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Innovative *in vivo* and *in vitro* bioassays for the establishment of toxicity thresholds of pollutants in sediment quality assessment using polychaetes and their immune cells

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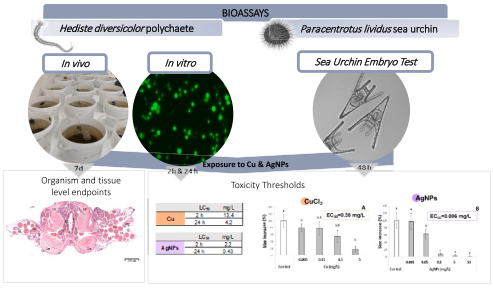
HIGHLIGHTS

- The bioassays with *H. diversicolor* accurately assess sediment-bounded pollutants effects.
- The toxicity of Cu and AgNPs were envisaged at very short-exposure times with coelomocytes.
- *In vitro* approaches with coelomocytes showed great potential for sediment ecotoxicology.
- The toxic responses of *H. diversicolor* were similar to the thresholds obtained with SET.

GRAPHICAL ABSTRACT



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ABSTRACT

Sediment toxicity testing has become a crucial component for assessing the risks posed by contaminated sediments and for the development of sediment quality assessment strategies. Commonly used organisms for bioassays with estuarine sediments include amphipods, *Arenicola marina* polychaetes and echinoids. Among the latter, the Sea Urchin Embryo test (SET) is the most widely used. However, one relevant limitation of this bioassay is the unavailability of gametes all year-round, particularly outside the natural spawning seasons. Consequently, the establishment of an appropriate and complementary model organism for a continuous assessment of sediment quality is recommended. A reliable assessment of the hazards resulting from pollutants in sediments or pore water, can be achieved with ecologically relevant species of sediment such as the polychaete *Hediste diversicolor*, which is widespread in estuaries and has the capacity to accumulate pollutants. The aim of this work was to develop reliable *in vivo* and *in vitro* bioassays with *H. diversicolor* and its coelomocytes (immune cells) to determine the toxicity thresholds of different contaminants bounded to sediments or resuspended into water. Polychaetes were exposed to sublethal concentrations of $CuCl_2$ (*in vivo*) and a non-invasive method for

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collection of polychaetes coelomocytes was applied for the *in vitro* bioassay, exposing cells to a series of $CuCl_2$ and AgNPs concentrations. Same reference toxicants were used to expose *Paracentrotus lividus* following the SET (ICES Nº 51; Beiras et al., 2012) and obtained toxicity thresholds were compared between the two species. *In vivo* exposure of polychaetes to high concentrations of Cu produced weight loss and histopathological alterations. After *in vitro* approaches, a significant decrease in coelomocytes viability was recorded for both toxicants, in a monotonic dose-response curve, at very short-exposure times (2 h). The toxicity thresholds obtained with polychaetes were in line with the ones obtained with the SET, concluding that their sensitivity is similar. In conclusion, *in vivo* and *in vitro* bioassays developed with *H. diversicolor* are accurate toxicity screenings of pollutants that could be bounded to sediments or dissolved in the pore water, and may complement the SET outside the spawning period of the echinoderms. The bioassays herein developed could be applied not only to establish the toxicity thresholds of individual compounds or mixtures, but also to assess the toxicity of field collected sediments.

1. Introduction

Estuaries have undergone important inputs of different chemicals over years due to industrial exploitation and urban wastes release (Brand et al., 2018; Ruiz et al., 2020). As a result, many estuarine sediments can be considered as reservoirs of persistent toxic substances introduced in coastal environments by human activities, acting as a sink and source of contaminants that can pose a risk for the environment (Irabien et al., 2018). A reliable assessment of the hazards resulting from pollutants in sediments can be achieved after integrating chemical analysis and ecotoxicological studies (Hansen et al., 2007; Chapman et al., 2013; Boehler et al., 2017). Sediment toxicity tests expose organisms to sediments under controlled conditions in order to give an estimation of the level of toxicity of contaminated sediments in the field (Simpson et al., 2017) and have the advantage to reflect the bioavailable fraction of contaminants, which can be very different from the total amount determined by chemical analysis (Bat, 2005). Despite significant advances have been made in the use of biological effects based toxicity indices in risk assessment (Altenburger et al., 2019; De Baat et al., 2019), the consideration of the risk for benthic biota is still poorly represented in sediment quality assessment strategies and improvements could be implemented. Sediment toxicity testing with ecologically relevant species should become a crucial component for assessing the risks posed by contaminated sediments and for the development of sediment quality assessment strategies (OECD, 2007; Scrimshaw et al., 2007; De Baat et al., 2019) and dredged material regulations (Hansen et al., 2007; CIEM, 2015).

The most commonly used benthic organisms for bioassays to assess the toxicity of marine and estuarine sediments include amphipods (EPA, 1994; Casado-Martinez et al., 2006), *Arenicola marina* polychaetes (Bat, 1998; Thain and Bifield, 2001) and echinoids (Hansen et al., 2007), being each model representative of different forms of exposure (e.g. solid or liquid phase). With echinoids, the sea urchin embryo test (SET) has been worldwide used as a rapid, sensitive, and cost-effective biological tool for the evaluation of seawater quality (Beiras et al., 2001; Losso et al., 2007; Saco-Álvarez et al., 2010). The SET has been also described for sediment toxicity screenings by exposing embryos to elutriates coming from the sediment and using size increase as a quantitative endpoint (Beiras et al., 2012). In addition, developmental abnormalities can be measured in larvae after exposure to sediment elutriates, since a high sensitivity of skeletogenesis to specific contaminants has been demonstrated (Carballeira et al., 2012). Some relevant limitations of this bioassay are the unavailability of sea urchins gametes all year-round, particularly outside the natural spawning seasons, at least in temperate and/or cold environments, and the need of 48 h to perform the test. Consequently, the establishment of an appropriate and complementary model organism for a continuous assessment of sediment quality is recommended. Most of the endobenthic organisms and polychaete worms in particular, are in close contact with sediments and ingest subsurface sediments that could have potential toxicants bounded. Among them, the common ragworm *Hediste diversicolor*, a deposit-feeder found in both sandy and muddy sediments of the European and the North American coast of the Atlantic Ocean (Smith et al., 1996), tolerates a broad range of salinities and temperatures in estuaries (Scaps, 2002; Ashley and Budd, 2016). They are usually available throughout the year and play important roles in the food chain and consequently, in the sediment community organisation (Bat, 2005). The bioturbation due to their activity is an important determinant for sediment biogeochemistry and element cycling, and also for oxygen availability in burrow environments that may affect growth and population sizes of the associated meioorganisms (Palomo and Iribarne, 2000; Scaps, 2002). The easy sampling and maintenance under laboratory conditions of ragworms (tolerance to temperature, salinity, and oxygen levels), and their high degree of responsiveness (quick response to environmental stressors), make *H. diversicolor* particularly suitable for ecotoxicological studies, likely to uptake metals through metal-bound particles and from pore water (Ghribi et al., 2019; Silva et al., 2020). Hence, several works demonstrated *H. diversicolor* to be sensitive to common contaminants (Moreira et al., 2006; Bouraoui et al., 2016), but also to chemicals of emerging concern in the sediment or in the water column (Cong et al., 2014; Fonseca et al., 2017; Silva et al., 2020; Abouda et al., 2022). These characteristics make this a suitable species for biomonitoring campaigns (Durou et al., 2007), for acute and metal accumulation pattern bioassays (Burlinson and Lawrence, 2007; Cong et al., 2014) and for the application of multi-biomarker approaches (Buffet et al., 2013; Catalano et al., 2012; De Marchi et al., 2017; Fonseca et al., 2017; Freitas et al., 2017; Ghribi et al., 2019). The biomarkers were mainly associated with cytotoxicity, oxidative stress, changes in metabolism, lipid peroxidation and neuro and genotoxicity; all involved in the toxic response to common anthropogenic chemicals. Similarly, observation of histological alterations and/or damage of somatic tissues such as the digestive epithelia (tissue-level biomarkers) could be expected in *H. diversicolor* under pollution conditions. Detailed histological studies about the microanatomy of the digestive epithelia in polychaetes are scarce (Rodrigo et al., 2015) and, moreover, histopathological alterations have not been widely used as tissue-level biomarkers to assess biological effects of pollution like in other marine species (i.e. mussels; Garmendia et al., 2010).

