Bioaccumulation and effects of metals bound to sediments collected from Gulf of Cádiz (SW Spain) using the polychaete, *Arenicola marina*

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Introduction

Sediments are widely recognized and employed in assessing the impact of contaminants on aquatic systems (Bryan and Langston 1992) as chemicals normally have considerable higher concentrations in sediments than in the water column. Given that aquatic sediments can act as reservoir for persistent contaminants (e.g. trace metals), organisms which live in and feed on sediment can accumulate considerable amount of contaminants. Thus, to assess the adverse effects of pollutants on the aquatic sedimentary environment, several authors have proposed the use of sediment toxicity tests (Chapman and Wang 2001). These kinds of tests aim to study the relationships between the sediment concentration of chemicals and any adverse biological effects on the biota resulting from exposure to these chemicals. Therefore, the choice of test organisms is essential for providing appropriate information of the hazards of chemical stressors (Chapman et al. 2002). Polychaetes, in particular *A. marina* have been used as biomonitors of littoral and estuarine contamination (Packer et al. 1980; Bat 1998; OSPAR 1995; Casado-Martínez et al. 2008; Morales-Caselles et al. 2008). Lugworms are abundant, tolerant to a wide range of environmental factors and ecologically important, moreover they live in direct contact with the sediment.

The majority of studies on metal availability in sediments are based on toxicity, however bioaccumulation may be a more accurate endpoint for assessing bioavailability in risk assessment (Ankley et al. 1996). Trace metals may be bioavailable even if toxicity is not observed since toxicity depends on various factors such as the sensitivity of organism, length of exposure period etc. Biological adverse effects of pollutants may also be manifested at the biochemical level in organisms or at higher levels of organisation and responses are measurable. Biomarkers have been incorporated into environmental toxicology research for decades, but they are still seldom used in a day to day management of the environment (Handy et al. 2003). The biomarker based on the measurement of metallothionein-like protein (MTLP) concentration in biological tissues represents a detoxification role for structures which have been reversibly impaired by inappropriate metal binding (Martín-Díaz et al. 2007). They have been considered as useful and suitable biomarkers of metal exposure in aquatic organisms (see review by Amiard et al. 2006).

The aim of this work was to assess the bioavailability of metals bound to sediments collected from twelve sampling sites along the Gulf of Cádiz, characterized with different types and degrees of metal contaminations. In order to assess the defence mechanisms against the accumulated metals, the induction of a typical biomarker, metallothionein-like proteins was determined in tissues sampled after 7 and 14 days of exposure. Data were integrated by multivariate analysis to correlate the concentrations of contaminants and biological responses in the biota.

Materials and Methods

Study area, sample collection and characterization

Sediments were collected from twelve sampling sites along the southwest coast of Spain (Table 1). Guadiana (sample 1, near the international bridge between Spain and Portugal and sample 2 in the mouth of the river) and Huelva estuaries (sample 3 is more influenced by the rivers Tinto and Odiel, and sample 4 in the Pedro Santo Channel) are influenced by the surrounding mining area, moreover an industrial complex is located in the proximity of Ría of Huelva. Sampling points in the Guadalquivir estuary (5 and 6) are impacted by maritime transport and urban activities, as well as samples from the

Bay of Cádiz (samples 7 and 8), while sample 9 is a comparatively clean area considered suitable as negative toxicity control according to previous studies (Riba et al. 2004a). The Bay of Algeciras (sample 10 and 12 in the mouth of Guadarranque River, near chemical processing plants; sample 11 in the mouth of river Palmones) is considered as an important harbour and receives considerable contaminant input derived from several industrial and commercial shipping activities.

Sediments were collected with a 0.025 m^2 Van Veen grab from approximately the top 20 cm of the sediments. Samples were transferred to the laboratory where they were homogenized, sieved through a 2 mm mesh and stored in dark at 4 ˚C no longer than 2 weeks before the start of testing. Total organic carbon (TOC) was determined using the El Rayis (1985) modification based on an acidification of the sediment sample, and grain size distribution was analysed by using a laser particle size Fristch (model Analysette 22) following the method reported by Riba et al. (2003).

