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Biological Adverse Effects on Bivalves Associated with Trace Metals Under Estuarine Environments

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Abstract Toxic effects of pollutants on marine organisms can be studied both by performing field measurements, and by undertaking laboratory simulation experiments. Here is described the effect of trace metals Zn, Cd, Pb and Cu on the clam *Scrobicularia plana* along a salinity gradient simulated in a hypothetical estuary using simulation experiments. The simulator produces a continuous entry of trace metals into the estuary through injection in the lower salinity tank of the system. The clams were exposed during two weeks to different concentration of trace metals to assess the bioaccumulation process along a salinity gradient. Bivalves were analysed for body tissue residue to determine the bioaccumulation factors related to each metal and the salinity influence was addressed. Differences among tanks were observed as a result of the salinity gradient. In the achieved assays, the mechanism of bioaccumulation of Zn and Cd in organisms was more efficient at high salinity values. Bioaccumulation factors for both metals showed a linear increase with the increase of salinity values. It seems that the mechanism of bioaccumulation of Pb and Cu in organisms was dependent on two simultaneous processes: the proximity to the input point of metals and the low salinity values.

Keywords Bioaccumulation · Clams · Estuaries · Metallothioneins · Simulation

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1 Introduction

Trace metals are natural constituents of the marine environment in trace concentrations. However, anthropogenic inputs have increased strongly the trace metal concentrations in coastal areas during the last century. One of the main access ways of trace metals to the marine environment is through estuaries, which receive human sewage of different origins (Blackstock, 1984). Therefore, in metal-polluted environments, a high availability of a trace metal will promote a high rate of metal entry into the body of any species exposed to this metal. If the rate of uptake exceeds the rate at which the metal can be detoxified or excreted, then the metal is available internally to exert toxic effects (Legras, Mouneyrac, Amiard, Amiard-Triquet, & Rainbow, 2000).

Toxic effects of pollutants on marine organisms can be studied both by performing field measurements, and by undertaking laboratory simulation experiments. Controlled laboratory experiments using an only substance (or a mixture of them) on a single biological species supply very useful information related to the knowledge of pollutant effects. The

development of this kind of bioassays is essential to assess the real impact produced by pollutants.

In this study, the adverse effects produced by trace metals on estuarine clams have been characterised using a dynamic simulator of estuaries by conducting different bioassays in which clams were exposed to a dynamic flow of trace metals. The idea of dynamic simulation is basically similar to that described by Bale and Morris (1981). Nevertheless, the dimensions and the features offered by this new simulator are considerably greater (for example, with this system tidal effects also can be simulated).

The metals selected for these experiments were Zn, Cd, Pb and Cu. Among benthic species, bivalve molluscs are characterised by being able to accumulate trace metals in concentrations proportional to those present in water or in sediments. Therefore, bivalve molluscs have been used to monitor bioavailability of such contaminants (Pavicic, Skreblin, & Raspor, 1987). The biological species selected in this study was *Scrobicularia plana* due to its ubiquity, local abundance and importance in the estuarine trophic chain (Ruiz, Bryan, Wigham, & Gibbs, 1995). In fact, *Scrobicularia plana* is an infaunal species commonly inhabiting the intertidal soft bottoms of Northeast Atlantic estuaries, from the Norwegian Sea into the Mediterranean and south to Senegal (Tebble, 1966).

The general objective of this study is to characterise the adverse effects produced by trace metals on estuarine clams by means of a dynamic simulator. For this, it has been necessary to quantify the trace metal concentrations in the soft body tissue from specimens of *Scrobicularia plana*, to calculate the bioaccumulation factors for the four trace metals selected and to determine the concentration of metallothioneins in specimens of *Scrobicularia plana* collected from each tank (as biological response determined).

2 Materials and Methods

2.1 Simulator description

The simulator system consists of eight tanks (Plexiglas, cylindrical, and of about 10 l capacity) interconnected under a hydrodynamic regime (Figure 1). The upper tank is supplied with fresh water. The lower tank is supplied with seawater sampled in a clean coastal area. From the lower to the upper tanks, there is a forced flow of water controlled by peristaltic pumps. In the inverse direction, filling the containers in series with fresh water generates a down flow. This permits a constant volume of 10 l to be maintained in each tank.

