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Evaluating metal toxicity in contaminated sediments in the South of Spain

Tesi di laurea in Fisiologia applicata all'ambiente

Relatore

Prof. Elena Fabbri

Presentata da

Bianca Maria Torre

Correlatore

Prof. Roberta Guerra

Correlatore

D. Ana R. Borrero Santiago

Correlatore

Prof. T. Angel Del Valls

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General abstract

Coastal zones are ecologically important areas subject to heavy anthropogenic pressures such as urbanization, fishing, commercial transports, as well as industrial and agriculture activities. In this context, Huelva, in the south of Spain, is a polluted area due to these reasons. In order to evaluate the consequences of anthropogenic impacts in the coastal area of Huelva, sediments from three different sites (Bay of Cádiz, Muelle de Capesa and Mazagòn) were assessed using an integrated model. This methodology integrates sediment physical-chemical parameters and biological responses in test organisms. Metal concentrations (Cr, Cu, Fe, Mn, Ni, Pb, Zn) and organic matter content were measured as physical-chemical parameters. Biological responses were assessed using three bioassays in two marine species: Mortality for *Ampelisca brevicornis* and Fertilization and Larval development for *Paracentrotus lividus*. The test organisms were exposed under two different route of exposure (whole sediment or elutriate). Acute effects associated with the presence of metals (Cr, Cu, Fe, Mn, Ni, Pb, Zn) were observed and resulted correlated with the abnormal fertilization and larval development of echinoderms exposed to the sediments.

The results here obtained were useful to confirm that the high level of metals contamination found in Huelva area, when compared to the control site (Bay of Cádiz), is likely to pose adverse effects on marine resident biota.

Chapter 1

Introduction

1.1 Metal pollution in marine ecosystems

There are many natural processes in the biosphere which are contributing to the chemical input in the environment such as volcanic activity and rock weathering (Jan et al., 2015). However, pollution caused by human settlements, industrial activities, soil exploitation, fishing, urban and vehicular emissions and non-sustainable actions is having a negative impact on the environment. Human settlements are often located in coastal zones, because of the large availability of natural resources that can be found in these areas. Therefore, the most polluted locations are the coastal areas.

The main toxic agents are classified based on their toxic process: metals and metalloids (Valvanidis et al., 2010), inorganic or organic toxicants (Sánchez-Rodríguez et al., 2012), pesticides (Katagi, 2010) and radionuclides (Anagnostakis et al., 2015). Metals can be divided in two groups: essential and non-essential. The first ones are required for optimal functioning of metabolic processes in the organisms, such as copper (Cu), nickel (Ni), iron (Fe), zinc (Zn) and manganese (Mn). For example, Fe is fundamental for haemoglobin, Cu for respiratory pigments; Zn and Mn for enzymes or cobalt for Vitamin B12. On the other hand, non-essential metals, with no known biological functions such as lead (Pb), mercury (Hg), cadmium (Cd) and tin (Sn), can be tolerated at low levels. However, at high concentrations they may be toxic (Bou-Abdallah et al., 2016). Metals are considered some of the most dangerous toxicants because they are very stable, highly toxic and can accumulate along the trophic chain and in living tissues. Some metals are available for uptake only as free ions, while others are transported over biological membranes as inorganic complexes (Domingos et al., 2015).

In marine ecosystems toxicants like metals can be found both in the water column and in sediments. However, metal concentration is usually higher in sediments than in the overlying water, because of their absorption capacity. The distribution of a metal in the sediment is modified by physical-chemical parameters such as pH, salinity, redox potential, temperature, organic matter and water (Chapman et al., 2001). Sediments can be a sink of contaminants from the water column (Riba et al., 2004). Under specific circumstances metals that are contained in the sediment can be released in the water column altering the normal balance, for example because of dredging activities (Zhang et al., 2014). There are many organisms which are living in the sediments and that are related to the food chain. In this sense, aquatic systems are very sensitive to metal pollution due to their complexity. There are many trophic levels in which the accumulation of contaminants is enhanced (Diop et al., 2016). Metal bioaccumulation increases the negative impacts of metals discharged in the water. Bioaccumulation of a chemical in a species varies in different taxa (De Jonge et al., 2010). It has also been demonstrated that exposure to contaminants create resistance to toxicants (Mano et al., 2016). In this sense, metals move up in the food chain, with a process that is known as "biomagnification". Bioaccumulation and biomagnification of metals in fish and other marine organisms may affect human health (Zahir et al., 2015). Metals can affect organisms both on cellular and whole organism level. In the first case, they can provoke damages such as the formation of Reactive Oxygen Species (ROS), breaking of plasma membranes, inhibition of Na, K-dependent ATPases and aminoacid transport, enzyme inhibition and alteration of RNA synthesis. On the whole organism they can be toxic via ingestion, respiratory pathways or by the skin, or influencing growth and development of the species (Jakimska et al., 2011).

Considering this, it is essential to evaluate the sediment quality and to monitor metals presence in aquatic ecosystems in coastal zone areas.

1.2 Integrated model

It is difficult to obtain realistic information about the quality of the environment, using a single technique such as the concentration of the contaminants (Del Valls et al., 2007). In order to get a global status of the environmental quality, it is necessary to answer three different questions: 1) What types of contaminants are in the environment, 2) levels or amount of the contaminants and 3) potential effects on organisms. In this context, an integrated method is the adequate option to have a global vision of the potential contamination in the total environment. An integrated model allows integrating all the information about all these questions.

The integrated model combines four different methodologies or weights of evidence (Figure 1):

-contamination of the study area, which is measured by the analysis of its physical-chemical characteristics;

-bioassays in the laboratory on different organisms;

- detection of *in situ* effects;

-bioaccumulation and biomagnification analysis.

In this study, physical-chemical characterisation of the sediment and laboratory bioassays, were considered as weight of evidence in order to evaluate the quality of sediment from Huelva.

Figure 1. Scheme of the integrated model with the four weight of evidence: contamination, laboratory and in situ effects, bioaccumulation/biomagnification. Source: Del Valls, 2007.

The bioassays, both acute and chronic toxicity tests, can be used in laboratory to establish the presence of a contaminant and to determine the potential toxicity of a specific contaminant or a mixture. These tests can be used to assess the quality of water, sediment and dredged material in a chosen location, by the evaluation of the relation between the contaminants levels and the concentration at which they cause adverse biological effects on the test organisms. This is obtained by the exposure of the biotic components to different levels of the contaminants, that can be short-term in case of an acute toxicity test that uses lethality as end point, or long-term in case of a chronic toxicity test that has sub-lethal end points during the organism life span, such as reproductive success or physiological and metabolic responses.

Benthic specimens are the ones that are mainly used to evaluate sediment quality and pollution, as they live closely related to sediment (Rodrìguez-Romero et al., 2013). According to the USEPA normative (USEPA 2002), at least two different species should be chosen, belonging to different levels of the trophic chain, because of the several exposure pathways and response that they can have. Toxicity tests can be conducted on the whole sediment, to assess the direct effect of the toxicant in the natural environment, or extracting elutriate from it, to see if the toxicant can affect the organism despite a low bioavailability in nature. Organisms from unpolluted locations are a useful tool to determine the lethal dose for organisms and health risks for humans (Rodriguez-Romero et al., 2013).

1.3 Study areas

The areas selected are located in the South of Spain. This is a very important area because of its high rate of industry, the large coastal human settlement, its importance for navigation routes and fishing. Dating techniques showed that the concentration of metals in the Gulf of Cádiz had an increase especially in the second half of the twentieth century, because of industrial activities and urban wastes. Pb, Zn and Hg exceed the concentration that is considered "moderately polluted" by the USEPA normative (Ligero et al., 2002). The highest metal concentrations were found for Zn, followed by Cu, Pb, As, Ni, Hg and Cd (Blasco et al., 2010). Despite this, the province has many protected areas surrounding.

For these reason, in order to evaluate the quality of the environment, three sampling sites in the Gulf of Cádiz were selected: Muelle de Capesa (H1) and Puerto deportivo of Mazagón (H2) located in Huelva area, and Bay of Cádiz (C1) located in the coast of Cádiz used as a control site (Figure 2).

