ADVANCES IN MARINE CHEMISTRY
J. Blasco and J.M. Forja (eds.)

Scientia Marina 74S1
December 2010, 143-152, Barcelona (Spain)
ISSN: 0214-8358
doi: 10.3989/scimar.2010.74s1143

Temporal variations in metallothionein concentration and subcellular distribution of metals in gills and digestive glands of the oyster *Crassostrea angulata*

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SUMMARY: The metallothionein levels and metal concentrations in whole body, digestive gland and gills of *Crassostrea angulata* were analyzed in field samples collected from the River Guadalquivir estuary over several years following a mining waste spill upstream. The subcellular distribution of metals was analyzed to determine the mechanisms involved in the detoxification process. The highest metallothionein levels were reported in the digestive gland shortly after the mining contamination event. In this organ, metals are stored preferentially in the non-cytosolic fraction when increased bioaccumulation takes place. In the cytosol of the gills, metals are associated with metallothionein, whereas in the digestive gland, the distribution of metals between metallothioneins and high molecular weight proteins is similar. Metallothionein variation cannot be explained by metals alone; other abiotic factors must be taken into account. In order to use metallothionein as a metal exposure biomarker in field studies, natural variability needs to be taken into account for the correct interpretation of results.

Keywords: metallothionein, metals, subcellular distribution, River Guadalquivir estuary, mining accident, *Crassostrea angulata.*

RESUMEN: Variación temporal de la concentración de metalotioneínas y distribución subcelular de metales en branquias y glándula digestiva del ostron *Crassostrea angulata*. – Los niveles de metalotioneínas y las concentración de metales en cuerpo total, glándula digestiva y branquias de *Crassostrea angulata* han sido analizados en muestras de campo recogidas en el estuario del Guadalquivir durante varios años después de un accidente minero. La distribución subcelular de metales se analizó con el fin de conocer los mecanismos implicados en los procesos de detoxificación. Los niveles más elevados de metalotioneínas se hallaron en la glándula digestiva de muestras recogidas después del accidente minero. En este órgano, los metales se almacenan preferencialmente en la fracción no citosólica cuando la bioacumulación aumenta. En el citosol de las branquias, los metales se hallan asociados a las metalotioneínas, mientras que en la glándula digestiva, éstos se encuentran distribuidos de manera similar entre las metalotioneínas y las proteínas de alto peso molecular. La variación de la concentración de metalotioneínas no puede ser explicada sólo en base a las concentraciones de metales, y otros factores abióticos pueden ser responsables del porcentaje de varianza no explicado. Por ello, el empleo de la concentración de metalotioneína como biomarcador de exposición de metales en *Crassostrea angulata* debe ser considerado con precaución y el conocimiento de la variabilidad natural deber ser tenido en cuenta para una correcta interpretación de los resultados.

Palabras clave: metalotioneinas, metales, distribución subcelular, estuario del río Guadalquivir, accidente minero, *Crassostrea angulata*.

INTRODUCTION

Coastal ecosystems of estuaries are vulnerable because they are subjected to both diffusive and periodic contamination from agricultural, industrial and urban activities. The Guadalquivir is the principal river in the SW of the Iberian Peninsula and its estuary is an important area in which many marine species are reared. This river has been impacted by metal contamination as a result of agricultural and mining activities and

urban sewage discharge (Blasco *et al*., 1999; García-Luque *et al*., 2003; Grimalt *et al.*, 1999).

Metallothionein-like proteins (MTLPs) are metalloproteins, which play a role in the homeostasis of the essential metals Zn and Cu and are involved in detoxification processes for non-essential trace metals such as Ag, Cd and Hg (Mason and Jenkins, 1995; Mouneyrac *et al*., 2001). These proteins are ubiquitous in vertebrates, invertebrates, microorganisms and plants (in the latter, a second type of metal-binding protein which participates in detoxification of heavy metals is phytochelatin, also very often called class III metallothionein) (Tomaszewska, 2002). The known induction of MTLP synthesis in aquatic organisms (fish, crustaceans and molluscs) exposed to an environment contaminated by metals has led to the suggestion that the determination of MTLP levels could represent a suitable monitoring procedure for assessing metal contamination in the marine environment (George and Olsson, 1994; Langston *et al*., 1998; Cosson, 2000; Cosson and Amiard, 2000; Cajaraville *et al*., 2000). Bivalves, mainly mussels (George and Olsson 1994) and oysters (Silva *et al*., 2001, Mouneyrac *et al.*, 1998), are considered the most appropriate species for use in monitoring the quality of coastal and estuarine waters and are recommended in numerous pollution monitoring programmes in Europe and the USA (the North Sea Task Force Monitoring Master Plan, the NOAA's National Status and Trends Programme and the Mediterranean Biomonitoring Programme). However, numerous studies have shown that levels of MTPLs are influenced by several factors of an environmental (salinity, season, location in the intertidal zone, etc.) and biological (sexual maturity, weight, etc.) nature (Hamza-Chaffay *et al*., 1999; Mouneyrac *et al*., 1998). This is especially relevant in field studies where changes in the concentration of MTPLs due to metal contamination need to be distinguished from those related to natural variations.

In this study concentrations of MTLP and metals (Cd, Co, Cu, Fe, Mn, Ni, Zn) were studied in the whole body, gills and digestive gland of the oyster *Crassostrea angulata* in the Guadalquivir estuary. Temporal variations in these concentrations were examined over a period of one year (from immediately after a mining accident) to evaluate seasonal changes. Finally, subcellular partitioning (pellet, cytosol and heat-stable fraction including MTLP) of the metals was studied to obtain information on the cellular distribution of metals and metallothionein induction.

