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Echinoderm larvae as bioindicators for the assessment of marine pollution: Sea urchin and sea cucumber responsiveness and future perspectives☆

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ABSTRACT

Echinoderms play a crucial role in the functioning of marine ecosystems and due to their extensive distribution, rapid response, and the high sensitivity of their planktonic larvae to a large range of stressors, some species are widely used as biological indicators. In addition to sea urchins, sea cucumbers have recently been implemented in embryotoxicity bioassays showing high potential in ecotoxicological studies. However, the use of this species is still hindered by a lack of knowledge regarding their comparative responsiveness. The present study aimed to investigate the responsiveness of different echinoderm species to environmental pollution in order to develop their integration in batteries of ecotoxicological bioassays. To this end, the embryos of two sea urchins (*Paracentrotus lividus* and *Arbacia lixula*) and two sea cucumbers (*Holothuria polii* and *Holothuria tubulosa*) were incubated with inorganic and organic toxicants (cadmium, copper, mercury, lead, sodium dodecyl sulphate and 4-n-Nonhyphenol) and elutriates from contaminated marine sediments, chosen as a case study model. The results obtained, expressed through the percentage of abnormal embryos and Integrative Toxicity Indices (ITI), indicated species-specific sensitivities to pollutants, with comparable and correlated responsiveness between sea urchins and sea cucumbers. More specifically, sea cucumber larvae exposed to elutriates appear to be more sensitive than sea urchins, especially when incubated with samples containing trace metals, PCB and TBT. These results indicate that toxic responses in embryos exposed to environmental matrices are probably modulated by interactions between different variables, including additive, synergistic and antagonistic effects. These findings suggest that performing a larval test using different echinoderm classes can integrate the interactive effects of bioavailable fraction of contaminants on various levels, providing sensitive, representative and all year-round batteries of bioassays to apply in ecotoxicological studies.

1. Introduction

Over the last few decades, the effects of industrialization, intensive agriculture and urban development have seriously impacted the quality of the marine environment (Bellas et al., 2005). About 90% of pollutants produced are transported by rivers to the sea, adding to direct releases associated to 70–80% of the word population living in coastal areas (Parra-Luna et al., 2020; Ventura et al., 2023). Sediments act both as a sink and a source of pollution, especially in harbour areas and coastal zones that are highly impacted by human activities (Ruocco et al., 2020). The resuspension of these sediments by storms or other anthropic perturbative events such as fishing and dredging operations, can promote the re-mobilization and bioavailability of toxic chemicals (Chiarore et al., 2020; Liberti et al., 2020), representing a potential hazard to local assemblages and populations. Marine invertebrates play a crucial role in the functioning of marine ecosystems and certain species have been selected as models for ecotoxicological investigations due to their important ecological role, known biology, and ease with which they can be collected and tested in laboratory conditions (Reguera et al., 2018). Echinoderms, in particular, are frequently chosen as biological

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Fig. 1. Piombino harbour area with sediment sampling sites for elutriate preparation.

indicators due to their extensive distribution, meroplanktonic development, rapid response and the high sensitivity of their planktonic larvae to a large range of contaminates (Sugni et al., 2007). Among echinoderms, sea urchins are a keystone species in hard bottom habitats, controlling the vegetal community dynamics (Grosso et al., 2021, 2022), and worldwide are considered an ideal tool for marine eco-toxicological tests since their embryos are sensitive to the adverse effects of a huge range of contaminates, including trace metals, organic compounds, microplastics, bioplastics, nanoparticles, pharmaceuticals, sunscreen products, in addition to complex environmental matrices such as marine waters and sediments (see Pagano et al., 2017a, 2017b; Gambardella et al., 2021 for a review). Various methodologies were used in the research, exposing sea urchin embryos to increasingly high concentrations of single contaminants in dose-response experiments, as well as a number of environmental stressors, to evaluate both developmental anomalies and biomarker response at molecular level (Chiarelli et al., 2021; Gambardella et al., 2021). Other studies used an ecotoxicological approach to perform embryo bioassays in an environmental risk assessment context (Broccoli et al., 2021). Both forms of investigation generally took into account anomalies in sea urchin embryo development, reporting the percentage of abnormally developed embryos. Some recent studies have developed new analytical indexes which also take in consideration the severity of such abnormalities (Morroni et al., 2016; Corinaldesi et al., 2017; Bonaventura et al., 2021) by using a selective criterion, such as detailed skeleton malformation (Carballeira et al., 2012) or through morphometrics criteria (Bertucci and Bellas, 2021). Sea cucumbers have also been proposed recently for embryo toxicity bioassays (Morroni et al., 2020b; Rakaj et al., 2021), showing their suitability as a new test species. Sea cucumbers are an important component of soft benthic communities, which play a key role in controlling organic matter dynamics and detrital pathways in marine sediments (Purcell et al., 2016; Rakaj et al., 2018, 2019; 2023; Boncagni et al., 2019; Grosso et al., 2023; Pensa et al., 2022). However, compared to sea urchins, our knowledge on the sensitivity of sea cucumbers to pollutants and environmental matrices is still in its infancy. Such information would be of a crucial importance to include echinoderms in batteries of ecotoxicological bioassays, using different species able to detect the full range of potential pollutants. Batteries of ecotoxicological bioassays are based on the contemporary use of species with different trophic levels, biological end-points and sensitivity, and their integration in a weight of evidence approach (WOE) has been validated in several case studies for environmental risk assessment associated with polluted sediments, harbour areas, or complex natural and anthropic impacts on the marine environment (Piva et al., 2011; Regoli et al., 2019, Morroni et al., 2020a).

The aim of the present study was to investigate the sensitivity of different echinoderm species (sea urchins and sea cucumbers) to environmental pollution, evaluating also their integrative use in batteries of ecotoxicological bioassays. To achieve this goal, the embryo-larval responsiveness to pollutants and environmental matrices were evaluated in two sea urchin species (*Paracentrotus lividus* and *Arbacia lixula*) and two sea cucumber species (*Holothuria polii* and *Holothuria tubulosa)*. Cadmium (Cd^{2+}), copper (Cu^{2+}), mercury (Hg^{2+}), lead (Pb^{2+}), sodium dodecyl sulphate (SDS) and 4-n-Nonhyphenol (NP) were selected as reference toxicants. Furthermore, in this study, embryo tests were carried out with real environmental samples using the elutriates of contaminated marine sediments from Piombino Harbour (Italy). Selected endpoints, including the percentage of abnormal embryos and the specific Integrative Toxicity Indices (ITI) and the association of different scores to various developmental anomalies (Morroni et al., 2016, 2020; Bonaventura et al., 2021; Rakaj et al., 2021), were used to assess the responsiveness of the assays.

2. Material and methods

2.1. Single contaminants – preparation of test solutions

Cadmium chloride, copper nitrate, lead nitrate, mercury chloride, Sodium Dodecyl Sulphate (SDS) and 4-n-Nonylphenol (NP) were used as reference toxicants (Sigma Aldrich srl, Milan, Italy - https://www.sigmaaldrich.com/IT/it). Stock solutions were prepared by dissolving reagent grade in bidistilled water (BDW) to obtain a concentration of 1000 mg/L and test solutions were obtained through dilutions in filtered seawater (FSW).

2.2. Sampling of marine sediments and preparation of elutriate solutions

Sediments were collected in 15 stations in Piombino harbour (Tyrrhenian coast, Italy), which was chosen as a case-study model (Fig. 1). This harbour is characterized by the presence of one of the largest European industrial metallurgy and steel complexes, carbon coke production, a thermoelectric energy plant and intensive maritime traffic related to industrial activities, commercial trade and tourism (Bocchetti et al., 2008). Previous investigations revealed the presence of pollutants deriving from steel processing, such as PAHs and trace metals (Valentina et al., 2021).