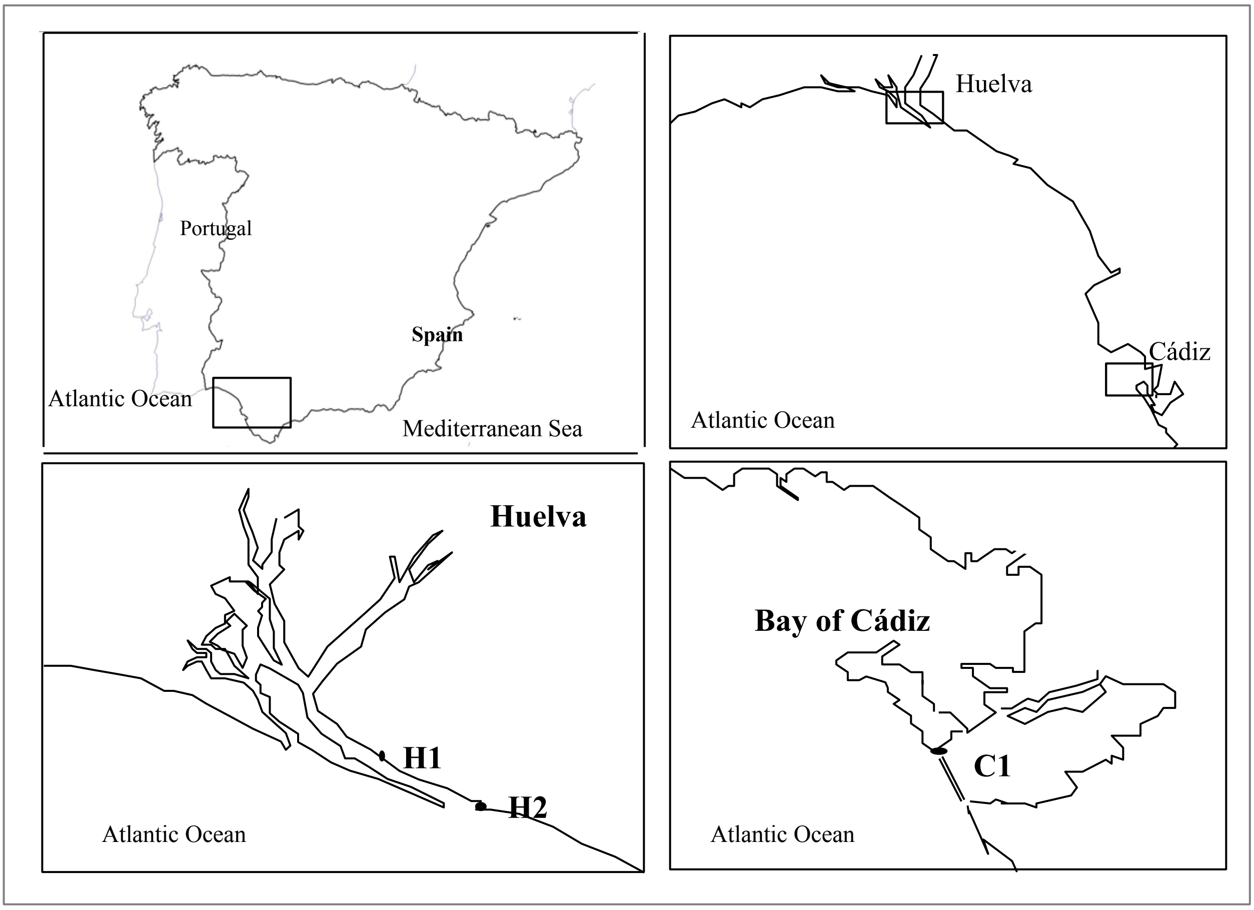

Figure 2. Map of the south-western Iberian Peninsula with the sampling sites used for the experiment for the three sediments: the first one in the Bay of Cádiz (C1), and two in Huelva, Muelle de Capesa (H1) and Puerto deportivo of Mazagón (H2).

1.3.1 Bahía de Cádiz

The Bay of Cádiz (Figure 3) is a zone located on the south-west coast of Spain, in the Gulf of Cádiz, with a singular configuration made during the years by geological, biological and anthropogenic factors. It is divided in three areas: the Maritime Bay, the Terrestrial Bay and the Amphibious Bay. The Maritime Bay can be divided in two basins, the Inner Bay and the Outer Bay. The second is deeper than the first, connected by the Puntales Channel. The maximum depth is 20 m. In the Bay of Cádiz area the predominant water dynamics are linked to tides, which allow the accumulation of fine sediments (silts and clays), which have a great absorption capacity for the substances solved in water (Ligero et al., 2002).

Despite its location in the centre of many maritime routes, the Bay of Cádiz has been proved to be a low contaminants area. This could probably be related to the particular surface circulation that this area has, given by the presence of the Atlantic Ocean and its geological structure. During most of the year and mainly during summer there is an anticyclonic circulation, which allows the dispersal of contaminants (Criado-Aldeanueva et al., 2009).

Figure 3. Map of the Bay of Cádiz with the sampling site (C1) used as a control station to compare its sediment with the one taken in Huelva area. Source: Google Earth © 2016

1.3.2 Huelva area

The Huelva area (Figure 4) is one of the most industrialized locations in Gulf of Cádiz and wastes generated by the activities and contaminants are discharged into the estuary of the Huelva River. The main sources of pollution are:

- The coastal currents that move from west to east, causing a process that transport water and sediment along the shoreline, carrying materials (Rosado et al., 2015).
- Mines exploitations: Rio Tinto mines have been exploited since 1000 BC, with the beginning of large-scale mining in 1873. Even the abandoned sites contribute to increase metal concentrations in water (Galàn et al., 2003; Morillo and Usero, 2008). Furthermore, the Tinto and Odiel river basins receive inputs from the Iberian Pyrite Belt (Nieto et al., 2006).
- High presence of industries, mainly chemical and petrochemical (Querol et al., 2002).

The Huelva estuary has the highest metal concentrations in Europe, and the most found are Cu, Cd, Pb, Zn and As. Furthermore, also the presence of elevated levels of PAHs has been confirmed in the sediment (Oliva et al., 2013).

Figure 4. Map of the Huelva area with the sampling sites in which the sediment was taken to evaluate the quality with the integrated model: the Muelle de Capesa (H1) and the Puerto deportivo of Mazagòn (H2). Source: Google Earth © 2016

1.4 Hypothesis and objectives

Human activities are altering the normal balance of metals in the environment, causing impacts on human health and marine organisms in Huelva. The hypothesis of this thesis is that Huelva area is an highly contaminated site, because of its particular coastal currents, the mining exploitation of the area and the presence of chemical industries. Therefore, the objective of this work is to evaluate sediment quality in Huelva, comparing it to the one taken from a control area, Bay of Cádiz, using an integrated model to get a global vision of the status in Huelva coastal area.

In order to accomplish the main objective, three sub-objectives were proposed:

1. Physical-chemical characterization of sediments from Huelva (polluted area) and Cádiz (control area) in order to obtain the concentration of organic matter and metals in the different sites (Fe, Mn, Zn, Pb, Cu, Cr, Ni).
2. Determinate the potential effects of metals in the embryo development on *Paracentrotus lividus* using elutriates as a route of exposure.
3. Evaluation of toxicity of metals in the fertilization process of *Paracentrotus lividus*.
4. Estimation of the effects on *Ampelisca brevicornis* using whole sediment as a route of exposure
5. Statistical analysis to estimate significant differences in terms of biological response for *Paracentrotus lividus* and *Ampelisca brevicornis*.
6. Analysis of the results obtained in order to determinate the rate of contamination given by human activities in the study areas.

1.5 MSC Thesis structure

The present Msc Thesis is divided into four chapters. The first one introduces an overview of the topic of this study, describing the problem of metal pollution in marine environment and coastal zones, types of metals and the main effects on the aquatic ecosystem, marine organisms and human health. The chapter analyses the integrated model and its parts, and explains the aim of this study and why the study areas were chosen. The second chapter presents the methodology used for the physical-chemical analysis of the sediment and for bioassays: *Paracentrotus lividus* fertilization and developmental tests and *Ampelisca brevicornis* survival test.

In the third part, results of the physical-chemical analysis and bioassays performed on different organisms are presented and discussed following previous works. The fourth chapter includes the main conclusions of this study.

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Chapter 2

Materials and methods

2.1 Sampling strategy

In order to evaluate the quality of sediments from Huelva, two different sitess were selected: Muelle de Capesa (H1) and Puerto deportivo of Mazagòn (H2) located in the Huelva area. Sediment from the Bay of Cádiz (C1) was selected as control site.

Sediment was collected during low tide (0−5 cm layer), grabbed and directly placed in plastic buckets. Before being used in the experiments it was sieved with a 1 mm Ø sieve to remove bigger particles. Surface clean seawater (±1 m depth) was collected in the Bay of Cádiz during high tide using 8 L plastic bottles. All the materials used for the experiment were previously washed with nitric acid and Milli-Q water to avoid any metal contamination.