MATERIALS AND METHODS

Sampling and fraction preparation

Oysters *(Crassostrea angulata)* were collected monthly over a period of one year (from April 1998 to April 1999) at two sampling sites in the Guadalquivir estuary, Punta Montijo (M) and Las Piletas (P). The sampling stations, shown in Figure 1, are relatively close to each other but are situated in the intertidal and subtidal zone, respectively. At station M, samples were also collected in 1999, 2000 and 2001. Sampling was carried out twice yearly, in the spring and autumn seasons of each year. Oysters collected in the field were transported to the laboratory and depurated for 48 h in seawater before being dissected into gills and digestive gland. The individual tissues were kept at -80°C until analysis. Tissues were homogenized in ice-cold 100 mM Tris HCl/Base (pH 8.1 at 4°C), 1 mM DTT and Protease Inhibitor Cocktail (SIGMA P2714). The ratio of buffer solution to the fresh tissue weight was 1:5 (v/w) for digestive gland and 1:3 (v/w) for gills. The homogenate was subsequently centrifuged (50000 g for 120 min at 4°C). The supernatant (S1) was separated from the pellet (P1), heat-treated at 95°C for 10 min and centrifuged (10000 g for 15 min at 4°C) (Dignam, 1990) to separate heat-stable compounds including MTLP (supernatant S2) from heat-unstable compounds (pellet P2). All the procedures were carried out at -4°C to avoid protein degradation. All glassware used was previously washed in HNO_3 at 10% for 24 hours, rinsed with MilliQ water and dried. The water used in all procedures (solution preparation and material washing) was pure water obtained using a MilliQ purification system.

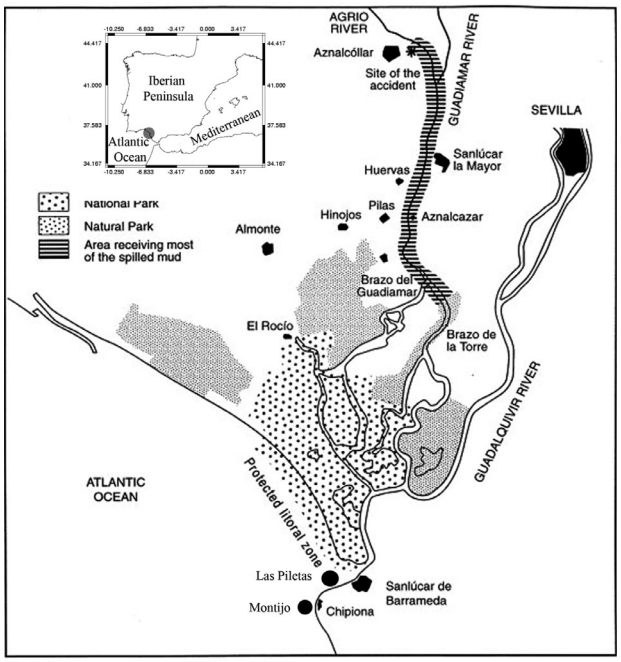

FIG. 1. – Map of Doñana Park and surrounding areas including Aznalcollar mine. Both sampling station were indicated with black circles (Las Piletas 36°47'06.35''N, 6°22'27.57''W, Montijo 36°45'41.01''N, 6°24'36.33''W).

MTLP analysis

The amount of MTLP was determined in the heat-treated cytosol (S2) by differential pulse polarographic analysis, according to the procedure described by Olaf-

son and Olsson (1991). A MetrohmTrace Analyzer with mercury drop electrode (SMDE) was used. The temperature of the cell was maintained at 7°C. The standard addition method was used for calibration with rabbit metallothionein I and II (Sigma M7641) in the absence of purified metallothionein from oyster. Results are expressed as μg MTLP/g dry weight of homogenized tissue.

Metal analysis

Metal analyses were performed on lyophilized tissue (whole body) and subsamples of the homogenate (S1, P1 and P2 fractions for gills and digestive gland) previously digested with suprapure HNO_3 and H_2O_2 according to an adaptation of the procedure of Amiard *et al*. (1987). Metal concentrations (Cd, Co, Cu, Fe, Mn, Ni, Zn) were determined using ICP-OES (Perkin Elmer 2000 DV). The quality of results was checked using certified reference material (*Mytilus edulis* EU BCR CRM278R). Metal concentrations (μg/g dry weight ± SD) obtained showed a good agreement with certified values. All metal concentrations are expressed as μg/g dry weight tissue.

Statistical analysis

One-way ANOVA, post-test for mean comparison (Student *t* test or Mann-Whitney test) and regression analysis (linear or multiple) were carried out using the SigmaStat 3.1 program. Metal contents were first normalized in order to standardize the different concentration ranges. The natural variables x_i were transformed into coded variables X_i.

$$MT = \alpha_0 + \sum_{i=1}^{n} \alpha_i X_i \qquad (1)$$

X_i max = +1 for the maximum value of the variable, and X_i min = -1 for the minimum value. The transformation of natural quantitative variables (Cu, Mn, Zn, Cd, Ni and Co) to coded variables is given by the following equation (Bebianno and Serafim, 2002):

$$X_i = \frac{x_i - \left(x_{i\max} + x_{i\min}\right)/2}{\left(x_{i\max} - x_{i\min}\right)/2} \qquad (2)$$

where $x_{i\ min}$ = the lowest value of the natural variable, and $x_{i\ max}$ = the highest value of natural variable.