The flow and temperature control is carried out by a personal computer using specific software developed in Visual Basic and an AID 21-bit translation card. The regulation of flow involves setting-up the peristaltic pumps (Masterflex, 7521-55) in phase with the flow meters (McMillan Company, 111). The temperature is controlled by means of coated dip heaters through thermistor probes. The water mixture is kept homogeneous in each tank by using variable-velocity mechanical stirrers. A more detailed description of this system has been previously reported by García-Luque, Forja, DelValls, and Gómez-Parra (2003).

After 15 days, adsorption of metals onto wall tanks can be neglected because this process has a kinetic order of about hours instead of days, reaching stationary state before the first 24 h.

2.2 Experimental design

The area of marine influence of a typical estuary was simulated. Specifically, the salinity gradient simulated was comprised between values of 10 and 36, to avoid osmotic impact on the clams.

Prior to the beginning of the assay, the clams were purified during 10 days in an aquarium of 70 l thanks to a continuos flux of oxygenated water. After this, the specimens of the clam *Scrobicularia plana* (10 per aquarium; average length: 4.4 cm; average wet weight: 2.5 g) were placed in each tank after two weeks of previous acclimatisation period to each salinity value under controlled conditions. Then, a solution with known concentration of the four metals was injected in the lowest salinity tank of the system in a continuous flow by means of a peristaltic pump. (The measured trace metal concentrations in this solution were Zn:Cd:Pb:Cu, 5:0.2:1:2 $mg \cdot l^{-1}$, respectively). This metal injection was diluted as it reached succeeding tanks. Dilution is result of two processes: the hydrodynamic regime and the inherent reactivity of trace metals along a salinity gradient. The exposure period of the bioassay was 15 days. After this, clams were collected to assess possible adverse effects (bioaccumulation, metallothionein synthesis) derived of their exposure to trace metals. Parallel to the

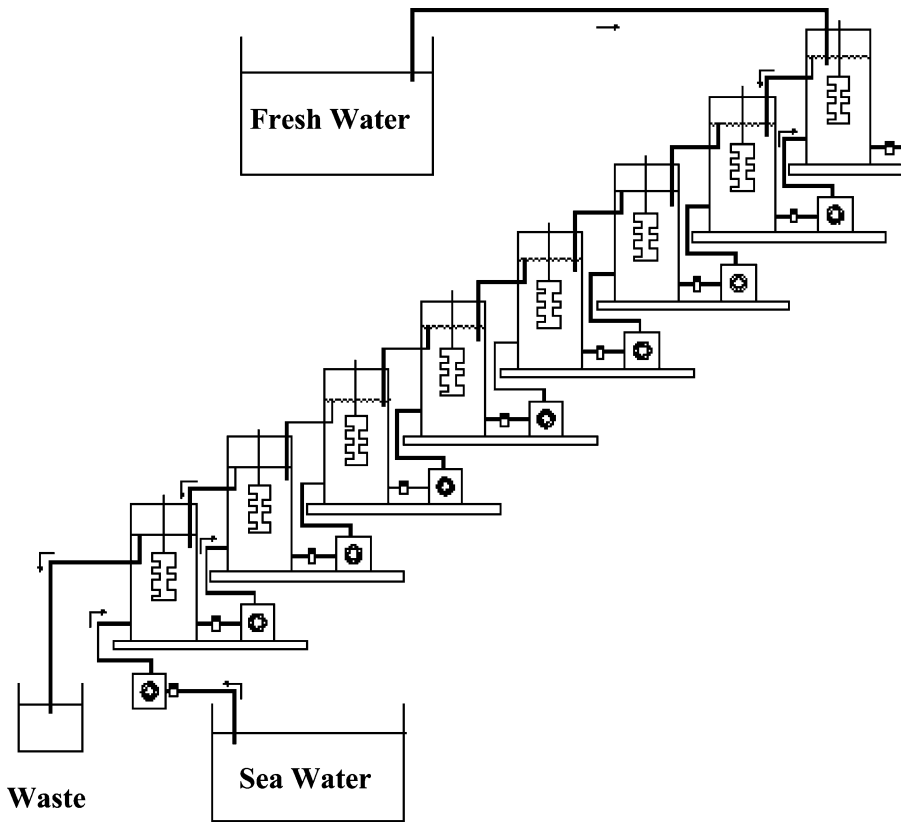

Figure 1 Schematic representation of the estuarine hydrodynamic simulation system (adapted from García-Luque et al., 2003).

experiment, 10 clams were maintained in a reference aquarium (absence of introduced metals).