2.2 Physical-chemical parameters

Metal concentrations for Fe, Mn, Zn, Pb, Cu, Cr and Ni were determined at the Centro Interdipartimentale di Ricerca per le Scienze Ambientali (CIRSA-UNICO) at the Ravenna Campus (University of Bologna). Subsamples of sediment (~1 g) from each sampling station were dried at <60°C overnight, disaggregated in a mortar and then sieved with a 0.63µm Ø sieve (Figure 5).

Figure 5. Sediment samples taken from the three different sampling sites: the control area, Bay of Cádiz, and contaminated area of Huelva, Muelle de Capesa (H1) and Mazagòn (H2) sites. Sediments were pretreated for acid dissolution and metal analysis.

Cu, Pb, Ni and Cr were analyzed by graphite furnace atomic absorption spectrophotometry (GF-AAS, Perkin Elmer A Analyst 100), while Zn, Fe and Mn were determined by flame atomic absorption (FAAS), according to the EPA methods 7010 and 7000B (USEPA, 2007a) after microwave-assisted digestion of sediment with an acid mixture (HCl, HNO_3, and HF, 3:1:1 v/v) (Figure 6).

Figure 6. Devices used to measure chemical parameters of the sediment. A) The microwave equipment that was used to treat sediment samples with an acid mixture (HCl, HNO_3, and HF, 3:1:1 v/v) and microwave-assisted digestion.
B) The graphite furnace atomic absorption spectrophotometry that was used to determine the metal concentration in the sediment for Cu, Pb, Ni and Cr. Zn, Fe and Mn were determined by flame atomic absorption (FAAS)

Reagent blanks, random sample replicates 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 procedures. Measured concentrations in Reference Material Sco-1 included in the analysis were within their certified ranges (Table 1). Accuracy (R%) and precision (CV) were ±10% and 15%, respectively, except for Mn.

Table 1. Certified and measured concentrations of Fe (%) and trace metal concentrations (mg kg^{-1}) in the Standard Reference Materials (Sco-1, Silty Marine Shale).

	Certified value		**Measured value**		**Statistics**		
	mean	**sd**	**mean**	**sd**	**CV**	**E%**	**R%**
Mn	430	30	348	63	18	19	81
Fe	3.59	0.13	3.3	0.02	0.5	4	92
Zn	100	8	101	16	15	17	101
Pb	31	3	29.8	2.6	9	11	103
Cu	29	2	31.5	0.8	2	10	102
Cr	27	4	27	3.5	13	20	100
Ni	68	4	63.3	1.4	2	6	93

Organic matter content in the sediment was estimated by loss of weight on ignition (Luczak et al., 1997). Sediment samples previously dried were placed in crucibles, weighed and ignited at 450°C in a muffle furnace overnight (THERMOLYNE model 47900). Percentage weight loss was calculated for each replicate. The weight loss at each interval are given as % loss on ignition (%L.O.I.).

2.3 Toxicity tests

2.3.1 Amphipod survival test

The amphipod toxicity test was carried out following standard procedures (Casado-Martinez et al., 2007). One liter glass jars were filled with approximately 250 ml of sediment taken from the different sampling sites and 750 ml of clean sea water taken from the control area of Bay of Cádiz, in a proportion 1:4 v/v.

Individuals of the species *Ampelisca brevicornis* (Cal/EPA, 2004) used for the 10 days toxicity test were collected from the control site located in the Bay of Cádiz. Once the organisms were collected, they were brought to the University of Cádiz laboratory for acclimation under laboratory conditions. Water quality and laboratory conditions are summarized in Table 2. The parameters were checked to ensure that the water quality and laboratory conditions were not affecting the organisms survival, as it is a non-renewal and static test in which the organisms are not fed (Basallote et al., 2012). Temperature and salinity were measured at the beginning and at the end of the bioassay, while ammonium was evaluated at day 3 and 8. Salinity was obtained using a refractometer ATAGO S/Mill Hand Refractometer, while pH using a portable pH meter (model: Phenomenal 1000H; accuracy ±0.005 pH) that was calibrated using pH buffer of 4.00 and 7.00. The Ammonium was measured using a commercial kit PRODAC test.

Table 2. Amphipod survival toxicity test. Water quality and laboratory conditions that were recorded during the static non-renewal test.

Parameters	**Conditions**
Temperature	20°C
pH	8
Salinity	38-40
Photoperiod	Natural of April (15-25/04)
Test chambers	Glass, 1l volume
Volume of sediment	250 ml
Volume of water	750 ml
Number of organisms per chamber	20
Test duration	10 days
Endpoint	Survival

An aquarium for acclimation was prepared with water and sediment (in a ratio 1:4 v/v) collected from the reference site of Bay of Cádiz. After one-week acclimation period, the toxicity test was performed. Three replicates were used for each sediment sampling site: Bay of Cádiz (as control), Muelle de Capesa (H1) and Mazagòn (H2) (Figure 7).

Sediments into the test chambers were left to settle with constant aeration for 24 hours prior to the addition of the amphipods. In each jar 20 animals were added. They were selected based on their good conditions. Males were preferably chosen because females could be pregnant affecting the final number of alive organisms. During the experiment the aeration was adjusted to not disturb the sediment surface. After 10 days the count of alive and dead animals was made.

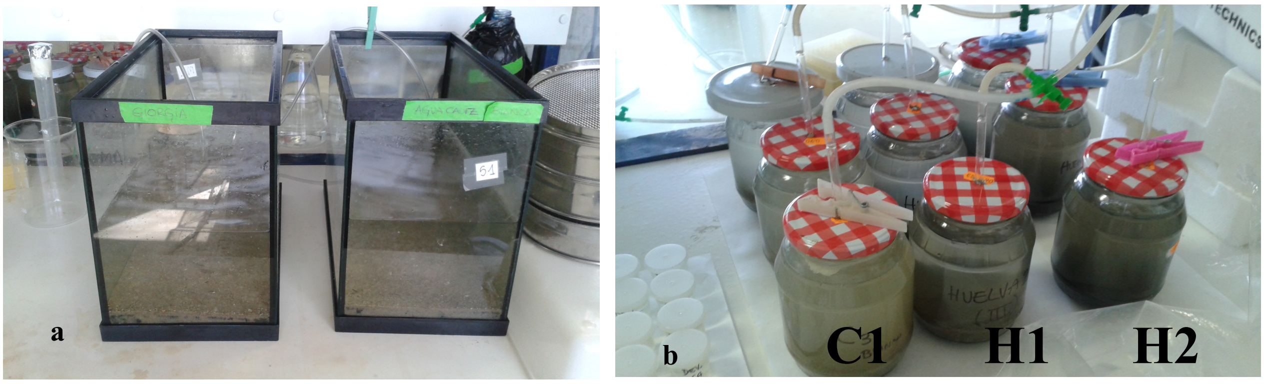

Figure 7. Amphipod survival toxicity test. Laboratory pictures of a) the acclimation aquariums that were used to put the *Ampelisca brevicornis* amphipods after they were taken on field and b) he jars that that were used for the Amphipod 10 days survival test, with the three sediments collected in the control site, Bay of Cádiz (C1), and in the contaminated sites: Muelle de Capesa (H1) and Puerto deportivo of Mazagón (H2). Water quality parameters were checked at the beginning and at the end of the experiment.

2.3.2 Sea urchin bioassays

The mature adults of *Paracentrotus lividus* used for the fertilization and larval toxicity tests were collected at low tide from a rocky intertidal platform in the control site of Bay of Cádiz in April (U.S. EPA 2002, Fernández et al., 2001). The echinoderms were transported to the laboratory at the University of Cádiz in cold and dry conditions (Figure 8).

Figure 8. Laboratory picture of some specimens of *Paracentrotus lividus* collected for the experiments at the site of

Bay of Cádiz. They were used for the extraction of eggs and sperm to get the fertilized eggs.

Once in the laboratory, the gametes from female and male were obtained by dissection on the equatorial axe and direct extraction from the gonads. In order to evaluate the conditions and the amount of 300 eggs (20-30 eggs per ml), they were observed on an inverted microscope OLYMPUS CKX4. Three drops of 10 µl of the water in which they were stored were put on the plate and observe with a 10x lens.

Two different tests were run with sea urchins:

1) the fertilization test, to evaluate if contaminants can affect the fertilization. The negative effect is related to the membrane formation.

2) the embryogenesis test, to see how the contaminants affect the embryo-larval development.

Fertilization

The fertilization test was conducted in polyethylene vessels containing filtered clean sea water as control in the toxicity test and elutriate for the different sites under analysis (Muelle de Capesa and Mazagòn). Filtered sea water (0.22 µm) was taken from Bay of Cádiz and used as control. The elutriates were made by 30 min rotation of sediment with clean sea water in proportion sediment-water 1:4. This method allowed the transference of contaminants from sediment to the water with a resuspension process. After waiting 24 hours to make it settle down, it was ready to be used (Figure 9).