Toxicity test

The test organisms, *Arenicola marina* were collected in the Cantabrian coast (North Spain) by hand digging and were transported to the laboratory in cool boxes containing clean seawater. Worms were transferred to 20 L tanks containing clean sediment (5 cm depth) and seawater and acclimated to laboratory conditions for a week prior to the test (17±1˚C, salinity 38). Tests organisms were placed in tanks containing only seawater to depurate the digestive tract 24 h prior to tests. The bioassay was performed in duplicate. Small tanks were filled with approximately 1 kg of sediments and 5 cm overlying clean seawater provided with gentle aeration. After the acclimation period 7 organisms were placed in every tank and after 24 h unburied worms were replaced. During the 14 days of exposure, the overlying water was replaced every two days and physical parameters (temperature, pH, salinity, dissolved oxygen concentration) were measured in each tank on the same day. Lugworms were sampled on day 0 to obtain the initial concentrations of tissues. After 7 and 14 days 4 individuals from each station were collected and 2 of them were immediately frozen at -80 ˚C (MTLP measurement), while the rest were placed for 24 h in clean seawater for depuration and stored at -20˚C until trace metal analysis. The mortality was also recorded during the experimental period.

Trace metal analyses

The whole tissues of lugworms were lyophilised and digested in concentrated HNO_3 and H_2O_2 (Suprapur, Merck) at 95˚C (García-Luque et al. 2007), while sediment samples after freeze-drying were extracted with 1 N HCl to provide more information about the bioavailability of metals (Luoma and Bryan 1982). In both cases, blanks were prepared simultaneously in the same way as the sample. Concentrations of Cd, Co, Cu, Ni, Pb and Zn in the acid digests were determined with an inductively coupled plasma-optical emission spectrometer (Optima 2000 DV, Perkin Elmer). The accuracy of the analyses was checked by digesting certified reference material (TORT-2, Lobster Hepatopancreas, National Research Council, Canada) and was considered satisfactory (Table 2). Data are expressed as μg g^{-1} dry weight.

MTLP induction

Tissue samples were homogenized with an ultraturrax homogenizer with Trizma-HCl/Trizma-Base 0.1M (pH 7.6) buffer (at a ratio of 1:3) on ice (4˚C) and centrifuged at 30 000g for 2 h at 4˚C. The supernatant (cytosol) was separated from the pellet and 0.1 mL of supernatant was added to 0.9 mL of NaCl (0.9%), heated at 95˚C for 4 min and centrifuged at 10 000g for 15 min at 4˚C. Metallothionein-like protein concentrations were determined in the supernatant by Anodic Stripping Voltametry (ASV), according to the procedure described by Olafson and Olson (1991), using purified rabbit metallothionein (Sigma-Aldrich, MT I-II). The total protein concentration was determined with a bicinchoninic acid protein assay (Smith et al. 1985).

Results and Discussion

Chemical characteristics of sediments

The concentrations of trace metals (Cu, Zn, Cd, Ni, Co and Pb) presented in the twelve sediments are shown in Table 3. Among all stations the negative control (sample 9) and sample 5 from the Guadalquivir estuary showed the lowest metal concentrations in sediments, whereas sediments from sample sites from Huelva estuary and Bay of Algeciras (samples 3, 4, 6 and 10) contained greater amount of trace metals. For instance, the highest concentrations of Cd, Zn, Cu and Pb were measured in sample 3. Total organic carbon content and grain size most probably control metal concentrations in sediments. The proportion of fines ranged between 30 and 90%, except samples 5 and 9 with a proportion of fines (referring to the proportion of sediment < 63µm) < 10%. Similarly, the TOC varied between 0.10 and 3%, except samples 5 and 9, which had TOC values < 0.10%. The correlation between the proportion of fines and TOC concentrations in sediments was statistically significant ($p<0.05$). It has to be mentioned that independently of the exclusion of the sandy sediment samples, this relationship behaved similar.

Biological responses in *Arenicola marina*

Organisms exposed to the sediment collected from the proximity of chemical processing plants in the Bay of Algeciras (sample 10) showed the highest accumulated mortality at the end of the exposure (66.6%), followed by those in Huelva estuary (sample 3) and the two sampling points in the Guadalquivir estuary (sample 5 and 6). Toxic responses measured in sediments from the mouth of Guadiana estuary (sample 2), Huelva estuary (sample 4) and mouth of river Palmones (sample 11) were lower, while the rest of the stations did not cause mortality.

