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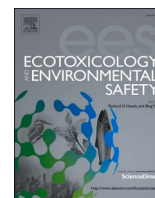
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Elutriate preparation affects embryo development test with *Paracentrotus lividus*: An in-depth study on the differences between two protocols and three different sediment/water mixing times

Davide Sartori^{a,*}, Simona Macchia^a, Nicolas Layglon^b, Sebastien D'Onofrio^b, Benjamin Misson^b, Maria Elena Piccione^a, Rosa Maria Bertolotto^c, Alice Scuderi^a, Fabiano Pilato^a, Silvia Giuliani^a, David Pellegrini^a, Andrea Gaion^d

^a Institute for Environmental Protection and Research, ISPRA, Via del Cedro, 38, 57123 Livorno, Italy

^b Université de Toulon, Aix Marseille Université, CNRS, IRD, Mediterranean Institute of Oceanography (MIO), UM110, France

^c Agenzia Regionale per la Protezione dell'Ambiente Ligure, ARPAL, Via Bombrini 8, 16149 Genova, Italy

^d University Centre South Devon, Vantage Point, Long Road, Paignton TQ4 7EJ, United Kingdom

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ABSTRACT

Coastal areas are under continuous and increasing pressure from different human activities. A mixture of contaminants (e.g. hydrocarbons, pesticides, persistent organic pollutants (POPs), emerging contaminants, and others), originating mainly from populated, industrialised and agricultural areas, can reach the marine environment through different means such as wastewater discharge, soil runoffs, leaching from agriculture, and volatilisation/deposition. In this context, marine sediments have increasingly been considered repositories for a variety of pollutants that can accumulate and be stored for long periods, acting as a secondary source of contaminants during subsequent dredging operation or vessel manoeuvring. Chemical and ecotoxicological analyses of sediments are routinely conducted to evaluate the potential hazard/risk to the environment, either on bulk sediment or elutriate. In general, sediment elutriates are commonly prepared according to ASTM Guide even if alternative protocols are proposed by USACE for the various condition that they have to represent. The goal of the present study was to determine if the toxicological properties of ASTM-prepared elutriates are comparable to those obtained from the USACE protocol. Sediment coming from 3 harbours (Olbia, Cagliari, and Toulon), as part of the "Se.D.Ri.Port" Interreg Project, were processed to obtain elutriates according to ASTM Guide and USACE Dredging Elutriate protocol and tested with the sea urchin *Paracentrotus lividus* embryo development test. Moreover, the significance of different stirring times of water/sediment mixture (1 h, 3 h, and 24 h) was tested with both the ASTM and USACE protocol. In addition to the biological analysis, for each sediment sample, heavy metals concentration, granulometry, and organic matter were determined. Even if for the ports of Toulon and Cagliari, the ASTM and USACE elutriates showed comparable results with *P. lividus* bioassay, for the port of Olbia the two protocols showed different criticalities. Preliminary results show that for the site Olbia elutriates prepared with the USACE protocol resulted in higher toxicity than elutriates obtained with ASTM ($p < 0.001$). In conclusion, differences in preparation protocols appear to be significant and can lead to different results in biological testing. To overcome this problem and to obtain more reliable evaluations of risk to the environment, standardisation and regulation must be the next goals in sediment management procedure.

1. Introduction

Marine sediments have increasingly become repositories for a variety of pollutants (Huerta Diaz 2006; Sahu et al., 2009) which can accumulate and remain in this matrix for long periods (Ruiz-Fernández et al.,

2012) and may be transferred through the food web, affecting marine biota and ultimately human health (Borgå et al., 2001).

A common approach in sediment risk assessment is to integrate chemical analysis and toxicological results (Chapman, 2000). Matrices generally taken into account to evaluate sediment toxicity are: whole

* Corresponding author.

E-mail address: davide.sartori@isprambiente.it (D. Sartori).

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sediment, pore water and elutriate. The whole sediment represents the most realistic exposure scenario however, bioassays on this matrix require a considerable investment of human resources, material and time (Haring et al., 2010). Pore water can represent a direct exposure route for many species but, depending on the grain size, it could be difficult to obtain the right volume to perform bioassays. Even the extraction methodology can lead to chemical-physical alterations in pH and redox potential of the pore water and in return can affect the real bioavailability of contaminants present (Chapman et al., 2002).

Tests with elutriates simulate exposure to contaminants released in the water column during dredging operation or open water disposal of dredged material (McDonald, 2005). According to Haring et al. (2012), elutriate preparation could reduce the toxicity of the sample by diluting contaminants and generally only hydrophilic contaminants can be extracted in elutriate. Elutriates are commonly prepared according to the ASTM E1391 (2014) method, which consists in mixing one part of sediment and four parts (in volume) of water. However, under the incentive of several research programmes related to dredging activities and management of dredged materials, the U.S. Army Corps of Engineers (USACE) has developed several elutriate preparation protocols using different water/sediment ratios to best simulate the environmental conditions that occur during handling activities such as sediment discharge into confined disposal facilities, offshore spillage, and dredging (Di Giano et al., 1995).

To our knowledge, as of today, there is no universally recognised and accepted method in the preparation of elutriates and, in recent years, numerous articles have been produced on this issue (Novelli et al., 2006; Haring et al., 2010, 2012; Vicinie et al., 2011). Similarly, Italian Decree 173/2016 (MD 173/2016), which established the technical criteria for the authorisation and management of dredged material, does not provide any indication about the method of preparation of elutriate but only indicates the 1:4 (w/v) water/sediment ratio.

There are reasonable grounds to believe that different elutriate methods can affect the bioavailability and toxicity of this matrix (Marin et al., 2001; Novelli et al., 2006), and consequently lead to different management options of sediments. For this reason, as a part of the Se.D. Ri.Port Interreg project, for the evaluation of the ecotoxicological effects related to the resuspension of sediments, the embryo development test with sea urchin *Paracentrotus lividus* was implemented to evaluate the toxicity of elutriates obtained from sediment sampled in the ports of Olbia (Italy), Cagliari (Italy), and Toulon (France) following a pollution gradient. In Toulon bay (South East France), surface and deep sediments are characterised by a strong anthropogenic contamination in heavy metal, (navy base, harbours, etc.); in particular, mercury appears to be the most enriched contaminant with a large pollution dispersion in the whole bay (Tessier et al., 2011).

Olbia harbour is the most important in Northern Sardinia for ferry traffic (<https://deims.org/>). Previous investigations have shown very low levels of contamination of both metals and organic contaminants (De Luca et al., 2005).