Figure 9. Elutriate preparation. a) The machine that was used to obtain the elutriates and b) the glass jars containing clean sea water and sediment taken from the three sampling sites: Bay of Cádiz (C1), Muelle de Capesa (H1) and Mazagòn (H2), that were used to obtain elutriates by rotation.

A volume of solution containing approximately 300 egg (20-30 eggs per ml) was added to the test vials. For the 20 minutes exposure time, the plastic glasses contained 10 ml of sediment elutriate, while for the 48 hours exposure time 20 ml were used. The liquid volume was higher because eggs need more space to develop (Figure 10).

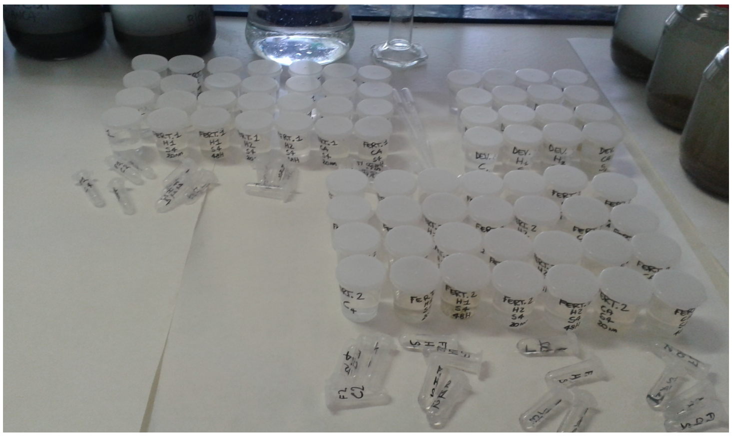

Figure 10. Laboratory pictures of the test vessels that were used for all the replicates in the fertilization and larval-development tests on *Paracentrotus lividus*. For the fertilization tests each plastic glass contained 10 ml of elutriate, or filtered sea water for the control; for the embryogenesis test, each plastic glass contained 20 ml of elutriate, or filtered sea water for the control, because to develop eggs need an higher volume of water.

For the fertilization test, the sperm was exposed for 1 hour to the three different elutriates separately. For the procedure 1 ml of elutriate was added to 10 µl of sperm. After 1 hour, 10 µl of the sperm exposed to the different elutriates were added to the vials.

At 20 minutes under 20°C, a volume of 500 µl of 36-40% formaldehyde was added to stop the fertilization process in four replicates for each sampling station (Carballeira et al., 2012). This is the time in which the fertilization membrane should be formed. After 48 hours under 20°C, a volume of 500 µl of 36-40% formaldehyde was added to stop the fertilization process in the other four replicates from each sampling point. During this time the larva should be completely formed (Gambardella et al., 2013; Basallote Sánchez, 2014). Percentage of fertilization was determined after counting 100 eggs and identifiable pluteus stages (Ward et al., 2006). The observations were made using an inverted microscope OLYMPUS CKX41 at 10x. Images were taken with a digital camera Olympus CAMEDIA C-5060.

Embryogenesis

For the embryo-larval development procedure the eggs extracted from *Paracentrotus lividus* were transferred in a 100 ml-test tube containing filtered clean sea water (Fernandez and Beiras., 2001). Sperm that was not exposed to the different elutriates was added to the eggs to obtain fertilization.

Fertilization success was checked with an inverted microscope OLYMPUS CKX41. Three drops of

10 µl of the water in which the fertilization process took place were put on the plate and observed with a 10x lens (Carballeira et al., 2012). Once it was confirmed (>95% fertilization rate), a 100 µl volume solution containing embryos was added to the replicates plastic glasses containing 20 ml of sediment elutriate (four replicates from each one of the test vessels). After the incubation of 48 hours under 20°C, the samples were fixed adding 500 µl of 36-40% formaldehyde (Kobayashi et al., 2004).

The development of 100 counted embryos was considered as the endpoint for the embryo-larval toxicity test (Ward et al., 2006). The toxicity was evaluated by ranking the different deformations as: 0 (none), 1 (slight), 2 (moderate) and 3 (high) following previous works (Carballeira et al., 2012; Gambardella et al., 2013) (Figure 11). The observations were made using an inverted microscope OLYMPUS CKX41 at 10x. Images were taken with a digital camera Olympus CAMEDIA C-5060.

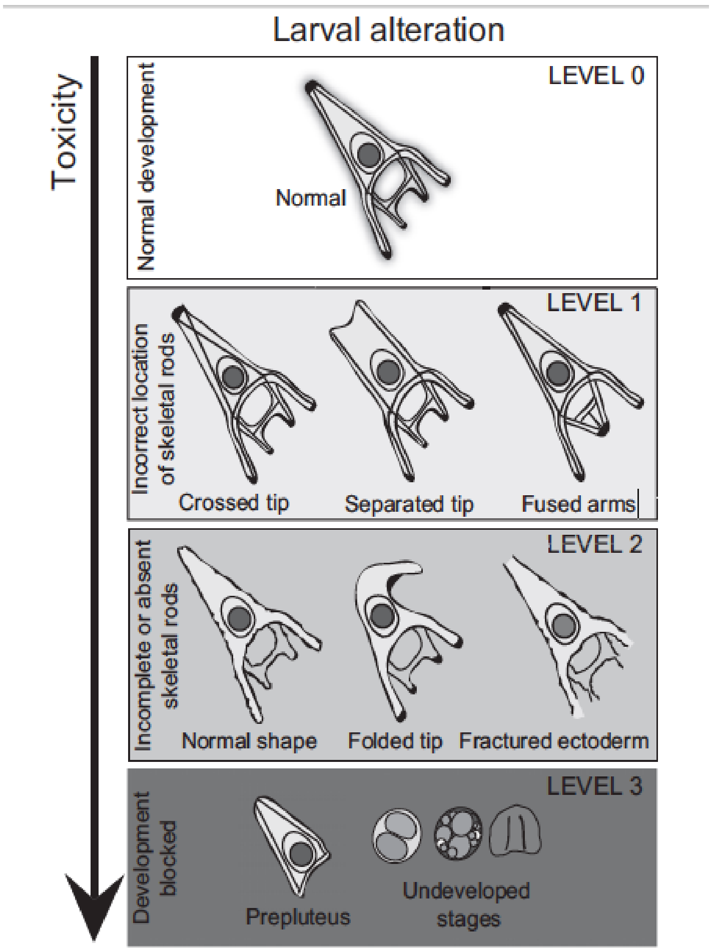

Figure 11. Altered larval stages that were noted during the experiments (Carballeira et al., 2012).

2.4 Statistical analysis

The statistical analyses included a 1-way ANOVA, Analysis of Variance (Anderson, 2001), with the post-hoc test of Bonferroni, to evaluate significant differences ($p<0.05$) in all the experiments. Significant differences were determined by 95% confidence intervals.

In addition a multivariate analysis was conducted using principal component analysis (PCA) to describe the distribution of the data with the minimum loss of information (Casado-Martinez et al., 2006). Before applying it, all the data were normalized.

All statistical analyses were performed using the IBM SPSS 21 Statistics software.

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Chapter 3

Results and discussion

3.1 Physical-chemical parameters

When assessing the toxicity of metals in a sediment-water environment, it should be considered that the mobility of the metals depends on their association with the solid phase to which they are bound (Basallote et al., 2014).

In this section of the study, physical-chemical parameters are discussed. As it can be observed in table 3 the sediment from Muelle de Capesa and Mazagòn sites in the Huelva area showed the highest metal concentrations. Mn was highest in Muelle de Capesa sediment, followed by Zn, Cu, Cr, Pb, Ni and Fe. Mn was also highest in Mazagòn sediment, followed by Zn, Cr, Cu, Pb, Ni and Fe. Fe and trace metals levels (Mn, Cr, Ni, Cu) found in the Bay of Cádiz were consistently lower, while Zn and Pb concentrations below the detection limit.

Table 3. Summarized results of the sediment physical-chemical characterization in the sediment samples taken from the three different sampling sites (Bay Cádiz, Muelle de Capesa and Mazagòn) and the geochemical background (González-Pérez et al., 2008). The results included metal concentrations expressed as percentage for iron (Fe) and as parts per million (mg/kg^{-1}) for the other metals, and the organic carbon content expressed as % loss on ignition (%L.O.I.). Not detected is expressed as n.d.

