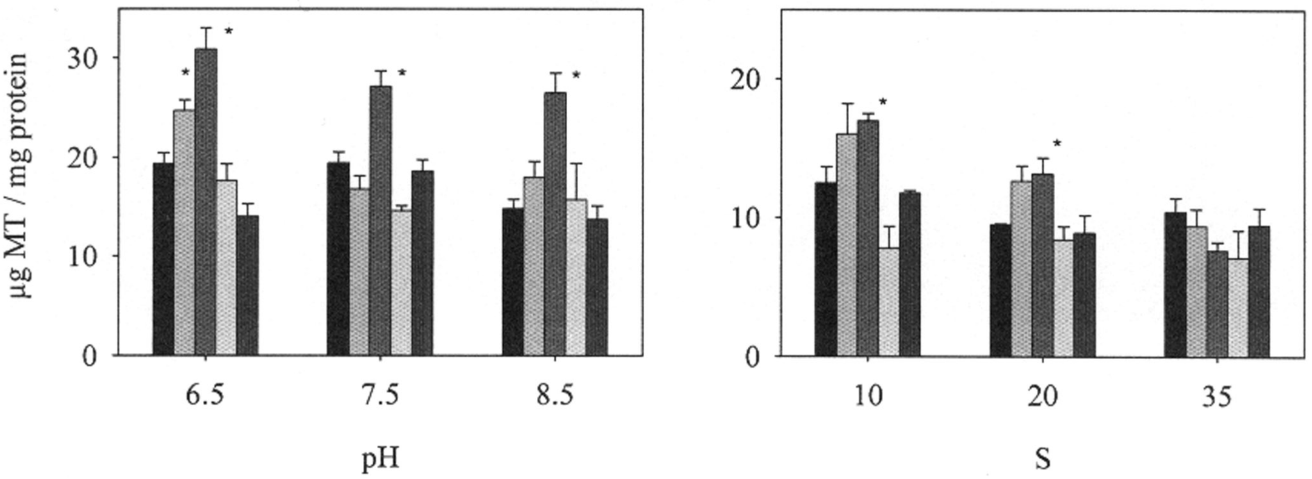

Figure 7 *Histogram of concentration of metallothioneins expressed as microgram per gram of total protein (mg) measured in the pH and in the salinity bioassays. Asterisks show those induction of metallothioneins significantly different (*P*<0.05) to those measured in control. Stations ordered from left to right in each pH and salinity values were: Bay of Cádiz (C), toxic mud dilution (0.3%), toxic mud dilution (7.9%), Ría of Huelva (H) and Guadalquivir estuary (GR).*

In general, the induction of MTs in the different salinity assays (Figure 7) shows a similar pattern than that mentioned for pH although with total concentration of inducted MTs lower than in the pH bioassay. In this sense, the highest toxic mud dilution was significantly different to the induction measured at the control treatments at salinity values of 10 and 20 and increasing with the decrease of the salinity values, being highest at the lowest salinity value assayed (10). The rest of the treatments show induction of MTs not significantly different among salinity values neither to the control treatments.

In summary, the highest toxic mud dilution (7.9%) shows an induction of MTs increasing with the decrease of salinity and pH values. Besides, the lowest concentration of toxic mud (0.3%) shows induction of MTs at lowest value of pH. None of the environmental stations show significant induction of MTs for any of the pH and salinity assays. These results are in concordance with the percentage of mortality measured in both bioassays, especially for both dilutions of toxic mud (0.3% and 7.9% dry weight of toxic mud) in which a correlation (r^2) higher than 0.89 was found between induction of MTs and measured mortality. It informs about the cause of the mortality, which is related to the concentrations of heavy metals that previously had induced the MTs.