The portual area of Cagliari showed heavy metal contamination, especially of As, Cd, Hg, and Pb (Schintu et al., 2016). Sediment coming from the S. Gilla lagoon, is rich in Hg because of important discharges from a petro-chemical plant that operated since the 1960s in the production of chloride (Contu et al., 1983; Frontalini et al., 2009). To assess the possible influence of sample preparation in the toxicity of sediment, in the present study elutriates were prepared following both the ASTM protocol and Dredging Elutriate method developed by USACE.

In addition to the biological analysis, for each sediment sample, heavy metals concentration, granulometry and organic matter content were determined. Furthermore, ammonium and nitrite concentrations in the elutriate, frequently regarded as confounding factors (Postma et al., 2002), have been checked prior to test execution to avoid interference with the results.

The goal of the present study was to determine if the toxicological properties of ASTM-prepared elutriates are comparable to those

obtained from the USACE protocol, considering the significance of 3 different stirring times of water/sediment mixture (1 h, 3 h, and 24 h).

2. Materials and methods

2.1. Sediment samples

The sediment samples for this study were collected from the harbours of Olbia, Toulon and Cagliari as part of the "Se.D.Ri.Port" Interreg project (See Supplemental Material, Figure OP.1) during the fall-winter season of 2019.

Sediments were sampled, in each port at three sites along the contamination gradient. For each Port, sediment collection at the indicated site occurred on the same day. In the Toulon Harbour, a sample was collected from presumably few contaminated aquaculture sites (Lazaret: 43° 05' 24.088" N, 05° 54' 58.922" E) and the other two-samples, in the inner and presumably most polluted part of the port (Corsica: 43° 06' 54.546" N, 05° 55' 33.666" E; Suffren: 43° 07' 10.193" N, 05° 54' 59.525" E).

In Olbia harbour, sediments were collected from three sites: P1 (40° 55' 26.777" N, 09° 31' 30.460" E) is located in the area where ferry boat daily resuspend sediment with their bow thruster; P2 (40° 55' 09.736" N, 09° 32' 26.332" E) is at the mouths of the Padrongian river, and P3 (40° 55' 23.984" N, 09° 33' 40.082" E) is located in the eastern unpolluted part of the harbour (reference site). In the Port of Cagliari, sediment samples were collected in the area of the fishing vessel's dock (Darsena: 39° 12' 39.045" N, 09° 06' 09.910" E), in the eastern part of the Port of Cagliari near the mouth of Channel Palma (S. Elmo: 39° 12' 03.375" N, 09° 07' 40.431" E; reference site), and in the area of the Rinascita Pier (39° 12' 41.807" N, 09° 06' 17.434" E) where the cruise terminal is located.

Surface sediments were collected with an 8-L van Veen grab, stored at 4 °C in capped polyethylene vessels and transferred to the laboratory for elutriate preparation and chemical analysis to be conducted within 2 weeks from sampling. Sediment samples, in order to minimise sources of contamination and the variation in the physical, chemical, or biological characteristics, were collected and processed following the indication reported in the technical manual U.S. EPA (2001).

2.2. Water Sampling

Elutriate samples were prepared using seawater collected from each sampling site 1 m above the sediment surface. Water was collected with a 4.2 L horizontal van Dorn type water sampler (Wildco, model Beta) decontaminated with a hydrochloric acid solution (10%) and then rinsed with Milli Q water the day before its use. On the sampling site, the water sampler was rinsed with acidified milli-Q (0.1% v/v HCl, Trace-Select, Fluka) and then rinsed three times with seawater from the site (Coclet et al., 2018; Layglon et al., 2020).

2.3. Sediment elutriate preparation

Sediment elutriate were prepared in 2L capped polyethylene vessel.

The Dredging Elutriate (Di Giano et al., 1995), developed by USACE to estimate the release of contaminants from sediment due to resuspension during dredging activity, consisted of mixing the sediment and site water at a total suspended solid concentration of 10 g L⁻¹ followed by the aeration of the slurry for 1 h. Aeration was performed in a thermostated room (4 ± 1 °C) equipped with a compressed air source with deionized water trap and tubing for bubble aeration of slurry. Glass tubes were attached to the aeration source and insert the vessel containing the sediment suspension. Vessel were covered on the top with a lid sealed with a parafilm strip. The flow rate was adjusted to agitate the mixture vigorously for 60 min. The mixture is allowed to settle for 60 min at 4 °C and subsequently the supernatant was centrifuged at 1200 x g for 20 min using a refrigerated ultracentrifuge (Thermo Scientific,

Mod. SL16R). According to ASTM protocol (ASTM, 2014), elutriates were prepared mixing 1 part of sediment with 4 parts of site water (1:4 v/v ratio), and mixed on a rotary shaker table, located in a thermostated room (4 ± 1 °C), for 1 h at a speed of 400 x g. After mixing, the samples were centrifuged at 1200 x g for 20 min to separate the water from the sediment using a refrigerated ultracentrifuge (Thermo Scientific, Mod. SL16R).

The aqueous fraction (elutriate sample) was stored without filtering in 50-mL polyethylene containers at 4 °C until used for sea urchin bioassay.

In addition to the procedures described above for both the ASTM and USACE protocols, additional elutriates were prepared from each sediment sample, by increasing the stirring time of the water-sediment mixture to 3 (ASTM 3 h, USACE 3 h) and 24 h (ASTM 24 h, USACE 24 h) following the same protocols described before.

2.4. Ammonium and nitrite determination

The Ammonium (NH₄) and Nitrite (NO₂) content in the elutriate was determined using Hach Lange kit LCK 304 and LCK341 respectively with a spectrophotometer Hach Lange DR3900. Briefly, the ammonium ions react at pH 12.6 with hypochlorite ions and salicylate ions in the presence of sodium nitroprusside as a catalyst to form indophenol blue. For nitrite determination, nitrites react with primary aromatic amines in acidic solution to form diazonium salts. These combine with aromatic compounds that contain an amino group or a hydroxyl group to form intensively coloured azo dyes.

2.5. Chemical analyses

At first step, considering the prevalent contamination of the ports involved in this work linked to inorganic contaminants (De Luca et al., 2005; Frontalini et al., 2009; Tessier et al., 2011; Schintu et al., 2016), it was decided to proceed only to the determination of heavy metals concentrations in sediments.