	Fe	**Mn**	**Zn**	**Pb**	**Cu**	**Cr**	**Ni**	**LOI**
Bay of Cádiz	0.74	381	n.d.	n.d.	3.3	14	6.8	1
Muelle de Capesa	1.8	439	279	32	113	33	4.9	1.03
Mazagòn	3.08	1219	166	23	41	51	4.2	0.68
Geochemical background	—	—	83	35	15	65	27	—

Low levels of trace metals (Pb, Cu and Ni), comparable to background levels, were found at the Bay of Cádiz area (Table 3). Previous works demonstrated that this sampling area is low contaminated, thus it has been used as a reference site (Martìn-Dìaz et al., 2008). One explanation of such low levels of contaminants could be that the inner Bay of Cádiz is not influenced by the surface anticyclonic circulation of the Gulf of Cádiz (Criado-Aldeanueva et al., 2009).

In contrast, higher concentrations of trace metals were found in the Huelva area sediments when compared to Cádiz sediments and the background levels. This result can be linked to the high load of metals that this area receives from industrial activities (Sánchez-Rodas et al., 2007), and to its particular coastal currents that move from west to east, causing the transport of materials along the shoreline (Rosado et al., 2015).

To sum up the results obtained, the chemical analysis of the sediments displayed the following order of concentration for trace metals: for Muelle de Capesa Zn > Cu > Cr > Pb > Ni, for Mazagòn Zn > Cr > Cu > Pb > Ni, and for Bay of Cádiz Cr > Ni > Cu (Table 3).

Metal concentrations in sediment from the Muelle de Capesa and Mazagòn sites were found to be different even if the sampling stations were both located within Huelva area. This may be linked to the fact that Muelle de Capesa is a site very close to the industrial area, while Puerto deportivo of Mazagòn site is located farthest away from the industries. On the other hand, previous works assessed that metals accumulation is influenced by the turnover of organic matter in sandy top soil (Quenea et al., 2009). Organic concentration of sediments is fundamental in studies of benthic ecosystems. It can be considered as a trophic source for the benthos, organic enrichment and a form of disturbance for the organisms.

In this study, organic matter content, expressed as % loss on ignition (% L.O.I.) showed very low values (Table 3), comparable to the ones recorded by Basallote et al. (2014). Nevertheless, in the Mazagòn sampling site, sediment showed a slightly lower content of organic matter when compared to the other samples. The higher Mn concentration was also observed in Mazagòn sediment. This could be linked to previous studies that assessed that shifts in the soil Mn equilibrium are correlated to the concentration of organic matter detected (Cotter et al., 1968).
In general in the Gulf of Cádiz the grain size characteristics of sediments provide an increased absorption capacity to the sediment of the elements dissolved in the water column (Ligero et al., 2002). The grain size distribution in the sampled sites is shown in Table 4. The percentage of sand and fines was used for sediment textural classification (Flemming et al., 2000). The percentage of fines (silt and clays < 0.2 μm) was low and similar for Bay of Cádiz and Mazagòn sites, but predominant in Muelle de Capesa sampling site. Previous works reported that propagation of metals is significantly influenced by grain size; the accumulation of the metal usually increases with decreasing of the grain size (Hanlon et al., 2003). This is in accordance with the data recorded, as Muelle de Capesa area showed a higher percentage of fines (smaller grain size) and a higher presence of trace metals.

Table 4. Range of mean grain size in sediments from the three sampling sites: Cádiz, Muelle de Capesa and Mazagòn. The results included percentages of sands (0.063-2 mm) and fines (<0.063 mm) (Basallote et al., 2014).

Grain size	Sand (%)	Fines (%)
Cádiz	54.80%	45.20%
Muelle de Capesa	33.57%	66.43%
Mazagòn	53.13%	46.87%

3.2 Amphipod survival test

Amphipods are largely used in toxicity tests because they are exposed to contaminants via ingestion of sediment particles, so that they can assess effects of both hydrophobic and more water-soluble contaminants (Cal/EPA, 2004).

In this study, the mortality results obtained with the 10-days acute toxicity test with *Ampelisca brevicornis* exposed to the three sediment treatments are presented. As this is a non-renewal static test, the water quality parameters were monitored to confirm the validity of results obtained (Table 5). The physical-chemical parameters recorded in the overlying water test chambers during the bioassays did not show significant alterations.

Table 5. Amphipod survival toxicity test. Water quality parameters: the table shows the averaged physical-chemical characteristics of the water (Salinity, pH and Ammonium) that were measured during the non renewal experiment in the three different sampling points: Bay of Cádiz, Muelle de Capesa and Mazagòn.

Water quality parameters	**Salinity**	**pH**	**Ammonium** (mg l^{-1})
Bay of Cádiz (control)	39±2	8±1	1±1
Muelle de Capesa	37±1	8±1	1±0
Mazagòn	37±1	8±1	1±0

Results of 10-days amphipods mortality are presented in Figure 12. A 1-way ANOVA analysis, with a post-hoc comparison with the Bonferroni method, was used to calculate the significant differences ($p<0.05$) between data.

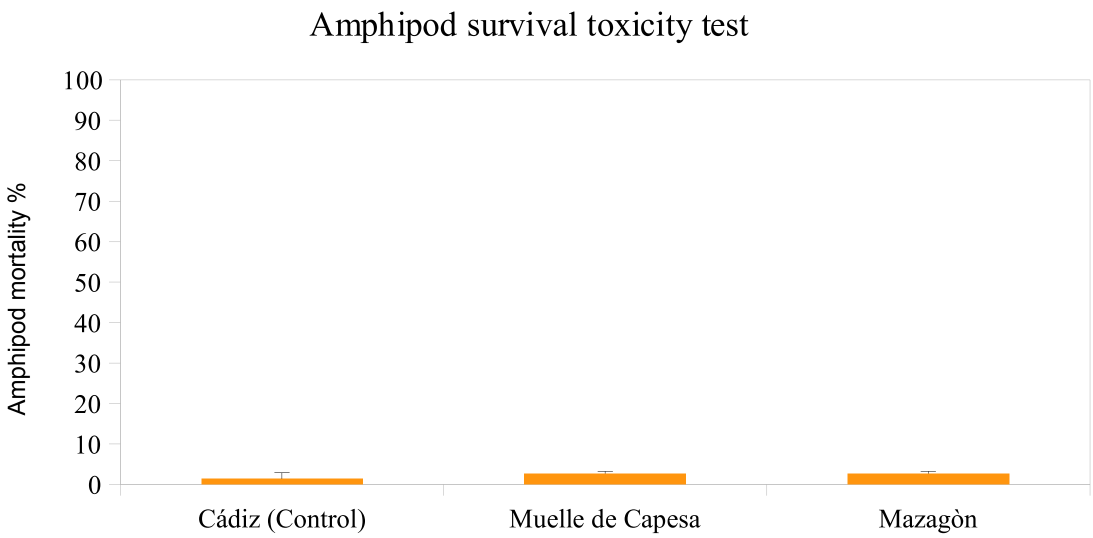


Figure 12. 10-day Amphipod survival toxicity test. Average and standard deviation (mean±sd) of amphipod mortality calculated after 10 days of exposure to the three different sediment from the Bay of C ádiz (control), and the Huelva area sampling sites, Muelle de Capesa and Mazagòn .

For Bay of Cádiz site, the mortality of amphipods was as low as 1%. On the other side, 3% of mortality was detected both for Muelle de Capesa and Mazagòn sediment. Sediment is considered toxic when the test shows mortality higher than 90%. The statistical analysis showed that amphipod mortality in sediment taken from Huelva area was not significantly different from the reference sediment (Bay of Cádiz). Therefore no toxicity effects were observed in sediments from the Huelva area sites. Further investigation is need to unveil eventual metal accumulation in amphipods in the studied sites. Previous works assessed the presence of metals bioaccumulation in benthic invertebrates (Kalman, 2009). The capacity of amphipods to accumulate metals could help to explain the fact that the results in this case showed no toxicity in Huelva area, which has been proved to be highly contaminated.

3.3 Sea urchin fertilization and larval toxicity test

3.3.1 Sea urchin fertilization test

Echinoidea are largely used for toxicity monitoring because the tests in which they are used are short-term and sublethal, and they are tolerant of Toxicity Identification Evaluation (TIE) procedures (U.S. EPA 2002). Fertilization test has been considered a reliable test to assess contaminants in sediment (Volpi Ghirardini et al., 2005), even if it is less sensitive than embryo development test (Xu et al., 2011).

Sea urchin fertilization test is an acute and static bioassay, in which the sperm is put in contact with clean eggs and elutriate extracted from the analysed sediment to see how it affects the fertilization process. In this study, results were divided between the ones obtained after 20 minutes and after 48 hours exposure time to the elutriates after the fertilization. For both data controls contained at least 90% of normal larvae with standard and skeletal criteria, indicating the validity of the test (Carballeira et al., 2012). A 1-way ANOVA analysis, with a post-hoc comparison with the Bonferroni method, was used to calculate the significant differences ($p<0.05$) between data.