RESULTS

Temporal variation in MTLP and metal concentrations

The temporal evolution of MTLP concentrations in gills and digestive gland for oysters from each of the two sampling stations is illustrated in Figure 2. MTLP concentrations for station M were higher than those for station P in the first six months (April- September) for both tissues; on the other hand, MTLP concentrations for station P were higher than those for the station M in the second six months (October-March). Even so, no significant differences were detected between the two locations. Furthermore, there was no statistically significant difference in the seasonal trend between the two stations. The highest MTLP concentrations were found in the digestive gland (nearly six times higher than in the gills) and there were significant differences between tissues (P<0.001). Overall, the values of MTLP concentrations recorded in 1998 were higher than those recorded in the next year (the mining spill occurred on 25 April 1998). For the digestive gland the highest value was in May 1998 (3659.4 ± 531.4 μg/g dry weight) and for gills in June 1998 (586.4 ± 68.1 μg/g dry weight). MTLP concentrations in the digestive gland decreased considerably in June and tended to increase in the following months to reach a maximum in October-November (this trend was less evident in gills). MTLP levels in gills were higher in

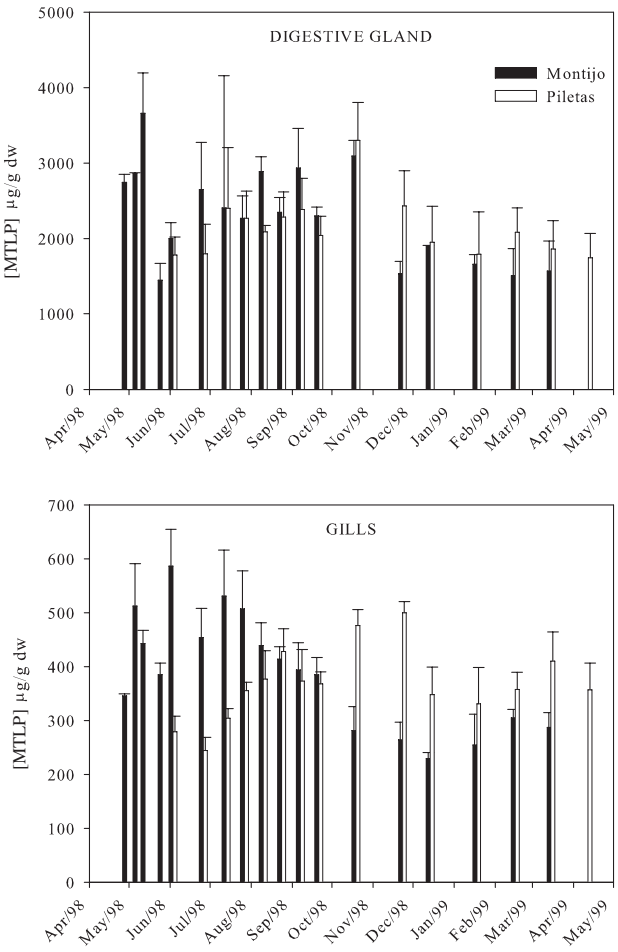

FIG. 2. – Seasonal evolution of MTLP concentrations in digestive gland and gills of oysters collected at both stations (Montijo, black columns; Piletas, white columns). MTLP values are expressed in μg/g dry weight.

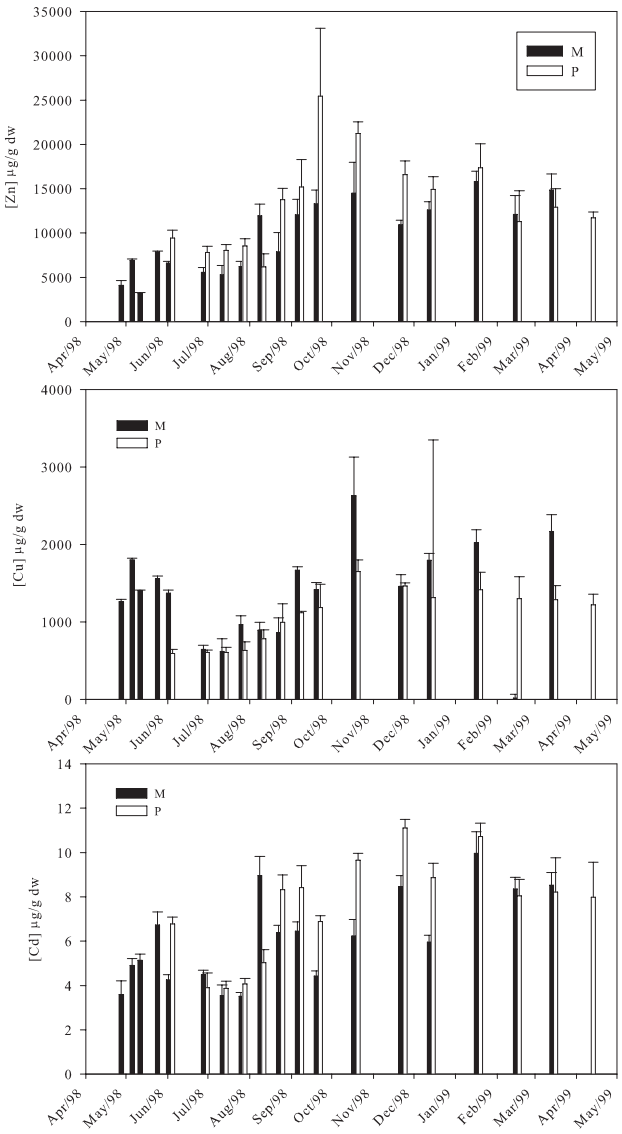

FIG. 3. – Temporal variation (April 1998-April 1999) of Zn, Cu and Cd concentrations (µg/g dry weight) in the whole body of oyster collected at Montijo (black columns) and Piletas (white columns).

