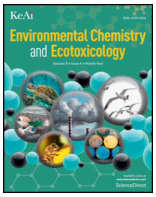
Environmental Chemistry and Ecotoxicology 1 (2019) 12–18

Contents lists available at ScienceDirect

Environmental Chemistry and Ecotoxicology

journal homepage: www.keaipublishing.com/en/journals/environmental-chemistry-and-ecotoxicology/



Trace metal levels and toxicity in the Huelva Estuary (Spain): A case study with comparisons to historical levels from the past decades

B.M. Torre [a,e], A.R. Borrero-Santiago [b,*], E. Fabbri [a,d], R. Guerra [a,c]

[a] *Centro Interdipartimentale di Ricerca per le Scienze Ambientali (C.I.R.S.A.), Università di Bologna, Via S. Alberto 163, 48123 Ravenna, Italy*
[b] *Norwegian University of Science and Technology, Department of Chemistry, 7491 Trondheim, Norway*
[c] *Department of Physics and Astronomy, Viale Berti Pichat 6/2, Bologna, Italy*
[d] *Department of Biological, Geological, and Environmental Sciences, Via Selmi 3, Bologna, Italy*
[e] *Faculty of Marine and Environmental Science, University of Cadiz, Polígono Río San Pedro s/n, 11510 Puerto Real, Cádiz, Spain*

ARTICLE INFO

Article history:
Received 27 May 2019
Received in revised form 24 June 2019
Accepted 3 July 2019
Available online 02 August 2019

Keywords:
Trace metals
Sediment
Paracentrotus lividus bioassay
Huelva Estuary
Toxicity

ABSTRACT

The Huelva Estuary, in the south of Spain, is a highly polluted area subject to heavy anthropogenic pressures such as coastal urbanization, fishing and tourism activities, commercial transports, as well as petrochemical, agrichemical and intense mining industry. Trace metal levels in sediments from the Huelva Estuary have been largely investigated over the last decades, but an evaluation through the years has not been considered yet. This study analyzed the current concentrations for Cr, Cu, Mn, Ni, Pb, Zn, Fe and organic matter content in sediments from two sampling sites (Muelle Capesa and Mazagón) and toxicity on fertilization and embryogenesis of sea urchin (*Paracentrotus lividus*). Results were compared with data from the past decades. Zn and Cu displayed higher levels and enrichment in sediments from the Muelle Capesa close to the Huelva industrial area. Mazagón sediment, despite moderate levels and enrichment in Zn and Cu, is proved to be affected by anthropogenic impacts, due to continuous inputs from acid mine drainage and alongshore current transport. In our experiments, sea urchin fertilization rates and larval development were significantly affected by exposure to elutriates from Huelva Estuary sediments. Therefore, the Huelva Estuary area shows a declining but chronic contamination in Zn and Cu, originating from point and diffused anthropic activities, which in turn, are likely to cause adverse effects on the coastal ecosystem.

1. Introduction

The city of Huelva, due to its prime location and excellent connection, as well as its close proximity to one of the most important ports in Spain, is a gateway to the Atlantic Ocean. Thanks to the coastal urban layout, the presence of petrochemical, agrichemical and mining industries, as well as tourism, agriculture and fishing activities, Huelva is one of the most important industrial and business hubs in Spain [1–3]. The Huelva Estuary is a highly polluted area, due to several contamination sources from the surrounding areas: trace metal loads from the Tinto and Odiel Rivers drain into the Iberian Pyrite Belt, one of the most important mine districts in Western Europe, industrial effluents from factories, and sewage from coastal towns [4,5]. During the past decades, a large number of investigations related to the sediment quality have been published due to the importance of the marine resources of this province of Spain [2,6–8]. High trace metal concentrations have been detected both in the water column and in sediments of the Huelva Estuary [5,9–11]. However, trace metal levels are usually higher in sediments than in overlying water, due to their adsorption capacity and physical-chemical parameters such as pH, salinity, redox potential, temperature, and organic matter content [12]. Metals can affect organisms both on the cellular and whole organism level [13].

Due to the high loads of trace metals discharged into the Huelva Estuary from urban, industrial and mining sources, several authors have conducted studies based on sediment chemistry and laboratory bioassays with a wide range of benthic organisms in order to investigate trace metal contamination and toxicity effects over the last decade [4,14–16]. However, there is a lack of knowledge about whether trace metal contamination is still ongoing, increasing or decreasing over time. The focus of this research work was to assess contemporary trace metal levels and toxicity on sea urchin bioassays in the Huelva Estuary sediments, thus contributing to the monitoring of trace metals contamination (Cr, Cu, Mn, Ni, Pb, Zn and Fe) over the last decades which are the more frequent ones in the Huelva province [17]. In order to achieve these objectives, adverse effects on the fertilization and embryo-larval development of the sea urchin *Paracentrotus lividus*, have

---

* Corresponding author.
E-mail address: ana.r.borrero@ntnu.no. (A.R. Borrero-Santiago).

Production and hosting by Elsevier on behalf of KeAi

http://dx.doi.org/10.1016/j.enceco.2019.07.002

been assessed at two sites within the Huelva Estuary characterized as medium and heavily contaminated by trace metals (Muelle de Capesa and Mazagón). Our results were compared with historical published data on the Huelva Estuary on polluted sediment and their adverse effects on target organisms.

2. Materials and methods

2.1. Sediment sampling

Huelva area is largely exploited for fishery activities, and thus, it is very important to monitor the possible effects on marine organisms that are intended for human consumption. Two representative sampling sites of pollution in the Huelva Estuary (SW, Spain) were selected based on the literature published of the area ([2,7,8,18–20]: Muelle de Capesa (H1) located in the proximity of the industrial sources, and Puerto deportivo of Mazagón (H2) on the outer estuary. In addition, sediments from the Bay of Cádiz (C1) were selected as a control area to address the contamination in these two areas (H1 and H2) (Figure 1). Previous authors have demonstrated that the Bay of Cádiz is a site characterized by the absence of significant metal contamination [1,19]. Surface sediments (0–5 cm layer) were collected with a Van Veen grab during low tide in April 2016 from both the Bay of Cádiz and the Huelva Estuary. After collection, sediment, which was in contact with the grab, was removed in order to avoid any metal contamination. Sediments were sieved in the field to remove large particles and shells and then transported in darkness to the laboratory for analysis. They were stored dried and frozen until the day of the experiment. The same sediments were used to obtain the elutriates for fertilization and embryogenesis bioassays.