Samples of sediment were dried at 60 °C, homogenised and sifted in a mortar, before chemical treatment. Trace metal analyses were performed with approximately 0.3 g of sample digested with a solution of 5 mL of HNO₃, 1 mL of HCl and 2 mL of ultrapure water, using high-pressure Teflon reactors in a microwave oven system (EPA, 3051 A).

The concentration of As, Cd, Cr, Cu, Ni, Pb, Zn was determined by inductively coupled plasma optical emission spectroscopy (ICP-OES 720, Agilent). Total mercury content was quantified by cold vapour atomic absorption spectrometry (CETAC, M-7600). Suitable internal chemical standards were used to calibrate the instrument and each reading was replicated 3 times. The analytical and quality assurance was performed using certified reference material (LGC6137, accepted % of recovery between 80% and 120%; APHA, 1992; EMEA, 2006), duplicate samples and procedural blanks. The external validation of the analytical

procedures is regularly assessed by participation in QUASIMEME (Quality Assurance of Information in Marine Environmental in Europe) interlaboratory exercises for trace metal analysis.

The limit of detection of the method and the limit of quantification for each metal analysed is given in Table 1.

A supplementary analysis was conducted on Olbia sediments to assess the presence of Polycyclic Aromatic Hydrocarbons (PAHs) and Polychlorinated Biphenyls (PCBs) in the matrices.

Polycyclic Aromatic Hydrocarbons (PAHs) were analysed using gas chromatography and mass spectrometry with a Soxhlet sample extraction technique (US-EPA 3540/8270D) (US-EPA, 2007b; US-EPA, 1996a). Quantifications of polychlorinated biphenyl (PCBs) were conducted according to the US-EPA 3660B/8082A (US-EPA, 2007a; US-EPA, 1996b).

2.6. Organic matter

The organic matter content was determined using the Loss on Ignition (LOI) methodology (Luckzak et al., 1997). Briefly, the sediment sample was subjected to three phases of combustion at increasing temperatures. Approximately 7 g of sediment was dried at 105 °C in weighted crucibles, for 24 h. Then, the sample was dried in a muffle, at 160 °C and 400 °C, for 6 and 4 h respectively. After each step weight was determined. The content of organic matter was expressed by the following relation:

$$\text{Organic Matter (g/kg)} = (\text{Net weight}_{160\text{ °C}} - \text{Net weight}_{400\text{ °C}}) * 100 / (\text{Net weight}_{105\text{ °C}})$$

2.7. Granulometry analysis

Granulometry analysis was conducted on 70 g of sediment. Each sample was treated with a solution of hydrogen peroxide and distilled water (1:8 v.v.), for 48 h at room temperature, to facilitate the separation of the granules. Afterward, moist sediment was separated on a 63 µm mesh with distilled water; the two fractions obtained were dried at 60 °C and subsequently weighed.

Sediment fraction > 63 µm was separated with a set of sieves (ASTM series) with mesh sizes that decrease downwards from – 1–4 phi with a range of 0.5 phi (1/2φ) and each fraction obtained was weighed.

2.8. Paracentrotus embryo-development test

P. lividus adults were collected from an intertidal rocky site along the southern coast of Livorno, Italy (43°25.6020' N e 10° 23.7800' E) and immediately transported in an insulated box to the laboratory. The embryo toxicity test with *P. lividus* was performed according to Sartori

Table 1

Heavy Metal Concentration (mg/Kg) and organic matter content (O.M.) (g/kg) in the Study Areas. Concentration are expressed as mean ± SD (n = 6). Limit of detection (LOD), Limit of quantification (LOQ) and Recovery (%) for each heavy metal analysed are listed in table. Accepted % of recovery between 80% and 120% (APHA, 1992; EMEA, 2006).

		As	Cu	Ni	Cr	Cd	Pb	Zn	Hg	O.M.
Toulon	Lazaret	21,53 ± 1,67	34,58 ± 0,48	15,73 ± 1,55	27,62 ± 0,05	0,05 ± 0,08	48,59 ± 1,65	121,49 ± 1,42	0,71 ± 2,08	10,16 ± 1,15
	Corsica	39,23 ± 1,78	462,90 ± 2,97	25,48 ± 0,86	52,23 ± 0,29	0,19 ± 0,29	195,08 ± 3,53	415,38 ± 1,24	9,85 ± 1,15	6,11 ± 0,68
	Suffren	16,11 ± 1,41	136,79 ± 1,64	25,47 ± 0,95	42,74 ± 0,95	0,41 ± 0,12	133,27 ± 1,25	396,45 ± 6,81	2,13 ± 1,38	2,81 ± 0,96
Olbia	P1	5,55 ± 1,70	26,99 ± 0,82	10,36 ± 0,82	17,43 ± 0,31	0,078 ± 0,46	21,73 ± 1,71	119,22 ± 1,42	0,058 ± 0,96	4,56 ± 0,38
	P2	4,61 ± 1,17	11,11 ± 0,58	5,12 ± 0,58	9,11 ± 0,58	0,068 ± 0,41	11,83 ± 2,08	65,95 ± 1,25	0,031 ± 1,70	1,78 ± 0,54
	P3	6,01 ± 1,00	6,04 ± 0,54	7,50 ± 0,49	13,25 ± 0,49	0,031 ± 0,41	10,34 ± 1,15	47,68 ± 1,24	0,022 ± 1,86	0,94 ± 0,58
Cagliari	Rinascita	9,32 ± 2,99	20,88 ± 2,00	7,74 ± 0,46	15,18 ± 0,86	0,39 ± 0,40	61,87 ± 1,38	94,19 ± 0,92	0,22 ± 0,88	1,69 ± 0,86
	Darsena	19,40 ± 0,05	117,18 ± 0,81	20,00 ± 0,47	32,59 ± 0,90	2,20 ± 0,05	304,47 ± 1,88	422,59 ± 1,76	0,26 ± 1,13	4,60 ± 0,96
	S. Elmo	5,45 ± 1,27	141,90 ± 1,27	21,11 ± 1,43	30,06 ± 0,95	1,084 ± 0,18	120,70 ± 0,96	461,50 ± 3,80	0,64 ± 1,28	14,73 ± 1,25
LOD (mg L-1)	0,0005	0,00004	0,0061	0,0035	0,0001	0,0071	0,0036	0,1672	–	–
LOQ (mg kg-1)	0,0441	0,0029	0,0512	0,2951	0,0004	0,5907	0,2998	13,93	–	–
Recovery (%)		89	93	102	93	97	102	96	98	–

et al. (2016). Controls were conducted as untreated negative controls (0.22 µm FSW, collected from the sea urchin sampling site) and positive controls with exposure to CuNO₃ * 3H₂O (Sigma Aldrich, USA). To obtain this reference value, embryos in the positive control group were exposed to the following Cu nominal concentration 12, 24, 36, 48, 60 µg/L. Elutriates were tested both undiluted (100%) and diluted at dilution values of 25% and 50%. Six replicates were prepared for each sample using sterile capped polystyrene six-well microplates. Embryos were incubated in a dark room at 18 °C for 72 h; at the end of incubation, samples were fixed with 1% Lugol solution, rinsed in Phosphate Saline Buffer (PBS) and observed with transmitted light microscopy.