For the data after 20 minutes the fertilization success was calculated as percentage (%) of the cells presenting a fertilization membrane. The egg fertilization after 20 minutes was not significantly different from the control for the Bay of Cádiz elutriate, but it showed significant differences for Muelle de Capesa and Mazagòn sediment elutriate ($p<0.05$). In particular, Muelle de Capesa elutriate had the heaviest toxic effect on fertilization (Figure 13). The two sites within the Huelva area showed very high sediment concentrations of Cu and Zn, particularly in Muelle de Capesa, far exceeding the background levels (Table 3), which could have been the cause of the lowest fertilization rate after 20 minutes exposure resulting in ~40% of eggs not fertilized. Previous authors found a decreased fertilization success in sea urchin in the presence of high concentrations of these metals (Vaschenko et al., 1999; Tualla and Bitacura, 2016).

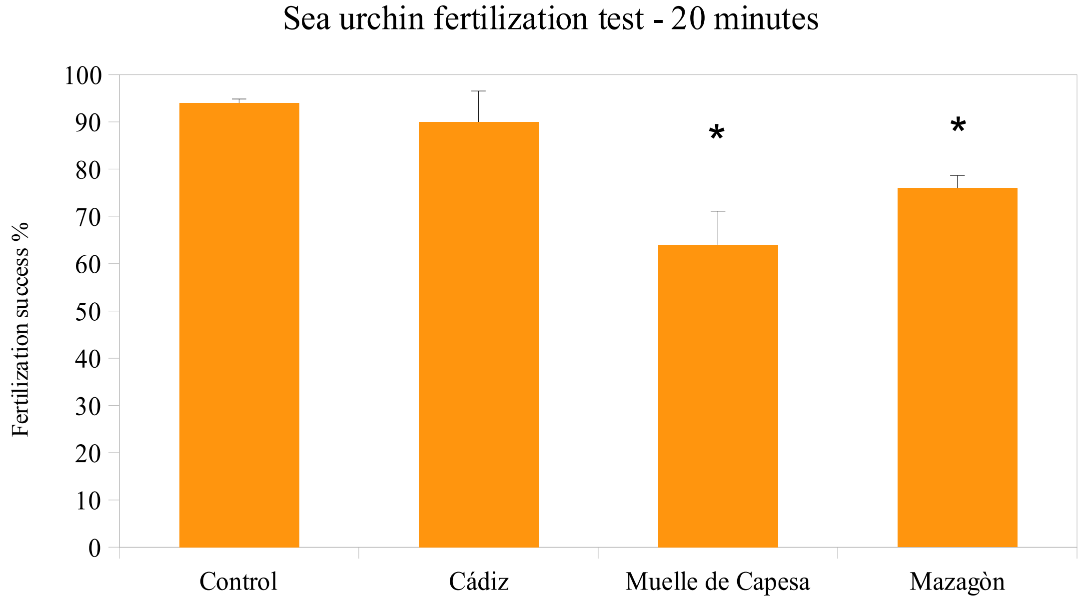


Figure 13. Fertilization success, calculated after 20 minutes of exposure to the three different elutriates. Fertilized eggs (the ones with fertilization membrane) calculated for quadruplicates (n=4) were expressed as percentage (%) with the standard deviation (mean±sd). Significant differences (*) ($p<0.05$).

Fertilization success after 48 hours was expressed as the number of the altered larval stages after fertilization with sperm in contact with sediment elutriates. The larval alterations were defined in three levels of toxicity (from 0 to 3), according to Carballeira et al. (2012) (Figure 14).

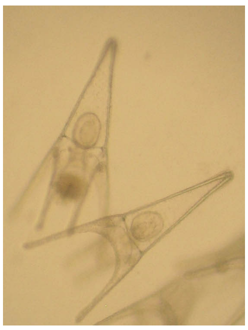
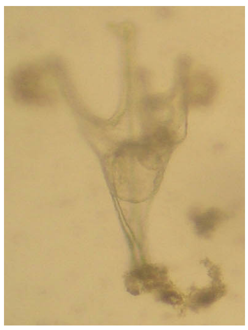
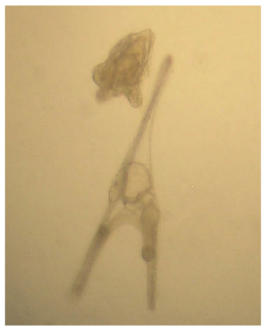
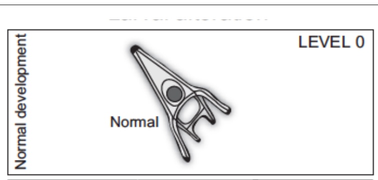
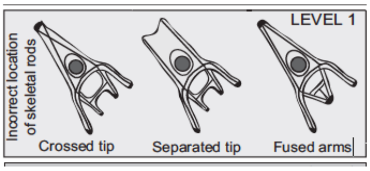
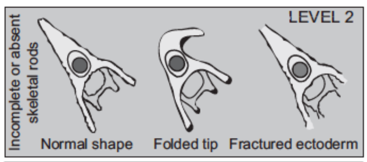
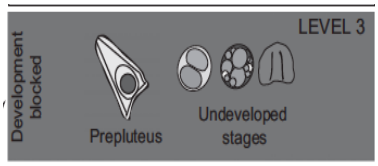
			
Level 0 – Normal shape	Level 1 - Crossed tip	Level 2 - Incomplete skeletal rods	Level 2 - Fractured ectoderm

Figure 14. Laboratory picture of types of normal and abnormal larval stages observed in this study after 48 hours incubation of the fertilized sea urchin eggs: normal (level 0), crossed tip (level 1), normal shape with incomplete or absent skeletal rods and fractured ectoderm (level 2).

The observations at the microscope showed a predominant number of level 0 larvae (normal shape) in the control and the Bay of Cádiz samples. An high percentage of level 2 (heavy abnormality) larvae was observed both in the Muelle de Capesa and Mazagòn sediment elutriates, with an high presence of larvae with incomplete skeletal rods. Level 1 (medium alterations) and level 3 (blocked development) larvae were found, but in a low percentage (Table 6).

Table 6. Fertilization success calculated after 48 hours of exposure. Larval stages are expressed as percentage (%) calculated for quadruplicates (n=4). The larval alterations are noted with a growing level of toxicity from 0 to 3, according to Carballeira et al., 2012.

Fertilization success (%) after 48 hours		**Control**	**Cádiz**	**Muelle de Capesa**	**Mazagòn**
		89%	87%	12%	30%
	Crossed tip	6%	6%	4%	12%
	Separated tip	—	—	—	—
	Fused arms	—	—	—	—
	Normal shape	2%	1%	81%	58%
	Folded tip	—	1%	—	—
	Fractured	—	—	—	—
		3%	5%	3%	—

The results of the metal concentration analysis suggest that the toxicity of the sediments from the Muelle de Capesa and Mazagòn stations may have contributed to the larval abnormalities results (Figure 15). Significant differences were found in Muelle de Capesa and Mazagòn sediment elutriates only in level 0 and level 2 larvae compared to the control ($p<0.05$).