Along the experiments, some physicochemical parameters (salinity, temperature, pH and dissolved oxygen) were measured in each tank of the simulator to ensure the correct development of the assays.

2.3 Data calculation and statistical analysis

The results obtained for salinity values, concentration of the four dissolved trace metals, concentration of trace metals bioaccumulated and concentration of metallothioneins were related by means of factor analysis, using principal components (PCA) as the extraction procedure, which is a multivariate statistical technique (MAA) to explore variable distributions. 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 factor analysis was performed on the correlation matrix, i.e., the variables were auto-scaled (standardised) so as to be treated with equal importance (DelValls & 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, Jennrich, & Sampson, 1985).

The sorted rotated factor loadings are coefficients correlating the original variables and the principal components in this analysis. The variables are reordered so the rotated factor loadings for each factor are grouped together. In this study, we selected to interpret a group of variables as those associated with a particular component where loadings were 0.35 or greater, corresponding to an associated explained variance of more than 35%.

2.4 Analytical techniques

Prior to sampling and setting-up the simulations, all equipment, filters, beakers and containers used were thoroughly cleaned with acid (10% HNO_3) and then rinsed in reagent grade water (Milli-Q).

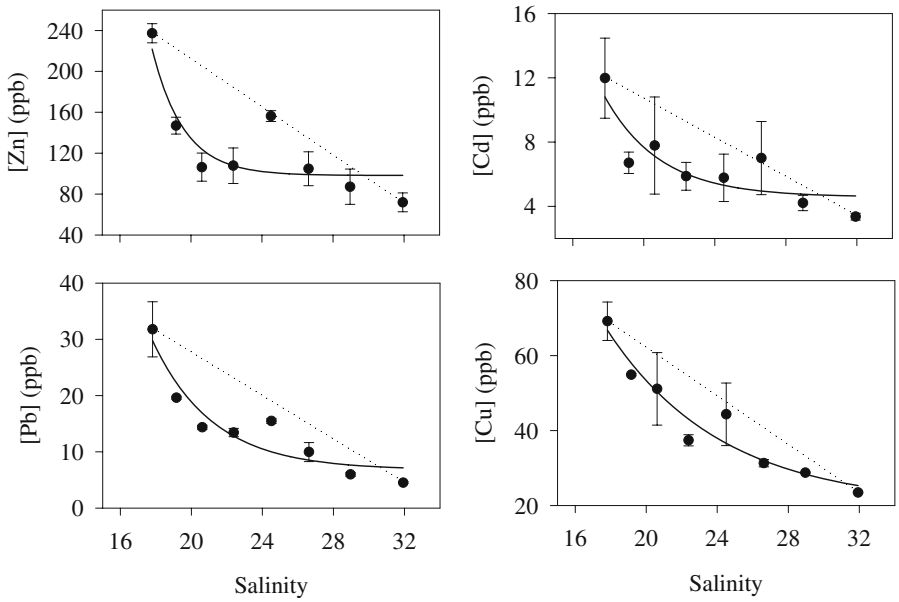

Figure 2 Concentration of dissolved Zn, Cd, Pb and Cu *along* the salinity gradient achieved at the bioassay. *Dotted line* shows the theoretical dilution line; *solid line* shows exponential fittings for variations of metal concentration with salinity values.