The percentage of normally developed plutei (P4) in each treatment was determined, following the criteria described in Pagano et al. (1986) by observation of 100 larvae. Plutei were classified as abnormal when presents skeletal aberration (apex spicules cross-linked or separated), deformation of gastrointestinal apparatus, arms deformation. The acceptability of the results was fixed at a percentage of normal plutei > 80% in negative control tests (Gaion et al., 2013).

Data have been expressed as Effective Concentration (EC) values using Probit analysis method described by Finney (1971).

2.9. Statistical analysis

In order to select the appropriate parametric (Anova and T tests) or non-parametric analysis (Kruskall-Wallis and Mann Whitney U test), data have been processed to assess normality and homoscedasticity. Data were considered normally distributed if the p value for the Shapiro Wilk test was ≥ 0.05 (Das and Rahmatullah Imon, 2013), or Skewness and Kurtosis were between - 1.96 and + 1.96 (Cosineau and Chartier, 2010). Homogeneity of variances was tested with Levene’s test (p ≥ 0.05), to select the appropriate post-hoc test in the analysis of variance

(Tuckey for homogeneous variances, Dunnett’s test otherwise). Additionally, to compute and measure the associations between the two sets of variables “Percentage of malformed plutei” and “Metals”, a Canonical Correlation Analysis (CCA) has been conducted for each individual protocol. Statistical significance for all the analytical procedures was set at values p < 0.05.

3. Results

With regards to the grain size composition, sediments of Toulon and Cagliari had a strong silt composition, generally over 45%, and exceeding the 90% in Corsica and Suffren site in the Port of Toulon and S. Elmo site in the Port of Cagliari (Fig. 1). The granulometric composition of the three sampling sites within each location (Toulon, Olbia and Cagliari) has been tested with a Chi-square Goodness of fit to assess the deviation of 2 sites (observed) from the remaining reference site (expected). All the individual comparisons resulted statistically different (p < 0.01) with the exception of Corsica vs Suffren in Toulon (p = 0.25) (see Supplementary Table, S4).

Except for the P1 site, where silt rises to 42.53% of the grain size fractions, samples collected in Olbia harbour had a predominant sandy composition (> 80%).

Table 1 shows organic matter content and heavy metal concentrations in the surface sediments of Toulon, Olbia, and Cagliari.

The highest concentrations of metals were found in the ports of Toulon and Cagliari. Among the metals analysed, high concentrations of mercury were found in Suffren and Corsica sediments (Toulon), where 2.13 and 9.85 mg/kg of Hg were measured respectively.

The highest Zinc concentration occurred in the Port of Cagliari at S. Elmo site, although similar concentrations were recorded, in the same harbour, in Darsena site, and for the Port of Toulon in the Corsica

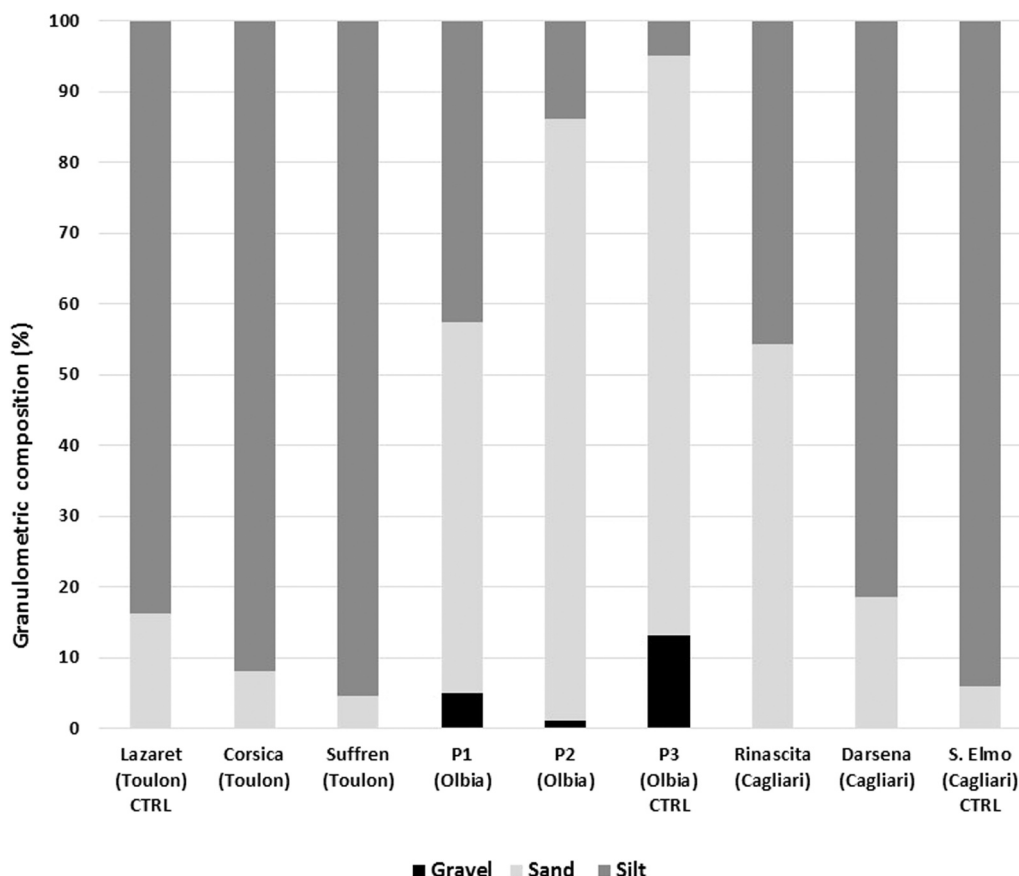


Fig. 1. Granulometric composition (%) of surface sediment collected in the Port of Toulon, Cagliari, and Olbia. “CTRL” indicates reference site for each study area.

sediment. For Lead, the highest concentration has been recorded in the Port of Cagliari, in the S. Elmo site, with 461 mg/kg although both in the Darsena site, in the port of Cagliari, and in the Corsica site, in the Port of Toulon, considerable concentrations have been measured (422.59 and 415.38 mg/kg respectively). Particularly critical in the two ports was the concentration of copper: in the Suffren and Corsica sites (Toulon), 136.79 and 462.90 mg/Kg were measured respectively, whereas in the Port of Cagliari, 141.90 and 117.18 mg/Kg of Cu were recorded in the S. Elmo and Darsena sites, respectively. In Olbia, concentrations of heavy metals are below the level of potential concern except for the P1 site, where Zinc was present with a high concentration of 119.22 mg/Kg. Chromium was found, in studied areas, with generally low concentrations (Table 1), whereas Nickel ranged, in Cagliari and Toulon harbour, between 7.74 and 25.48 mg/Kg.