A variety of standardized immunoassays have been developed with soil earthworms in order to assess the immunotoxic potential of toxicants based on cell-mediated and humoral responses (Hayashi and Engelmann, 2013; Irizar et al., 2014, 2015; Engelmann et al., 2016; Garcia-Velasco et al., 2019). Marine polychaetes possess similar immune mechanisms and cells (coelomocytes) to those of earthworms which could serve for the development of adequate and sensitive bioassays for toxicity screening of sediments (Cuvillier-Hot et al., 2014; Arredondo et al., 2020). In fact, in previous works with *H. diversicolor* cellular endpoints have been quantified in coelomocytes after *in vivo* exposure, with good results for environmental health assessment (Catalano et al., 2012; Cong et al., 2014; Fonseca et al., 2017, 2019). Moreover, the use of coelomocyte primary cultures would be a promising tool to determine sediment pollution effects in a rapid, simple and non-invasive form, since these cells, unlike sea urchin embryos, are available all along the year. The aim of this work was to develop quick, reliable and innovative

in vivo and *in vitro* sediment toxicity tests with ecologically relevant species such as *H. diversicolor* and its coelomocytes. Two model toxicants $CuCl_2$ and silver nanoparticles (AgNPs) were used as test substances, and the results of the bioassays were compared with the toxicity thresholds obtained by the standardized bioassay SET (Beiras et al., 2012) performed with species recommended in regulatory guidelines, the sea urchin *Paracentrotus lividus*. The improvement of ecologically relevant bioassays could contribute to render more robust sediment toxicity tests/protocols: combining multiple species to be developed all year round, varying exposure pathways and including outcomes (histopathology and cytotoxicity) complementary to acute endpoints (mortality, growth, behaviour).

2. Materials and methods

2.1. Test organisms

H. diversicolor polychaetes, purchased from the commercial dealer Cebocuc® (Galicia, Spain) were received in the laboratory one day after manual collections in the Galician coasts and readily placed in the acclimation tanks (30 L). The latter were filled with a layer of clean sediment (6 cm) collected in Plentzia (43°40′6.746″ N, 2°95′1.060″ W; Butroe estuary) and seawater directly taken from the Plentzia Bay, naturally filtered by sand in the uptake wells aided with a pump that sent the water to the marine station. The tanks had a constant seawater flow with a salinity range of 32–33‰ and constant aeration. The acclimation laboratory had a controlled temperature of 18 °C, with a photoperiodic system of 12/12 h of artificial light/darkness. Ragworms were kept in the acclimation tanks for 7 days prior to the bioassay, being fed with commercial fish food (Vipagram granular, 41.9% protein and 8.7% fat) twice a week.

Adult *P. lividus* were collected in a rocky shore in Armintza (43°43′3.625″ N, 2°89′9.262″ W; Bay of Biscay), in April 2019. The organisms were transported alive to the laboratory and were placed in aquaria with seawater at controlled temperature (16 ± 1 °C) for 2–3 weeks prior experimentation. Macroalgae were given weekly as food supply until spawning induction.

2.2. In vivo experiments with H. diversicolor

2.2.1. Sediment spiking procedure and characterization

Test sediments were collected in the same sampling point as those used for the acclimation tanks of polychaetes (Plentzia, Butroe estuary). According to previous metal characterization of sediments from Plentzia collected in September 2018 (Supplementary Material-Table S1) and in 2019 (Table 1), and to Blanco-Rayón et al. (2019), this site showed no significant metallic contamination and even showed reduced values for all the metal analysed between both years. A physico-chemical characterization, including oxidable organic matter (OM) content, texture (% of sand, silt and clay) and water holding capacity (WHC) was performed in CSR laboratories (Jaén, Spain). In addition, pH, electrical conductivity (EC) and salinity were also determined (Table 2).

Sediments were sampled and stored according to Thain and Bifield (2001) and King et al. (2004) with some modifications (sieved at 4 mm after air drying). Part of the dried sediments were artificially contaminated with copper (Cu) by spiking them with filtered seawater solutions containing Cu salts ($CuCl_2{\cdot}2H_2O$, Sigma Aldrich, prepared in volumetric flasks, 1 M HCl-washed glassware) in different quantities to obtain the desired concentrations (0, 50, 150 and 300 mg Cu/kg). Copper was selected as model contaminant and because is the reference toxicant in sea urchin embryo test. Copper concentrations were selected on the basis that 50 mg Cu/kg represent low pollution levels (Menchaca et al., 2012), and 150 mg Cu/Kg and 300 mg Cu/kg produced chronic and acute toxic effects, respectively (Blaise and Férard, 2013; Watson et al., 2018). Spiked sediments were thoroughly homogenized with a clean polyethylene spatula and were left to stabilize for 14 days, by mixing them every 2 days (King et al., 2004). Copper concentrations were measured in spiked sediments (58, 134, 235 mg/kg) and were similar to the nominal ones (50, 150, 300 mg/kg) (Table 1), indicating a proper spiking procedure and stabilization period.

Table 1
Metal characterization of sediments collected in Plentzia, showing metal concentrations of 20 elements in control and artificially polluted sediments with three $CuCl_2$ concentrations (50, 150 and 300 mg Cu/Kg).