The salinity was determined by means of an induction salinometer (Beckman RS-10). The total concentration of dissolved trace metals (Zn, Cd, Pb, Cu) was assessed in water samples using a differential pulse anodic stripping voltametry (DPASV), after a digestion procedure with UV radiation (4 h) at 85°C (Metrohm, 705UV). Measurements were made with static drop mercury electrode (SMDE), using a Metrohm 693 processor as reported by Gómez-Parra, Forja, DelValls, Sáenz, and Riba (2000). The analytical procedure for dissolved metals was checked using reference material CASS3 with an accuracy of ±90%. All samples were measured at the end of the experiment. Nevertheless, the system had reached stationary state when the injection of the trace metal solution to the system was carried out. Therefore, measured concentrations at the end of the assays are similar than those found during the experiment.

To analyse the residue of tissue body for trace metals and to address the bioaccumulation process, organisms were lyophilised and pounded prior to an acidic digestion with nitric acid and hydrogen peroxide during 1 h at 95°C. Samples (previously filtered) were analysed by atomic absorption spectrophotometry. The concentrations of trace metals Zn and Cu in the samples were determined with a Perkin-Elmer 2100 Flame atomic absorption spectrophotometry. The trace metals Cd and Pb were measured by graphite furnace atomic absorption spectrophotometry (Perkin-Elmer, 4100 ZL).

The concentration of metallothioneins was analysed on the total body of the organisms at the end of the experiment through Anodic Stripped Voltammetry in the heat stable fraction (the supernatant of the second centrifugation), based on the protocol outlined by Olafson and Olsson (1991). Rabbit liver metallothionein (Sigma) was used as reference for the standard addition calibration curve. Total content of protein determination was based on the Bradford method (1976). Metallothionein concentration was expressed in relation to total protein content.

3 Results and Discussion

Figure 2 shows the concentrations of dissolved trace metals along the salinity gradient obtained during the bioassay. In all cases, it can be observed how obtained values are below the theoretical dilution line, showing a non-conservative behaviour for all the metals assessed. Therefore, a high chemical reactivity is shown mainly at low salinity values. The non-conservative behaviour of these metals has been reported for other estuaries (e.g., Benoit et al., 1994; Windom et al., 1991).

Although the behaviour of the assessed metals in the simulation assay is non-conservative, the decreases in concentration in the dissolved phase with salinity show a similar pattern. The trace metal

Table I Fitted parameters corresponding to Equation (1) (and correlation coefficients) calculated for the trace metal (Zn, Cd, Pb and Cu) concentrations *versus* salinity values during the bioassay

	a	*b*	*c*	r^2
Zn	98.23	$263.7 \cdot 10^5$	0.56	0.745
Cd	4.53	1539.94	0.31	0.739
Pb	6.76	4197.42	0.29	0.885
Cu	20.11	743.18	0.15	0.932

concentration gradients *versus* the salinity increase are described by the following exponential equation:

$$C = a + b \cdot e^{-c \cdot S} \quad (1)$$

where C is the trace metal concentration in the dissolved phase, S is the salinity, and a, b, and c are the fitted parameters for each metal. Values for these parameters as well as the correlation coefficients for the equation are shown in Table I.

The trace metal concentrations in the soft body tissue from three specimens of *Scrobicularia plana* collected in each tank of the simulator also was quantified. Figure 3 shows the concentrations of bioaccumulated Zn, Cd, Pb and Cu along the salinity gradient in the clams (concentration of metals in biological tissues is expressed taking into consideration wet weight of the tissues). In this figure, two different behaviour patterns can be observed. The concentrations of Zn and Cd do not show a clear pattern with the salinity increase. On the other hand, the concentrations of Pb and Cu exhibit a decrease with the salinity increase. In fact, bioaccumulation for both metals (Pb and Cu) decreases exponentially with salinity describing an equation similar to expression (1), with correlation coefficients of 0.889 for Pb and 0.988 for Cu.

Bioaccumulation factors (BCFs) for the four trace metals have been calculated also. These factors are calculated as the ratio between the concentration of the bioaccumulated metal and the concentration of the dissolved metal. Obtained results are showed in Figure 4.

The bioaccumulation factors for Zn and Cd increase following lightly a linear pattern with the salinity increase. It indicates that the bioaccumulation of Zn and Cd is more effective when salinity increases. Therefore, at high salinity values, the amount of bioaccumulated trace metals (Zn and Cd) is about the same that at low salinity values (Figure 3), although Zn and Cd concentrations in water decrease with the salinity increase (Figure 2).