The rate of increase in metal concentrations (K_{metal}) from days 0 to 14 in *A. marina* was described by a linear kinetic approach (Martín-Díaz et al. 2007) and was fitted with the results as follows:

$\ln [M] = \ln [C_0] + K_t$

where C_0 is the concentration of metal or MTLP at t = 0; M is the concentration of metal or MTLP at time t minus at the initial time (0); K is the constant rate of increase. The positive K values obtained from these equations demonstrated that lugworms had potential to accumulate trace metals during the exposure time. In general, trace metal concentrations were higher in specimens sampled from the contaminated sites. On day 14, elevated concentrations of Zn, Cu, Ni and Co were recorded in individuals from the Bay of Algericas (sample 10), as well as Cu, Zn in lugworms exposed to sediments from Huelva estuary (samples 3 and 4). Part of the samples were accidentally lost during the analysis, therefore no data for K_{Pb} are available.

The rate of metallothionein-like protein increase (K_{MT}) demonstrated the induction of these proteins in organisms along the 14 days of exposure. Lowest MTLP synthesis was observed in lugworms from samples 2 and 9, whereas increased concentrations were elevated in various samples, such as 3, 5, 10 and 11.

Multivariate analysis

In order to observe the relationships between the chemical concentrations in sediments and biological effects on test organisms, multivariate analysis was applied. The initial data matrix contains 12 cases, the twelve stations and 15 variables, the geochemical characteristics (fines and total organic carbon), the metal concentrations in sediments (Cd, Zn, Ni, Co, Cu and Pb), the toxicity measured as mortality, the rate of increase of MT concentrations (K_{MT}) and the rate of increase of bioaccumulation of metals in tissues (K_{Cd}, K_{Zn}, K_{Ni}, K_{Co}, K_{Cu}). The factor analysis with varimax normalized permitted after the reduction of 15 variables to three principal factors, which explains 79.14 % of the total variance (Table 4).

The first principal factor (F1) was predominant and accounted for 41.75% of the variance. This factor combines concentrations of Cd, Zn, Cu and Pb in sediment and shows relationship with the induction of MTs (K_{MT}) and with the increase in bioaccumulation of Zn and Cu (K_{Zn}, K_{Cu}). The second principal factor (F2) accounts for 21.35% of the total variance and has positive loadings for the sediment concentrations of Ni and Co, the fine particles and total organic carbon of sediments and associates with toxicity to *A. marina*. The third principal factor (F3) accounts for 16.04% of total variance and combines the bioaccumulation of Cd, Zn, Ni, Co. This factor suggests that the bioaccumulation of these trace metals was not associated neither with the biological responses that we have studied nor with the concentrations of these metals measured in sediments.

Estimated factor scores are to explain the prevalence of every component for each station and used to confirm the factor descriptions (Figure 1).