Three replicates of sediment samples were collected in each station with a Van Veen grab of 0.1 m^3 and stored at 4 °C. A total of 1 L of sediment was taken from each replicate (3 L for each sampling site). Within 10 days the elutriates were prepared according to USEPA (1991) guidelines and literature studies (Volpi Ghirardini et al., 2005). Sediment samples were mixed in a 1:4 (v/v) ratio of sediment to 0.45 filtered sea water (FSW) and placed on a rotary shaker table for 1 h, at a speed of 300 rpm, at room temperature. The dilutions were made up with FSW collected in a long-term monitored reference site located far from human activities. After mixing, the samples were centrifuged (Thermo Scientific SL 16 R, Rodano, Italy) for 20 min at 3000 rpm (4 °C) and the aqueous fractions (elutriate samples) were poured off and stored for 30 days at −20 °C until used for the toxicity tests.

2.3. Sea urchin embryo test

Adults of *P. lividus* and *A. lixula* were collected at Santa Marinella in the central Tyrrhenian Sea, Italy (42°3′0″N, 11°49′9″E). After collection, the sea urchins were transported in insulated containers to the Laboratory of Experimental Ecology and Aquaculture (University of Rome Tor Vergata) and acclimatized for up to one week in flowing seawater at a temperature of 15 °C ± 1, salinity 35 and natural photoperiod (12:12 h). Embryotoxicity tests were performed in accordance with Morroni et al. (2018). Three males and three females were induced to spawn by injecting 1 ml of 0.5 M KCl into the sea urchin body cavity through the peristomial membrane around the mouth. Eggs were collected by placing spawning females on 100 ml beakers with 0.45 μm filtered seawater (FSW) which had been collected from the same site as the sea urchins. Females with eggs that were not round, immature forms or debris were discarded. Sperm was collected "dry" from the gonopores of male sea urchins and was kept on melting ice until use (<1 h). After checking sperm motility and the success of fertilization (>95%), 50 μl of sperms were diluted in 5 ml of FSW and added to 350 ml of egg suspension (1000 eggs/ml), sperm/egg ratio 50:1 (Morroni et al., 2018). After fertilization, a period of 20 min was allowed to pass before starting the incubations with test solutions in 10 ml sterile capped polystyrene six-well micro-plates (1 ml per well, corresponding to a final density about 100 embryos/ml), at a temperature of 20 °C in a dark room for 48 h. Control embryos were exposed to FSW only. At the end of the experiment, samples were preserved by adding 100 μl of 40% buffered formalin and morphological evaluation was performed. Finally, embryo morphology was evaluated under an inverted microscope. Tests were accepted if the percentage of control embryos (negative control) was 70% (Morroni et al., 2016). The toxicity results of Cu were used as positive controls, and tests were accepted if they fell within the laboratory acceptability ranges (Morroni et al., 2020a; Rakaj et al., 2021).

2.4. Sea cucumber embryo test

Adults of *H. polii* and *H. tubulosa* were collected at Santa Marinella in the central Tyrrhenian Sea, Italy (42°3′0″N, 11°49′9″E). After collection, the sea cucumbers were transported in insulated containers to the laboratory and acclimatized inside 30 L tanks equipped with aerators and two ice packs of 0.5 L (to maintain the temperature below 28 °C) and acclimatized for up to 1 week in a recirculating aquaculture system (RAS) at 24 °C. Embryotoxicity tests were performed in accordance with Morroni et al. (2020) and Rakaj et al. (2021). Before spawning induction, the adults were kept in substrate-free aquaria for 48 h in order to void their gut contents. After this period, spawning was induced using a thermal shock method, and the gametes from at least 3 males and 3 females were collected. To perform this operation, the sea cucumbers were individually transferred to 10 L spawning tanks with 0.45 μm filtered and UV-sterilized seawater (FSW) and the water temperature was rapidly increased by 3–5 °C (from 24 °C to 27–29 °C). This temperature was maintained for 1.5 h, and then decreased back to the initial temperature. Eggs from each female were collected from the bottom of spawning tanks and re-suspended in separate beakers with FSW.

Before pooling the eggs, females with eggs which were not round, immature forms and debris were discarded. Males were identified after the first spawn and removed from the spawning tanks. Sperm was collected dry from at least three males and was kept on melting ice until use. Sperm motility and fertilization success were checked to provide an acceptability threshold higher than 95%. The sperm was then diluted in an egg suspension of 60 eggs/mL for *H. polii* and 200 eggs/ml for *H. tubulosa*, in order to obtain a final density of 1×10^4 spermatozoa/mL and a final ratio of 166:1 and of 50:1, respectively. These values were determined by Morroni et al. (2020) for *H. polii* and by Rakaj et al. (2021) for *H. tubulosa* in accordance with egg size differences between the two species.

After fertilization, the embryos were washed three times by decantation, removing the sperm in the supernatant and adding FSW. The experiments were set up using 10 ml sterile capped polystyrene six-well microplates, carrying out 6 replicates per experimental condition. Embryos were incubated in a dark room at a temperature of 26 °C for 72 h. At the end of the incubation period, the samples were fixed and preserved by adding 50 μl of 40% buffered formalin. Finally, embryo morphology was evaluated under an inverted microscope.

Tests were accepted if the percentage of control embryos (negative control) was 60% for *H. polii* and 90% for *H. tubulosa*. The toxicity results of Cu were used as positive controls, and tests were accepted if they fell within the laboratory acceptability ranges (Morroni et al., 2020a; Rakaj et al., 2021).

2.5. Endpoints and toxicity criteria

A minimum of 100 randomly chosen embryos were photographed using a digital camera and analysed. The degree of toxicity was calculated using the integrative toxicity index (ITI) and the standard criteria of evaluation based on the calculation of the percentage of normal versus abnormal embryos. ITI was calculated by assigning a different weight to various embryonic malformations depending on both their severity and the stage at which malformations (delayed and/or abnormal embryo morphologies) appeared, then quantified based on a ranking of severity defined by Bonaventura et al. (2021) and Rakaj et al. (2021). Lower toxicity values were given to delayed embryos (embryos with a delay in development and absence of malformations) and higher scores were attributed to abnormal embryos (embryos with a delay in development and malformations) with no chance to recover development (Morroni et al., 2018a).