The BCF for Pb does not show a clear trend with the salinity, and varies around an average value ($850\ \mathrm{l\,kg^{-1}}$). Therefore, the bioaccumulation of Pb could not depend on salinity values, being controlled by the amount of dissolved Pb along the salinity gradient. Thus, at high salinity values, where dissolved Pb

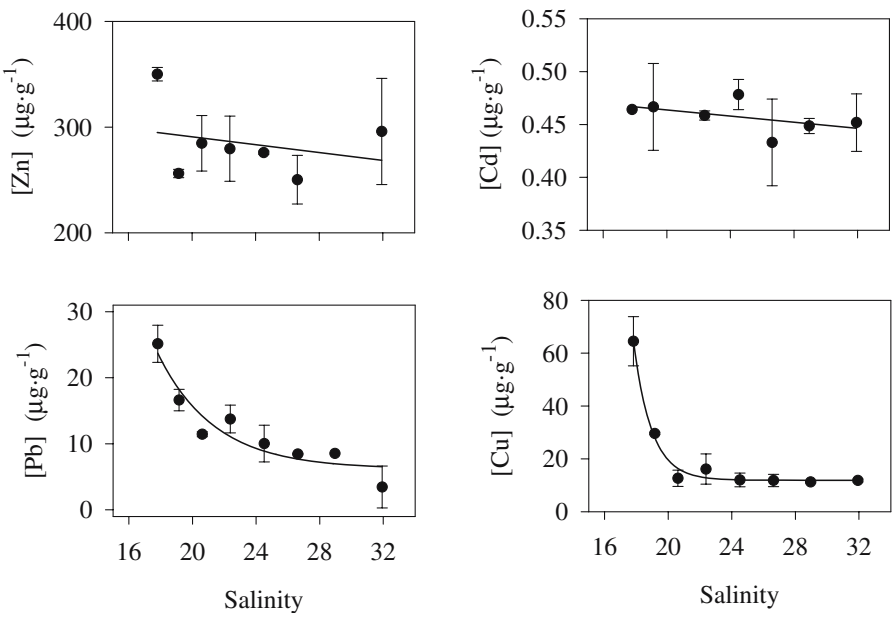

Figure 3 Concentration of Zn, Cd, Pb and Cu bioaccumulated by specimens related to their total weight *along* the salinity gradient achieved at the bioassay (metal concentration is expressed taking into consideration wet weight of the tissues).

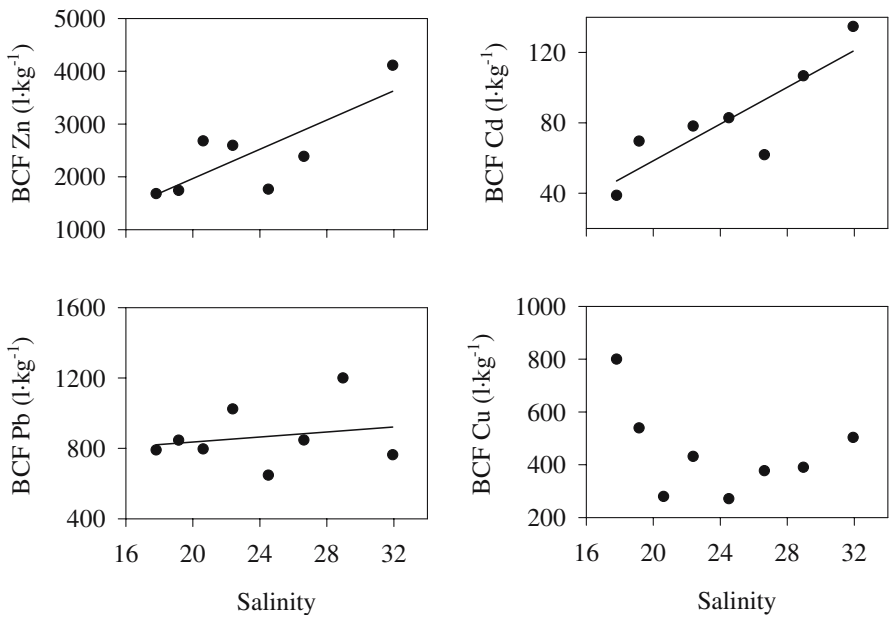

Figure 4 Bioaccumulation factors (BCFs) calculated for Zn, Cd, Pb and Cu along the salinity gradient achieved at the bioassay.

concentration is low, it is measured the lowest bioaccumulation for Pb (Figure 3).