Metal characterization (mg/kg)	Control (Plentzia)	50 mg Cu/kg	150 mg Cu/kg	300 mg Cu/kg
Cu	25.87 ± 3.07	57.77 ± 3.33	134.25 ± 5.88	235.93 ± 16.76
Li	16.00 ± 0.12	15.76 ± 0.72	16.87 ± 1.57	15.51 ± 2.7
Mo	1.28 ± 0.37	0.85 ± 0.04	1.60 ± 0.86	0.85 ± 0.22
Ag	0.18 ± 0.03	0.16 ± 0.01	0.18 ± 0.01	0.18 ± 0.02
Sn	4.28 ± 0.94	4.46 ± 0.95	4.06 ± 0.13	4.33 ± 3.53
Sb	0.36 ± 0.04	0.20 ± 0.12	0.40 ± 0.09	0.41 ± 0.04
Ba	83.2 ± 3.87	89.15 ± 8.41	94.24 ± 8.51	80.88 ± 6.57
W	0.25 ± 0.00	0.20 ± 0.02	0.35 ± 0.15	0.25 ± 0.18
Hg	0.46 ± 0.15	0.38 ± 0.04	0.41 ± 0.06	0.42 ± 0.05
Tl	0.17 ± 0.01	0.18 ± 0.01	0.17 ± 0.02	0.17 ± 0.02
Pb	29.90 ± 1.95	28.68 ± 0.77	30.01 ± 4.94	29.93 ± 0.80
Ti	104.12 ± 1.74	109.54 ± 8.20	116.20 ± 16.03	119.75 ± 7.69
Co	5.55 ± 1.74	6.37 ± 1.64	5.83 ± 0.33	5.74 ± 0.34
Zn	138.33 ± 9.52	134.77 ± 6.32	146.35 ± 6.2	142.77 ± 2.21
As	13.83 ± 0.44	16.45 ± 2.76	17.36 ± 0.07	14.35 ± 0.88
Cd	0.32 ± 0.10	0.41 ± 0.19	0.32 ± 0.09	0.35 ± 0.07
V	20.61 ± 0.28	20.72 ± 0.71	22.33 ± 0.90	20.23 ± 2.12
Cr	12.7 ± 0.21	12.89 ± 0.47	13.91 ± 0.65	12.78 ± 1.11
Ni	9.29 ± 0.11	10.43 ± 2.00	9.76 ± 0.47	9.54 ± 0.31
Se	0.62 ± 0.09	0.88 ± 0.10	0.89 ± 0.11	0.76 ± 0.08

2.2.2. Experimental set up and performance

The experimental procedure was designed following the guidelines outlined by Thain and Bifield (2001), with small modifications. Test tanks (5 L, polyethylene) were filled with 1 Kg of stabilized sediments (resulting in 3 cm layer) and 3.75 L of seawater (0.2 μm-filtered, 30 ‰-3.5 L seawater +250 mL distilled water-to obtain a final salinity of 32–33‰ after contact with sediment), reaching 12 cm in height and obtaining a sediment-overlying water depth ratio of 1:4. All test tanks were allowed to settle over a period of 24 h.

H. diversicolor polychaetes of similar weight (3.0–3.3 g) were weighted in pools of five individuals, and maintained in tanks with control (CT) and artificially polluted sediments with Cu, for 7 days. Three replicates were prepared for each treatment. Continuous airflow, 18 °C and photoperiod (12/12 h light: dark) conditions were kept during the experiment, being the physico-chemical parameters of the seawater (pH, temperature, salinity and dissolved oxygen-DO-) monitored with a multiparametric probe (YSI Professional Plus). Ragworms were not fed during the 7 days of exposure.

At the end of the experiment, sediment samples were collected to determine the concentration of 20 metals (Li, Mo, Ag, Sn, Sb, Ba, W, Hg,

Table 2
Physico-chemical characterization (oxidizable organic matter in %p/p, texture as % of sand, silt and clay, Water Holding Capacity-WHC in %p/p, pH, electrical conductivity in mS/cm and salinity in g NaCl/L) of the sediment used for experimentation.

SEDIMENT SITE	SAMPLING DATA	OXIDIZABLE O.M (%p/p)	TEXTURE				WHC (%p/p)	pH	EC (mS/cm)	SALINITY (g/L)
			Sand	Silt	Clay	USDA Soil Class.				
Plentzia	Sept 2018	3.07	53	35	12	Loamy-Sand	22.3	7.59	15.79	8.83

Tl, Pb, Ti, Co, Zn, As, Cd, V, Cr; Mn, Ni, Se) by ICP-MS (NexION 300, PerkinElmer). Briefly, about 0.5 g of each dried and sieved sample was subjected to an extraction procedure with 3.9 mL of nitric acid (69%, Tracepur, Merck), 6.2 mL of hydrochloric acid (37%, Tracepur, Merck) and 9.8 mL Milli-Q quality water (Millipore, Billerica, MA, USA). Ultrasound energy was applied for 6 min by means of a HD 2070 Sonopuls Ultrasonic Homogenizer (Bandelin, Germany) equipped with a 6 mm glass tip. The acid extract was filtered using a 0.45 μm PVDF filter and diluted in Milli-Q water. Different dilutions were prepared in order to match the element concentrations to the dynamic range of the instrument. In all the cases the concentration of HNO_3 in the dilutions was adjusted to 1%.

2.2.3. Organisms and tissue level endpoints

At the end of the experiment, polychaetes were removed from the tanks, mortality checked and pools of organisms were again weighted to determine weight loss. A midgut body portion (segments 20–25) of the polychaetes (n = 5) was dissected out, fixed in formalin (10% commercial Formaldehyde in Phosphate Buffer Saline-PBS-with 0.17 M NaCl, stored at 4 °C) and routinely processed for histology (Martoja and Martoja-Pierson, 1970). Histopathological alterations were addressed in transversal paraffin sections stained with Haematoxylin-Eosin, analysed under a light microscope (Nikon ECLIPSE Ni). Polychaetes collected from the acclimation tank at the beginning of the experiment (referred as CT0) and after 7 d in non-spiked sediments (CT7) were used as reference of the anatomical and physiological states of the purchased animals. In addition, metal accumulation in tissues was measured in 5 polychaetes of all treatments.

2.3. In vitro approaches with H. diversicolor coelomocytes

2.3.1. Coelomocyte extrusion and seeding in microplate

Coelomocytes were extruded together with coelomic fluid following a non-invasive procedure based on a previously developed method for earthworms (Irizar et al., 2014). Polychaetes were kept voiding their gut contents in constant seawater flow during 24 h and were thoroughly cleaned to remove any sediment particle attached to the body. Organisms were individually placed in glass petri dishes and coelomocytes were extruded by electric stimulation (9 V) using 0.35 M NaCl PBS, with 0.02% EDTA and 1% antibiotics (Antibiotic antimycotic solution (×100): 10,000 units penicillin, 10 mg streptomycin and 25 μg amphotericin B per mL). Extrusion solution was adjusted to the salinity of polychaetes coelomic fluid (0.17 M NaCl) taking into account the dilution factor after exposure in the microplate wells. The presence of gametes in the solution was checked under light microscope and only the cell solutions extruded from females (containing oocytes) were collected. In order to avoid the oocytes, the coelomocyte solution was filtered through a 40 μm strainer in a centrifugation step (500 rpm, 2 min). Cell solutions extruded from 8 to 10 females were pooled together, coelomocytes were counted with the aid of a Neubauer chamber and the cellular concentration adjusted to 5×10^5 cells/mL. Coelomocytes in coelomic fluid based medium were then seeded in microplates (100 μL, 5×10^4 cells/well) and were left stabilizing for 1 h at 18 °C before exposure. For each pollutant (Cu, AgNPs) and exposure time (2 and 24 h), three microplates were prepared (filled with cell solutions from different pools, Supplementary Material-Fig. S1).