Regarding the embryo development test with *P. lividus*, the percentage of well-developed plutei recorded in negative control (0.22 FSW) was 87% while the EC₅₀ value obtained with the reference toxicant (Cu(NO₃)₂ * 3H₂O) was 24.019 (21,135 –23.709) µg/L. This value falls within the range of laboratory control chart (22.60–68.34 µg/L) and agrees with the data reported in literature for this species with this reference toxicant (Arizzi Novelli et al., 2003; Sartori et al., 2016).

In the Port of Toulon, as reported in Fig. 2a, in the Corsica site, elutriate prepared according to ASTM, showed higher toxicity with respect to USACE ones (Fig. 2a), with a percentage of abnormal plutei of 50% and 20% respectively (p < 0.01). A significant difference (p < 0.01), in the percentage of malformed plutei, for ASTM and USACE method, was observed in the Suffren site as well (33.33% and 64.67% respectively). For the samples from the Port of Cagliari, elutriates prepared according to the ASTM method, showed the highest criticalities, with a percentage of well-developed plutei ranging between 0% and 6%. Contrary to what observed in the Port of Cagliari, in Olbia, the samples prepared following the USACE, showed the highest toxicity with a significantly higher percentage of malformed embryos (p < 0.05) in all three studied areas (Table 2).

The percentage of abnormal larvae of *P. lividus* increased along with the stirring time (3 h) in the ASTM method (ASTM 3 h), and values > 50% were reached with the S. Elmo, Rinascita, Darsena (Cagliari) and Corsica (Toulon) samples respectively (Fig. 2b). The highest percentages of abnormal plutei were recorded in the Port of Cagliari with values rising the 100% in Darsena and S. Elmo elutriates.

Samples prepared based on the USACE protocol increasing the stirring time to 3 h (USACE 3 h), showed similar trends of malformed plutei observed with the unmodified USACE method. An increase in the percentages of malformed plutei was observed with the USACE 24 h protocol, especially for the port of Olbia where the percentages of well-developed embryos < 30% in all samples (Table 2).

The total ammonium and nitrite concentrations (see Supplementary Table S3) detected in elutriates were not significant, and therefore not sufficient to justify the presence of confounding factors, assuming that the inhibition is the result of the presence of anthropogenic toxicants.

Both CCA analyses, resulted in a model significant at p < 0.001; in particular, CCA for protocol ASTM showed a total correlation coefficient for the two sets of data of 0.935 whereas with regards to USACE protocol the model resulted in a correlation coefficient of 0.662. Partial correlation coefficients and significances are reported in Table 3.

4. Discussion

The environmental sediment quality was discussed in comparison to the L1 (the lowest chemical level of reference) and L2 (the highest chemical level of reference), defined by the Italian Ministerial Decree 173/2016 which establishes criteria and methodological procedures for characterising marine and brackish sediments to be dredged, their classification and identification of appropriate management options and monitoring". In addition, metal concentration was evaluated considering the Probable Effect Level – PEL established in the Canadian

Sediment Quality Guidelines for the Protection of Aquatic Life (CSQG) by the Canadian Environmental Quality Guidelines Canadian Council of Ministers of the Environment (CCME, 1995).

In this study, the levels of Cu, Pb, Zn in the ports of Cagliari and Toulon were noteworthy and above the L1, L2 for the Italian legislation, and the PEL values reported in the CSQG (CCME, 1995) derived from studies on field-collected sediments that measured chemicals concentrations and associated biological effects. Samples from these locations presented fine grain size predominance (Fig. 1) and this fraction was strongly correlated with Cu, Pb, and Zn concentrations (Fig. 3), suggesting the occurrence of metal complexation processes. The observation that sediment grain size has a pronounced effect on heavy metal concentrations agreed with other studies, which reported the strong affinity of heavy metal with clay fraction (Roussiez et al., 2005; Liu et al., 2019). The high surface area of fine sediment particle serves as a foundation for increased adsorption of heavy metals (Mantie and Sappington, 1994) which can return to the water column, due to dissolution process caused by physical-chemical changes after resuspension (Maddock et al., 2007; Machado et al., 2011). In the port of Toulon considerable concentrations have been found also for Hg, whereas in the port of Cagliari sediments showed high level of contamination for Cd. Mercury concentration was noteworthy in the port of Toulon; in particular, in Suffren and Corsica sites, the levels of Hg detected were 2.13 and 9.85 mg/kg respectively, one order of magnitude higher than the PEL value and L1 and L2 values. The contamination found in the two ports is consistent with the findings of Tessier et al. (2011) for the port of Toulon and Schintu et al. (2016) for the port of Cagliari. The Port of Olbia did not show any particular criticality with regards to heavy metal, except for Zn which is particularly high in the sediments of the P1 site which showed a percentage of silt greater than 40%.

Considering the effects of metals on the embryonic development of *P. lividus* reported in the literature, EC₅₀ values vary between 0.02 and 0.11 mg/L and 0.5 and 11.24 mg/L for copper and cadmium respectively; whereas the EC₅₀ values for zinc ranged from 0.02 to 0.058 mg/L (Fernández and Beiras, 2001; Gaion et al., 2013; Manzo et al., 2013; Sartori et al., 2016). The concentrations of these metals found in the sediments from the ports of Cagliari and Toulon are well above the EC₅₀ values reported in the literature for this species and this finds a confirmation in the toxicity of elutriates (Table 4). As a result, possible additive and synergistic effects should be considered, as reported by Beiras and Fernandez (2001) with regards to different combinations of Hg with Cu, Pb and Cd.