The variation of BCF for Cu along the salinity gradient is shown in Figure 4. The BCFs are higher at low (16–22) than at high salinity (24–32) values. It could inform that bioaccumulation of Cu is higher at low than at high salinity values.

To assess possible adverse effects derived from the exposure of clams to trace metals at different salinity values, the concentration of metallothioneins in specimens of *Scrobicularia plana* collected from each tank has been analysed. Summarised results of metallothionein concentrations and total proteins concentrations in analysed clams are shown in Table II. The metallothionein concentration shows intersite differences that could be associated with the salinity gradient in the simulated estuary. Recent studies (e.g., Legras et al., 2000; Mouneyrac, Amiard-Triquet, Amiard, & Rainbow, 2001) indicate the existence of a relationship between salinity and metallothionein concentration. Salinity gradient in an estuary influences on the chemical speciation of the trace metals and, therefore, on its bioavailability.

The results obtained for salinity values, concentration of the four dissolved trace metals, concentration of trace metals bioaccumulated and concentration of metallothioneins were linked by means of a MAA. The application of PCA to the chemical data represents the original variables by four new variables, or principal factors (Table III). These factors explain 94.5% of the variance in the original data set. The loadings following varimax rotation for the four factors are given in Table III. Each factor is described according to the dominant group of variables.

Table II Concentration of metallothioneins (MTs, μg) normalised to the total content of proteins (Tot. prot., mg) measured in the soft body of individuals of the clam *Scrobicularia plana* collected from each tank in the simulator

		Tank 1	Tank 2	Tank 3	Tank 4	Tank 5	Tank 6	Tank 7	Tank 8
MTs/Tot. prot. (μg mg^{-1})	Specimen A	70.43	47.27	30.97	33.48	45.13	74.51	99.33	78.92
	Specimen B	45.80	48.86	49.42	37.29	65.56	68.69	111.90	67.21
	St. dev.	17.42	1.13	13.05	2.69	14.45	4.12	8.89	8.27
Total proteins (mg ml^{-1})	Specimen A	1.63	2.48	2.33	2.22	4.69	1.62	2.60	1.06
	Specimen B	1.34	2.11	1.41	1.96	1.63	2.19	1.22	1.42
	St. dev.	0.20	0.26	0.65	0.19	2.16	0.41	0.98	0.26

Standard deviation values are also expressed.

Table III Sorted rotated factor loading (pattern) of 10 variables on the bioassay

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

Variable	Factor 1 (70.41%)	Factor 2 (13.41%)	Factor 3 (7.12%)	Factor 4 (3.56%)
Salinity	−0.8248	−0.4823	–	–
Diss. [Zn]	0.8069	–	–	0.4216
Diss. [Cd]	0.7973	–	–	0.4294
Diss. [Pb]	0.8422	–	–	0.3810
Diss. [Cu]	0.8351	–	–	–
Bioacc. [Zn]	–	–	–	0.9142
Bioacc. [Cd]	–	–	0.9397	–
Bioacc. [Pb]	0.8698	–	–	–
Bioacc. [Cu]	0.7348	–	–	0.6160
[MTs]	–	−0.9491	–	–

The first principal factor, #1 is predominant and accounts for 70.41% of the total variance; this factor could be called 'Influence of salinity on concentration of dissolved and bioaccumulated trace metals.' This component shows positive loadings on the concentration of dissolved Zn, Cd, Pb and Cu, and the concentration of bioaccumulated Pb and Cu. A negative loading has been founded for the salinity. Clearly, this component relates inversely the concentration of the four dissolved trace metals and the concentration of bioaccumulated Pb and Cu with salinity. Therefore, at high salinity values, low concentrations of dissolved trace metals and low concentrations of bioaccumulated Pb and Cu are quantified.