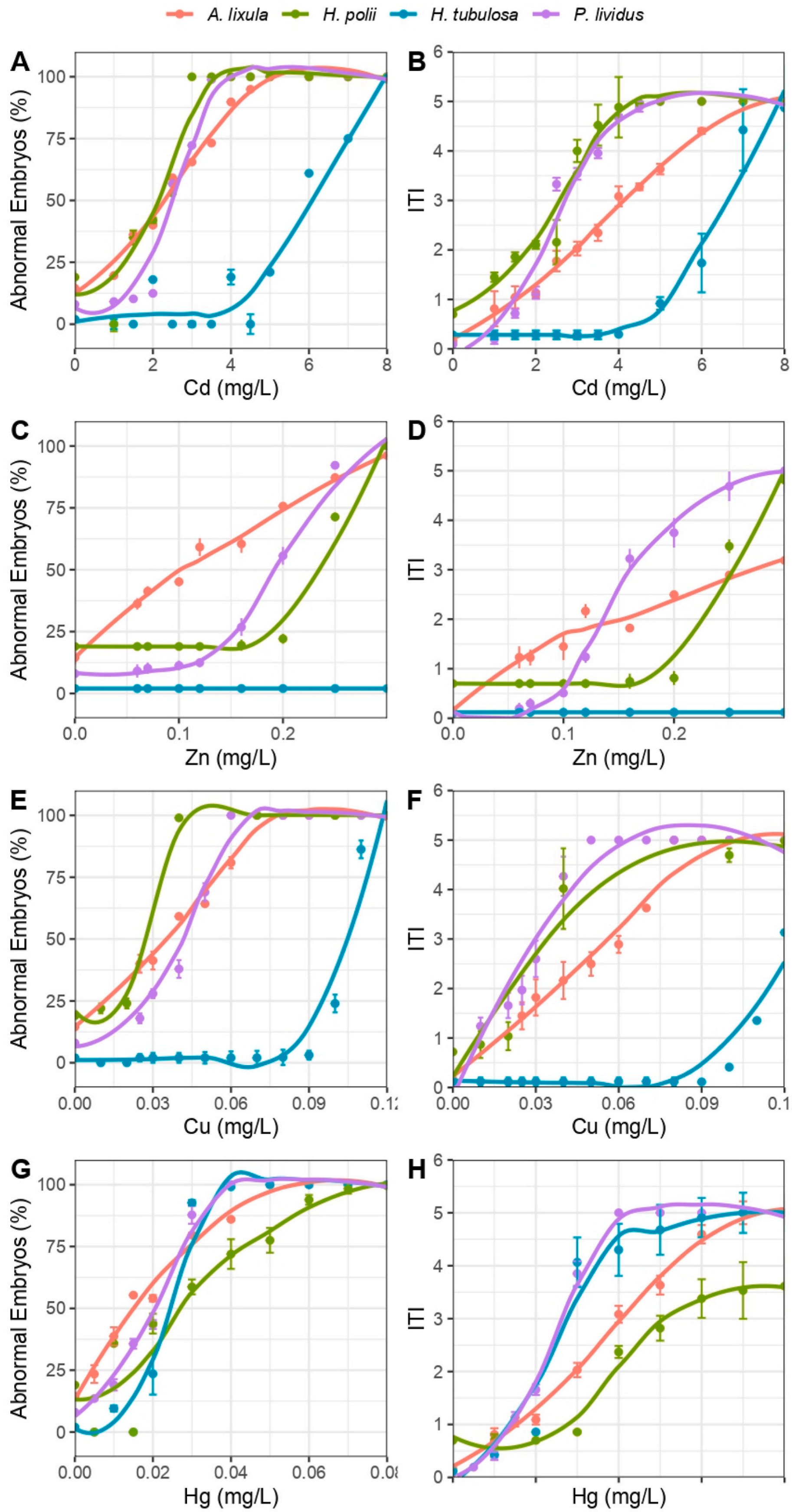

Fig. 2. Dose-response curves of embryos of *A. lixula*, *P. lividus*, *H. polii* e *H. tubulosa* exposed to Cd (A, B), Zn (C, D), Cu (E, F), Hg (G, H), Pb (I, J), NP (K, L), SDS (M, N). Data are expressed as percentage of abnormal embryos (A, C, E, G, I, K, M) and ITI (B, D, F, H, J, L, N).

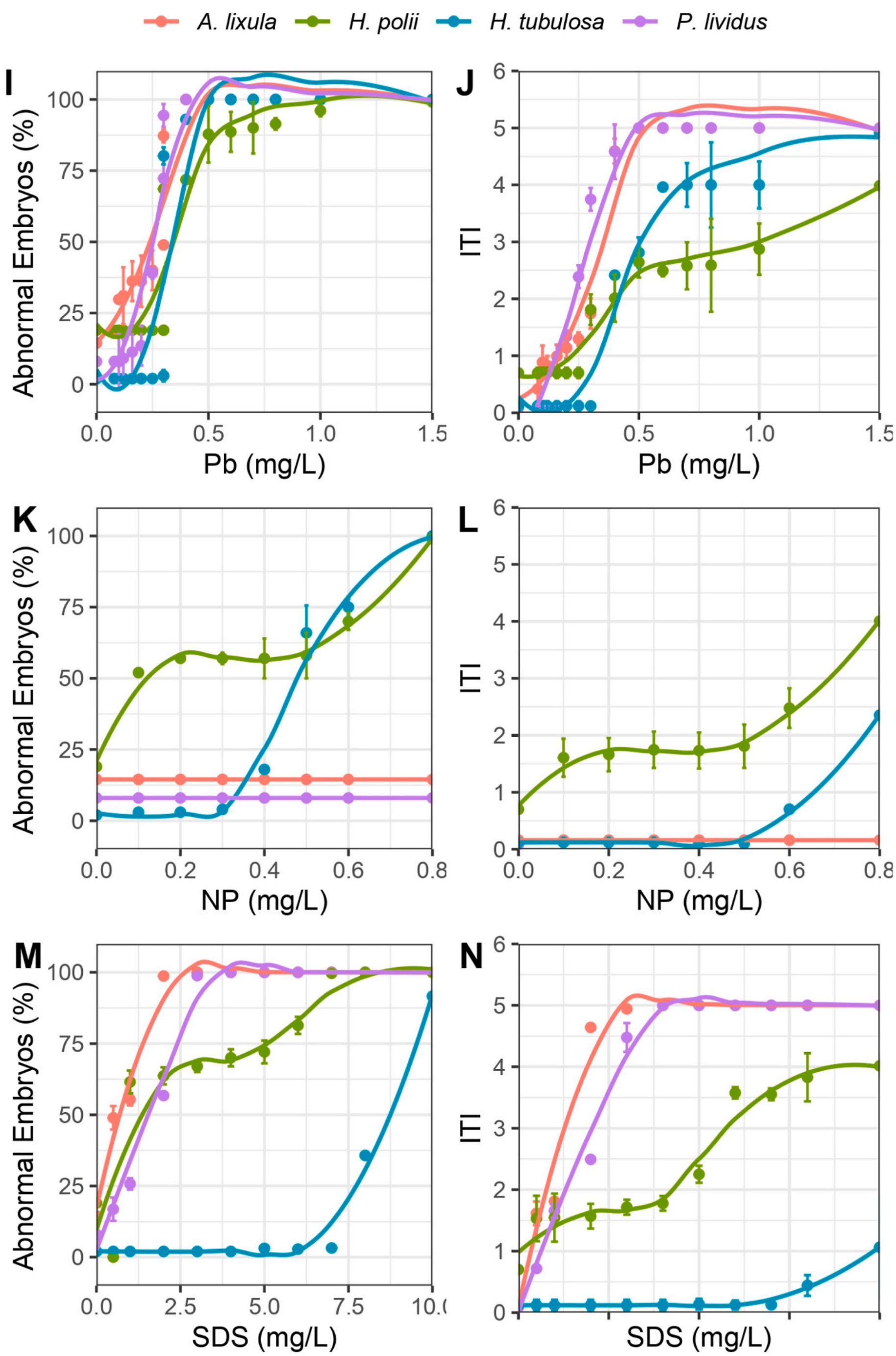

Fig. 2. (*continued*).

The ITI was calculated as follows:

$$\mathrm{ITI} = \sum \mathrm{ni} = 5(\mathrm{Si} * \mathrm{Fi}) \Big/ 100$$

where Si is the score associated with each abnormality and Fi is the frequency observed for that abnormality (i = 5).