The total ammonium concentrations detected in the elutriates were not significant (see supplementary table, S3) and fall below the EC₅₀ values reported in the literature for *P. lividus* and other sea urchin species (Arizzi et al., 2003; Saco-Álvarez et al., 2010) and therefore not sufficient to justify the presence of confounding factor.

Analysing the results of the embryotoxicity test with *P. lividus*, it can be observed that the increase in mixing time in the preparation of elutriates contributed to increasing the toxicity of the samples themselves, although with sometimes significant differences between the two protocols and between the three ports under investigation. In particular, for the port of Toulon in the Lazaret site, the ASTM 3 h and ASTM 24 h protocols showed higher toxicity for sediments compared to the USACE unmodified method, with an EC₂₀ value of 72.95% and 67.38% respectively. Also, the increase in mixing time between water and sediment from the Corsica site led to an increase in the toxicity of the samples analysed, albeit with opposite trends. Indeed, the ASTM 3 h and USACE 24 h protocol showed higher toxicity for sediments in this area, with EC₅₀ value of 77.91% and 78.74% respectively. These results confirm what reported by Arizzi Novelli et al. (2006) which showed that the commonly used 1:4 sediment: water ratio (ASTM) has not always been useful in detecting sediment toxicity, generally demonstrating a lower discriminatory capability than elutriate prepared with lower suspended solid concentration (1:20, 1:50, and 1:200 sediment: water ratio). However, if for Cu there is a rapid reduction in the amount of

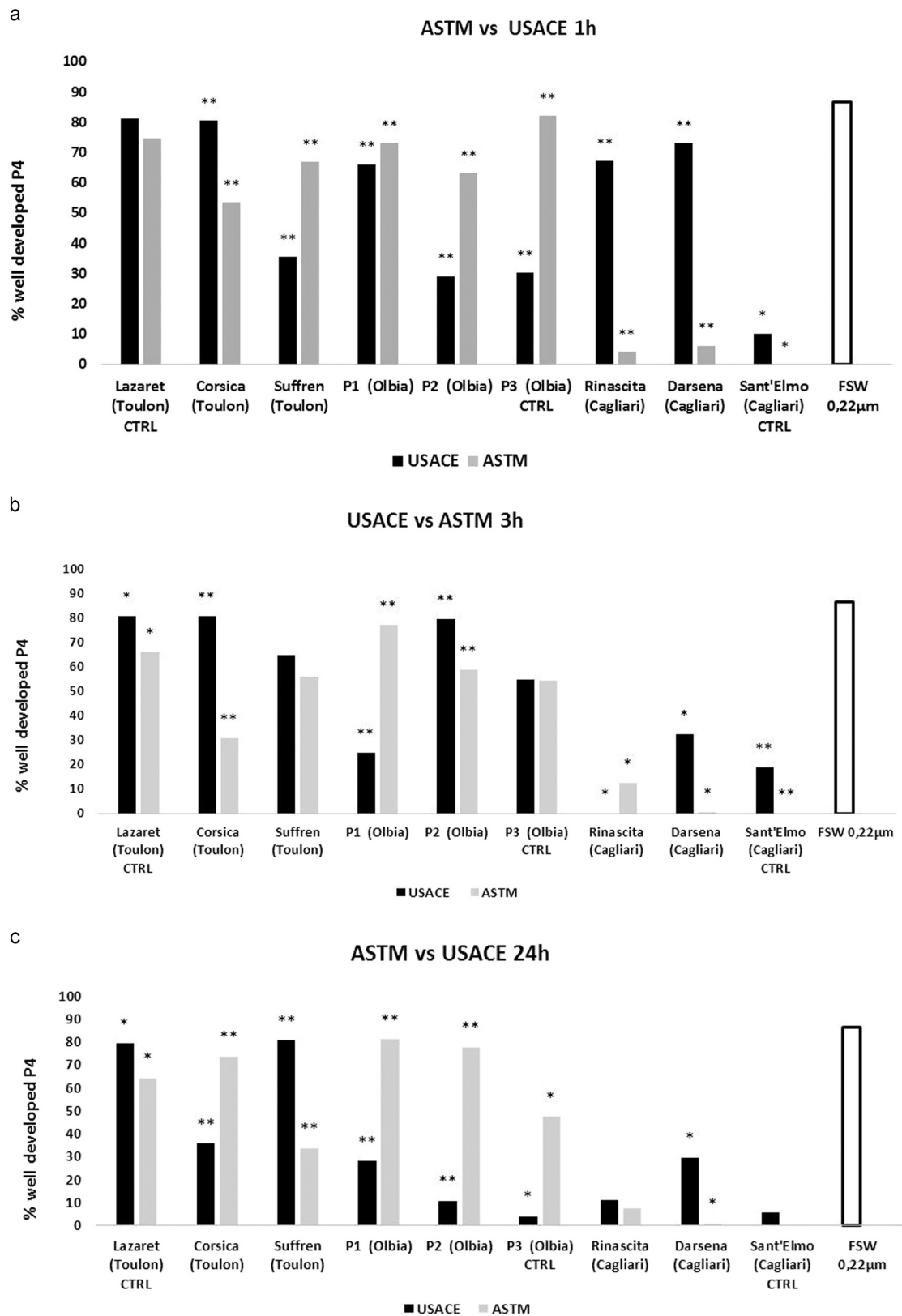


Fig. 2. Percentage (%) of well developed plutei (P4) in sediment elutriates prepared according to: standard ASTM and USACE method (a), ASTM and USACE modified method with 3 h of stirring time (b), and ASTM and USACE modified method with 24 h of stirring time (c). Percentage of normal plutei are expressed as mean among six replicates (n = 6) ± SD (vertical bars). “CTRL” indicates reference site for each study area, *Indicates statistical significance p < 0.05 (independent samples t-test, between results obtained from 3 elutriate dilution, six replicate each for ASTM and USACE elutriates), **Indicates statistical significance p < 0.01 (independent samples t-test, between results obtained from 3 elutriate dilution, six replicate each for ASTM and USACE elutriates). Lack of * indicates no statistical difference.

Table 2

Percentage (%) of normal developed plutei (P4) observed in sediment elutriate (undiluted) prepared according to ASTM and USACE protocol, with three stirring times (1, 3 and 24 h). Concentration are expressed as mean ± SD (n = 6).