2.3.2. Coelomocyte exposure and viability assays

H. diversicolor coelomocyte responses *in vitro* were obtained by exposing cells to a range of concentrations (0, 0.05, 1, 5, 10, 50 mg/L) of $CuCl_2$ and to AgNPs. AgNPs were PVP-PEI coated with a size of 5.08 ± 2.03 nm, and Z-potential of 18.6 mV. Exposures were performed by addition of 100 μL of exposure solution, filling eight wells per concentration. As control, 0.17 M NaCl PBS was used. After 2 and 24 h of exposure, coelomocyte viability (Calcein-AM) was assessed through a microplate assay. The schematic diagram of the experimental design followed for the *in vitro* exposure of *H. diversicolor* coelomocytes to Cu and AgNPs is illustrated in SM-Fig. S1.

The Calcein-AM Viability assay provides a simple, rapid, and accurate method to measure cell viability and cytotoxicity. The calcein acetoxymethyl ester (Calcein AM) permeates live cells and is hydrolyzed by intracellular esterase to calcein, a hydrophilic and strongly fluorescent compound that is well retained in the cell cytoplasm (Bratosin et al., 2005). The Calcein-AM viability test was performed according to Garcia-Velasco et al. (2019), with slight modifications (removal of washing steps). In half of the plates wells, 2 μl of Calcein-AM Working solution (2.5 μM Calcein/well) were added. In the remaining wells, 2 μl of PBS were added in order to overcome background noise (SM-Fig. S1). After 40 min of incubation (18 °C, darkness), fluorescence was read at 490/20 nm excitation, and 520/20 nm emission in Cytation 3 Cell Imaging multi-mode reader (BioTek Inc.). Supposing that the control group has the maximum calcein retention capacity (100%), the relative values were calculated. In addition, the median lethal concentration (LC_{50}) was calculated.

2.4. Sea urchin embryo test (SET): reference bioassay in sediment toxicology

In parallel, the SET (Beiras et al., 2012) was performed, exposing fertilized eggs of *P. lividus*, to the same reference toxicants at a range of exposure concentrations ($CuCl_2$: 0, 0.005, 0.05, 0.5, 5 mg Cu/L; AgNPs: 0, 0.005, 0.05, 0.5, 5, 50 mg/L). After 48 h, embryo size increase and developmental abnormalities were measured. Size increase consisted on the measurements of the maximum dimension of 50 embryos per vial, while malformations were recorded on 100 embryos/vial following Carballeira et al. (2012). A general index of toxicity (IT), that weight the degree of developmental abnormalities by the frequency, was then calculated for each treatment. With size increase results, the median effect concentration (EC_{50}) was calculated for each toxicant.

2.5. Statistical analysis

The statistical analysis of the data was carried out with the aid of the SPSS statistical package (IMB SPSS Statistics 23). Shapiro-Wilk ($n < 30$) and Levene's tests were performed to study normality and equality of variances of the datasets, respectively. Datasets were analysed with Kruskal-Wallis followed by Dunn's post-hoc test and significant differences were established at $p < 0.05$. In order to estimate the median lethal and effect concentration (LC_{50}, EC_{50}) the Probit model was used.

3. Results

3.1. In vivo experiments with H. diversicolor

3.1.1. Sediment characterization

The sediments collected in Plentzia and used for the *in vivo* exposure of polychaetes were classified as a loamy to sand matrix (USDA, 1999), presenting 3.07% organic matter and a water holding capacity of 22.3% (Table 2). The concentrations of metals measured in these sediments (Table 1) were low and similar to those measured previously in sediments collected in September 2018 (SM-Table S1), reinforcing this site as a clean site for metallic contamination (Legorburu et al., 2013; Blanco-Rayón et al., 2019). Concerning the sediments from Plentzia spiked with Cu, the real exposure concentrations were linearly correlated with the nominal ones. Hence, at the end of the exposure a nominal concentration of 50 mg Cu/kg resulted in 58 mg/kg, 150 mg Cu/kg in 134 mg/kg and 300 mg Cu/kg resulted in 236 mg/kg (Table 1).

3.1.2. Organism level effects

In vivo exposure of *H. diversicolor* to Cu caused increased mortality (around 30%) after exposure to 150 mg Cu/Kg, and a significant dose-response weight loss compared with the control group, with a maximum (>30%) for the highest dose (300 mg Cu/Kg, Fig. 1).

Net metal accumulation did not occur in control polychaetes (CT7) maintained in sediments of Plentzia compared with CT0. In spiked sediments, *H. diversicolor* accumulated mainly Cu, Cd and Ag in tissues (Fig. 2). The concentration of Cu measured in the organisms maintained in 50 mg Cu/Kg treatment were similar ($p > 0.05$) to CT0 values (14.94 ± 3.90 μg/g and 8.25 ± 1.42 μg/g, respectively). After exposure to 150 and 300 mg Cu/kg, higher Cu concentrations were measured, 48.65 μg/g and 51 ± 8 μg/g, respectively (Fig. 2 A). Enrichments of Cd and Sn occurred in ragworms maintained in the highest concentration of Cu (Fig. 2 B and D), while highest Ag accumulations were recorded in 50 and 300 mg Cu/Kg treatments (Fig. 2 C).

3.1.3. Effects at tissue-level

Control polychaetes (CT7) exhibited a normal body wall structure (tegument, epidermis) and digestive epithelium integrity after 7 days of experiment (Fig. 3A). Exposure to the highest Cu concentrations (150 and 300 mg Cu/Kg) showed clear blood accumulation in the intestinal areas when compared to CT0 and control (CT7) polychaetes (Fig. 3). In the case of polychaetes exposed to 300 mg Cu/Kg, conspicuous brown deposits were clearly observed in digestive epithelia (Fig. 3D). Parasites (spores of a Mesomycetozoea parasite, unicellular amoeba-like protists; Fig. 3D) were observed in the digestive epithelium of ragworms irrespective of treatments. The infection with parasites does not seem to be serious for the animals since no inflammation signs were recorded.