For sea urchins, embryos were classified as normal only when they satisfied all the following morphological criteria: (1) correct schedule in reaching the developmental endpoint, (2) left/right and dorso/ventral embryonic axis symmetry, (3) differentiation of oral/aboral ectoderm and endoderm (Morroni et al., 2016, 2018). The ITI range between 0 (non) and 5 (maximum toxicity). In the toxicity scale developed in this study, a score of 0 was assigned to pluteus stage (Pl), 2 when observing delayed stages corresponding to early pluteus (ePl), toxicity scores increase for Pluteus (3), Prism (4) and Blastula/Gastrula/Morula (5) stages when abnormal morphotypes are observed.

For sea cucumbers, the mid-auricularia stage was selected as the developmental endpoint when they satisfied all the following morphological criteria: (1) correct schedule in reaching the developmental endpoint, (2) left/right and dorso/ventral embryonic axis symmetry, (3) correct differentiation of axohydrocoel, somatocoele and digestive system (mouth, stomach and anus). The toxicity was quantified by counting the frequency of delayed and/or abnormal embryo morphology and by quantitatively ranking the severity of effects from 0 (none) to 5 (high). The toxicity score of 0 was assigned when normal mid-auricularia (mAu) was observed. A score of 1 was assigned for well-developed early-

Table 1
Sensitivity of *H. polii*, *H. tubulosa*, *P. lividus*, *A. lixula* to Cd, Cu, Hg, Pb, Zn, SDS, NP. Data are expressed as EC50 (±95% confidence limits) (mg/L). Slope: ■: 0–1; ■■: 1–3; ■■■: 3–6; ■■■■: 6–10; ■■■■■: >10.

	Holothuria polii		Holothuria tubulosa		*Paracentrotus lividus*		Arbacia lixula	
	EC50 (±95% confidence limits) (mg/L)	Slope	EC50 (±95% confidence limits) (mg/L)	Slope	EC50 (±95% confidence limits) (mg/L)	Slope	EC50 (±95% confidence limits) (mg/L)	Slope
Cd	2.260 (1.691–3.311)	■■	4.978 (3.884–5.628)	■■■■■	2.510 (2.223–2.841)	■■■	2.361 (2.023–2.370)	■■■
Cu	0.026 (0.023–0.038)	■■■■■	0.104 (0.103–0.105)	■■■■■	0.042 (0.036–0.048)	■■■■	0.039 (0.032–0.047)	■■
Hg	0.025 (0.019–0.033)	■■	0.023 (0.022–0.025)	■■■■■	0.019 (0.015–0.024)	■■■	0.017 (0.012–0.023)	■■
Pb	0.242 (0.177–0.329)	■■	0.372 (n.c.)	■■■■■	0.217 (0.194–0.244)	■■■■	0.341 (0.236–0.494)	■■
Zn	0.236 (0.221–0.251)	■■■■■	No effect up to 0.350	–	0.371 (0.232–0.612)	■■	0.134 (0.092–0.213)	■■
SDS	4.022 (3.448–4.693)	■■■■	8.501 (8.302–8.514)	■■■■■	1.580 (1.174–2.120)	■■■	1.231 (0.294–5.130)	■
NP	0.491 (0.424–0.5689	■■■■	0.710 (0.683–0.734)	■■■■■	No effect up to 10	–	No effect up to 10	–

auricularia (eAu) larvae, thus with only a delay in development, but without malformations. Toxicity values increased for malformed embryos and larvae, depending on the stage of development: higher toxicity values are attributed to malformed mAu (score 3), even higher for delayed and malformed early-auricularia larvae (score 4) and, finally, the highest score at Gastrula/Blastula/Morula stage (G/Bl/M) (score 5) (Rakaj et al., 2021).

2.6. Chemical analysis

Nominal concentrations of single contaminants were compared with measured concentrations. In particular, trace metals were analysed by inductively coupled plasma optical emission spectrometry (ICP-OE, Agilent Technologies 7900, Santa Clara, CA, USA). The concentration of NP was measured via an HPLC-fluorescence detection method (Cruceru et al., 2012), while SDS was measured as methylene blue active substance (MBAS) using a PerkinElmer Lambda 45 spectrophotometer (George and White, 1999). Regarding Piombino harbour, elutriates, concentrations of trace metals (Al, As, Cd, Cu, Cr, Hg, Ni, Pb, Zn), Polycyclic Aromatic Hydrocarbons (PAHs), Organochlorine Pesticides (OPs), Organotin Compounds were determined in marine sediments. High performance liquid chromatography (HPLC) with diode array (DAD) and fluorimetric detection were used for PAHs, atomic absorption spectrophotometry (AAS) for trace metals (Benedetti et al., 2014). PCBs and OPs were analysed using the EPA 3545a (extraction), EPA 3630 (clean-up) and EPA 8270D methods for analytical determination. Organotin compounds (TBT) were analysed according to ICRAM (2001). Total concentrations/levels of ammonia and nitrite were determined by spectrophotometry (HACH LANGE GmbH DR 2800 using kit 304 and 340 HACH LANGE GMBH LCK) on elutriate samples.

2.7. Data analysis

To evaluate the toxic effect of reference toxicants and elutriate samples, the percentages of abnormal embryos were considered, estimating EC_{50} values which were then compared with literature data available for other test species. EC values with 95% confidence limits were calculated following the Trimmed Spearman-Karber statistical method. Responses in each experimental condition were corrected for effects in control tests by applying Abbott's formula (Hamilton et al., 1978). For each contaminant, Dose-response curves (LOESS) of ITI and Abnormal Percentage were plotted in R environment employing ggplot2 Package V3.4.1 (Wickham et al., 2023) (Fig. 1). Significant differences between the percentage of abnormal larvae in FSW (control) and in the reference substance solutions were determined by one-way ANOVA followed by a Dunnett's test for multiple comparison (Carballeira et al., 2012; Murado and Prieto, 2013). Two levels of significance were established: $p < 0.05$ and $p < 0.01$. The effect of each toxicant was determined by a parametric Pearson correlation test. Significance was established at 95% ($p < 0.05$). A correlation test to compare the sensitivity of sea urchins and sea cucumbers to different pollutants was also performed. Both ANOVA and Pearson correlation tests were performed using PAST statistical software (Hammer et al., 2001).

Multivariate data analysis was performed by principal component analysis (PCA) in order to visualize the correlations among larval responsiveness, granulometric and chemical variables, and observations.

3. Results

3.1. Sensitivity to single contaminants

Chemical analyses of the test solutions revealed that the measured concentrations varied less than 13% compared to the nominal concentrations, which are thus considered for the following evaluations.

Single contaminants induced significant abnormalities ($p < 0.01$) in embryos of both echinoderm species in a dose-dependent-manner, as reported in Fig. 2.

The effects of Cd^{2+} on embryo development resulted in an EC50 (±95% confidence limit) of 2.260 (1.691–3.311) in *H. polii*, 4.978 (3.884–5.628) in *H. tubulosa*, 2.796 (2.470–3.164) in *P. lividus*, 2.365 (2.023–3.783) in *A. lixula* (Table 1). Specifically, *H. tubulosa* exhibited lower sensitivity to Cd with respect to *H. polii*.

Concerning sea urchins, in *A. lixula* the developmental anomalies increased more gradual than in *P. lividus*, which quickly reach 100% of anomalies at 4 mg/L (Fig. 2).