All the materials used for the collections and experiments were previously washed with nitric acid (HNO_3, 10%) to avoid metal contamination.

2.1.1. Sediment elutriates

Sediment elutriates were obtained by 30-min rotation (60 rpm) of the sediment with control seawater mixture (ratio 1:4 *v*/v; [21]) and left to settle in darkness overnight [22]. The supernatant was siphoned and filtered (0.22 μm). Elutriates were prepared the same day that the sediment was collected. Control seawater (non-polluted seawater) used in the experiments and for the elutriates was collected in the Bay of Cádiz in the same station where sediment was collected during high tide (1 m depth) using 8 L plastic bottles and transported to the laboratory.

2.1.2. Physical-chemical parameters

Sediment samples ($\sim$1 g) were dried at <60 °C overnight, disaggregated in a mortar and then sieved at 0.63 μm Ø. Concentrations of iron (Fe), manganese (Mn), zinc (Zn) were determined by flame atomic

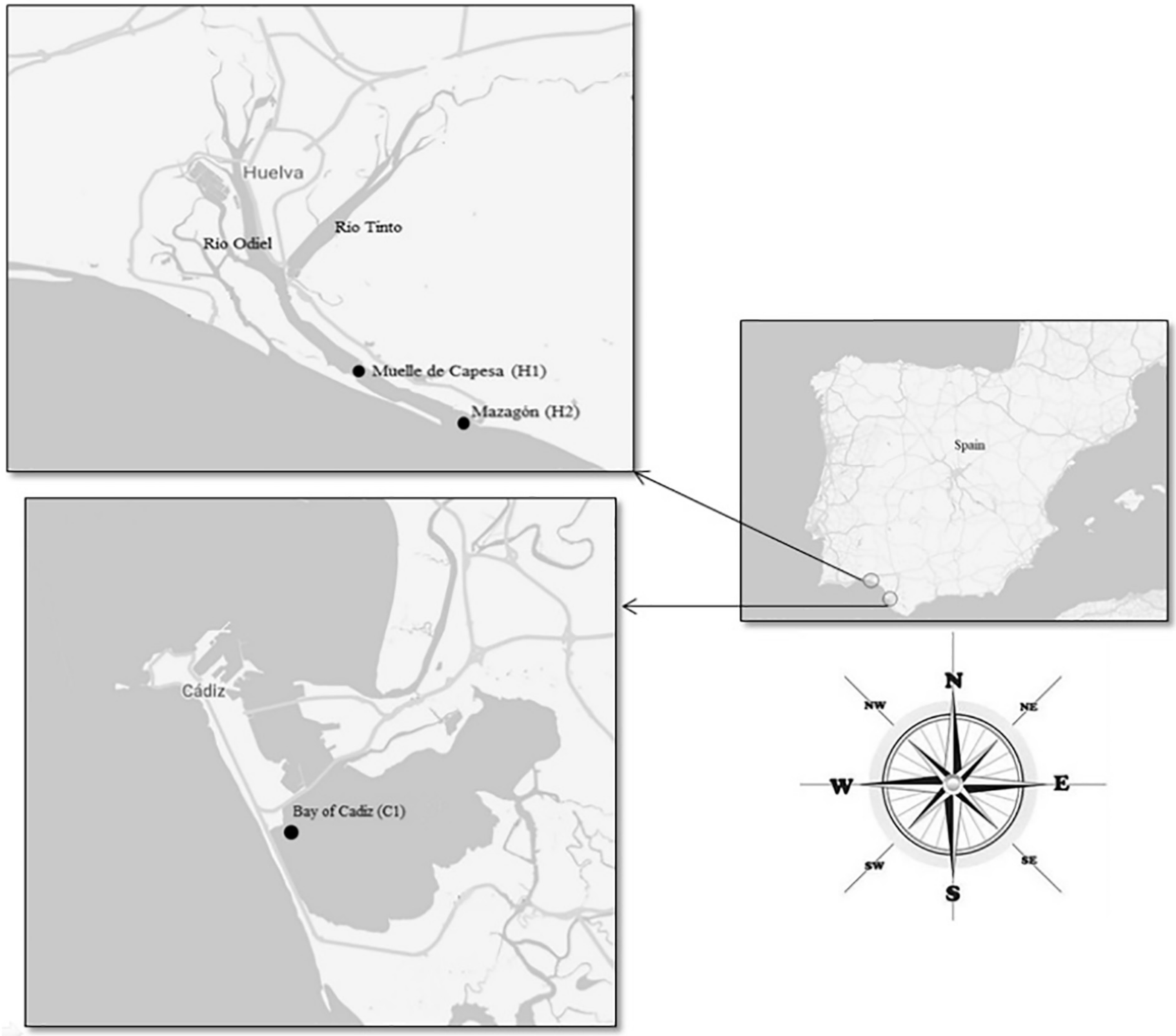


Fig. 1. Map of the south-western of Spain with the three sampling locations: the Bay of Cádiz (C1, 36°29′24″N, 6°15′59″W), Muelle de Capesa (H1; 37°9′43″N, 6°53′50″W) and Puerto deportivo of Mazagón (H2; 37°7′56″N, 6°49′56″W).

absorption (FAAS); and copper (Cu), lead (Pb), nickel (Ni) and chromium (Cr) by graphite furnace atomic absorption spectrophotometry (GFAAS, Perkin Elmer A Analyst 100), according to the EPA methods 7010 and 7000B [23] after microwave-assisted digestion of the sediment with an acid mixture (HCl, HNO_3, and HF, 3:1:1 v/v). Reagent blanks, duplicate samples, and certified reference material (Sco-1 Silty Marine Shale from the United States Geological Survey, USGS) were analyzed in parallel with each batch of samples to ensure the accuracy of the analytical procedure. Measured concentrations in Reference Material Sco-1 included in the analysis were within their certified ranges. Accuracy (R%) and precision (CV) were ± 10%.

Organic matter content in the sediment was estimated by percentage loss of weight on ignition (LOI) at 450 °C for 8 h after drying at 80 °C for 24 h [24] in a muffle furnace (THERMOLYNE model 47,900).