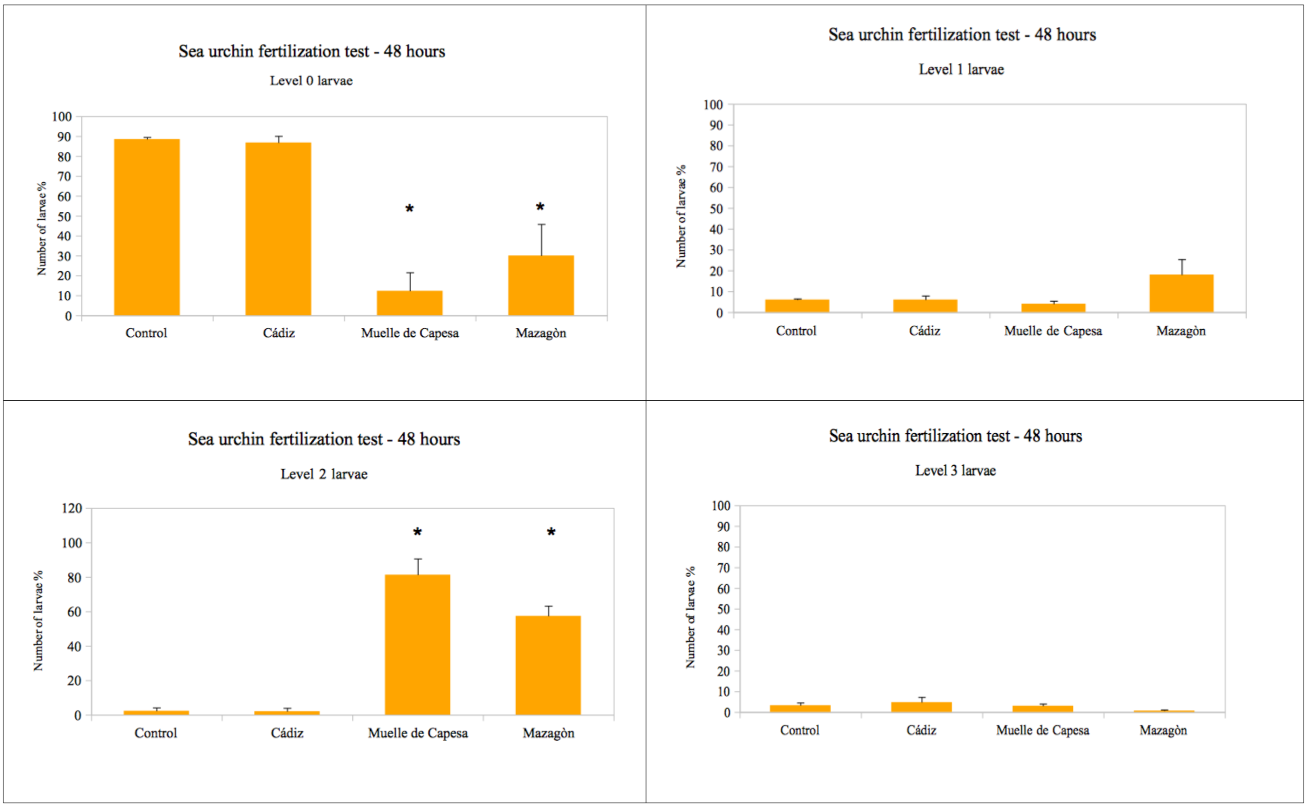


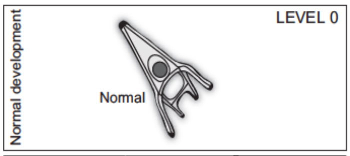
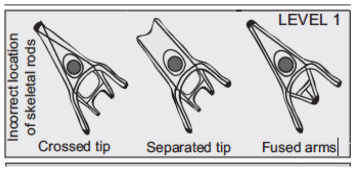
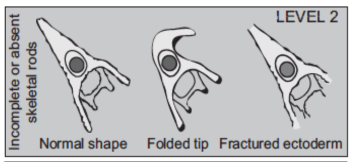
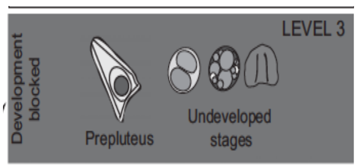
Figure 15. The bar plot shows the fertilization success, calculated after 48 hours of exposure. Different levels are expressed as percentage (%) of the type 0, 1, 2 and 3 larvae calculated for quadruplicates (n=4), with the standard deviation (mean±sd). Significant differences (*) ($p<0.05$).

3.3.2 Sea urchin larval toxicity test

Sea urchin larval toxicity test is a chronic and static bioassay in which larval development is evaluated after the fertilization with clean sperm and 48 hours of exposure to the contaminated elutriates. The developmental success is calculated as the number of the altered larval stages, expressed as percentage (%). The larval alterations were here noted with a growing level of toxicity from 0 to 3, according to Carballeira et al. (2012). The control contained at least 90% of normal larvae with both standard and skeletal criteria, indicating the validity of the test.

The observations at the microscope showed a high number of level 0 larvae (normal shape) in the control and the Bay of Cádiz samples (Table 7). A predominant percentage of level 2 (heavy abnormality) larvae was observed both in Mazagòn and Muelle de Capesa sediment elutriates, with a high presence of larvae with incomplete skeletal rods and fractured ectoderm in lower percentage. Also a consistent number of level 1 larvae (medium alterations) was found in the Mazagòn elutriate. Level 3 (blocked development) larvae were found in a not significant number in all the samples.

Table 7. Fertilization success calculated after 48 hours of exposure. Larval stages are expressed as percentage (%) calculated for quadruplicates (n=4). The larval alterations are noted with a growing level of toxicity from 0 to 3, according to Carballeira et al., 2012.

Developmental larval stages %		**Control**	**Cádiz**	**Muelle de Capesa**	**Mazagòn**
		94%	90%	10%	17%
	Crossed tip	3%	8%	17%	30%
	Separated tip	—	—	1%	—
	Fused arms	—	—	—	—
	Normal shape	2%	2%	40%	34%
	Folded tip	—	—	—	—
	Fractured	—	—	25%	16%
		1%	—	7%	3%

The larval development showed significant differences from the control for Muelle de Capesa and Mazagòn ($p<0.05$) sediment elutriates for level 0 and level 2 larvae. For level 1 larvae only Mazagòn elutriate was significantly different from the control ($p<0.05$). For level 3 larvae no significant presence was found (Figure 16). These results revealed statistical differences ($p<0.05$) between sampling stations in Bay of Cádiz and Huelva area for level 0, 1 and 2 larvae. Based on this, the sediments had different levels of toxicity on *P. lividus* larval development.

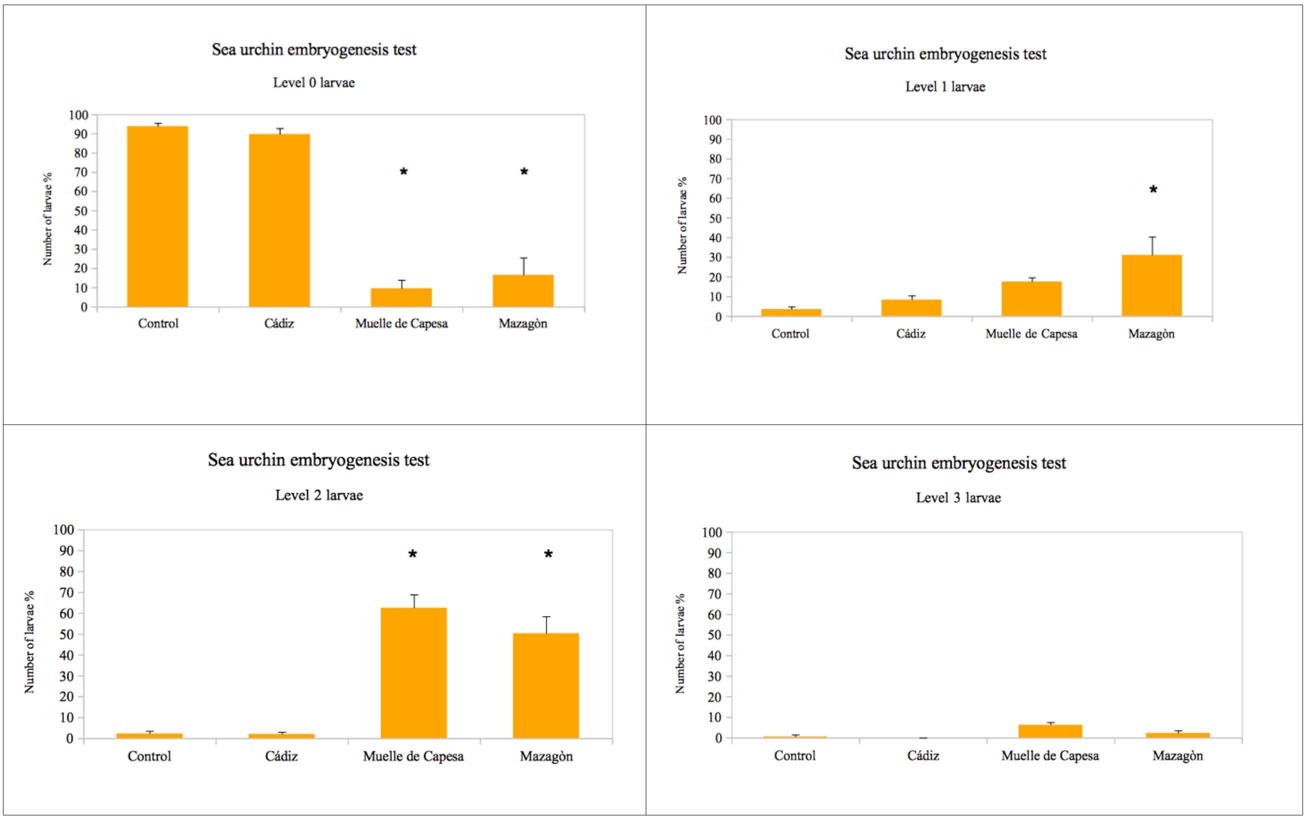


Figure 16. The bar plots show the larval development, calculated after 48 hours of exposure to the three different elutriates, expressed as percentage (%) of the type 0, 1, 2 and 3 larvae calculated for quadruplicates (n=4), with the standard deviation (mean±sd). Significant differences (*) ($p<0.05$).