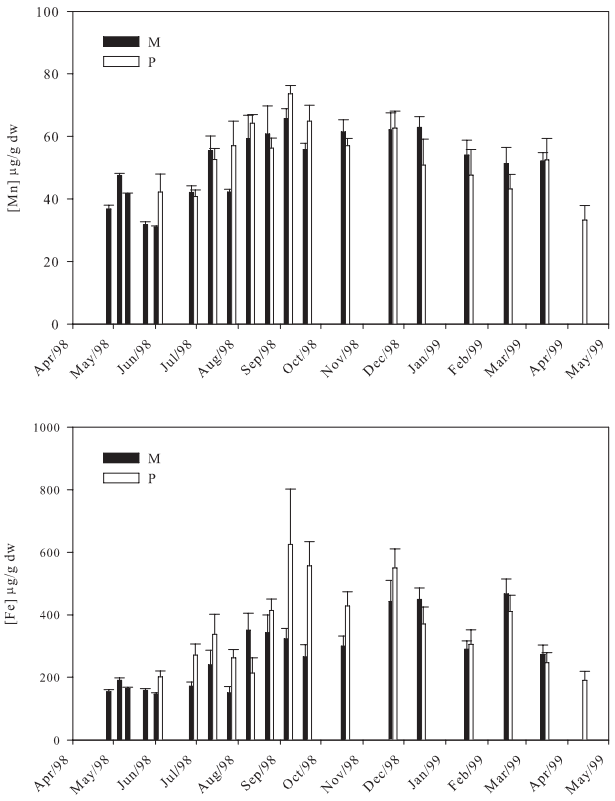

FIG. 4. – Temporal variation (April 1998-April 1999) of Mn and Fe concentrations (µg/g dry weight) in the whole body of oyster collected at Montijo (black columns) and Piletas (white columns).

the spring-summer period (from May to October) than in the remaining six months. In both tissues, the levels for April-May 1999 were lower than during the same period in 1998.

Metal concentrations in whole body of *C. angulata* collected at the two sampling stations from April 1998 to April 1999 are shown in Figures 3 (Zn, Cu and Cd) and 4 (Mn and Fe). The differences between stations M and P were not statistically significant. As with MTLP, high concentrations of Cu, Cd and Mn were recorded in May 1998, followed by a decrease in July; this trend, however, was not evident for Zn and Fe. From July, levels of all the metals showed a tendency to increase, reaching maximum values in September or October (depending on the metal). From October, the levels of Mn decreased, while Cd and Cu levels remained unchanged. At both stations, Zn was the metal that showed the highest concentrations in whole body and in the two tissues (Table 1). The other metals were present in lower concentrations, according to the sequence Cu>Fe>Mn>Cd. Seasonal changes for metal concentrations in digestive gland from 1998 to 2001 at the "Montijo" sampling station are plotted in Figure 5. Zn, Mn, Cd and Ni increased in the year of the accident from April to October, while Cu and Fe did not show significant changes. Data for April 1998 for Co are not available but an increase in concentration was observed from October 1998 to April 1999. Metal concentrations in 1998 were generally lower than in the following years.

Subcellular distribution of metals and relationship to MTLP

Figure 6 shows, for Cu and Mn, the relationship between total metal concentration (S1+P1) and the concentration in cytosolic (S1) and non-cytosolic fractions (P1) in digestive gland and gills. The parameters of the equations of the linear regressions between soluble (cytosolic) and insoluble (non-cytosolic) metal versus total metal are shown in Table 2 for all the metals. In the digestive gland, with increasing total concentrations, metals tend to be stored in the insoluble fraction, since it can be observed that the slope of the equation is always significantly higher (P<0.001) for this fraction

TABLE 1. – Average and range (in brackets) (μg/g dry weight) of metal concentrations in the digestive gland and gills of oysters from the two stations. These data represent the sum of S1 and P1. No data for Zn are available for the Piletas station. DG: digestive gland; G: gills; n.a.: not available.

Station	Tissue	Zn	Cu	Cd	Mn	Fe	Ni	Co
Montijo	DG	7213 (1676-16548)	1176 (622-1910)	7.5 (4.4-11.6)	19 (14-23)	386 (180-805)	4.3 (1.1-8.1)	1.6 (0.4-4.0)
	G	6128 (1931-10398)	1152 (568-1692)	3.44 (1.9-7.5)	39 (24-54)	253 (118-492)	4.7 (2.8-6.8)	1.2 (0.3-2.5)
Piletas	DG	n.a.	998 (465-1803)	13.1 (5.3-30.6)	19 (13-29)	612 (248-1627)	2.8 (1.7-3.6)	2.5 (0.6-7.9)
	G	n.a.	925 (472-1285)	5.7 (2.5-12.5)	32 (25-53)	364 (136-908)	3.6 (2.1-4.5)	1.9 (0.4-4.4)