Besides, it confirms that the values of bioaccumulated Pb and Cu are related to the concentrations of dissolved Pb and Cu in a direct way. Amiard, Amiard-Triquet, Berthet, and Metayer (1987) described a similar behaviour for both metals. They indicated that field and laboratory studies conducted using individuals of the clam *Scrobicularia plana* and another species show that the bioaccumulated trace metals depend mainly on environmental levels of these metals.

On the other hand, the bioaccumulation of Zn and Cd is not associated with this factor, so could be prevailing at high salinity values as has been discussed previously for the BCF of these two metals.

The second factor, #2 accounts for the 13.41% of the total variance and could be called "Influence of the physicochemical variable 'salinity' on metallothionein synthesis." This component shows negative loadings on the salinity and the concentration of metallothioneins. This component relates lightly these two variables. It could indicate that, under the conditions of this bioassay, a physicochemical variable (salinity) shows higher influence in the induction of metallothioneins than the concentration of trace metals (dissolved or bioaccumulated).

The third factor, #3 accounts for the 7.12% of the total variance (a low loading of the total value) and could be called 'Bioaccumulation of Cd in soft body tissue clam.' This factor shows only positive loading on the concentration of bioaccumulated Cd. It could inform that the influence of the different variables was not addressed under the conditions of this bioassay, so other processes should be affecting the bioaccumulation of this metal.

The fourth factor, #4, could be called 'Bioaccumulation of Zn in soft body tissue clam.' It is the only factor in which the concentration of Zn in clams is

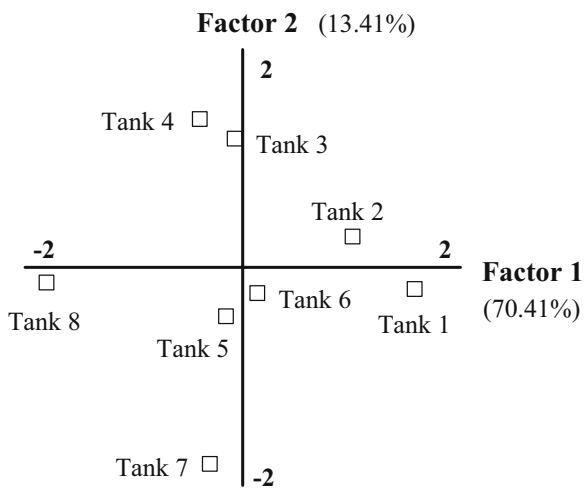

Figure 5 Distribution of the different cases (represented by the tanks of the simulator) in the *space* defined by factors 1 and 2 (F1: 70.41% of the total variance and F2: 13.41% of the total variance).

included, but with the lowest variance explained (3.56%). So, its description should be taken with caution.

Figure 5 shows the ordination of the cases (represented by the tanks of the simulator) in the space defined by factors 1 and 2 (83.8% of the total variance, as a whole). Tanks 1 and 8 represent two extreme situations. Tank 1 shows the highest score for Factor 1, so the highest dissolved concentrations for the four metals and the highest concentrations of Pb and Cu in clams can be found at salinity value of Tank 1 (18). Tank 8 represents the opposite situation: dissolved concentrations for the four metals and concentrations of Pb and Cu in clams show the lowest values. The rest of the tanks show intermediate situations. This behaviour is related to the distance of each tank to the input point of metals into the system. On the other hand, Tanks 5, 6 and 7 show a relatively significant induction of metallothioneins, being Tank 7 where this process is specially marked (This Tank shows the highest score for Factor 2).

The other two factors (3 and 4) are not shown in Figure 5 owing to the low proportion of the total variance explained by them.

4 Conclusions

In this study, adverse effects provoked by trace metals on estuarine clams have been characterised using a dynamic simulator. The bioaccumulation factors related to each metal and the salinity influence were addressed. Under conditions employed in these bioassays, it could be inferred that:

(1) The mechanism of bioaccumulation for Zn and Cd in clams was more efficient at high salinity values. Therefore, bioaccumulation factors for both metals showed a linear increase with the increase of salinity values.
(2) It seems that the mechanism of bioaccumulation for Pb and Cu in bivalves was dependent on two simultaneous processes: a) the proximity to the input point of trace metals employed and b) the low salinity values.
(3) The process of induction of metallothioneins in this experiment is more related to existing salinity values than concentration of the metals employed.