2.2. Sea urchin sampling and tests

Sexually mature specimens of sea urchins (*Paracentrotus lividus*) were collected from a reported clean area [25,26] in a rocky subtidal environment in Bay of Cádiz in April 2016. Animals were immediately transported to the laboratory. For both bioassays (egg fertilization and sea urchin embryogenesis/larval development), gametes were obtained by dissection on the equatorial axe of a single pair of adult sea urchins and direct extraction from the gonads from three male and three female sea urchins into clean seawater which was previously filtered through 0.22 μm. After gamete emission, gamete viability (spherical oocytes and motile spermatozoa) was evaluated with an inverted microscope (model OLYMPUS CKX4), and maintained in suspension in polyethylene vessels containing filtered clean seawater. Both fertilization test and embryogenesis bioassays were performed in quadruplicate ($n = 4$).

2.2.1. Fertilization test

Fertilization with *P. lividus* was conducted following the procedure of Fernández and Beiras [27]. Approximately 300 eggs (20–30 eggs per mL) were added to the test vials with 10 mL of sediment elutriates, plus the filtered seawater control. Sperm were collected directly from the gonad and were exposed to 1 mL of different sediments elutriates for 1 h. Then, 10 μL of sperm suspension (2500 ± 500 spermatozoa per 1 μL), was added to the test vessels [28]. After 20 min exposure, sample suspensions were fixed with 500 μL of 40% formaldehyde, and the percentage of fertilized eggs (those fully surrounded by a fertilization membrane) in 100 eggs was recorded under an inverted microscope (OLYMPUS CKX41) [29]. The egg fertilization bioassay was considered successful when the percentage of fertilization in the control seawater was at least 90%.

2.2.2. Embryogenesis test

For the embryo-larval bioassay, the same procedure for fertilization test was followed in order to get the sperm and eggs suspensions. Embryo-larval bioassay was conducted in polyethylene test vessels filled with a 20 mL of sediment elutriates and filtered clean seawater as control. Approximately 300 fertilized eggs (20–30 eggs per mL) were placed in each vessels, and incubated for 48 h at 20 °C under natural photoperiod [27], after which the larvae were fixed adding 500 μL of 40% formaldehyde [30]. The embryogenesis test was considered successful when the percentage of normally developed plutei in the control was at least 90%. The percentage of abnormally developed plutei (those who do not have well-developed arms), or failure rate, was taken as an endpoint of sediment elutriate toxicity. The alterations were evaluated by ranking the malformations as follows: 0 (none), 1 (slight), 2 (moderate) and 3 (high) according to Carballeira et al. [25]. An index of toxicity (IT) was then calculated for each sampling site. The IT evaluated the degree of malformations by their frequency observed in 4 replicates from each sampling point as follows:

$$IT = [0 \times \%\text{Level } 0 + 1 \times \%\text{Level } 1 + 2 \times \%\text{Level } 2 + 3 \times \%\text{Level } 3]/100.$$

The IT of all the replicates ranged from 0 (no toxicity) to 1 (slight toxicity).

2.3. Statistical analyses

Analysis of Variance (ANOVA) following of a post-hoc test of Bonferroni was used in order to determine significant differences ($p \leq 0.05$) both on trace metals concentrations detected in sediment from the different sampling points, and on the sea urchin fertilization and embryo-larval development. Homogeneity of variance was checked before parametric statistics according to Levene's test and normality was studied using Shapiro–Wilk test. The Spearman rank correlation method was used to determine the correlation between the sediment elutriate toxicity and malformations observed in *P. lividus* bioassays.

3. Results and discussion

3.1. Physical-chemical parameters

All the metals tested showed significant differences ($p < 0.05$) of their concentrations between the sampling points located in Huelva area and the one in Bay of Cádiz. Trace metal concentrations have then been compared to previous levels detected in the Huelva Estuary and the Bay of Cádiz in order to eventually detect changes in contamination over the years (Table 1). Our results showed that Fe and Mn levels were higher in sediments from the Mazagón site (3.08% and 1291 mg kg^{-1}, respectively), followed by Zn, Cr, Cu, Pb, and Ni (166 mg kg^{-1}, 51 mg kg^{-1}, 41 mg kg^{-1}, 23 mg kg^{-1}, and 4.2 mg kg^{-1}, respectively). Conversely, Mn and Fe were slightly lower (1.80% and 439 4.2 mg kg^{-1}, respectively), and Zn and Cu levels consistently higher in sediments from the Muelle de Capesa sampling site (279 mg kg^{-1} and 113 mg kg^{-1}), followed by Cr ≥ Pb > Ni (33 mg kg^{-1}, 32 mg kg^{-1}, and 4.2 mg kg^{-1}, respectively). However, the apparent increase in Fe and Mn levels detected in sediments from the Huelva Estuary over the years is not indicative of increased contamination/pollution, due to their redox-sensitive behavior in aquatic environments, which is strongly dependent on processes of oxidation and reduction, which in turn resulted either in precipitation or (re)dissolution in the solid phase [38].

In contrast, trace metals such as Cr, Cu, Ni, Pb and Zn can have either a natural or anthropic origin in sediments [39]. Despite the higher levels of Cr and Pb found in recent sediments from the Huelva Estuary when compared to the Bay of Cádiz sediment, their concentrations remain close to their background levels, thus indicating a natural contribution from weathering of source rocks rather than anthropogenic inputs in this area of investigation over the years. Conversely, Cu and Zn far exceeded their background values in recent sediments, thus indicating a decreasing but continuous anthropic enrichment of these trace metals within the Huelva Estuary sediments over the years.

Trace metal levels detected in the sediment from the Bay of Cádiz are consistently lower than those found in the Huelva Estuary sediments (Table 1). The low levels of Cr, Cu, Ni, Pb and Zn found in this work are in agreement with sedimentary levels of trace metals reported in a previous work on the Bay of Cádiz [36]. Several works have demonstrated that this sampling site within the Bay of Cádiz is not contaminated and has been extensively used as a control site for bioassays [1,19]. The concentrations of Cr, Cu, Ni, Pb and Zn detected in the Bay of Cádiz were found to be comparable and/or at their background values [33] and this is indicative of a natural contribution rather than anthropogenic inputs to the sediments. These findings are in agreement with the absence of point contamination sources and to the geological structure and hydrodynamics of this area reported by [10].