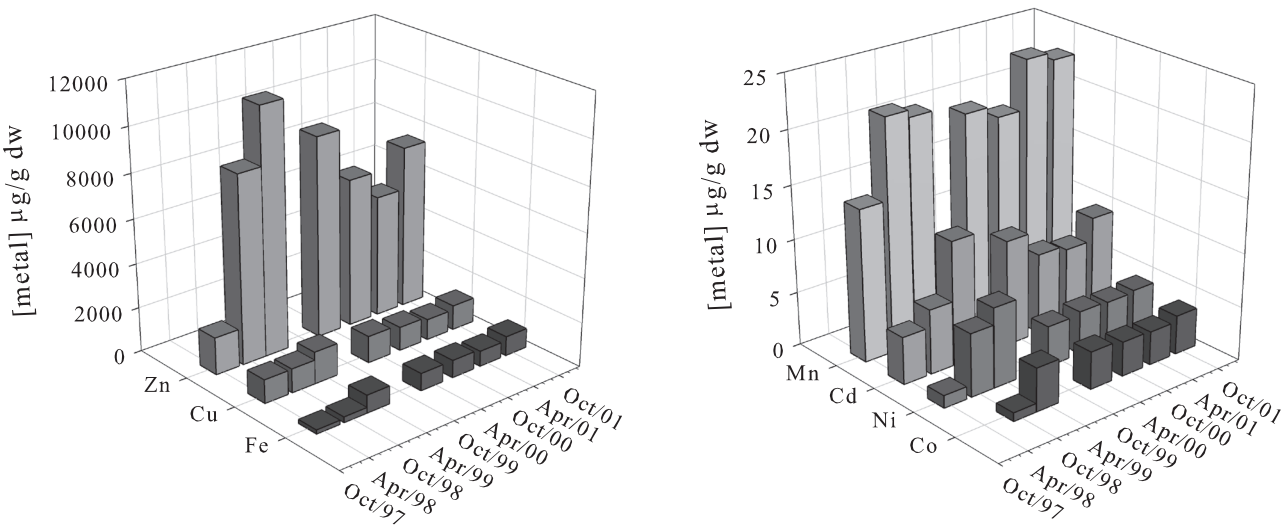

FIG. 5. – Temporal evolution (April, October) in the years 1999, 2000 and 2001 for metal concentrations (μg/g dry weight) in digestive gland of oysters from Montijo.

TABLE 2. – Soluble (S1) and insoluble (P1) fractions vs. total metal contents in digestive gland and gill of *C. angulata*: slope, intercept and correlation coefficients of regression straight lines. (n=36, for Zn n=21).

Metals	Fraction	Digestive gland			Gills		
		Slope	Intercept	*r*	Slope	Intercept	*r*
Cu	P1	0.853	65.735	0.885*	0.826	98647	0.824*
Cu	S1	0.066	40.952	0.267	0.169	-78.096	0.313
Zn	P1	0.804	-370.196	0.830*	0.821	-822.72	0.786*
Zn	S1	0.173	699.875	0.724*	0.134	1053.49	0.608
Mn	P1	0.872	1.911	0.993*	0.435	6.307	0.830*
Mn	S1	0.128	0.217	0.771*	0.408	-0.691	0.754*
Fe	P1	0.985	-48.171	0.998*	0.996	-22.767	0.996*
Fe	S1	0.013	48.297	0.307	0.015	24.032	0.439
Cd	P1	0.919	-0.073	0.994*	0.960	-0.142	0.985*
Cd	S1	0.069	0.223	0.769*	0.048	0.257	0.383
Ni	P1	0.846	-0.596	0.943*	0.688	-0.598	0.861*
Ni	S1	0.215	0.429	0.713*	0.348	0.496	0.708*
Co	P1	0.998	-0.356	0.999*	1.002	-0.204	0.996*
Co	S1	0.001	0.364	0.007	0.015	0.198	0.214

* significant at the 99% level

than for the soluble fraction (Table 2). In the gills, only for Cu, Zn, Fe, Co and Cd is the slope for the equation consistently higher for the insoluble fraction (Table 2) whereas for Ni and Mn, as shown by the parameters of the equation, the metal tends to accumulate equally in both S1 and P1.

Figure 7 shows the relationship between metal contents in S2 (heat-stable proteins including MTLPs) and