		Normal developed P4 (%)					
		ASTM 1 h	USACE 1 h	ASTM 3 h	USACE 3 h	ASTM 24 h	USACE 24 h
Toulon	Lazaret	74,67 ± 1,53	81,00 ± 1,00	66,00 ± 1,15	81,00 ± 0,58	64,33 ± 1,53	79,33 ± 1,52
	Corsica	53,33 ± 2,08	80,33 ± 0,58	31,00 ± 1,00	81,00 ± 2,08	73,67 ± 1,53	35,67 ± 0,58
	Suffren	66,67 ± 1,15	35,33 ± 1,53	56,00 ± 2,65	65,00 ± 1,53	33,67 ± 1,53	80,67 ± 0,58
Olbia	P1	73,00 ± 1,53	66,00 ± 0,58	77,33 ± 0,58	25,00 ± 1,53	81,00 ± 1,00	28,00 ± 1,00
	P2	63,00 ± 1,15	29,00 ± 0,58	58,67 ± 1,53	79,67 ± 1,53	77,67 ± 1,53	10,67 ± 0,58
	P3	82,00 ± 1,00	30,00 ± 1,00	54,33 ± 1,53	54,67 ± 1,15	47,33 ± 1,15	4,00 ± 1,73
Cagliari	Rinascita	4,00 ± 1,15	67,00 ± 2,08	12,33 ± 2,08	0,00 ± 0,00	7,67 ± 1,53	11,33 ± 1,15
	Darsena	6,00 ± 2,65	73,00 ± 1,15	0,33 ± 0,58	32,33 ± 2,51	1,00 ± 1,00	29,67 ± 1,53
	Sant'Elmo	0,00 ± 0,00	10,00 ± 0,58	0,00 ± 0,00	19,00 ± 3,60	0,00 ± 0,00	5,67 ± 0,58

Table 3

Summary of the results from the CCA analysis showing the correlation between heavy metals and sea urchin larvae malformation for ASTM and USEPA protocol.

Metal	ASTM Protocol (p < 0.001; r = 0.935)		USEPA Protocol (p < 0.001; r = 0.662)	
	Loadings	Corr. Coeff. with % malformed	Loadings	Corr. Coeff. with % malformed
As	-0.047	n.s.	-0.649	-0.430
Cu	-0.020	n.s.	-0.337	-0.223
Ni	0.280	0.262	-0.381	-0.252
Cr	0.151	n.s.	-0.529	-0.350
Cd	0.883	0.825	0.461	0.305
Pb	0.610	0.570	-0.177	n.s.
Zn	0.515	0.481	-0.045	n.s.
Hg	-0.095	n.s.	-0.498	-0.329

dissolved metal as a result of the adsorption and precipitation processes, for Pb there is a continuous release from the sediments, until reaching the maximum concentration in solution after 6–9 h. Similarly, in a recent study testing a model combining geochemical and kinetic processes, Ciffroy et al. (2019) showed a release of Pb from oxides and carbonates from sediment during the first 10 h. In the long-term period, there is a diametrically opposed trend for these two metals, with the concentration of dissolved Cu increasing again and those of Pb decreasing (Dang et al., 2020).

For the port of Cagliari, elutriates prepared with both protocols showed high toxicity, in line with the high contamination of Pb, Zn, Cu and Cd occurring in these areas. The toxicity of the elutriates remained almost unchanged also by increasing the resuspension time of the sediments (3 and 24 h) with the exception of the Darsena and Rinascita samples which did not show any particular criticality, based on the unmodified USACE protocol. If results obtained in the Rinascita site were expected, considering the low contamination and the prevalent sandy composition (more than 50%) of the sediments, the high toxicity

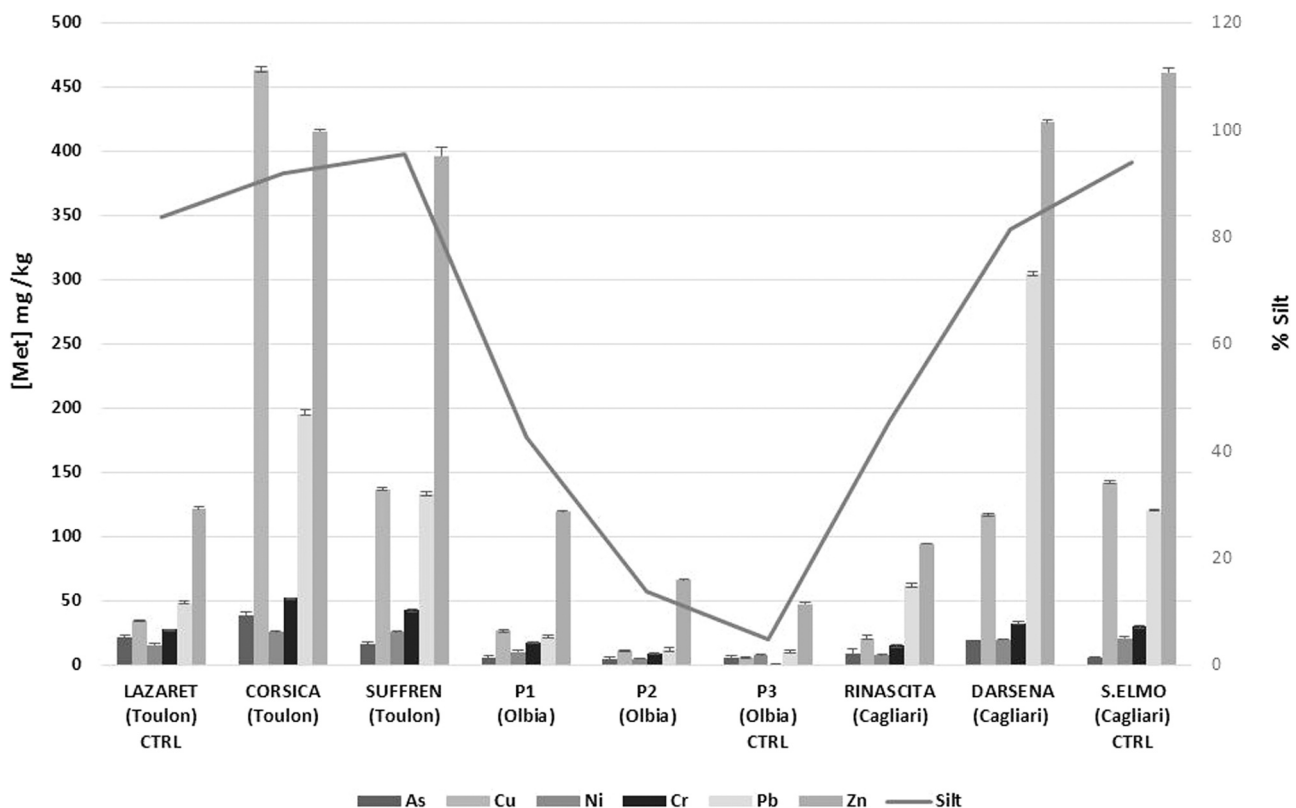


Fig. 3. Correlation between concentration of As, Cu, Ni, Cr, Pb, and Zn (mg/kg) and percentage (%) of silt in sampled sediment. Trace metal concentration are expressed as mean ± SD (vertical bars). “CTRL” indicates reference site for each study area.