The contemporary levels of Zn and Cu found in the sediment from Muelle de Capesa, and to a lesser extent, in the sediment from Mazagón, when compared to historical levels show a decrease over the last decades [34]. Previous studies found that Zn, Pb and Cu are particularly linked to vehicle and traffic-related emissions [40–42]. Yet, intensive chemical and

Table 1
Physico-chemical characterization of sediments from the Bay of Cádiz and the Huelva Estuary. Concentrations are reported in mg kg^{-1} except Fe (%) and organic matter (OC %).

Area	Fe	Mn	Cu	Cr	Ni	Pb	Zn	OC	Reference
Bay of Cádiz	–[a]	–	2	nd	nd	6	6	nd	[31]
	–	–	1.34–48.7	3.47	1.7	4.59–41.3	6.28–140	0.71–2.27	[15]
	19.63–43.87	86–407	6.68–202.80	0.10–14.94	0.06–21.25	2.28–89.90	21.27–378.25	1.07–24.33	[19]
	–	–	15.6–169	nd	8.9–29.3	12.2–99.2	18.3–360	1.10–2.60	[32]
	0.74	381	**3.3**	**14**	**6.8**	**7.0**	**28**	**1.0**	**Present work**
		448	*9*	*24.4*	*21.5*	*11.6*	*35.9*	*0.50–3.24*	*Background values*; [33]
Huelva Estuary	–	–	6.5	nd	nd	6.0	42	–	[31]
			22–1830	35–150	< 10–46	31–926	75–2300	–	[34]
	–	–							
			25–2990	19–165	–	11–4860	58–3330	–	[35]
	–	–	979–2438	43.5–73.5	21.4–37.2	270–433	1310–2695	2.59–3.95	[15]
	–	–	20–3304	8–119	1–55	19–2873	61–3300	0.42–2.06	[36]
	41.25–65.75	304–383	150–1939	8.1–32.9	7.1–129	218–385	1176–2458	6.30–20.27	[19]
	–	–	789–1989	nd	21.2–97.2	198–406	987–2010	1.10–3.90	[32]
	1.4–8.2	189–416	363–2420	72–118	17–40	290–2420	200–2920	1.57–3.43	[8]
			1116–3334	–	–	37–1167	603–1737	–	[2]
	1.8–3.08	439–1219	**41–113**	**33–51**	**4.2–4.9**	**23–32**	**166–279**	**0.68–1.03**[b]	Present work
			15–21.7	17.1–65	5–27	23.6–35	65–103.7		Background values; [37]; [36]

[a] Not determined.

[b] Loss of ignition (LOI).

petrochemical industries and fertilizer production are located within the Huelva Estuary area. According to the findings of Morillo et al. [43], the main sources of metal pollution in this area are industrial wastes and acid mine drainage from the Odiel and Rio Tinto Rivers [37]. As expected, trace metals levels and their anthropic enrichment resulted higher for Zn and Cu at the Muelle de Capesa sampling site, which is close to the Huelva industrial area. Although lower levels and moderate anthropogenic contribution were reported in terms of Zn and Cu, the Mazagón sediment is currently displaying a decreasing but continuous anthropogenic signal over the time. Mazagón has been previously identified as an area receiving point and diffuse sources of trace metal pollution from industrial wastes and acid mine drainage, respectively [34,37]. The fact that Mazagón sampling site is still recording an anthropogenic signal despite decreasing levels of trace metals recorded in recent sediments may be a consequence of south-westerly dominant winds that generate a current parallel to the coastline, carrying more enriched suspended material from acid mine drainage sources that finally accumulate Zn and Cu, and to a lesser extent Pb, in the Mazagón sediment [14].

A slightly lower organic matter content was observed in Mazagón and Cádiz Bay sediments in tandem with higher Mn levels. Shifts in the Mn equilibrium have been previously correlated to the organic matter content in sediment [44]. On the other hand, considering the grain size characteristics detected by Basallote et al. [45], the percentage of fines (silt and clays < 0.2 μm) was low both in Bay of Cádiz and Mazagón sites. Conversely, fines were predominant in Muelle de Capesa sediment. It is well known that trace metals tend to accumulate in fine-grained sediment [46], and this is in accordance with our findings, where Muelle de Capesa showed higher levels of Zn, Cu and Pb and the higher organic matter content. In addition, the organic matter content itself seemed to be influenced by the sediment grain size distribution [47]. Previous studies assessed that fines percentage is usually related to the organic matter content [48]. In this case a slightly lower presence of organic matter may be linked to a lower percentage of fines, and thus lower levels of trace metals in Bay of Cádiz and Mazagón sediments, while a slightly higher organic matter content in Muelle de Capesa sediment is likely be correlated to higher percentage of fines and as a consequence, to the higher levels of trace metals recorded in this work.

3.2. Sea urchin fertilization and embryo-larval development bioassays

The percentage of fertilization success (%) is shown in Fig. 2. Sea urchin fertilization was always >90% in the control seawater, which is a good indication of fertilization success according to U.S. Environmental Protection Agency [49] protocol. The comparison of fertilized eggs between the control and the Bay of Cádiz elutriate was not significantly different. Fertilized eggs of *Paracentrotus lividus* after 20-min exposure were significantly lower in sediment elutriates from the Huelva Estuary (H1 and H2; $p < 0.05$) with respect to the to the control seawater, with Muelle de Capesa elutriate (H1) showing the lowest fertilization rate (~40%).

Previous authors found a decreased fertilization success in sea urchins in the presence of high concentrations of Cu and Zn [50,51]. Both Mazagón and Muelle Capesa locations showed high concentrations of these metals in sediments, which could explain the lower fertilization rate.

Exposure to elutriates from Huelva Estuary sediments caused detrimental effects on sea urchin larval development (Table 2). The observations in the microscope were based on the abnormalities described by Carballeira et al. [25]. Both the control and the Bay of Cádiz samples showed the highest percentage in level 0 (normal shape). These results confirmed that the sediment collected from the Bay of Cádiz station used in this study was not toxic. On the other hand, larval development was found to be affected by the elutriates obtained from Huelva area sediment. Statistical differences ($p < 0.05$) between sampling stations in Huelva area and the control for level 2 (no skeletal rods larvae) and 3 (undeveloped stages) larvae were revealed by the Analysis of variance (ANOVA). A significantly higher number ($p < 0.05$) of level 1 larvae (crossed tip larvae) was found in the Mazagón sediment elutriate, while a significant ($p < 0.05$) higher number of level 2 larvae (no skeletal rods and fractured larvae) was found in with Muelle de Capesa sediment elutriate.