The first factor (F1), which is representative of the contaminants (Cd, Zn, Cu, Pb) in sediment, the bioaccumulation of Zn and Cu and the MTLP induction in tissues, has positive loadings in samples 3 and 4 from the Huelva estuary. Both samples are characterized by high concentrations of trace metals due to mining and industrial activities (Riba et al. 2004a). The elevated concentrations of Zn and Cu measured in tissues of *A.marina* according to F1 did not cause toxicity. The most likely explanation could be that Cu and Zn are considered as strong inducers of MTLPs, as these proteins can bind them via the sulfhydryl group of cysteine residues. Therefore MTLPs were probably involved in protection against trace metal toxicity. The induction of MTLPs in polychaetes after metal exposure has been confirmed by several authors. In the Cd-exposed *Neanthes arenaceodentata* an increase in the metal concentration associated with MTLPs was observed by Jenkins and Mason (1988). Ng et al. (2008) found no increase in MTLP concentrations, but higher MTLP turnover (synthesis and breakdown) in the nereid polychaete, *Perinereis aibuhitensis* after Cd pre-exposure. These findings indicate that measuring the MTLP concentration alone can be misleading. The synthesis of MTLPs was observed in *Eurythoe complanata* after exposure to Cu or Zn (Marcano et al. 1996). However these authors reported no increase in the concentrations of MTLPs in the combined Cu-Zn treatments, suggesting the possible interaction between the metals. Trace metals in sediments from the Huelva estuary also caused MTLP induction in female shore crabs, *Carcinus maenas* (Martín-Díaz et al. 2009) after 28 days of exposure. Numerous studies support the idea of using MTLP concentrations in organisms as biomarkers in environmental monitoring programme. It has to be mentioned that the expression of MTLPs can be influenced by various natural factors that affect the metal accumulation, such as salinity (Leung et al. 2002) or temperature (Serafim et al. 2002). In some species the presence of MTLPs has been confirmed but their levels were not correlated to the local metal bioavailability due to the ability to regulate their metal burden (Mouneyrac et al. 2003; Poirier et al. 2006). Thus, additional research on the relationship between metal toxicity and MTLP induction in this species is required before their routine use in biomonitoring programs. Conversely, the Ni and Co sediment concentrations, which were comprised in F2 and showed positive factor scores in sample 3, 6, 10 and 11 (Figure 1) did not cause MTLP induction, but related to high mortality to *A. marina*. F2 is mainly prevalent in sample 10, collected from the Bay of Algeciras, continuously impacted by industrial effluents and maritime activities. Morales-Caselles et al. (2007) previously reported high toxicity to *Corophium volutator* exposed to sediment collected from the same area due to Ni and Co contaminants associated with PAHs in oil spills. *A. marina* was chosen as an organism known to be sensitive to hydrocarbons, but no measurements of these contaminants were included in the present work. These two metals obviously could originate from any other sources, however Riba et al. (2004b) reported hydrocarbon contamination in sediments from the Huelva estuary (sample 3). Cesar et al. (2007) found that contaminations by PAHs and Ni were closely related to amphipods (*C. volutator*) mortality in the Bay of Algeciras as well as in the Huelva estuary. The characterization of sediments can provide some explanations for the metal accumulation in the organisms. Usually the trace metal concentrations increase with decreasing grain size of the sediment. In the case of fine sediments, the surface binding sites per unit mass of the particle increase. Organic matter can contain functional groups that form complexes with metals and decrease their bioavailability to organisms. Negative correlations between bioavailability of some metals to *A. marina* and the proportion of fines and total organic material have been considered by Casado-Martínez (2006). It has been shown that deposit-feeding invertebrates can adjust their rates of ingestion according to the quality of the sediment (Cammen 1980). Thus, lower organic matter content may lead to an increase in ingestion rate to satisfy their nutrient requirement. In the present study, lowest TOC were recorded for two samples (5 and 9), in which low concentrations of metals were measured, and as a consequence the bioaccumulation in lugworms exposed to these two sediments was obviously low. Although higher TOC values were related to greater amounts of accumulated metals by organisms (e.g. samples 3 and 10), these metals generally are associated with organic materials in the sediments, that lugworms feed preferentially.

The third factor demonstrates the bioaccumulation of Cd, Zn, Ni, Co in the tissues without toxic or sublethal effect on the test organisms. The capacity of lugworms to accumulate trace metals from sediments has been addressed by several authors (Packer et al. 1980; Bat 1998; Bat and Raffaelli 1998; Casado-Martínez et al. 2008). Time course of metal uptake was also revealed in three body compartments (body wall, intestine and blood) of *A. marina* after 25 days of copper sediment exposure (Everaarts 1986).

Conclusions

The results of this survey confirm that MTLPs induction in *A. marina* was related to increased sediment metal concentrations in samples collected from the Huelva area. The rates of increase in MTLP and accumulated metal concentrations along the exposure time seemed to be good and suitable tools for evaluation of metal bioavailability in sediments. Results suggest that MTLP induction in tissues played an important protective role, therefore lower toxicity was detected. In our study, the test organism, *Arenicola marina* due to its capacity to accumulate metals from sediment was found as a possible indicator species for sediment quality assessment. The use of multivariate analysis appeared to be a useful tool to link the chemical concentrations and biological responses.

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Figure captions

Figure 1. Factor loadings for the three principal factors in each of the twelve cases. The factor scores quantify the prevalence of every factor for each station and are used to establish the description of each factor.