Table 4

Correlation between silt abundance (%) in sediment sample, heavy metal of potential concern and elutriate protocol which showed the greatest and the least effect on embryo development test with *P. lividus*.

Site	Sample	Silt (%)	Protocol with > Effect in <i>P. lividus</i> test	Protocol with < Effect in <i>P. lividus</i> test	Heavy metal of potential concern
Toulon	Lazaret	83,7	ASTM 24 h	USACE; USACE 3 h	Zn, Hg
	Corsica	91,95	ASTM	USACE; USACE 3 h	As, Cu, Cr, Pb, Zn, Hg
	Suffren	95,44	ASTM 3 h	USACE 24 h	Cu, Pb, Zn, Hg
Olbia	P1	42,53	USACE 3 h; USACE 24 h	ASTM 24 h	Zn
	P2	13,81	USACE; USACE 24 h	USACE 3 h; ASTM 24 h	–
	P3	4,96	USACE 24 h	ASTM	–
Cagliari	Rinascita	45,62	ASTM 3 h; ASTM	USACE	Pb, Zn
	Darsena	81,48	ASTM; ASTM 3 h; ASTM 24 h	USACE	Cd, Cu, Pb, Zn
	S. Elmo	94,01	ASTM; ASTM 3 h; ASTM 24 h	USACE 3 h	Cd, Cu, Pb, Zn

revealed by *P. lividus* assay in Darsena elutriate was unexpected.

Noteworthy was the result obtained in the port of Olbia where the USACE protocol highlighted the most critical issues with the *P. lividus* bioassay, despite the low contamination of heavy metals. To better interpret the ecotoxicological results, PAHs and PCBs analysis on the sediments of the port of Olbia (see [Supplementary Methods](#)) were conducted. The analysis of organic contaminants did not detect the presence of important concentrations of PAHs in the sediments of the investigated area (see [supplementary Table S1](#)). Similarly, with regards to PCBs, it was found that some congeners were present although with extremely low concentrations (see [supplementary Table S2](#)) and far below the PEL levels reported in the CSQG and the L1 values reported in Decree 173/2016. The toxicity detected in Olbia sediments could be due to the presence of emerging contaminants (such as fragrances, flame retardants, sunscreens, etc.) which, for the Gulf of Olbia, characterised by a strong increase in human pressure during the spring-summer season, can considerably increase their concentration in the water column and consequently accumulate in the sediment.

In [Table 4](#), the percentages of silt in sediment samples and the corresponding elutriate protocol are reported; these factors resulted in the highest and lowest toxicity in the *P. lividus* bioassay. In general, for sediments with a percentage of silt greater than 80%, (Lazaret, Corsica and Suffren in Toulon; Darsena and S. Elmo in Cagliari) the protocol that highlighted the greatest toxicity is the ASTM protocol, whereas, for sandy samples (P1, P2 and P3 in Olbia), the most suitable protocol to highlight the greatest criticality is the USACE protocol. Furthermore, it is important to stress that samples with a high level of toxicity in elutriates prepared according to the ASTM method are characterised by high concentrations of Cu, Pb and Zn. These results show that the ASTM protocol, although with different intensities linked to the water-sediment mixing time, is the most suitable to reveal toxicity due to the presence of high concentrations of heavy metals. Further investigations should be conducted on this issue; however, these preliminary results suggest to consider an accurate choice of elutriate methodology for ecotoxicological bioassays based on the granulometric characteristics of the samples themselves.

From the analysis of CCA of our results, it appears that ASTM protocol is more efficient in extracting metals from the sediment evenly. The higher general correlation coefficient compared to USACE method (0.935 vs 0.662, respectively) is further confirmed by the higher number

of metals which positively correlated with malformed plutei. Indeed, in the first model, Ni, Cd, Pb and Zn all correlated importantly with the embryotoxicity, with particular emphasis on Cd and Pb whose coefficients are 0.825 and 0.570. With regards to the CCA of the USACE protocol, only Cd positively correlated with malformed plutei, with a lower coefficient than its counterpart (0.305).

5. Conclusions

The two methodological protocols used in the preparation of the elutriates provided partially comparable results for the ports of Toulon and Cagliari, whereas for the port of Olbia the data obtained were sometimes discordant. In general, the results of the embryo-development bioassay with *P. lividus* showed an increase in toxicity related to the increased mixing time between water and sediment during the preparation of elutriates, both for the ASTM and USACE protocols. This fact suggests a better extraction capability of hydrophilic compounds in the aqueous phase as the contact time between water and sediment increases. The variation of toxicity as a function of time can be related to the different kinetics of trace metals migrating from sediment to the aqueous phase during the preparation of elutriates ([Dang et al., 2020](#)), or with the synergistic toxicity of some heavy metals (such as Hg, Pb, and Cu) to the larval development of *P. lividus* ([Fernández and Beiras, 2001](#)).

The results obtained in this paper showed that the elutriate protocols provide a different biological response depending on the grain size composition, with a higher sensitivity given by the ASTM protocol for sample with high percentage of silt.

In Italy, dredged sediments can be classified, in five classes (MD n.173/2016) based on the integration of chemical analyses with the outcome of toxicological tests (weight of evidence criteria); this classification can be the discriminant factor for the management strategy of the sediments, ranging from nourishment of submerged coastal areas to disposal, and consequent handling precautions, into confined disposal facilities (CDF). An incorrect ecotoxicological classification of sediment due to inappropriate methodological procedures can result in loss of millions of Euros as well as representing a potentially environmental critical scenario.

A correct protocol which can improve the interpretations of the results obtained from bioassay, the uniformity, and the standardisation of the matrices preparation must necessarily be an objective for the future. Standardisation will permit to have comparable results obtained from different areas and different times, allow better management of sediments without compromising ecosystems of particular naturalistic value and productive activities, such as aquaculture facilities, whose damage can in turn create a risk to human health.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ecoenv.2021.112010](https://doi.org/10.1016/j.ecoenv.2021.112010).

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