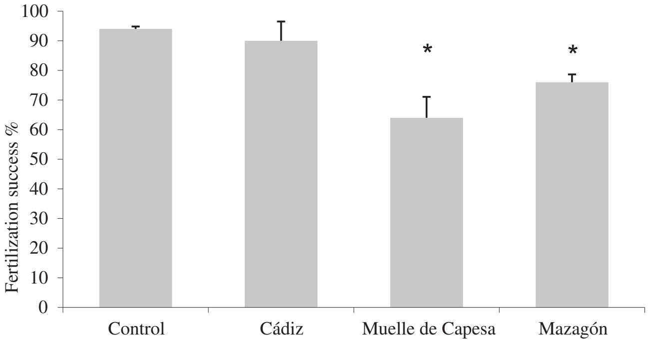


Fig. 2. Fertilization success (%), calculated after 20 min of exposure to the three different sampling points (Bay of Cadiz, C; Muelle de Capesa, H1; Mazagón, H2) and the control. Fertilized eggs were calculated for quadruplicates ($n = 4$) (mean ± sd). Significant differences (*) ($p < 0.05$).

Table 2
Percentage of embryonic stages and developmental abnormalities of sea urchin *Paracentrotus lividus* calculated for quadruplicates ($n = 4$). The larval alterations are noted with a growing level of toxicity as: 0 (none), 1 (slight), 2 (moderate) and 3 (high) according to [25]. Not detected larval stage is expressed as (—).

P. lividus larval stages (%)		Control	Bay of Cádiz	Muelle de Capesa	Mazagón
Level 0		94	90	10	17
Level 1	Crossed tip	3	8	17	30
	Separated tip	–	–	1	–
	Fused arms	–	–	–	–
Level 2	No skeletal rods	2	2	40	34
	Folded tip	–	–	–	–
	Fractured	–	–	25	16
Level 3	Undeveloped stages	1	–	7	3

Table 3
Toxicity of sediment from the three sampling points according to the toxicity index calculated for quadruplicates ($n = 4$) in order to understand level of larval alterations.

Sampling point	Toxicity Index	Toxicity
Control	0.11	Absent
Bay of Cádiz	0.12	Absent
Muelle de Capesa	1.62	Moderate
Mazagón	1.37	Moderate

Based on the malformations, different levels or index of toxicity (IT) were found (Table 3). The IT index calculated for each sampling point confirmed the quantitatively analysis: the percentage of malformed larvae in Bay of Cádiz (0) was the same as the one calculated in the control, while Muelle de Capesa and Mazagón sampling sites sediments showed the presence of a moderate toxicity on larvae (>1). Previous works ranked metals with a decreasing order of toxicity on sea urchins as follows: $Cu > Zn > Pb > Fe > Mn$ assessing that their combined action may be either synergetic or antagonistic [27,52]. In the present work, the sediment from the Muelle de Capesa site reported higher concentrations of trace metals $Zn > Cu > Cr$, while in Mazagón $Zn > Cr > Cu$. Considering the higher toxicity recorded in Muelle de Capesa sampling site, the most severe toxicity seems to be correlated with Zn and Cu concentrations in sediment. The Spearman rank correlation method confirmed that the toxic effects detected on *P. lividus* were correlated to the presence of Zn and Cu in sediments.

These results have been compared with previous studies in order to address the toxicity over the years (Table 4). In particular, the sediment from Muelle de Capesa site displayed toxic effects on testing organisms in previous works, while sediment from Mazagón showed an increasing toxicity on different organisms during the past decades. It is interesting to highlight that despite the decreasing trends of trace metals over the last decades, both Muelle de Capesa (H1) and Mazagón (H2) sediments still show toxicity on organisms. This could be linked to bioavailability of metals in sediment, as recent studies in the same zone found out that metal bioavailability is higher in the Estuary when compared to other sampling points in the Huelva area [53]. Also anthropogenic impacts may have contributed to Pb, Zn and Cu enrichment, as these trace metals are among the most common elements that tend to be released and accumulated in sediment by human activities [54,55]. The peculiar decreasing but continuous anthropogenic signal over time in the Mazagón area sediment observed, could instead be linked to the south-westerly dominant winds that tends to cause the accumulation of toxic metals [14] and to the different metals bioavailability that varies between the Estuary and the other sampling points in Huelva area [53]. In this area Mn concentration showed the most significant change, and this has been linked to the higher organic matter content [44] and sediment grain size [47].

4. Conclusions

Changes in trace metals levels and anthropic sources have been studied by comparing recent sediment chemistry and toxicity outcomes with historical data in the Huelva Estuary. Our work revealed that there has been a

Table 4
Summarized toxic effects detected in different organisms exposed to sediments collected in different years at the three sampling points of Bay of Cádiz (C1), Muelle de Capesa (H1) and Mazagón (H2). Incidence of effects: (−) no toxic effect detected on organisms, (+) toxic effect detected on organisms, (nd) no data on this sampling site.

Organisms	Source		Site	
		C1	H1	H2
Clam Ruditapes philippinarum	[15]	−	+	−
Amphipod *Ampelisca brevicornis*	[15]	−	+	+
Rotifer *Brachionus plicatilis*	[15]	−	+	−
Bacteria Vibrio fischeri	[15]	−	+	−
Fish *Solea senegalensis*	[15]	−	+	−
Clam *Chamelea gallina*	[16]	nd	+	+
Clam Ruditapes philippinarum	[16]	nd	+	+
Bacteria Vibrio fischeri	[16]	nd	+	+
Clam Ruditapes philippinarum	[19]	−	+	nd
Crab *Carcinus maenas*	[19]	−	+	nd
Fish Solea senegalensis	[20]	nd	+	nd
Sea urchin *Paracentrotus lividus*	[18]	−	+	+
Bacteria Photobacterium phosphoreum	[8]	nd	+	+
Sea urchin Paracentrotus lividus	Present work	−	+	+

change in terms of concentrations of trace metals in sediments from the Huelva Estuary over the last decades, when compared to historical levels recorded in previous works. Overall, our results on recent sediments underlined four different results: (1) the Bay of Cádiz showed Cr and Ni, as well as Cu, Pb and Zn levels, are comparable to their background values, and thus there is no indication of anthropic inputs and/or changes over the years; (2) Zn and Cu displayed higher levels and enrichment in sediments from the Muelle Capesa close to the Huelva industrial area; (3) despite moderate levels and enrichment in Zn and Cu, the sediment from Mazagón still records an anthropogenic signature due to continuous inputs from acid mine drainage and alongshore current transport; and (4) sea urchin fertilization rate and larval development were significantly affected by exposure to elutriates from Huelva Estuary sediments.