These results were similar to the ones of the fertilization test after 48 hours, but with a lower level of contamination, probably because in the fertilization test the sperm was exposed to the sediment elutriates before the fertilization process.

Previous works ranked metals with a decreasing order of toxicity on sea urchins as follows: Hg > Cu > Zn > Pb > Fe > Mn (Fernandez et al., 2001; Kobayashi et al., 2005), assessing that their combined action may be either synergetic or antagonistic.

A higher number of abnormalities of level 2 larvae was found in Muelle de Capesa elutriate compared to Mazagòn one. These results may be explained by the higher concentrations of heavily toxic metals (Cu, Zn, Pb) that were detected in Muelle de Capesa sampling site, far exceeding the background levels (Table 3).

Previous studies confirmed the toxic effect of these metals on larval stages. Zn is known as one of the most active metal affecting early stages of echinoids (Kobayashi et al., 2004). Its action can also be increased by the presence of other metals such as Mn, Pb and Fe (Kobayashi et al., 2005). It has also been demonstrated that Ni and Cu are highly toxic for the pluteus stages at low concentration (Bielmyer et al., 2005). Pb can also affect the early larval stages causing abnormal morphology and problems to the skeletogenesis (Ghorani et al., 2013).

While observing the larvae it was possible to see that dimensions was variable among different samples, with the larvae from Muelle de Capesa and Mazagòn smaller than the Bay of Cádiz ones. This could be linked to the fact that it has previously seen that some metals such as Pb can affect larvae size (Vahideh et al., 2012). Most of the larvae exposed to the sample from the Bay of Cádiz showed a normal shape with no alterations, concomitant with non detectable Pb concentrations, while most of the larval stages observed in the site of Muelle de Capesa showed alterations (Figure 17).

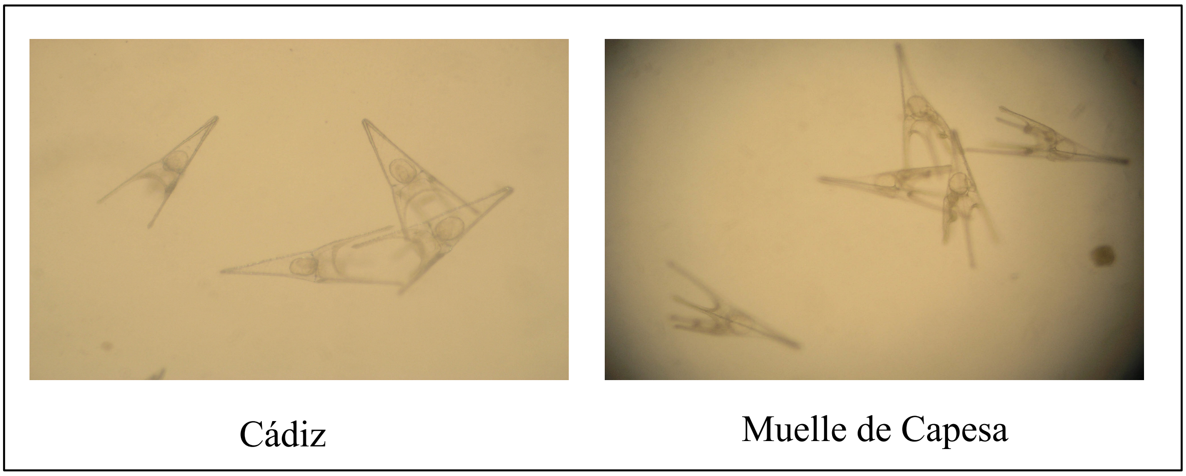


Figure 17. Laboratory pictures that **c**ompare the observed larval stages in Cádiz and Muelle de Capesa.

The results of *P. lividus* bioassays, in contrast with the ones obtained by *A. brevicornis* 10-days survival toxicity test, can be linked to the fact that early larval stages are more vulnerable than mature organisms when exposed to a complex mixture of sediments (Khosrovyan et al., 2013). This could be linked to interspecies differences in toxicants (metals) tolerance, or the different effects that the combination of metal contaminants has in the sediment or in the elutriate, and yet to the bioavailability of the metals in the different sediment phases (Haring et al., 2009)

3.4 PCA

In order to understand the correlation between toxicity results and metal concentrations, a principal component analysis (PCA) was made starting from a correlation matrix (Table 8). Two new factors were obtained from the original values: metals concentrations in sediment, amphipods survival test, sea urchin fertilization (after 20 minutes) and larval-development test (for type 0 larvae) results.

The two factors, taken together, explained more than 80% of the total variance. Factor 1 accounted for 53% of the total variance and linked amphipods mortality, sea urchin larvae responses (fertilization and larval development) with the metals Cu, Zn and Pb. The second factor (Factor 2) accounted for 40% of the total variance and showed a correlation between Fe, Mn and Cr in the different sediment elutriates. The factor 2 showed the different presence of major and trace metals (Fe, Mn and Cr) in the sediment taken from the three sampling sites, however it is not correlated with toxicity test response in the test organisms. This analysis suggested that Cu, Zn and Pb are associated to toxicity, controlling the amphipod mortality and the reduction of egg fertilization and larval development success. The results obtained are in line with previous works were Ni, Cu and Pb resulted highly toxic for the pluteus stage larvae, causing abnormal morphology and problems to the skeletogenesis (Bielmyer et al., 2005; Ghorani et al., 2013). The PCA results confirmed that the different metals concentrations among the sampling stations affected sea urchin fertilization and larval development in Huelva area samples. Other studies reported that coast of Huelva is a highly polluted area (Ramos-Gómez et al., 2009; Casado-Martinez et al., 2006).

Table 8. Rotated component matrix with components loadings of the original variables for the two principal factors after principal component analysis. Only the values greater than ±0.5 are shown in the table.

	Factor 1 (53,831%)	Factor 2 (40,368%)
Fe	–	0,939
Mn	–	0,998
Zn	0,967	–
Pb	0,938	–
Cu	0,996	–
Cr	–	0,927
Ni	0,872	–
Sea urchin fertilization	-0,969	–
Sea urchin development	-0,862	–
Amphipod mortality	0,529	–
Sampling site	–	0,927

3.5 References

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Chapter 4

Conclusion

1. The physical-chemical characterization of sediment showed higher concentrations of metals (Fe, Mn, Zn, Pb, Cu, Cr, Ni) in Muelle de Capesa and Mazagòn sampling sites, compared to the one in Bay of Cádiz. This confirmed the major contamination level in Huelva area, where Zn and Cu concentrations far exceeded the background levels.

2. *Ampelisca brevicornis* showed an high tolerance in the 10-days survival toxicity test, and its mortality was not affected by the presence of contaminated sediment. Further studies on bioaccumulation are recommended.

3. *Paracentrotus lividus* specimen was affected by the presence of metals both for fertilization and larval-development bioassays. Statistical analysis showed significant differences ($p<0.05$) between treatments and control, indicating that the presence of metals can affect the fertilization and larval development. Both Huelva sediment sampling sites presented the higher number of malformations, and the high concentratiosn of Cu and Zn found in Muelle de Capesa sediment could have had an effect on the highest abnormal development observed.

4. As it has been seen before, the Bay of Cádiz was confirmed to be a reference site. Instead in Huelva the levels of metal contaminants could explain the high toxicity effects to the sea urchin fertilization and development.

5. In *Paracentrotus lividus* bioassays organisms were affected by exposure to Huelva area sediment elutriates, while no significant difference was observed on *Ampelisca brevicornis* mortality. The toxic effect varied between the two bioassays, depending on the use of elutriate or whole sediment. This result can be linked to the fact that usually larval stages (*P. lividus*) are more sensitive than adult organisms (*A. brevicornis).* Furthermore, the experimental exposure routes can affect in different ways metal accumulation and detoxification mechanisms in aquatic organisms. This indicates that a suite of different toxicity tests methods is the best way to get an environmental assessment, because of the different exposure routes they have.

6. In this study, the high contamination Zn and Cu levels detected in the Huelva area when compared to the control area of Bay of Cádiz, was assessed by the use of an integrated

model. Therefore it has been observed the importance of running different bioassays on more than one organism with different exposure routes, because of the different responses organisms can have.

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