Embryos exposed to Cu exhibited EC50 values (±95% confidence limit) of 0.026 (0.023–0.038) (*H. polii*), 0.104 (0.103–0.105) (*H. tubulosa*), 0.182 (0.166–0.200) (*P. lividus*) and 0.038 (0.032–0.047) (*A. lixula*) (Table 1). More details on developmental anomalies are reported in Fig. 2. In particular *H. polii* passed quickly from 22, 24% of abnormal embryos (ITI: 0.87, 1.04) at 0.02 mg/L 100% (ITI: 4.02) at 0.05 mg/L. On the contrary, *H. tubulosa* showed the lowest sensitivity among the echinoderm species, exhibiting significant effects on embryo development only at 0.09 mg/L and reaching 100% of abnormal embryos at 0.12 mg/L (ITI: 3.13).

Hg was the most toxic of the contaminates tested, with EC50 values (±95% confidence limit) of 0.025 (0.019–0.033) (*H. polii*), 0.023 (0.022–0.025) (*H.tubulosa*), 0.021 (0.016–0.026) (*P. lividus*), 0.017 (0.012–0.023) (*A.lixula*) (Table 1). *H. polii* exhibited a very gradual increase in developmental anomalies, while *H. tubulosa* exhibited a rapid increase in malformations, with 92% of abnormal embryos at 0.4 g/L. The sea urchin species showed a similar dose-response curve, with higher sensitivities than the sea cucumbers.

Pb caused EC50 values (±95% confidence limit) of 0.242 (0.177–0.329), 0.372 (n.c.), 0.494 (0.259–0.976), 0.434 (0.290–0.650) in *H. polii, H. tubulosa, P. lividus, A. lixula*, respectively (Table 1). Observing the effects on embryo development in greater detail (Fig. 2), *H. polii* exhibited the lowest effect concentrations (LOEC) at 0.3 mg/L, rapidly increasing from 19% (at 0.25 mg/L) to 68% of abnormal embryos (ITI of 0.70 and 1.81), with a more gradual increase in developmental anomalies up to the highest tested concentration of 0.8 mg/L, with 91% of abnormal embryos (ITI of 2.87). *H. tubulosa* showed the same LOEC as *H. polii* but a faster increase in toxic effects at increasing

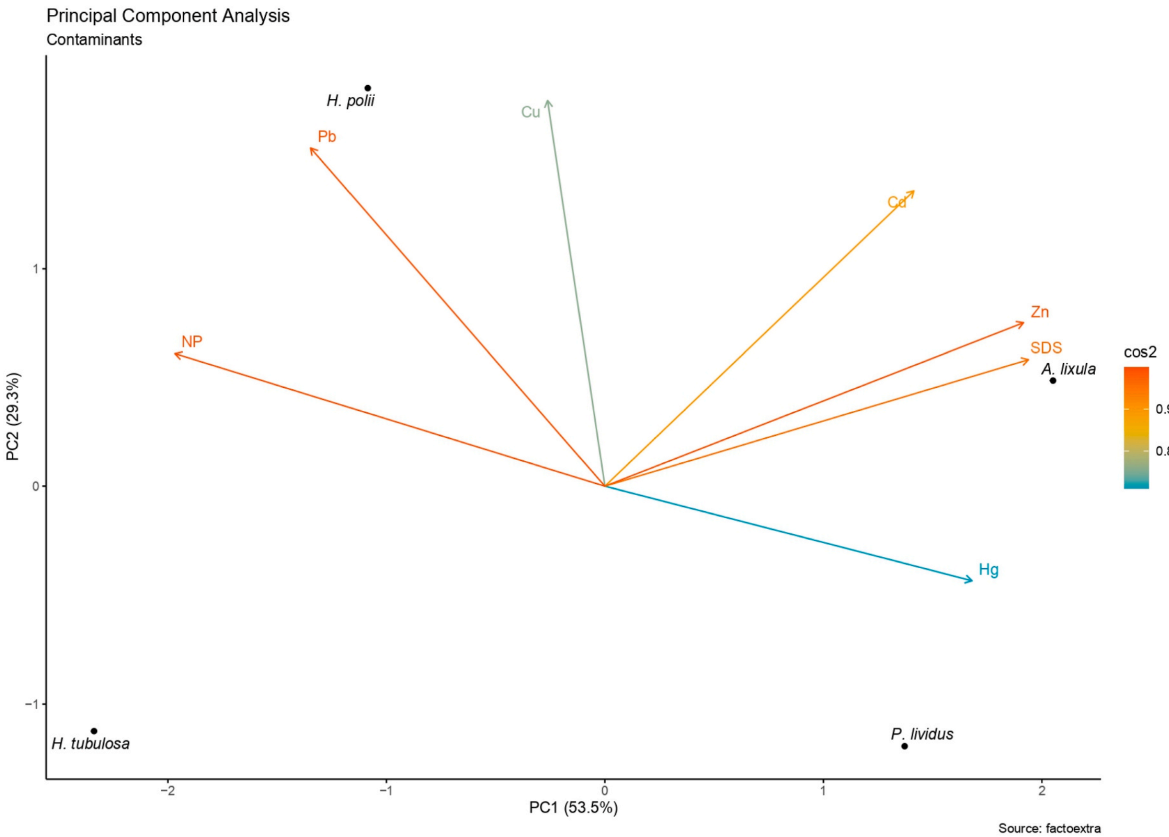

Fig. 3. Principal component analysis biplot of echinoderm species sensitivity to environmental pollutants of the first two principal components (PC 1 and PC 2). Cos 2 indicates the square of the cosine of the angles between the object (from the origin of the PCA plane) and a given axis. Similar colour of the axis indicates the position in the same plane. Species sensitivity is reversed indicating higher sensitivity with array direction. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Pb concentrations, reaching 100% of abnormal embryos (ITI: 4) at 0.5 mg/L. Regarding sea urchins, a similar trend and sensitivity was seen, as observable in the dose-response curve reported in Fig. 2 I,J.

Embryos exposed to Zn showed EC50 values (±95% confidence limit) of 0.236 (0.221–0.251), 0.192 (0.165–0.225), 0.114 (0.090–0.143) for *H. polii, P. lividus,* and *A. lixula,* respectively. Concerning *H. tubulosa* the absence of effect was observed at all tested concentrations. *H. polii* did not show any effects up to 0.2 mg/L, with 22, 71 and 100% of malformed embryos (ITI: 0.81, 3.47, 4.81) at 0.2, 0.25 and 0.3 mg/L (Fig. 2). Sea urchins showed a more gradual increase in toxicity with respect to *H polii*, in addition to a higher sensitivity.

Among the organic pollutants, SDS showed EC50 values (±95% confidence limit) of 4.022 (3.448–4.693), 8.501 (8.302–8.514), 2.013 (1.306–3.103), 1.223 (0.290–5.131) in *H. polii, H. tubulosa, P. lividus,* and *A. lixula*, respectively (Table 1). *H. polii* exhibited a gradual increase in toxic response up to 7 mg/L, while *H. tubolosa* exhibited higher effects concentrations and a rapid increase in toxic effects between 8 and 10 mg/L. Sea urchins appeared to be more sensitive to SDS than sea

Table 2
Sensitivity of *H. polii, H. tubulosa, P. lividus, A. lixula* to elutriate of marine sediments from Piombino harbour. Data are expressed as EC50 (±95% confidence limits) (mg/L).