Sediments from the Huelva Estuary have shown a continuous and high level of toxicity on several organisms from different trophic levels over the past decades, and this fact is indicative of the ongoing although declining Zn and Cu anthropogenic contamination affecting this coastal ecosystem.

Despite the fact that the Water Framework Directive (2000/60/EC) provisions include measures against pollution by groups of chemicals presenting a significant risk to or via the aquatic environment nearly two decades earlier, the Huelva Estuary area shows a declining but chronic contamination in Zn and Cu originating from point and diffuse anthropic activities, which in turn, are likely to cause adverse effects on the coastal ecosystem.

According to the Directive 2008/105/EC, Environmental Quality Standard (EQS) are required for the chemical status of surface waters including marine waters. Further research is needed to establish site-specific sediment EQS in the Huelva Estuary in order to detect earlier or delayed/chronic signals of adverse effects on marine organisms, and to implement timely control measures to reduce pollution effects over the years.

Acknowledgements

Bianca Maria Torres would like to thank the Erasmus Plus Program for the exchange studentship to the University of Cádiz. Also thanks are due to Giulia Leardini and Marilia Lopes for assistance in sediment analysis. Dr. Borrero-Santiago would like to express gratitude to the Research Council of Norway for the financial support by CLIMIT program (TrykkCO2 grant 254777/E20). Additionally, authors also thank A. A. Vold for her help with language corrections.

References

[1] J. Blasco, T. Gomes, T. García-Barrera, A. Rodríguez-Romero, M. Gonzalez-Rey, F. Morán-Roldán, M.J. Bebianno, Trace metal concentrations in sediments from the Southwest of the Iberian Peninsula, Sci. Mar. 74 (S1) (2010) 99–106, https://doi.org/10.3989/scimar.2010.74s1099.

[2] M. Oliva, C. Gravato, L. Guilhermino, M.D. Galindo-Riaño, J.A. Perales, EROD activity and cytochrome P4501A induction in liver and gills of Senegal sole *Solea senegalensis* from a polluted Huelva Estuary (SW Spain), Comp Biochem Physiol Part - C Toxicol Pharmacol 166 (2014) 134–144, https://doi.org/10.1016/j.cbpc.2014.07.010.

[3] X. Querol, A. Alastuey, J. De la Rosa, A. Sànchez-de-la-Campa, F. Plana, C.R. Ruiz, Source apportionment analysis of atmospheric particulates in an industrialized urban site in southwestern Spain, Atmos Env 36 (2002) 3112–3125, https://doi.org/10.1016/S1352-2310(02)00257-1.

[4] D. Rosado, J. Usero, J. Morillo, Assessment of heavy metals bioavailability and toxicity toward *Vibrio fischeri* in sediment of the Huelva estuary, Chemosphere 153 (2016) 7–10, https://doi.org/10.1016/j.chemosphere.2016.03.040.

[5] A. Sainz, J.A. Grande, M.L. de la Torre, Characterisation of heavy metal discharge into the Ria of Huelva, Environ. Int. 30 (2004) 557–566, https://doi.org/10.1016/j.envint.2003.10.013.

[6] M.D. Galindo-Riaño, M. Oliva, J.A. Jurado, D. Sales, M.D. Granado-Castro, F. López-Aguayo, Comparative baseline levels of heavy metals and histopathological notes in fish from two coastal ecosystems of south-west of Spain, Int J Environ Res 9 (2015) 163–178, https://doi.org/10.22059/IJER.2015.886.

[7] J. Ramos-Gómez, M.L. Martín-Díaz, T.A. Delvalls, Acute toxicity measured in the amphipod *Ampelisca brevicornis* after exposure to contaminated sediments from Spanish littoral, Ecotoxicology 18 (2009) 1068–1076, https://doi.org/10.1007/s10646-009-0383-5.

[8] D. Rosado, J. Usero, J. Morillo, Application of a new integrated sediment quality assessment method to Huelva estuary and its littoral of influence (Southwestern Spain), Mar. Pollut. Bull. 98 (1–2) (2015) 106–114, https://doi.org/10.1016/j.marpolbul.2015.11.057.

[9] C. Barba-Brioso, J.C. Fernández-Caliani, A. Miras, J. Cornejo, E. Galán, Multi-source water pollution in a highly anthropized wetland system associated with the estuary of Huelva (SW Spain), Mar Poll Bull 60 (2010) 1259–1269, https://doi.org/10.1016/j.marpolbul.2010.03.018.

[10] R.A. Ligero, M. Barrera, M. Casas-Ruiz, D. Sales, F. López-Aguayo, Dating of marine sediments and time evolution of heavy metal concentrations in the Bay of Cádiz, Spain, Environ. Pollut. 118 (2002) 97–108, https://doi.org/10.1016/S0269-7491(01)00209-3.

[11] J.M. Nieto, A.M. Sarmiento, M. Olías, C.R. Canovas, I. Riba, J. Kalman, T.A. Delvalls, Acid mine drainage pollution in the Tinto and Odiel rivers (Iberian Pyrite Belt, SW Spain) and bioavailability of the transported metals to the Huelva Estuary, Environ. Int. 33 (2007) 445–455, https://doi.org/10.1016/j.envint.2006.11.010.

[12] P.M. Chapman, F. Wang, S.S. Caeiro, Assessing and managing sediment contamination in transitional waters, Environ. Int. 55 (2013) 71–91, https://doi.org/10.1016/j.envint.2013.02.009.

[13] M. Jaishankar, T. Tseten, N. Anbalagan, B.B. Mathew, K.N. Beeregowda, Toxicity, mechanism and health effects of some heavy metals, Interdiscip. Toxicol. 7 (2014) 60–72, https://doi.org/10.2478/intox-2014-0009.