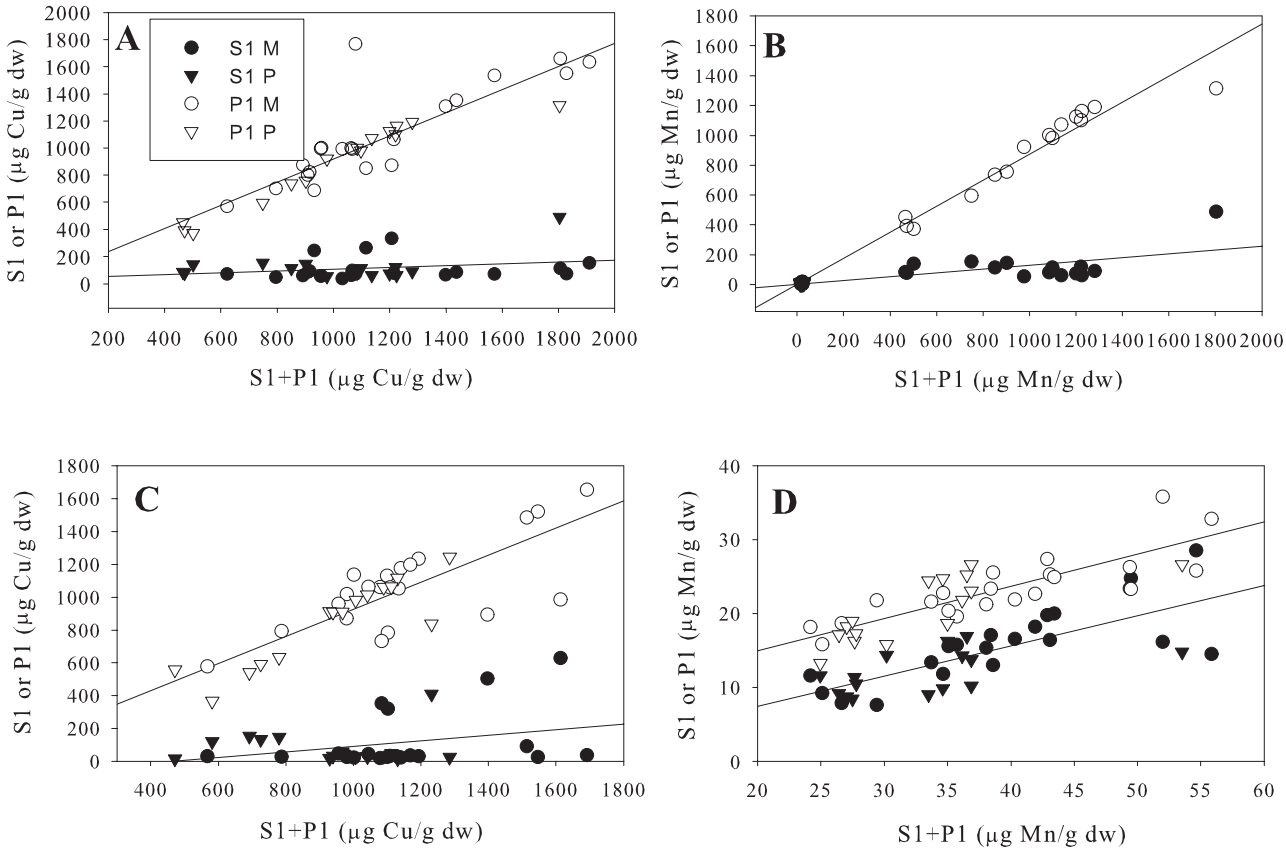

FIG. 6. – Soluble (S1) and insoluble (P1) fractions vs total metal content (S1+P1) for Cu and Mn in the digestive gland (A, B) and gills (C, D) of *C. angulata.* Values for both sampling stations (M=Montijo, P=Piletas) were considered together. Metal concentrations are expressed in µg/g dry weight. The parameters of the straight line equations are shown in Table 2.

TABLE 3. – Parameters of the equation* MTLP concentration = f (metal concentration) for metals in the soluble S1 fraction at two sites, Montijo and Piletas, in the Guadalquivir estuary. Only those metals significantly related to MTLP concentration (P<0.005) are included in this table. Number of samples: Montijo=17, Piletas=14. There are no data for Zn for the Piletas station.

Site	Organ	Metals (regression coefficients α_1, α_2, α_3, …)							Constant (α_0)	R^2_{Adj}
		Cu	Cd	Mn	Zn	Fe	Ni	Co		
Montijo	Digestive gland	n.r.	742. 652 (P=0.008)	1444. 295 (P<0.001)	n.r.	-618. 539 (P=0.020)	-726. 876 (P=0.025)	-932. 193 (P=0.050)	2333.773	0.510
	Gills	n.r.	96. 952 (P=0.017)	120. 241 (P=0.007)	-187. 568 (P<0.001)	n.r.	n.r.	n.r.	478.247	0.507
Piletas	Digestive gland	-653. 894 (P=0.036)	616.187 (P=0.033)	n.r.	n.a.	349.800 (P=0.014)	-127.716 (P=0.339)	445.613 (P=0.004)	1953.901	0.694
	Gills	n.r.	n.r.	n.r.	n.a.	61.117 (P=0.063)	n.r.	n.r.	365.201	0.183

P = probability for a significant correlation between MTLP and soluble metal concentration based on *t* test.
n.r.= not reported.

P2 (proteins and other molecules that precipitate during heat-treatment) in the gills. In this tissue, when metal concentrations in the cytosolic fraction (S1) are low, the metals are distributed equally between S2 and P2 fractions. When cytosolic concentration of the metal increases, the metal is binding to the thermostable proteins contained in the S2 fraction. The amount of metal present in the P2 fraction remains unchanged, except for Fe and Co where a negative slope is reported for both metals. In the digestive gland, only Fe, Mn and Co show a tendency to bind to the S2 fraction, when the metal concentration increases in the S1 fraction. However, Cu, Cd and Ni concentrations increase in both fractions (but without significant differences between S2 and P2).

The relationship between MTLP and metal concentrations (for Cd, Co, Cu, Fe, Mn, Ni and Zn) was determined using multiple regression analysis (best subset regression). Independent variables were standardized and expressed according to the following function:

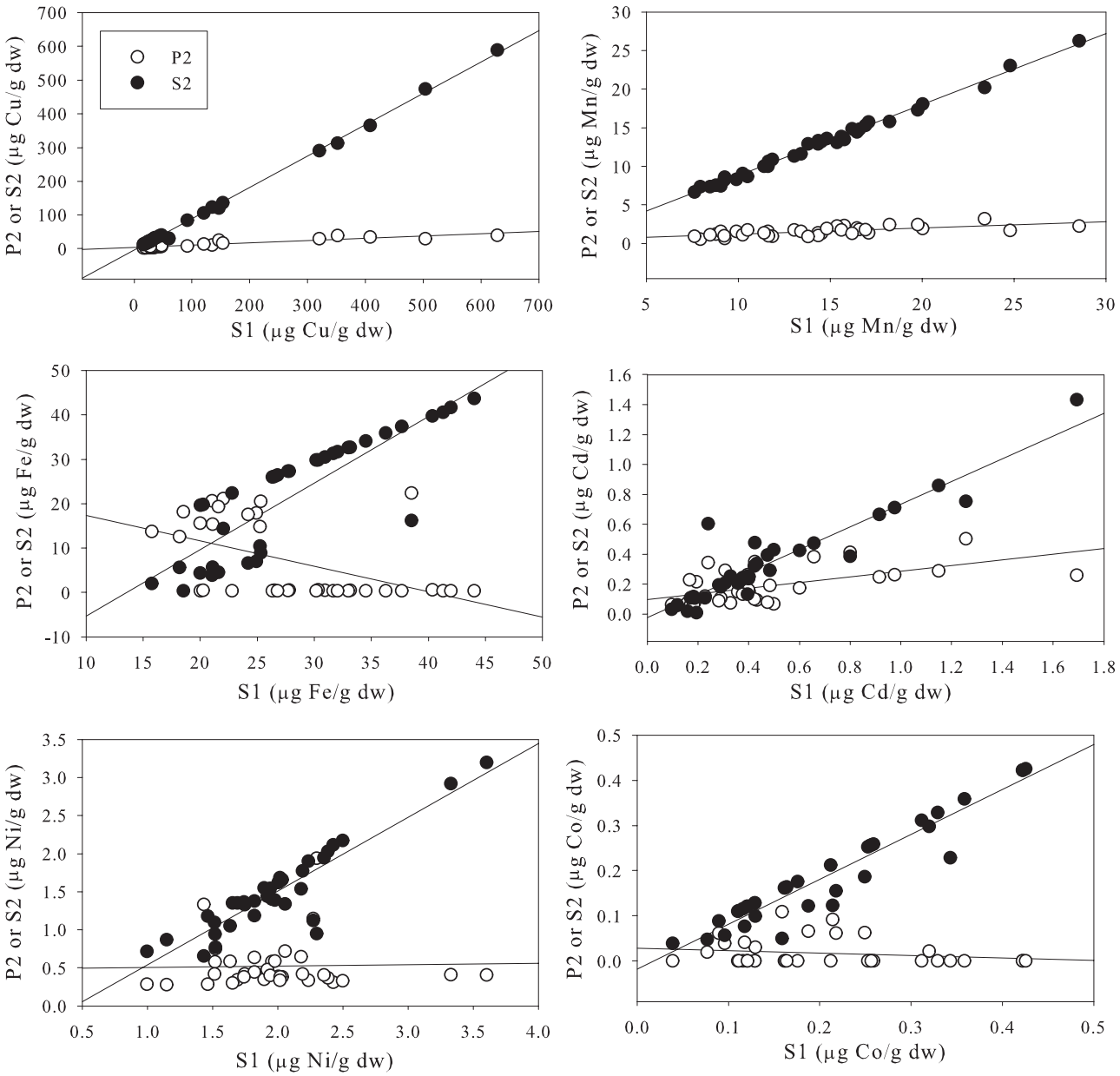

Fig. 7. Relationship between metal content in P2 and S2 fractions and total metal in cytosol of gill. Data for Zn not are available. For each metal values relative to both stations are considered together. Metal concentrations are expressed in µg/g dry weight.

MTLP = *f* (Cd, Co, Cu, Fe, Mn, Ni, Zn). This model was applied for the digestive gland and gills separately. Because MTLP is a cytosolic heat-stable protein, it seems appropriate to consider the relationship between MTLP concentration in a particular type of tissue, and metal concentration in the supernatant obtained after heat-denaturation of the cytosol (S2). However, during heating, the distribution of metals among cytosolic compounds may be modified (Bragigand and Berthet, 2003). Consequently, in the present study, the relationship between MTLP concentration and metal content in the supernatant before heat treatment (S1) was examined. The parameters for the regression are summarized in Table 3. At station M, Cd, Mn, Fe, Ni and Co together explain 51% (R^2_{Adj} = 0.510) of the variance in MTLP concentration observed in the digestive gland: Cd and Mn were positively correlated with MT concentration, whereas Fe, Ni and Co were negatively correlated. In the gills, only Cd, Mn and Zn contribute to explaining 50.7% (R^2_{Adj} = 0.507) of the variance in MTLP, and only Zn presented a negative correlation. At station P, Cu, Cd, Fe, Ni and Co explain 69.4% (R^2_{Adj} = 0.694) of the variance in MTLP (Cu and Ni were negatively correlated) in the digestive gland, whereas in the gills, only Fe contributes, and explains only a small percentage,18.3%, (R^2_{Adj} = 0.183) of the variance in MTLP. No strong correlation between MTLP and metals was observed, as indicated by the low values of $R^2_{Adj.}$

DISCUSSION

Oysters (including *Crassostrea angulata*) and other species of molluscs are employed in marine environment biomonitoring programmes in many parts of the world, particularly for metal contamination (Rainbow, 1995, O'Connor, 1994). The organism shows a high capacity to accumulate metals in its soft parts, proportionally to metal concentration and bioavailability in the environment (Bryan *et al*., 1985). It also uses detoxification mechanisms (such as induction of MTLPs) to regulate internal concentration of metals and prevent cellular damage. In this study, the levels of metals in whole body, gills and digestive gland increased during 1998 as the result of metal inputs to the Guadalquivir estuary originating from the Aznalcóllar accident (Gómez-Parra *et al*., 2000). The maximum levels were observed in 1999, and there was a tendency to decrease or to remain unchanged in subsequent years, depending on the metal (Blasco *et al*., 2003)