Sample	*Holothuria polii*		*Holothuria tubulosa*		*Paracentrotus lividus*		*Arbacia lixula*	
	EC50 (±95% confidence limits) (%)	Slope	EC50 (±95% confidence limits) (%)	Slope	EC50 (±95% confidence limits) (%)	Slope	EC50 (±95% confidence limits) (%)	Slope
P1	35.7 (31.7–40.1)	3.2	53.2 (50.7–57.4)	0.3	>100		15.7 (11.7–19.3)	1.380
P2	53.8 (50.8–59)	*0.6*	52.8 (50.6–56.5)	*1.3*	84.5 (80.4–88.9)	*5.0*	12.6 (8.66–17.2)	0.437
P3	64.5 (58.3–70.7)	*3.2*	52.6 (50.6–55.8)	*2.0*	55.2 (53.5–57.2)	*6.1*	>100	
P4	63.2 (35–105)	*0.4*	52.3 (50.5–55.2)	*2.1*	>100		16.7 (12.5–20.9)	1.343
P5	56.5 (9.9–102)	*0.29*	>100		>100		18 (12.7–23.7)	0.851
P6	50.9 (32–79.7)	*0.7*	53.4 (50.8–57.7)	*0.7*	35 (32.2–37.8)	*3.0*	17.2 (14.4–19.5)	3.270
P7	58.2 (31.1–104)	*0.4*	>100		71 (66.8–75.7)	*3.7*	>100	
P8	19.1 (14.7–23.2)	*1.6*	53.2 (50.7–57.1)	*0.7*	>100		>100	
P9	41.7 (23.6–80)	*0.4*	28.2 (25.4–30.9)	*1.1*	74.7 (69.7–80.4)	*3.4*	18.9 (13.2–25.1)	0.754
P10	36.4 (31.6–41.6)	*2.6*	40.2 (37.8–42.7)	*3.5*	64.3 (61–67.9)	*4.5*	14.4 (10.1–19.2)	0.602
P11	73.4 (54–105)	*0.9*	33.4 (30.4–36.5)	*0.2*	>100		>100	
P12	30.8 (25.1–36.7)	*1.9*	34.8 (33.2–36.9)	*9.9*	>100		>100	
P13	36.4 (29.3–44)	*1.7*	18.2 (15.6–20.2)	*3.2*	>100		15 (11.2–18.6)	1.532
P14	35.5 (29.5–42)	*2.0*	42.8 (40.3–45.7)	*3.9*	>100		>100	
P15	30.4 (26.8–34.2)	*3.2*	53.2 (50.7–57.1)	*0.7*	>100		>100	

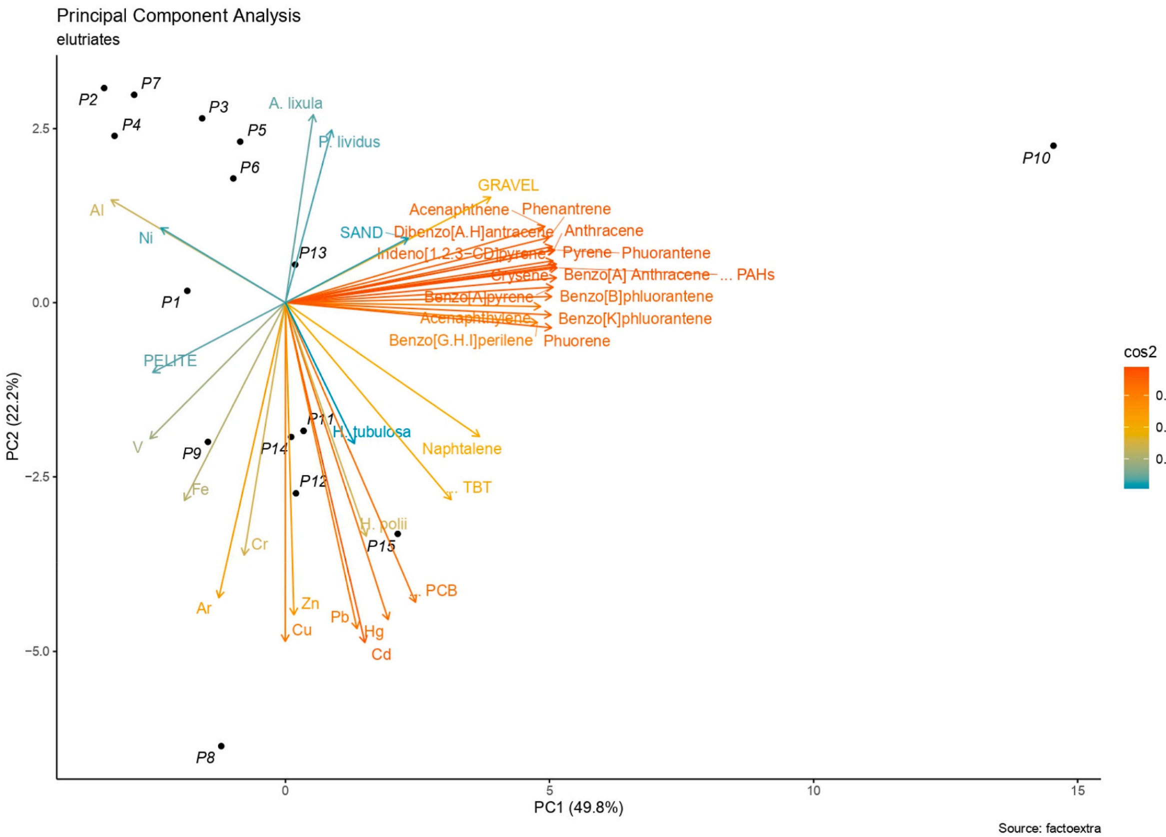

Fig. 4. Principal component analysis biplot on elutriates from sediment samples of Piombino harbour, organic and inorganic contaminants, sediment granulometry, and species responsiveness with loadings and scores in the coordinates of the first two principal components (PC 1 and PC 2). Cos 2 indicates the square of the cosine of the angles between the object (from the origin of the PCA plane) and a given axis. Similar colour of the axis indicates the position in the same plane. Species sensitivity is reversed indicating higher sensitivity with array direction. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cucumbers.

With regards to NP, EC50 values (±95% confidence limit) in sea cucumbers were 0.491 (0.424–0.5689) (*H. polii*) and 0.710 (0.683–0.734) (*H. tubulosa*), while no effects were observed in sea urchins at all tested concentrations, without the possibility to estimate the dose-response curve (Table 1). *H. polii* exhibited a dose response curve with a more gradual slope than those estimated for *H. tubulosa* (Table 1), characterized by an initial increase in toxicity between the control and 0.1 mg/L exposed embryos (52% of alterations, ITI of 1.60) and a second rapid increase between 0.6 and 0.8 mg/L, reaching 70% and 99% of abnormal larvae (ITI: 2.57 and 4) (Fig. 2K and L).

The PCA (Fig. 3) allowed us to analyse the multivariate relationship among echinoderm species and their dose-responses to the contaminants (in terms of EC-50% for Cu, Pb, SDS, NP, Hg, Zn). The first two principal components (PC1 and PC2) accounted for 53.5% and 29.3% of the variation, respectively, representing 82.8% of the whole database variation. The PCA (Fig. 3) was employed to highlight the multivariate relationship among echinoderm species (*P. lividus, A. lixula, H. polii, H. tubulosa*) and contaminants (Cu, Pb, SDS, NP, Hg, Zn). The ordination of the observations showed a heterogeneous responsiveness to the tested contaminants among echinoderm species. In particular, regarding sea urchin species, *A. lixula* was seen to correlate (high sensitivity) to Hg, Zn, SDS and Cd, while *P. lividus* correlated with Hg. On the other hand, *H. polii* was more closely correlated to Cu, Pb and NP, whereas *H. tubulosa* was inversely correlated (low sensitivity) to SDS, Zn and Cd.