A central aspect of the role of MT as a defense system/stress response thus concerns its rate of synthesis, turnover and affinity by metals: if the protein is to be effective as a detoxifying agent, synthesis must be sufficiently rapid, and binding sufficiently strong, to prevent excessive binding to more sensitive molecules (Langston *et al.*, 1998). Metallothionein can be also induced besides by heavy metals by other stress agents, such as strong variations of temperature, oxygen and salinity that can increase the cellular concentrations of MT (Viarengo *et al.*, 1988). The toxicity of some metals, as Cu and Fe, is related to their prooxidant effect; in marine organisms these metals can induce oxyradical production leading to lipid peroxidation. In mussels it has been verified that MT play a role as defense mechanism to oxidative stress (Viarengo *et al.*, 1999). Although it is not absolutely addressed to link change in MT levels to injury at cellular organism levels (Oost *et al.*, 2003) the correlations found in this work between induction of MTs and mortality in the clam used in the assay demonstrate that once the concentration of the metals have reached the binding capacity of the detoxification agent (MTs) the mortality of the clam is measured.

Linking biological responses and chemical speciation

From the obtained results in the association of the heavy metals compared to the biological responses measured in the different bioassays it can be identified the chemical specie responsible for the effect measured. The total concentration of heavy metals measured in water can not explain alone all the effects measured in the assays. Although the effects determined in the highest dilution of toxic mud could be related to the dissolved concentration of the metals in this treatment (see β, Figures 2 and 3) it can not easily discriminates which is the metal responsible of the effects. Furthermore, the total concentration of some metals measured in water exposed to sediments from the estuary located in the Ria of Huelva (H) at lowest values of pH and salinity are higher or similar than those measured in the lowest toxic mud dilution (0.3%) but with absence of biological effect. To better explain the effect measured in both dilutions of toxic mud but not detected in the water exposed to sediments from treatment 'H' it is important to note different pattern in the speciation of some metals between both treatments. From Figures 4 and 5 it can be observed that the percentage of Zn^{2+} at both treatments of salinity and pH reaches values of percentage higher than those measured in the treatment of Huelva and in the control sediments. It could be appreciated the same pattern for the heavy metal Cu but in this case, the free ion does not reaches higher percentages than those obtained in the control treatments (64%) for any of the toxic mud dilutions neither in the 'Ria of Huelva' treatment.

In order to compare the set of data from the chemical concentrations and speciation of metals and the two biological effects measured, a multivariate analysis approach was applied. The analysis uses the values obtained of these 10 original variables in 15 different cases for each pH and salinity bioassays in the negative control of toxicity, two toxic mud dilutions (0.3% and 7.9%) and two environmental stations located in the Ria of Huelva (H) and in the Guadalquivir estuary (GR).

The application of PCA to the original 10 variables at the pH bioassay indicates that they can be grouped in two new factors. These new variables explain more than 96% 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 loadings were 0.35 or higher (Table 1), corresponding to an associated explained variance of more than 30%. This approximates Comrey's 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 loadings of the original variables. The loadings following varimax rotation for the two components are given in Table 1. Each factor is described according to the dominant group of variables and based on their scores for each of the cases studied and shown in Table 2. Both factors, group the heavy metal concentrations (total and free ion) except in factor

Table 1 Sorted rotated factor loading (pattern) of 10 variables on the two and three new components for the pH and salinity bioassay respectively. The loading matrix has been rearranged so that the columns appear in decrease order of variance (in brackets) explained by factors. Only loading greater than 0.35 is shown. Factors (F) are numbered consecutively from left to right in order of decreasing variance explained

	pH		Salinity		
	F1 (90%)	F2 (6.52%)	F1 (69.6%)	F2 (11.6%)	F3 (10.1%)
Cd	0.69	0.68	–	0.87	–
Cu	0.89	0.44	0.42	0.77	–
Zn	0.85	0.49	0.79	0.40	0.41
Pb	0.79	0.60	0.59	0.58	0.54
Cd^{2+}	0.81	0.57	0.53	0.58	0.55
Cu^{2+}	0.91	–	–	–	0.88
Zn^{2+}	0.89	0.44	0.81	–	0.52
Pb^{2+}	0.88	0.47	0.80	–	0.52
MTs	0.37	0.91	0.88	–	–
Mortality	0.43	0.87	0.71	0.56	–