[14] V. Funes, J. Alhama, J.I. Navas, J. López-Barea, J. Peinado, Ecotoxicological effects of metal pollution in two mollusc species from the Spanish South Atlantic littoral, Environ. Pollut. 139 (2) (2006) 214–223, https://doi.org/10.1016/j.envpol.2005.05.016.

[15] I. Riba, C. Casado-Martínez, J.M. Forja, T.Á. Del Valls, Sediment quality in the Atlantic coast of Spain, Environ. Toxicol. Chem. 23 (2) (2004) 271–282, https://doi.org/10.1897/03-146.

[16] J. Usero, J. Morillo, H. El Bakouri, A general integrated ecotoxicological method for marine sediment quality assessment: application to sediments from littoral ecosystems on Southern Spain's Atlantic coast, Mar. Pollut. Bull. 56 (12) (2008) 2027–2036, https://doi.org/10.1016/j.marpolbul.2008.08.009.

[17] F.J. Rodríguez-Ttovar, F.J. Martín-Peinado, Lateral and vertical variations in contaminated sediments from the Tinto River area (Huelva, SW Spain): incidence on tracemaker activity and implications of the palaeontological approach, Palaeogeogr. Palaeoclimatol. Palaeoecol. 163 (2014) 489, https://doi.org/10.1016/j.palaeo.2014.09.022.

[18] A. Khosrovyan, A. Rodríguez-Romero, M.J. Salamanca, T.A. Del Valls, I. Riba, F. Serrano, Comparative performances of eggs and embryos of sea urchin (*Paracentrotus lividus*) in toxicity bioassays used for assessment of marine sediment quality, Mar. Pollut. Bull. 70 (2013) 204–209, https://doi.org/10.1016/j.marpolbul.2013.03.006.

[19] M.L. Martín-Díaz, N. Jiménez-Tenorio, D. Sales, T.Á. Del Valls, Accumulation and histopathological damage in the clam *Ruditapes philippinarum* and the crab *Carcinus maenas* to assess sediment toxicity in Spanish ports, Chemosphere 71 (10) (2008) 1916–1927, https://doi.org/10.1016/j.chemosphere.2008.01.022.

[20] M. Oliva, J. José Vicente, C. Gravato, L. Guilhermino, M. Dolores Galindo-Riaño, Oxidative stress biomarkers in Senegal sole, *Solea senegalensis*, to assess the impact of heavy metal pollution in a Huelva estuary (SW Spain): seasonal and spatial variation, Ecotoxicol. Environ. Saf. 75 (2012) 151–162, https://doi.org/10.1016/j.ecoenv.2011.08.017.

[21] U.S. Environmental Protection Agency, Evaluation of dredged material proposed for ocean disposal testing manual, US Environ Prot Agency (1991) 1–13.

[22] T.A. DelValls, P.M. Chapman, 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 (3) (1998) 313–336, https://doi.org/10.7773/cm.v24i3.753.

[23] U.S. Environmental Protection Agency, Framework for metals risk assessment, US Environ Prot Agency (2007) 1–172.

[24] C. Luczak, M.A. Janquin, A. Kupka, Simple standard procedure for the routine determination of organic matter in marine sediment, Hydrobiologia 345 (1997) 87–94, https://doi.org/10.1023/A:100290262.

[25] C. Carballeira, J. Ramos-Gómez, L. Martín-Díaz, T.A. DelValls, Identification of specific malformations of sea urchin larvae for toxicity assessment: application to marine pisciculture effluents, Marine Environ Res 77 (2012) 12–22, https://doi.org/10.1016/j.marenvres.2012.01.001.

[26] L.A. Maranho, M.C. Garrido-Pérez, T.A. DelValls, M.L. Martín-Díaz, Suitability of standardized acute toxicity tests for marine sediment assessment: pharmaceutical contamination, Water Air Soil Pollut 226 (65) (2015) 1–14 doi: 0.1007/s11270-014-2273-6.

[27] N. Fernández, R. Beiras, Combined toxicity of dissolved mercury with copper, lead and cadmium on embryogenesis and early larval growth of the *Paracentrotus lividus* sea-urchin, Ecotoxicology 10 (2001) 263–271, https://doi.org/10.1023/A:1016703116830.

[28] C. Gambardella, M.G. Aluigi, S. Ferrando, L. Gallus, P. Ramoino, A.M. Gatti, M. Rottigni, C. Falugi, Developmental abnormalities and changes in cholinesterase activity in sea urchin embryos and larvae from sperm exposed to engineered nanoparticles, Aquat. Toxicol. 130–131 (2013) 77–85, https://doi.org/10.1016/j.aquatox.2012.12.025.

[29] T.J. Ward, J.R. Kramer, R.L. Boeri, J.W. Gorsuch, Chronic toxicity of silver to sea urchin (*Arbacia punctulata*), Environ. Toxicol. Chem. 25 (2006) 1568–1573, https://doi.org/10.1897/05-299R.1.

[30] N. Kobayashi, H. Okamura, Effects of heavy metals on sea urchin embryo development. 1. Tracing the cause by the effects, Chemosphere 55 (10) (2004) 1403–1412, https://doi.org/10.1016/j.chemosphere.2003.11.052.

[31] R.D. Stenner, G. Nickless, Heavy metals in organisms of the Atlantic coast of S.W. Spain and Portugal, Mar. Pollut. Bull. 6 (6) (1975) 89–92, https://doi.org/10.1016/0025-326X(75)90151-4.

[32] R.B. Choueri, A. Cesar, D.M.S. Abessa, R.J. Torres, R.D. Morais, I. Riba, C.D.S. Pereira, M.R.I. Nascimento, A.A. Mozeto, T.Á. DelValls, Development of site-specific sediment quality guidelines for North and South Atlantic littoral zones: comparison against national and international sediment quality benchmarks, J. Hazard. Mater. 170 (1) (2009) 320–331, https://doi.org/10.1016/j.jhazmat.2009.04.093.

[33] M.R. Rodríguez-Barroso, J.L. Garcìa-Morales, M.D. Coello Oviedo, J.M. Quiroga Alonso, An assessment of heavy metal contamination in surface sediment using statistical analysis, Environ. Monit. Assess. 163 (2010) 489, https://doi.org/10.1007/s106661-009-0852-6.