3.2. Sensitivity to elutriates of marine sediments

The results of the chemical analysis conducted on sediment samples of Piombino Harbour are reported in Table S1 and Table S2. The results regarding the sensitivity of the different echinoderm species to 15 elutriate of marine sediments from Piombino harbour, expressed as EC50, are reported in Table 2. *H. polii*, *H. tubulosa*, *P. lividus*, *A. lixula* showed comparable results, with values generally falling in the same order of magnitude, although some specific differences were observed. In particular, *A. lixula* appeared to be more sensitive than other species towards the samples P1–P7 (located in northern-east part of the harbour), with EC50 generally between 12.6 and 18%, with the exception of P3 and P7 with values > 100%. On the contrary, in the sampling stations P8–P15 (located in the south-eastern area of the harbour) sea cucumbers appeared to be more sensitive than sea urchins, with EC50 higher than 100% in P11, P12, P13, P14, P15. Sea cucumber embryos exposed to the same elutriate samples showed EC50 ranging between 18.2% and 73.4%. The slopes of the dose-response curves appear to be similar, with the highest values in *P. lividus*, with data ranging from 3 to 6.1.

A Principal Component Analysis (PCA) was performed on elutriate dose-responses (in terms of EC-50%) to visualize the correlations between toxicity, granulometry, chemical composition and species in relation to the different harbour areas of the matrix sediments tested. The PCA highlighted the multivariate relationship existing among variables (toxicity, granulometry, chemical variables and species) and observations (different harbour elutriates) (for chemical data see Table 2). The first two principal components (PC1 and PC2) accounted for 49.8% and 22.2% of the whole database variation. Among these components, organic compounds are mainly shown on PC1, while trace metals (i.e., Cu, Cd, Zn, Pb etc.) are mainly described on PC2, showing a correlation among them.

Interestingly, some organic compounds, such as PCB, TBT and naphthalene, are distributed between the two axes, and Al and Ni was seen to be inversely correlated with the other metals. The PCA showed two distinct dose-response groups based on the observed distributions and an inverse correlation between sea urchin and sea cucumber species. In fact, as shown in Fig. 4, the sea urchin species were found to be more sensitive to the elutriates from the north-west harbour sites, showing lowest EC-50. Conversely, the sea cucumber species appeared to be more sensitive to the elutriates from the south-east harbour sites, characterized by high concentrations of trace metals.

4. Discussion

4.1. Sensitivity of echinoderm species to single contaminants

The sensitivity of echinoderm larvae towards single contaminants was compared by exposing the embryos to five trace metals and two organic pollutants (see paragraph 3.1). Our results showed a good responsiveness of the test species, with an increase in teratogenic effects in a dose dependent manner for all the tested chemicals. The toxicity results, expressed as EC50, showed comparable values among the echinoderm species, falling within the same order of magnitude, with slight species-specific differences. In particular *H. tubulosa* seems to be less sensitive than other species, with EC50, on average, higher than those obtained for sea urchins and *H. polii*, corroborating previous results obtained by Rakaj et al. (2021). Dose-response curves revealed a higher control value in *H. polii* and *A. lixula* than in *H. tubulosa* and *P. lividus* (15.19% vs 2–8% of abnormal larvae), and higher slopes which caused a comparable but slightly higher sensitivity toward tested contaminants.

These data are consistent with previous findings on embryos and larvae of other marine invertebrates commonly used in bioassays. For *P. lividus*, the EC50 obtained in this study for Cd, Cu, Hg, Pb, Zn (2.510, 0.042, 0.019, 0.217, 0.371 mg/L, respectively) are comparable with those of literature studies, ranging between 0.230 and 9.240 mg/L for Cd, 0.045–0.068 ml/L for Cu, 0.014–0.017 mg/L for Hg, 0.110–19 for Pb and 0.028–0.380 mg/L for Zn (Warnau et al., 1996; Fernandez and Beiras, 2001; Arizzi Novelli et al., 2003; Manzo et al., 2010; Morroni et al., 2018). The echinoderms responsiveness to trace metals observed in this study is also comparable to those of other embryo-larval models. The mussel *Mytilus galloprovincialis* exhibited an EC50 for Cu of 0.007–0.018 mg/L (Prato and Biandolino, 1997; Beiras and Albentosa, 2004; Arnold et al., 2010; Boukadida et al., 2016) and a Cd effect concentration of 1.93 mg/L (Beiras and Albentosa, 2004). For this metal, EC50 values of 0.037–0.212, 0.180 and 0.790 were observed in *Crassostrea gigas, Hydroides elegans* and *Ficopomatus enigmaticus* respectively (His et al., 1999; Mai et al., 2012; Gopalakrishnan et al., 2008; Oliva et al., 2018). These values are lower than those obtained in this study but on the same order of magnitude, with a higher comparability of Cd dose-response between *M. galloprovincialis* (1.930 mg/L) and echinoderms (2.260–4.980 mg/L). Also for Pb, the EC50 found in echinoderms (0.217–0.372 mg/L) falls within the literature range of *P. lividus* of 0.068–1.250 (Fernandez and Beiras, 2001; Arizzi Novelli et al., 2003; Manzo et al., 2010; Morroni et al., 2018), being slightly lower than values reported for *H. elegans* (1.130 mg/L) and *C. gigas* (0.660 mg/L) (Gopalakrishnan et al., 2008; Xie et al., 2016). Despite the different embryotoxicity of Cu, this metal seems to cause a delay in development in both sea cucumber and sea urchin species at lower concentrations, in line with the effects observed on sea urchin embryo development (Arizzi Novelli et al., 2003; Morroni et al., 2018). Moreover, in sea cucumbers this metal caused a significant larval length reduction in the mid auricularia development stage, compared with control embryos. Moreover, at higher concentrations, a malformation in the early auricularia stage caused a-typical body shape associated with exposure to Cu and Hg. This peculiar malformation, which was also seen in *H. polii,* highlights a developmental anomaly that is typical of sea cucumber larvae in specific stress conditions (Rakaj et al., 2021). Concerning the organic compounds, sea urchins did not respond to NP, differently from sea cucumbers, which showed EC50 of 0.491 (*H.polii*) and 0.710 (*H. tubulosa*), values almost ten times lower than those reported for *F. enigmaticus* (6.810 mg/L) (Oliva et al., 2018), and higher compared to the bivalve *M, galloprovincialis*, with 0.140 mg/L (Tato et al., 2018). The EC_{50} of SDS revealed a higher sensitivity of sea urchins (1.231–1.580 mg/L) than sea cucumbers (4.022–8.501 mg/L), with values comparable to those of *F. enigmaticus* (8.680 mg/L), *M. galloprovincialis* (2.253 mg/L) and *Ciona intestinalis* (5.145 mg/L (Bellas et al., 2005; Beiras and Bellas, 2008).

From the PCA (Fig. 3), responsiveness variations to the tested contaminants emerged among the four echinoderm species, which indeed are distributed in different quadrants. In fact, *A. lixula* was found to have a higher sensitivity (inversely correlated) to Hg, Zn, SDS and Cd than *P. lividus*, which instead was more closely correlated to NP and Pb (lower sensitivity). On the other hand, *H. polii* showed higher sensitivity (inversely correlated) to Cu, Pb and NP than *H. tubulosa* which was correlated to SDS, Zn and Cd (lower sensitivity). This responsiveness suggests that the integration of species belonging to the Echinoidea, and Holothuroidea class, results in a gain in terms of weight of evidence in ecotoxicological batteries.