3.2. In vitro approaches with H. diversicolor coelomocytes

The Calcein-AM viability assay performed in coelomocytes extruded from *H. diversicolor* and exposed to $CuCl_2$ and AgNPs showed a significant dose-response decrease in calcein retention (Fig. 4). Cells exposed to longer time (24 h) showed less retention capacity than after 2 h of exposure to Cu and AgNPs (Fig. 4). Coelomocytes viability significantly decreased from 5 to 0.5 mg/L onwards for Cu (Fig. 4A) and AgNPs (Fig. 4B), respectively. The calculated LC_{50} values after Cu exposure were 13.4 (2 h) and 4.2 mg/L (24 h), and for AgNPs 2.2 (2 h) and 0.43 mg/L (24 h).

3.3. Sea urchin embryo test (SET)

The SET showed that the exposure to $CuCl_2$ caused decrease in embryo size, being significant after exposure to 5 mg Cu/L (Fig. 5A). After exposure to AgNPs, embryos showed more marked effects at lower concentrations, with a significant decrease in size starting at 0.05 mg/L (Fig. 5B). The EC_{50} values for size impairments were established at 0.38 mg/L and 0.086 mg/L for Cu and AgNPs, respectively. The values of the Index of toxicity (IT), were higher after exposure to the highest concentrations of Cu (5 mg Cu/Kg) and from 0.5 onwards in the case of exposure to AgNPs (Fig. 5C and D).

4. Discussion

In the last 15 years an exponential growth in the understanding of contaminants in estuarine sediments has occurred. Accordingly, sediment quality guidelines (SQG) have recently been introduced in different countries and regions although the application of these guidelines is not based on a scientific consensus. SQG can be understood as trigger values or threshold concentrations (Chapman and Anderson, 2005). Below this threshold the possibility to produce deleterious biological effects is very low. Moreover, Chapman and Anderson (2005)

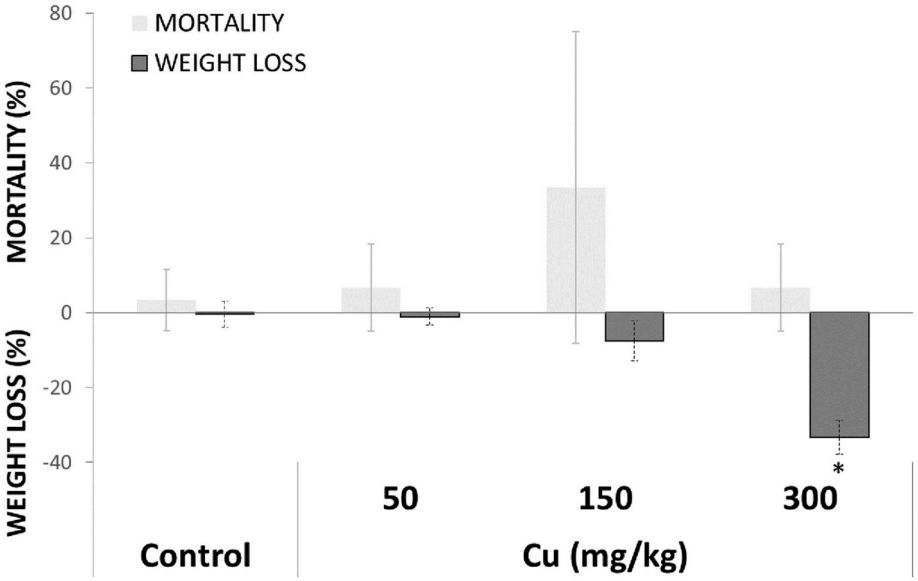

Fig. 1. Mortality and weight loss (in %) of *H. diversicolor* after *in vivo* exposure to control and Cu spiked (50, 150 and 300 mg/kg) sediments. Values are represented as average ± standard deviations and significant differences ($p < 0.05$ with Kruskal-Wallis) between treatments are represented by asterisk.

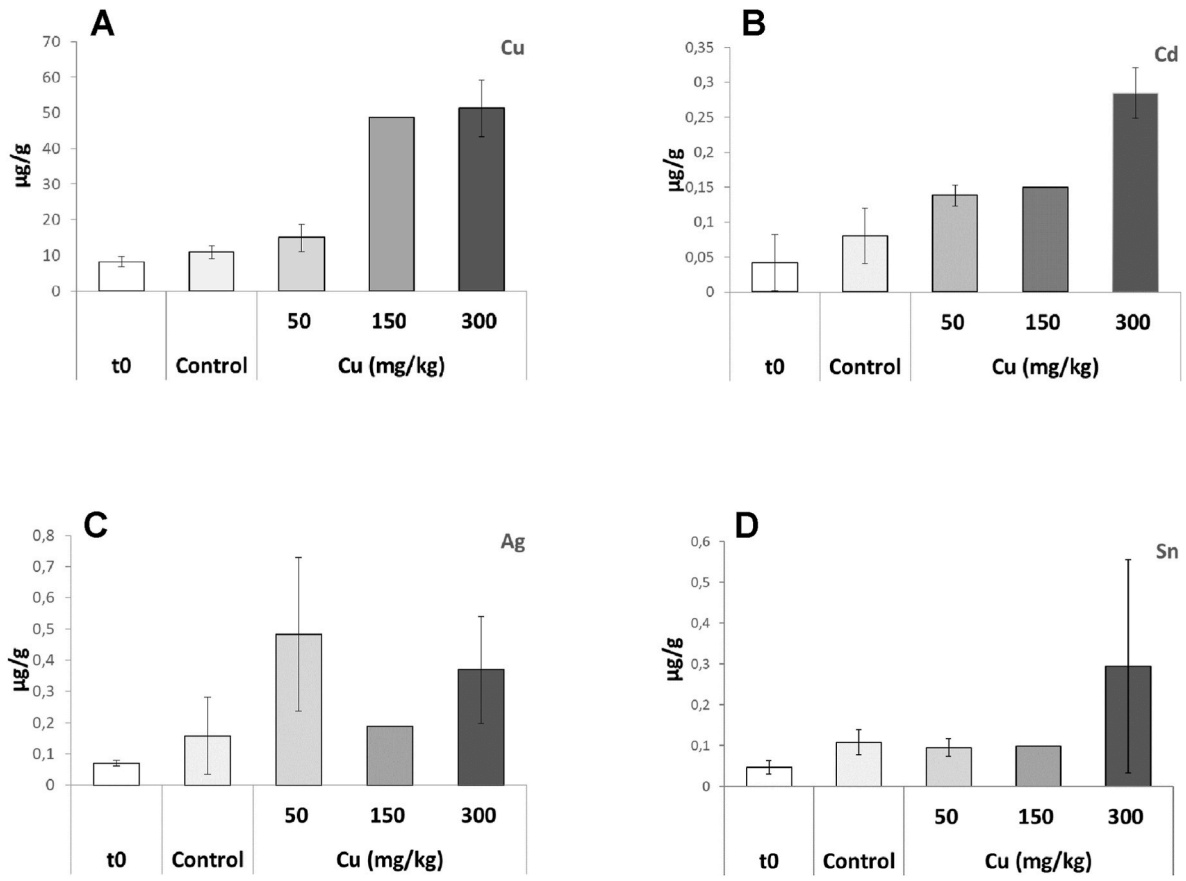

Fig. 2. Metal accumulation (Cu, Cd, Ag, Sn) in tissues of *H. diversicolor* after 7 d *in vivo* exposure to $CuCl_2$ (50, 150 and 300 mg Cu/Kg) spiked in the sediments. Values are represented as average ± standard deviations.