Zinc and copper showed higher accumulation in all tissues analyzed, in agreement with high metal concentrations recorded by Achterberg *et al*. (1999) in the estuary after the mining spill at Aznalcollar. *C. angulata* shows considerable ability to accumulate Zn and Cu; this is characteristic of oysters and is due to hemocyanin respiratory pigment (Shulkin *et al*., 2003; Ruelas-Inzunza *et al*., 2000). Metal bioaccumulation in oysters shows a temporal variability which can be affected by biotic factors such as abundance of food and weight changes due to biological cycle, particularly reproductive cycle (Raspor *et al*., 2005; Pytharopolou *et al*., 2006), and by abiotic factors such as salinity and remobilization of sediment-bound trace metals, which affect both the total dissolved metal concentrations and their bioavailability (Riba, 2005; Bebianno and Serafim, 2003; Ke and Wang, 2001). Tidal changes do not seem affect the metal bioaccumulation and MTLP responses in oysters because the range of concentration found was similar at the two sampling stations despite being subjected to a different tidal regime.

MTLP and some of the metals showed similar seasonal variability. The importance of natural factors in MTLP concentrations was confirmed by multiple regression analysis, which showed that soluble metals only partially (18.3–69.4%) explain the MTLP variability. Other natural factors can affect MTLP levels.

The values recorded in May 1998 for metals concentrations are almost certainly related to the high environmental metal concentrations due to the mining waste spill. In fact, studies carried out in the oyster *C. gigas* regarding seasonal evolution of metal content in unpolluted areas did not increase in these months (Langston and Spence, 1995). However, differences in metal levels are associated with the reproductive cycle and this influence may be modified depending on the latitude at which the oysters are collected (Frías-Espericueta *et al*., 1999a; Páez-Osuna *et al*., 1995).

Metals were differently accumulated in the two target tissues analyzed: Mn and Ni were accumulated in gills, and Fe, Cd and Co mainly in the digestive gland, whereas Cu and Zn showed similar concentrations in both tissues. Similar results were obtained in oyster *C. iridescens* (Frías-Espericueta *et al*., 1999b) and *M. galloprovincialis* (Irato *et al*., 2003; Simkiss and Taylor, 1995). Fisher and Reinfeld (1995) pointed out that the elements associated with particulate material are accumulated in soft tissues like the digestive gland, whereas soluble metals accumulate in the gills: thus, metal accumulation in different tissues are related to the form in which the metal is available in the environment (soluble, particulate, etc.). Gills and digestive gland showed different patterns of MTLP levels: the concentration of proteins in gills was about six times less than in the digestive gland, because the latter is the main organ for detoxification.

In both gills and digestive glands, all the metals were stored mainly in the insoluble fraction, which is in agreement with the biological processes (compartmentalization into lysosomes, granules and membrane-bound vesicles) proposed by Langston and Spence (1995) and Langston *et al*. (1998). The insoluble fraction of bioaccumulated metals is the result (at least partly) of the biomineralization into different kinds of granules (Ettajani *et al*., 2001; George, 1990; Mason and Jenkins, 1995); through this process, metals are no longer active in the intracellular metabolism (Geffard *et al*. 2005). Another mechanism for controlling intracellular toxicity of metals is binding with cytosolic proteins (MTLP in soluble fraction S2) (Viarengo and Nott, 1993; Roesijadi, 1992). As the cytosolic metal concentration increases, this causes the metals to bind to the heat-stable fraction S2; this may be indirectly related to the increase of total MTLP concentration induced by high intracellular levels of metals (Bonneris *et al*. 2005), thus confirming the primary role of these proteins in the internal control of metals.

Cd, Zn and Cu are trace metals normally associated with MTLP (Amiard *et al*., 2006; Mouneyrac *et al*., 2001). Cd, which is soluble in the S2 fraction, showed a positive correlation with MTLP concentration in both tissues at the intertidal sampling station (Montijo), whereas there was a negative correlation between soluble Zn and MTLP concentration in gills at Montijo, and between Cu concentrations and MTLP in the digestive gland at the subtidal station, Piletas. Occasionally, MTLP may bind to other metals such as Co and Ni (Stegeman *et al*., 1992). More surprising is the correlation observed with Fe and Mn, which may be explained by high levels of these metals in the environment after the accident. Transition metals, such as Fe, can cause oxidative stress in marine organisms. Viarengo *et al*. (1999) observed a small but significant increase in MTLP in digestive gland tissue of mussel after treatment with Fe, suggesting that MTLP plays a role in protecting against Fe-induced oxidative stress.

Numerous authors have stated that monitoring MTLP in oyster is useful as an indicator of environmental quality (Mouneyrac *et al*., 2001). However, in most cases the natural variability of MTLP levels in molluscs tends to conceal any changes due to metal pollution. Moreover, a high level of contamination may produce results different from those expected, such as the binding to MTLP by metals (Co, Ni, Fe and Mn) that, in an unpolluted environment, only occasionally bind to these proteins. From the results of this work it is clear that the variations in MTLP levels are not exclusively associated with metal concentration and that other environmental factors could explain a percentage of MTLP variability. Therefore, care is needed in the interpretation of metallothionein levels from field studies.

ACKNOWLEDGEMENTS

We would like to thank the Junta de Andalucía (Research Plan for Andalusia, RNM306) for funding this research. Chiara Trombini has a fellowship under the Erasmus Programme.

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Received November 1, 2008. Accepted July 26, 2010.
Published online November 13, 2010.