4.2. Sensitivity of echinoderm species to elutriates in marine sediments

The sensitivity of echinoderm larvae towards sediment elutriates was compared by exposing the embryos to different sediment matrices collected from 15 areas around the commercial and industrial harbour of Piombino. This harbour was chosen as a case study for the sediment elutriate test, since it has a marked contamination heterogeneity between sub-areas, hence providing heterogeneous contaminations to test the species' responsiveness (Broccoli et al., 2021). The harbour of Piombino is characterized by the presence of an industrial pole and by commercial and tourist traffic towards the islands (Elba, Sardinia, and Corsica). The pollutants found in the harbour include those deriving from steel processing coal and all the products derived from its distillation, namely PAHs, tars, as well as heavy metals contained in ferroalloy minerals that are added to steel (Valentina et al., 2021). Metals appear abundant especially in the southern area of the port, near the "Lucchini" steel plant, with the majority of PAHs observed in the northern area of the port, near the industrial plant and the pier for commercial vessels. In addition, especially in the northern part of the harbour which is already subjected to intense merchant/commercial maritime traffic, the level of sea pollution is exacerbated by the high contribution of solid and liquid intake from the Cornia river (east of Piombino).

The toxicity results showed, on average, a higher degree of sensitivity in sea cucumbers, especially for samples located in the southern area of the harbour. The more in-depth Principal Component Analysis (PCA) highlighted two distinct dose-response groups based on the observed distributions and an inverse correlation between sea urchin and sea cucumber species. In fact, sea urchin species were found to be more sensitive to the elutriates of north-west harbour sites, showing the lowest EC-50, than the sea cucumber species which instead were sensitive also to the elutriates of the south-east harbour sites, which were characterized by high trace metal concentrations. This pattern suggested a different species sensitivity for elutriates on the basis of their specific contaminants. In fact, the samples from P1 to P7, located in the northern-west of the harbour, were characterized by high values of Ni and Al and by lower values of other metals and organic compounds; the samples from P8 to P15, located in the south-east of the harbour, were characterized by high values of heavy metals including Cu; Zn; Pb; Cd; Hg and organic compounds such as PCB; TBT and Naphtalene. Among these observations, P10 emerged as an outlier, since it was characterized by the highest concentrations of organic compounds. These results showed that the integration of these species in a battery of bioassays

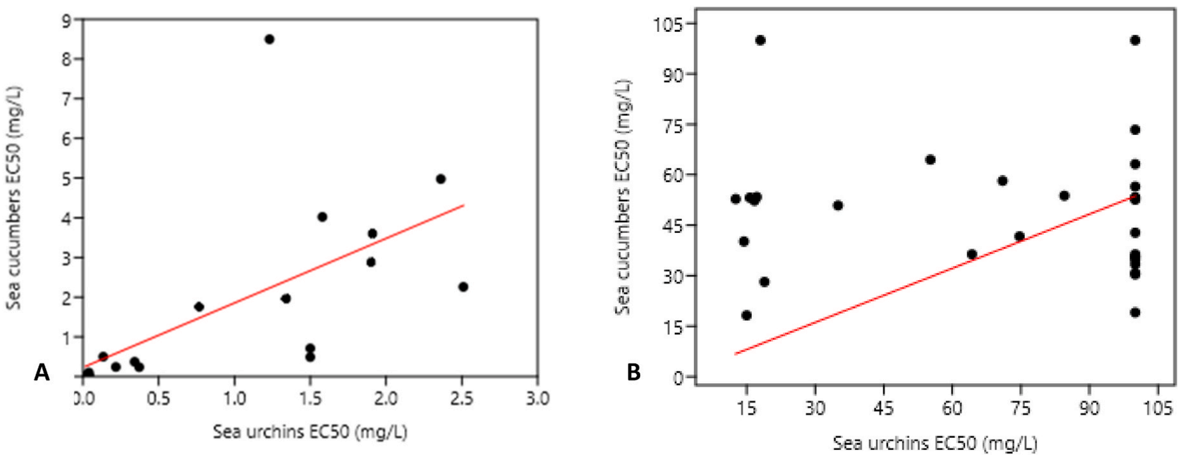

Fig. 5. Correlation between sea cucumbers EC50 (X axis) and sea urchin EC50 (Y axis) for environmental pollutants trace metals (Cd, Cu, Hg, Pb, Zn, SDS, NP) (A) and 15 elutriate samples of marine sediments from Piombino harbour (B).

represents an effective tool also for environmental assessments.

4.3. Echinoderm responsiveness and general implications

The responsiveness of echinoderm larvae towards single contaminants revealed comparable results, with a slightly higher sensitivity of *A. lixula* and *H. polii* with respect to *H. tubulosa*, which appear to be the least sensitive species. Moreover, *A. lixula,* which appears to have a higher sensitivity to Hg, Zn, SDS, Cd than *P. lividus*, and *H. polii,* seems to respond more to Cu, Pb and NP than *H. tubulosa*. In this contest, the responsiveness of sea urchins and sea cucumbers to pollutants yielded a statistically significant correlation ($r = 0.68$, $p < 0.05$), as shown in Fig. 5A, confirming that these species produce comparable results. However, although the responsiveness to single contaminants was similar among echinoderm species, their sensitivity to environmental matrices, which are a mix and synergy of substances, varied. Indeed, incubating echinoderm embryos with elutriates of marine sediment from Piombino harbour, sea cucumbers appear to be more sensitive than sea urchins, especially when exposed to the samples taken from the southern area of the port containing trace metals, PCB and TBT. Moreover, in the case of environmental matrices, the correlation between sea urchin and sea cucumber response (Fig. 5B) does not appear to be significant ($p = 0.72$).

These results indicate that toxic responses in embryos exposed to environmental matrices are probably modulated by interactions between different variables, including additive, synergistic and antagonistic effects. For this reason, the chemical determination of pollutants, in a complex mixture of unknown composition, such as the elutriate of marine sediments, does not allow for a significant estimation of toxicity. For such samples, our results confirmed that the bioassay approach is one of the most suitable investigative methods that can be employed (Bandard et al., 2006) and also suggested that both sea cucumber and sea urchins are excellent organisms for integration into ecotoxic batteries to evaluate the effects of all contaminants, including interactive effects, providing valuable information on the bioavailable fraction of contaminants.

The sensitivity of the echinoderm species to single contaminants and environmental matrices from an applicative point of view, points to the potential integration of sea urchin and sea cucumbers bioassays, which could provide a differentiated and high-ecological value response to pollution.

5. Conclusions

Our results evidenced a very good responsiveness of Echinoderm embryo-larvae to single contaminants, with sensitivities of the same order of magnitude as those of commonly employed marine invertebrates, but with species-specific differences. Moreover, the incubation of embryo-larvae with elutriates of marine sediments, evidenced that sea cucumbers appear to be more sensitive than sea urchins, especially when exposed to samples characterized by high densities of trace metals, PCB and TBT.

These results indicate that toxic responses in embryos exposed to environmental matrices are probably modulated by interactions between different variables, including additive, synergistic and antagonistic effects. Hence, in this complex system of interactions, the performance of embryo/larval tests with both Echinoids and Holothuroids can provide a sensitive and representative response to assess marine pollution using a battery of bioassays. For these reasons, sea cucumber bioassays also represent a promising tool that could be adopted in future ecotoxicological studies using a more integrated WOE approach for the ecological risk assessment of marine sediments.

Author statement

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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

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.122285.

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