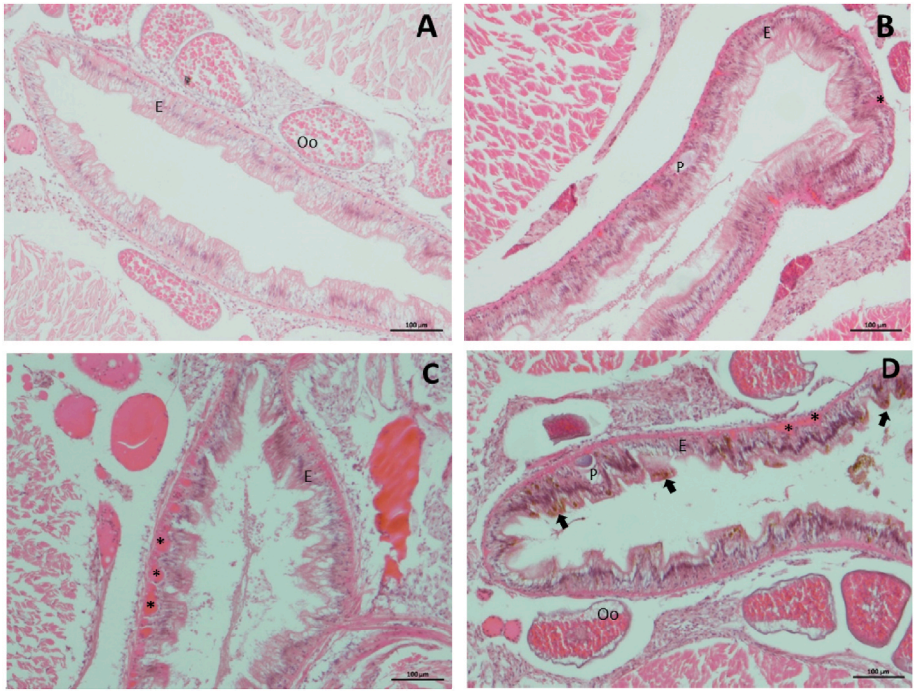

Fig. 3. Digestive tract in transversal sections of *H. diverscolor* at the beginning of the experiment (t0) (A), after 7 days in control sediment (B), and after 7 d exposure to 150 mg Cu/Kg (C) and 300 mg Cu/Kg (D). Blood irrigation is labelled with an asterisk (*) and brown deposits with an arrow. Epithelium (E), Oocytes (Oo), Parasites (P). Scale bar: 100 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

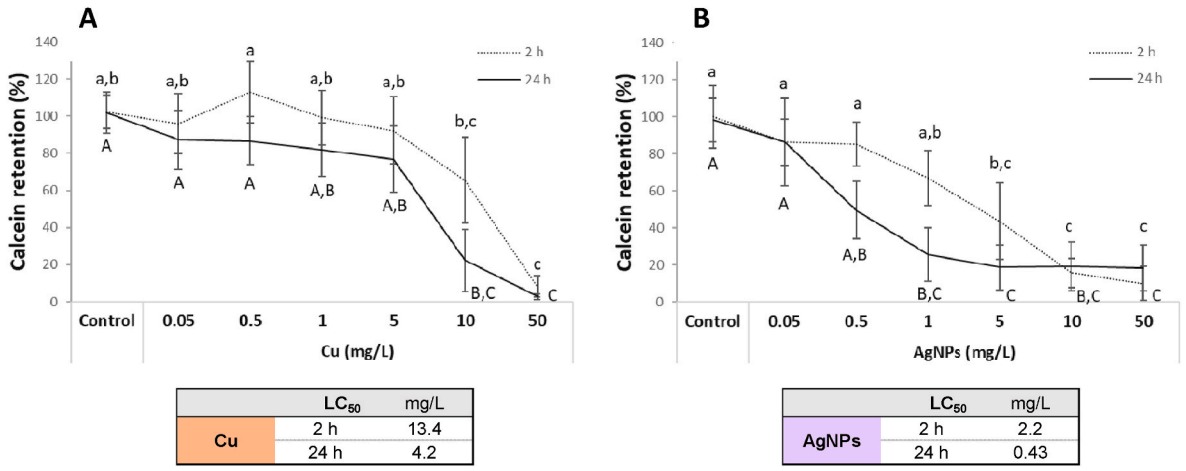

Fig. 4. Cell viability (Calcein AM viability assay, represented by calcein retention in % relative to the control) in primary cultures of *H. diversicolor* coelomocytes exposed to $CuCl_2$ (A) and AgNPs (B) (n = 3). Values are represented as means ± standard deviations and the significant differences ($p \leq 0.05$ with Kruskal-Wallis) are represented by letters (a,b,c for 2 h and A,B,C for 24 h).