[34] J.C. Fernández-Caliani, F. Ruiz Muñoz, E. Galán, Clay mineral and heavy metal distributions in the lower estuary of Huelva and adjacent Atlantic shelf, SW Spain, Sci. Total Environ. 198 (2) (1997) 181–200, https://doi.org/10.1016/S0048-9697(97)05450-8.

[35] F. Ruiz, Trace metals in estuarine sediments from the Southwestern Spanish coast, Mar. Pollut. Bull. 42 (6) (2001) 481–489, https://doi.org/10.1016/S0025-326X(00)00192-2.

[36] J.A. González-Pérez, J.R. de Andrés, L. Clemente, J.A. Martín, F.J. González-Vila, Organic carbon and environmental quality of riverine and off-shore sediments from the Gulf of Cádiz, Spain, Env Chem Lett 6 (2008) 41–46, https://doi.org/10.1007/s10311-007-0107-0.

[37] F. Ruiz, M. González-Regalado, J.M. Muñoz, M. Abad, A. Toscano, M.I. Prudencio, M.I. Dias, Distribution of heavy metals and pollution pathways in a shallow marine shelf: assessment for a future management, Int. J. Environ. Sci. Technol. 11 (2014) 1249–1258, https://doi.org/10.1007/s13762-014-0576-1.

[38] D.J. Burdige, The biogeochemistry of manganese and iron reduction in marine sediments, Earth-Science Reuiews 35 (1993) 249–284.

[39] B.S. Lollar, H.D. Holland, K.K. Turekian, Environmental Geochemistry, Treatise on Geochemistry, Elsevier Science, 2005.

[40] M.D. Jiménez, R. De Torre, I. Mola, M.A. Casado, L. Balaguer, Local plant responses to global problems: *Dactylis glomerata* responses to different traffic pollutants on roadsides, J. Environ. Manag. 212 (2018) 440–449, https://doi.org/10.1016/j.jenvman.2017.12.049.

[41] X. Li, C. Poon, P.S. Liu, Heavy metal concentration of urban soils and street dusts in Hong Kong, Appl. Geochem. 16 (2001) 1361–1368, https://doi.org/10.1016/S0883-2927(01)00045-2.

[42] N. Omar, M. Abas, N. Rahman, N. Tahir, A. Rushdi, B. Simoneit, Levels and distributions of organic source tracers in air and roadside dust particles of Kuala Lumpur, Malaysia, Environ. Geol. 52 (2007) 1465–1500, https://doi.org/10.1007/s00254-006-0593-6.

[43] J. Morillo, J. Usero, I. Gracia, Heavy metal distribution in marine sediments from the southwest coast of Spain, Chemosphere 55 (2004) 431–442, https://doi.org/10.1016/j.chemosphere.2003.10.047.

[44] D.J. Cotter, U.N. Mishra, The role of organic matter in soil manganese equilibrium, Plant Soil 29 (3) (1968) 439–448, https://doi.org/10.1007/BF01348975.

[45] M.D. Basallote, M.R. De Orte, T.Á. Del Valls, I. Riba, Studying the effect of CO_2 - induced acidification on sediment toxicity using acute amphipod toxicity test, Environ Sci Technol 48 (15) (2014) 8864–8872, https://doi.org/10.1021/es5015373.

[46] T. Hanlon, Y.N. Kwon, S. Suresh, Grain size effects on the fatigue response of nanocrystalline metals, Scr. Mater. 49 (2003) 675–680, https://doi.org/10.1016/S1359-6462(03)00393-2.

[47] B.A. Bergamaschi, E. Tsamakis, R.G. Keil, T.I. Eglinton, D.B. Montluçon, J.I. Hedges, The effect of grain size and surface area on organic matter, lignin and carbohydrate concentration, and molecular compositions in Peru Margin sediments, Geochim. Cosmochim. Acta 61 (1997) 1247–1260, https://doi.org/10.1016/S0016-7037(96)00394-8.

[48] A.R. Quiroga, D.E. Buschiazzo, N. Peinermann, Soil organic matter particle size fractions in soils of the Semiarid Argentinian Pampas, Soil Sci. 161 (1996) 104–108, https://doi.org/10.1097/00010694-199602000-00004.

[49] U.S. Environmental Protection Agency, Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms, US Environ Prot Agency (2002) 1–107.

[50] I.P.B. Tualla, J.G. Bitacura, Effects of cadmium and zinc on the gamete viability, fertilization, and embryonic development of Tripneustes gratilla (Linnaeus), Scientifica 2016 (2016)https://doi.org/10.1155/2016/8175213.

[51] M.A. Vaschenko, Z.P. Zhang, P.K.S. Lam, R.S.S. Wu, Toxic effects of cadmium on fertilizing capability of spermatozoa, dynamics of the first cleavage and pluteus formation in the sea urchin *Anthocidaris crassispina* (Agassiz), Mar. Pollut. Bull. 38 (1999) 1097–1104, https://doi.org/10.1016/S0025-326X(99)00116-2.

[52] N. Kobayashi, H. Okamura, Effects of heavy metals on sea urchin embryo development. 2. Interactive toxic effects of heavy metals in synthetic mine effluents, Chemosphere 61 (2005) 1198–1203, https://doi.org/10.1016/j.chemosphere.2005.02.071.

[53] D. Rosado, J. Usero, J. Morillo, Ability of 3 extraction methods (BCR, Tessier and protease K) to estimate bioavailable metals in sediments from Huelva estuary (Southwestern Spain), Mar. Pollut. Bull. 102 (1) (2016) 65–71, https://doi.org/10.1016/j.marpolbul.2015.07.008.

[54] S. Audry, J. Schäfer, G. Blanc, J.M. Jouanneau, Fifty-year sedimentary record of heavy metal pollution (Cd, Zn, Cu, Pb) in the Lot River reservoirs (France), Environ. Pollut. 132 (2004) 413–426, https://doi.org/10.1016/j.envpol.2004.05.025.

[55] L. Xiandong, Z. Shen, O.W. Wai, Y.-S. Li, Chemical forms of Pb, Zn and Cu in the sediment profiles of the Pearl River Estuary, Mar. Pollut. Bull. 42 (2001) 215–223, https://doi.org/10.1016/S0025-326X(00)00145-4.