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References

Amiard, J. C., Amiard-Triquet, C., Berthet, B., & Metayer, C. (1987). Comparative study of the patterns of bioaccumulation of essential (Cu, Zn) and non-essential (Cd, Pb) trace metals in various estuarine and coastal organisms. *Journal of Experimental Marine Biology and Ecology*, *106*, 73–89.

Bale, A. J., & Morris, A. W. (1981). Laboratory simulation of chemical processes induced by estuarine mixing: The behaviour of iron and phosphate in estuaries. *Estuarine, Coastal and Shelf Science*, *13*, 1–10.

Benoit, G., Otkay-Marshall, A., Cantu, A., Hood, E. M., Coleman, C. H., Corapcioglu, M. O., et al. (1994). Partitioning of Cu, Pb, Ag, Zn, Fe, Al and Mn between filter-retained particles, colloids, and solution in six Texas estuaries. *Marine Chemistry*, *45*, 307–336.

Blackstock, K. J. (1984). Biochemical metabolic regulatory responses of marine invertebrates to natural environmental change and marine pollution. *Oceanography and Marine Biology: Annual review*, *22*, 263–313.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–254.

DelValls, T. A., & Chapman, P. M. (1998). Site-specific sediment quality values for the Gulf of Cádiz (Spain) and San Francisco Bay (USA), using the sediment quality triad and multivariate analysis. *Ciencias Marinas*, *24*, 313–336.

Frane, J., Jennrich R., & Sampson, P. (1985). Factor analysis. In: W. J. Dixon (Ed.), *BMDP statistical software* (pp. 480–500). Berkeley: University of California.

García-Luque, E., Forja, J. M., DelValls, T. A., & Gómez-Parra, A. (2003). The behaviour of heavy metals from the Guadalquivir estuary after the Aznalcóllar mining spill: Field and laboratory surveys. *Environmental Monitoring and Assessment*, *83*, 71–88.

Gómez-Parra, A., Forja, J. M., DelValls, T. A., Sáenz, I., & Riba, I. (2000). Early contamination by heavy metals of the Guadalquivir Estuary after the Aznalcóllar mining spill (SW Spain). *Marine Pollution Bulletin*, *40*, 1115–1123.

Legras, S., Mouneyrac, C., Amiard, J. C., Amiard-Triquet, C., & Rainbow, P. S. (2000). Changes in metallothionein concentrations in response to variation in natural factors (salinity, sex, weight) and metal contamination in crabs from a metal-rich estuary. *Journal of Experimental Marine Biology and Ecology*, *246*, 259–279.

Mouneyrac, C., Amiard-Triquet, C., Amiard, J. C., & Rainbow, P. S. (2001). Comparison of metallothionein concentrations and tissue distribution of trace metals in crabs (*Pachygrapsus marmoratus*) from a metal-rich estuary, in and out of the reproductive season. *Comparative Biochemistry and Physiology. Part C*, *129*, 193–209.

Olafson, R. W., & Olsson, P. E. (1991). Electrochemical detection of metallothionein. *Methods in Enzymology*, 205, 205–213

Pavicic, J., Skreblin, M., & Raspor, B. (1987). Metal pollution assessmet of the marine environment by determination of metal-binding proteins in *Mytillus* sp. *Marine Chemistry*, *22*, 235–248.

Ruiz, J. M., Bryan, G. W., Wigham, G. D., & Gibbs, P. E. (1995). Effects of tributyltin (TBT) exposure on the reproduction and embryonic development of the bivalve *Scrobicularia plana*. *Marine Environmental Research*, *40*, 363–379.

Tebble, N. (1966) *British bivalve seashells* (p. 212). London: British Museum Natural History.

Windom, H., Byrd, J., Smith, R., Hungspreugs, M., Dharmvanij, S., Thumtrakul, W., et al. (1991). Trace metal–nutrient relationships in estuaries. *Marine Chemistry*, *32*, 177–194.