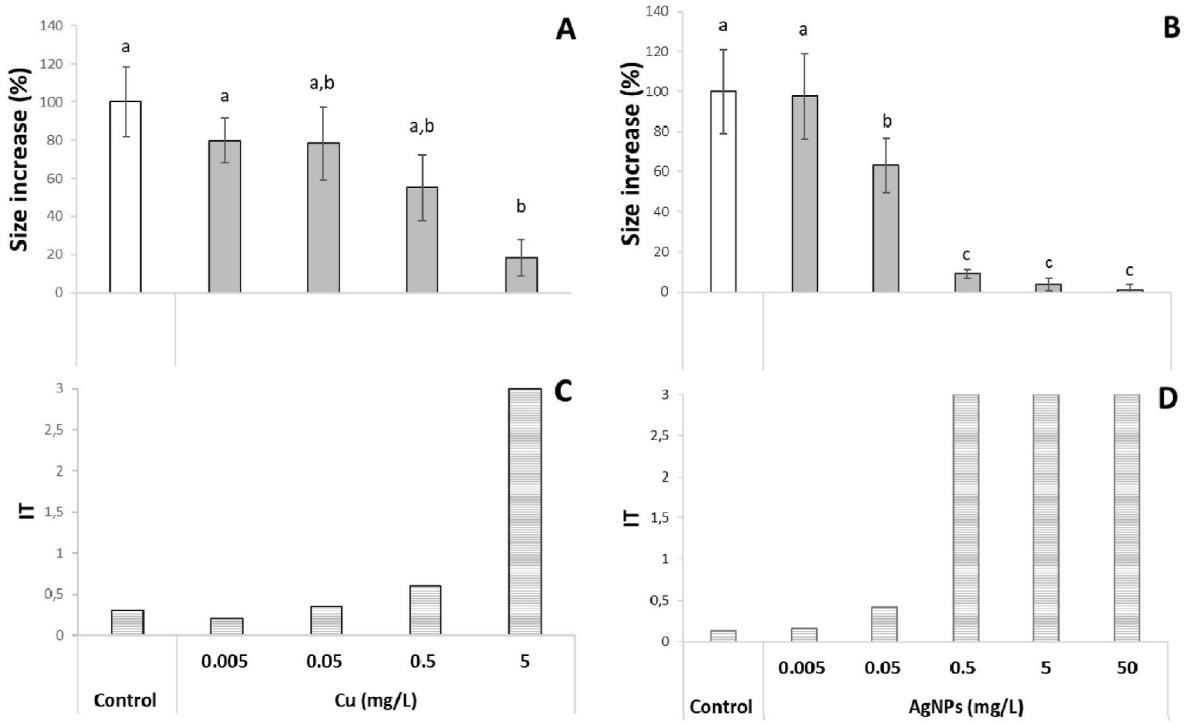

Fig. 5. Size increase (% to control; A, B) and the index of toxicity (IT; C, D) calculated from the frequency of malformations detected in embryos exposed to Cu (A, C) and AgNPs (B, D). Significant differences ($p \leq 0.05$ with Kruskal-Wallis) are represented by letters (a,b,c).

suggested that investigations should combine sediment chemistry (including contaminant bioavailability and bioaccumulation), benthic ecology and toxicity testing. All this information will offer a rapid and accurate tool in order to perform a proper management of polluted sediments.

The world-wide used SET test provides a general view of the toxicity exerted by chemicals bioavailable in seawater by quantifying (in terms of pluteus larvae size) the degree of developmental completion achieved by the embryos (Beiras et al., 2012). This bioassay endpoint is a quantitative, observer-independent, automatically readable response, and have been standardized and amended under several international guidelines and directives (ASTM, 1990; USEPA, 1995; Environment Canada, 1997; CETESB, 1999; ASTM, 2002). Presently, SET was highly sensitive to determine the toxicity thresholds for $CuCl_2$ and AgNPs after 48 h of exposure. In the case of Cu exposure, the EC_{50} (for the size increase endpoint) was estimated in 0.38 mg/L. This value is very similar to the normalized SQGs calculated for the Basque coast using three toxicity tests including SET, AMBI and Microtox that was 0.55 mg Cu/L (Menchaca et al., 2012). In the same line, Chapman et al. (1999) and Choueri et al. (2009) proposed that SQG should be used in the region where they were developed to better predict the toxicity of contaminants for each specific coastal environment. The coincidence of this calculation suggest that this value is the reference threshold for Cu in the Basque Coast. The case of AgNPs is not so clear due to the lack of works regarding toxicity thresholds calculated on the basis of toxicity tests. Presently, the EC_{50} for AgNPs was estimated in 0.086 mg/L, much lower that for $CuCl_2$, suggesting that the SQGs should be more restrictive for this contaminant. In conclusion, the sensitivity of early developmental

stages of sea urchin allows to evaluate the potential effects of Cu and AgNPs that may potentially become available to the water column (or release to the water column due to sediment resuspension) and to establish toxicity thresholds for both pollutants.

One of weaknesses, when planning toxicity testing with sea urchins in the framework of biomonitoring programs, is the lack of continuous availability of suitable biological material (gametes) outside the natural spawning season of the species. Therefore, the development of bioassays with species that could complement the SET in seasons outside the spawning period would be very welcome. One recommendation is the use of epi-/benthic test species (ECHA, 2014), which are in direct contact with the sediment and pore water, with a broad geographical distribution, compatible with selected exposure methods and endpoints and have a toxicological database demonstrating sensitivity to a range of contaminants (Simpson et al., 2005). Endobenthic aquatic polychaetes such as *H. diversicolor* fulfil above mentioned requirements. Hence, *in vivo* and *in vitro* assays have been developed with polychaetes exposed to reference toxicants ($CuCl_2$, AgNPs), being the suitability of the assays validated through comparison with the SET reference bioassay (Beiras et al., 2012).

After the *in vivo* bioassay, in which *H. diversicolor* were exposed during 7 d to loamy sediments spiked with different Cu concentrations, they accumulated Cu in tissues following a dose-dependent pattern. The massive accumulation was related with mortality rates in 150 mg/kg exposure and weight losses recorded for doses ranging 150–300 mg Cu/Kg, suggesting the onset of acute toxicity responses. Weight losses have been previously related with induced dehydration of specimens as a result of enhanced mucus generation and excretion (exocytosis) from the tegument to form a protective layer over its surface under stress conditions (Berthet et al., 2003; Murray et al., 2012). Conversely, lower Cu concentrations (50 mg Cu/Kg) did not produce any effect (mortality or weight loss). In contrast, exposure of other polychaete species such as *A. marina* to doses slightly higher than 14 mg Cu/Kg induced lethality (LC_{50} = 20 mg/kg; Bat and Raffaelli, 1998). Moreover, Cu spiked in the water column induced mortality in *Nereis* at concentrations higher than 2.3 mg Cu/L (Bryan, 1976). This might suggest different sensitivity of polychaetes to pollutant insult depending on their feeding habits (macrophagic feeders-*Arenicola*-vs. predators –*Nereis*-), or the phase in which the pollutant is spiked (sediment vs. water column).

Under some specific conditions competitive displacement reactions occurred after the addition of metals, such as Cu and Cd in estuarine sediments (Simpson et al., 2000). Interestingly, Cu exposure (300 mg Cu/kg) produced a concomitant increase in Cd and Sn levels although the levels reached were very low. Simpson et al. (2000) suggested that in the Cu-spiked sediments, Cd (present in the original sediment) was displaced into the pore waters becoming more available for polychaetes. However, it cannot be discarded that the burrowing or bioturbation activity of polychaetes can increase the release of contaminants in sediments to the water column (Scaps, 2002; Martinez-Garcia et al., 2015). The extent to which metal additions affect sediment pH and redox conditions, and disrupt the equilibrium partitioning of sediment constituents, is poorly understood and deserves further investigation (Luoma and Bryan, 1982; Simpson et al., 2000).

Assessment of the histopathological alterations produced in target organs of polychaetes after exposure to pollutants has been previously reported although the amount of research in this field is still very low (Geracitano et al., 2004; Murray et al., 2012). Anatomically the polychaete possess a large central digestive epithelium surrounded by the coelom cavity filled with gametes and coelomocytes, the nerve cord, the dorsal and ventral vessels (Rodrigo et al., 2015). After exposure to Cu (high dose), an increased blood irrigation close to the digestive tract and massive accumulation of brown deposits (probably lipofuscin granules) were observed in digestive epithelia. These deposits are membrane-bound structures that can contain by-products of the digestive process that are released to the lumen of digestive tract at more advanced stages of digestion (Costa et al., 2014). Histochemical examinations of *H. diversicolor* after exposure to acetylsalicylic acid have shown important increase of mucous cells in the tegument (Gomes et al., 2019), but up to now, these kind of histopathological alterations have not been recorded in the digestive system of polychaetes. These alterations may indicate that changes at tissue level might occur under environmental stress situations and deserves more attention for future sediment toxicological works.