Table 2 Estimated factor scores for the two and three factors determined in the pH and salinity bioassays respectively. These factors are determined in each of the cases studied: control (C), two dilutions of toxic mud, 0.3% and7.9% and in the two environmental samples (Ría de Huelva (H) and Guadalquivir estuary (GR)) and for each pH (6.5, 7.5, 8.5) and salinity (10, 20, 35) values to the centroid of all cases for the original data. The factor scores quantify the prevalence of every factor for each station and are used to establish the description of each factor

	pH			Salinity		
	F1	F2		F1	F2	F3
C 6.5	–0.3365	–0.2732	C 10	–0.0068	–0.5511	–0.9617
0.3% 6.5	–1.0362	1.4438	0.3% 10	1.2833	0.1450	–1.5790
7.9% 6.5	3.4112	0.8714	7.9% 10	2.9183	0.3164	1.7429
H 6.5	–0.033	–0.3808	H 10	–0.4321	–0.2544	–0.1095
GR 6.5	–0.0037	–0.9376	GR 10	–0.0588	–1.1924	0.6489
C 7.5	–0.3572	–0.2536	C 20	–0.2888	–0.4358	–0.6400
0.3% 7.5	–0.2856	–0.1836	0.3% 20	0.5162	0.2069	–1.2001
7.9% 7.5	–0.03047	1.7633	7.9% 20	–0.1079	1.9922	0.6006
H 7.5	–0.0044	–0.8125	H 20	0.3160	–0.2663	–0.0820
GR 7.5	–0.2807	–0.3870	GR 20	–0.7537	–1.1461	1.1882
C 8.5	0.0000	–0.8919	C 35	–0.1636	–0.4723	–0.6825
0.3% 8.5	–0.3158	–0.1918	0.3% 35	–0.1717	0.1201	–0.7508
7.9% 8.5	–0.8794	1.9306	7.9% 35	–1.2778	2.1320	0.7182
H 8.5	–0.0458	–0.7060	H 35	–0.5975	–0.0006	–0.2601
GR 8.5	–0.0004	–0.9912	GR 35	–0.7537	–1.1461	1.1882

2 the concentration of Cu^{2+}, and the two biological effects. However, in the factor 1 the variable loadings associated with the free ion metals were slightly higher than the dissolved total concentrations of the four metals. The biological effects show higher loadings in the factor 2 and associated with slightly higher loadings of the total dissolved concentrations of metals. The factor score of the first factor was positive only at the highest dilution of toxic mud (7.9%) at the lowest pH value (6.5). The factor 2 shows positive values at the highest dilution of toxic mud at any pH value, and at the lowest dilution of toxic mud at the lowest pH value (6.5). For the rest of the treatments negative scores were measured (Table 2).

These associations relate the biological effects to the total dissolved concentrations of metals and with the chemical form of the free ion for all the metals. It confirms that the acidification of the medium provokes a mobilization of the metals in the toxic mud dilutions that were associated with the biological effect measured. It is specially interesting the relationship measured at the lowest dilution of toxic mud in the treatment of the highest concentration of protons (pH=6.5).

The application of PCA to the original 10 variables at the salinity bioassay indicates that they can be grouped in three new factors (Table 1). These new variables explain more than 91% of the total variance in the original data set. In this bioassay the loading cut off selected was equal than that described for the pH assay. The loadings following varimax rotation for the three components are given in Table 1. Each factor is described according to the dominant group of variables and based on their scores for each of the cases studied and shown in Table 2. Only factors 1 and 2 include association of both chemical and biological variables. The first factor includes both biological effects with highest loadings than in any of the other new factors. These biological effects were related to the concentrations of metals Zn and Pb and the highest loading was obtained for their concentration in the form of free ions (Zn^{2+}, 0.81; Pb^{2+}, 0.80). The second factor only associates mortality and with lower loading than in the first factor. This factor relates the biological effect to the concentration of metals, both total and free ion although the loadings of total concentrations in this factor were higher than the concentrations of free ion metals. The third factor only associates chemical concentrations in it and was not discussed here.