The presence of parasites is indicative of a depressed immunological system due to a variety of environmental factors including pollutants in aquatic animals (Benito et al., 2019). Parasites were observed, mainly in the digestive epithelium of the gut, although they were present in the majority of individuals regardless the treatment. There is a lack of literature on polychaetes parasites, however, according to their size, general aspect (unicellular amoeba-like protists) and smooth capsule, the parasites found in polychaetes seem like the active spores of a Mesomycetozoea, according to Dr. Pedro Costa (Nova Univ. Lisbon, pers. comm).

The use of primary cultures of immune cells of invertebrates to investigate the impact of pollutants in the environment have been recently outlined with promising results. In fact, annelids (oligo and polychaetes) have developed cellular immunity (phagocytosis, encapsulation against parasites) and possess humoral components of immunity which are essentially represented by antibacterial proteins (Dhainaut and Scaps, 2001; Catalano et al., 2012; Hayashi and Engelmann, 2013; Cong et al., 2014; Cuvillier-Hot et al., 2014; Irizar et al., 2015; Garcia-Velasco et al., 2019). Presently, *in vitro* approaches were optimized for marine worms (polychaetes *H. diversicolor*) based on a previously developed method for earthworms (Irizar et al., 2014). The modifications in the protocol included the use of coelomic based medium (with PBS adjusted to polychaetes coelom osmolarity) to maintain the cells in culture, the shortening of the exposure to 2 and 24 h (avoiding medium replacements) and the removal of washing steps when performing the Calcein-AM viability test. *In vitro* exposure of coelomocytes to a series of Cu concentrations produced a significant dose-response decrease in the viability of cells. Likely, chronic copper exposure impaired the immunological response of the polychaete *Eurythoe complanata* with changes in coelomocytes viability, number and phagocytosis capacity (Nusetti et al., 1998). Exposure to AgNPs exerted more toxic effects than Cu according to LC_{50} values after 2 and 24 h. Similarly, exposure to AgNPs caused cytotoxicity in coelomocytes of *Eisenia fetida* being EC_{50} 6 mg AgNP/L (Garcia-Velasco et al., 2019). Additionally, these authors found a selective toxicity in amoebocytes more than in eleocytes (subpopulations of coelomocytes), that was the opposite for non-particulated contaminants (ionic Cu) in which the target were the eleocytes (Irizar et al., 2015). More work is needed to assess the differential role in metal handling regarding the two subpopulation of coelomocytes in polychaetes, although it can be concluded the capacity of the test to predict impairments caused by pollutants (toxicity thresholds) and provide rapid and valuable information for (cyto)toxicology.

The sensitivity of this innovative *in vitro* bioassay based in coelomocyte viability reflected the same response pattern obtained using the SET with similar sensitivity. That is, Cu exposure exerted a significant toxicity effect on cell viability already after exposure to 10 mg Cu/L, while SET showed toxicity between 5 and 10 mg Cu/L. The response of coelomocytes could be envisaged after 2 h (with similar results after 24 h) while SET was showing toxicity after the 48 h of duration of the test. The results obtained after exposure to AgNPs were similar but with more power of discrimination for SET vs. coelomocyte culturing: 0.5 mg AgNP/L vs. 1–5 mg AgNP/L, respectively. Maybe, dissimilar dissolution rates of AgNPs in seawater media and coelomic fluid based culture media (10‰ salinity) could be responsible of this slight discrepancy between methods. Nevertheless, the values of the IT, calculated from the frequency of malformations detected in exposed sea urchin embryos, were in line with the responses obtained for coelomocytes. Hence, it can be concluded that the sensitivity of the size endpoint in SET is slightly

higher although coelomocyte bioassay showed faster responses that can be used for a quick screening of toxicity. Furthermore, primary cultures of coelomocytes may render high-throughput amount of data in a short time when cells are seeded and exposed in wells and read in a microplate. Finally, it is suggested that this toxicity screening could be applied not only to establish the toxicity thresholds of individual compounds or mixtures, but also to assess the toxicity of elutriates coming from field collected sediments.

4. Conclusions

The bioassays developed with *Hediste diversicolor* (*in vivo*) and their coelomocytes (*in vitro*) are useful tools to assess the effects of pollutants that can be bounded to sediments or resuspended into water. The toxicity thresholds obtained with both assays were in line with the ones obtained with the sea urchin embryo test (SET), concluding that their sensitivity is similar. The optimization of the methodology for primary cultures of *H. diversicolor* coelomocytes (non-invasive extrusion method, use of coelomic fluid based medium, viability assay with removal of washing steps) can be of great importance for sediment toxicity assessment since it allows performing rapid and simultaneous screening of toxicants (including individual compounds or mixtures and elutriates of real sediments). *In vivo* and *in vitro* bioassays with polychaetes and their coelomocytes may be cost-effective tools for toxicity screening and may complement the SET outside the spawning period of the echinoderms when gametes are not available to perform the test.

Author contributions statement

Garcia-Velasco, Nerea: Investigation (samplings, *in vivo* and *in vitro* experiment design and development), Methodology (optimization of *in vitro* method with coelomocytes), Data acquisition and curation (graphs, tables and statistics), Writing (original draft, reviews and editing). **Carrero, Jose Antonio:** Investigation (chemical sample processing and analysis), Methodology, Data curation (tables), Writing (review and editing). **Urionabarrenetxea, Erik:** Investigation (samplings, support for *in vitro* experiment), Methodology (support in the optimization of *in vitro* method with coelomocytes), Writing (review). **Doni, Lucia:** Investigation (samplings, support for *in vivo* experiment), Writing (support for the original draft). **Zaldibar, Beñat:** Data curation, Supervision, Writing (review and editing), **Izagirre, Urtzi:** Investigation (breeding of laboratory animals, experimental design in aquaria), Data curation, Supervision, Writing (review and editing). **Soto, Manu:** Funding acquisition, Project administration, Resources, Supervision, Data curation, Writing (original draft, reviews and editing).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2022.136935.

Table S1
Initial metal characterization of the sediments collected in Plentzia in September 2018.

SEDIMENT METAL CHARACTERIZATION (mg/kg)	Cu	Li	Mo	Ag	Sn	Sb	Ba	W	Hg	Tl
	32.34 ± 3.92	21.07 ± 0.12	1.52 ± 0.18	0.17 ± 0.01	5.35 ± 0.44	0.82 ± 0.06	111.62 ± 15.15	0.19 ± 0.03	0.53 ± 0.04	0.27 ± 0.02
	Pb	Ti	Co	Zn	As	Cd	V	Cr	Ni	Se
	42.59 ± 5.24	345.59 ± 25.84	16.31 ± 0.86	243.45 ± 32.32	19.32 ± 3.00	0.36 ± 0.00	57.02 ± 1.99	38.29 ± 3.64	24.83 ± 2.38	3.67 ± 0.23

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