The factor score obtained for factor 1 shows that the free ion concentration of Zn can explain the different bioavailability of metals based on the free ion concentrations that provokes the biological effect measured at lowest salinity value for the toxic mud dilution of 0.3%. In this sense, the concentration of free ion Zn^{2+} could be associated with the mortality of the individuals of *Ruditapes philippinarum* exposed to this toxic mud dilution. Although the free ion of lead was included in the factor its low total dissolved concentrations can neglects its effect compared to that associated with the metal Zn. The score of factor 2 was positive in the toxic mud dilution and confirms the biological effect measured in these treatments. In this case, the biological effect is related to the total dissolved concentrations of heavy metals that were mobilized from the sediments.

From these results, it is confirmed that the toxicity identified in the bioassays was related to the mobilization of metals from sediment to water then related to the total concentration of metals (specially Zn) in water. It is also shown that the free ion can explain the differences in the toxicity of the same sample when values of salinity change. Nevertheless, the total concentration of metals in water is the main responsible of the toxicity measured. The environmental significance of these results can be related to the different toxicity that can be measured associated with the same sample in different areas of an estuary. Then, in the areas with low salinity and pH values the mobilization of some metals (Zn, Pb and Cu) can be toxic if the threshold level of the organisms (clam in this study) is overlapped. However, the same sample can be not toxic if we are in the marine influenced area of the estuary with higher values of pH (7.5) and salinity (20).

CONCLUSIONS

The mobilization of heavy metals from sediment to water assayed shows a different pattern for the pH and the salinity bioassays. Whereas in the salinity assay the heavy metal mobilization varies from metal to metal depending on the relative importance of two counteractive processes, desorption from sediments to water or coagulation, flocculation, and precipitation; in the pH assays the acidification of the medium provoked an increases of the mobility of metals with the decrease of the pH values.

The chemical speciation of the different heavy metals varies depending on the metal and the assay using different values of pH and salinity. Acidification of the sediment samples produces high percentages of free ion for all the metals. Salinity affects highly the speciation of all the metals and specially related to cadmium and lead for the environmental stations located in the estuaries of the Gulf of Cádiz. This pattern could be associated with a decrease of the bioavailability of these metals at higher values of salinity and in the marine areas of the estuary (S about 35).

Low values of pH and salinity show mortality and induction of MTs associated with heavy metals in the different dilutions of toxic mud with clean sediments. In this sense, the decrease in the pH and salinity values increases the toxicity associated with the heavy metals bound to sediments assayed. The values of these two key-variables in the estuarine systems such as those assayed determine both the chemical speciation of the heavy metals and the toxicity associated with them in two different ways: (a) affecting to the chemical speciation of the metals producing more bioavailable species at lower values of pH and salinity; and (b) influencing to the sensitivity of the organisms through the increase of diffusion of some metals through the protein membranes and to the organism. Both, low values of salinity and pH increase the concentration of metal in water in the form of free ions, specially Zn^{2+}. This increase permits a more easily transported through the plasmatic membrane producing an increase of the MTs. Once the threshold of binding to MTs is exceeded at low values of salinity and pH and in the station of toxic mud dilution, the metal produces the mortality observed in the clams.

The heavy metals from the mining spill bound to the toxic mud are more bioavailable at low pH and salinity values and it could explain previous results that identified toxicity in low hydrodynamic areas of the estuary with similar values of pH and salinity as those tested in this study (Riba *et al.*, in press). Furthermore, it can explain that although the concentrations of heavy metals in the dilutions of toxic mud are much lower than those monitored in the Ría of Huelva they are associated with biological responses such as induction of metallothionein and lethal toxicity. It gives an interesting indication of the fundamental difference between the long-term effects of continuous heavy metal discharge over centuries and the effect of an isolated, albeit very large, single discharge.

ACKNOWLEDGEMENTS

We thank Dr. John Roitz for critically reading an earlier version of this manuscript, also Rosa Vázquez and the staff of the aquaculture facility at the Faculty of Marine and Environmental Sciences, University of Cádiz. This research was supported by grant REN2002-01699 from the Spanish National Plan for research, innovation and development (Ministerio de Ciencia y Tecnología). Inmaculada Riba thanks the 'Consejería de Educación y Ciencia de la Junta de Andalucía' for her Doctoral fellowship (F.P.D.I.).

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