Ecotoxicity of marine sediments: Sampling and laboratory artifacts and their impacts on risk classification

Serena Anselmi [a], Paolo Pastorino [b,*], Francesca Provenza [a,c], Monia Renzi [c,d]

[a] *Bioscience Research Center, via Aurelia Vecchia 32, 58015 Orbetello, Italy*
[b] *Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, via Bologna 148, 10154 Torino, Italy*
[c] *Department of Life Sciences, University of Trieste, via L. Giorgieri 10, 34127 Trieste, Italy*
[d] *Consorzio Nazionale Interuniversitario per le Scienze del Mare, CoNISMa, Piazzale Flaminio 9, 00164, Roma, Italy*

A B S T R A C T

Assessing the ecotoxicological risk of marine sediments is now a critical factor in deciding how to treat dredged material in harbor and coastal areas. Although ecotoxicological analyses are routinely required by some regulatory agencies in Europe, laboratory skills necessary to perform them are often underestimated. According to the Italian Ministerial decree No. 173/2016, ecotoxicological tests are performed on the solid phase and elutriates, and the classification of sediment quality is defined using the "Weight of Evidence" (WOE) approach. However, the decree does not provide adequate information regarding the preparation techniques and laboratory skills. As result, a wide variability among laboratories occurs. An error in the classification of ecotoxicological risk has a negative impact on the whole environmental quality and/or the economy and management of the interested area. Thus, the main aim of this study was to determine if such variability can affect the ecotoxicological outcomes of tested species and WOE associated classification, producing different options for the management of dredged sediments. Four different sediment types were selected to assess the ecotoxicological responses and their changes as a function of variability of the following factors: a) the storage time laps (STL) for both the solid phase and the elutriates; b) the methods used to prepare the elutriates (centrifugation *vs.* filtration), and the conservation method used for the elutriates (freshly prepared *vs.* freezing). Results suggest a wide variability of ecotoxicological responses among the four sediment samples here considered, differentiated according to chemical pollution, grain-size texture, and macronutrient contents. The storage time laps significantly affect the physicochemical parameters and the ecotoxicity of both the solid phase test and elutriates. For the elutriates preparation, centrifugation is preferred to filtration to preserve a better representation of sediment heterogeneity. Freezing of elutriates does not seem to show any significant effects on the toxicity. Findings allow to define a weighted schedule of the storage time of sediments and elutriates useful for laboratories to scale analytical priority and strategies related to different sediment types.

1. Introduction

In aquatic ecosystems, sediment is an important component supporting biodiversity associated to a large quantity of ecological niches and covering an important role for trophic webs (Burton, 1992a; ASTM, 2014). Surficial layers represent the sedimentary "active zone" because they interface between the deepest and biologically inactive layers and the water column (Burton, 1992b). Active layers of bottoms can be resuspended by bioturbation (Aller and Cochran, 2019), natural storms (Nowacki and Ganju, 2018) and human activities as well as dredging, that is able to suspend also deeper sediment layers (Burton, 1992b;

Wilber and Clarke, 2001) and other organic materials (Renzi et al., 2022). Dredging of harbors is the most common periodically recurring action in their management, as the high sedimentation rates occurring in harbor water led to rapid closure of channels and to the loss of their function (Bortone et al., 2004). Much of the world's shoreline is eroding, and shoreline loss is a widespread phenomenon (Houston, 1995; Cipriani et al., 2013). In the United States, this type of issue is the primary concern for summer tourism-based economies (Houston, 1995). To cite just a few data on this type of problem, in Tuscany Region (Italy) 113 km out of 230 km of coast are endangered (Cipriani et al., 2013). In this region alone, 2 million cubic meters of sand are needed annually to fill regressing beaches, while 150–200 million m^3 (100 cubic meters per

meter of beach) are needed to stabilize 4500 km of Italian coastline suffering from erosion (Bigongiari et al., 2015). Since the use of river sediments for these purposes has not been possible since the 1970s, sediments dredged from ports could be a real resource to reduce the high raw material costs that must be buy for beach replenishment activities, costs that can be around 20–40 euros/m^3 (i.e., Bigongiari et al., 2015). The Italian law, Ministerial Decree No. 173/2016 (MD, 2016), has recognized this importance and made it mandatory to use sediments from harbor bottoms, provided they are of good quality (Class A according to MD No. 173/2016), for beach replenishment.

Sediments are heterogeneous materials characterized by different composition in terms of granulometric structure and content of macronutrients and chemicals of natural origin (Renzi et al., 2009, 2011, 2015; Bigongiari et al., 2015). Chemical pollution of sediments in human-managed structures such as harbors is well known in the literature (Ausili et al., 1998; Fortune, 2006; Renzi et al., 2009; Ausili et al., 2012; ASTM, 2014). Some of them are particularly affected by human activities that can have specific impacts on sediment pollution (Renzi et al., 2009, 2011), while other harbors are in geographic areas that have natural anomalies due to their geological origin and where natural levels of trace elements are significantly higher than the average values of larger regions (Renzi et al., 2011, 2015). In addition, some areas are characterized by a high proportion of detritus composed by large amounts of materials of biological origin such as bioclasts and decomposing mass of phanerogam leaves (Renzi et al., 2022).

Most chemical reactions in sediments are related to organic matter and other biogenic materials such as calcium carbonates and silicates and affect pH and redox potential (Eh) in sediments (Pepi et al., 2020). In literature it is well known that well-oxygenated bottom sediments with low organic matter content and high gravel content usually have positive Eh values, whereas silty and organically enriched sediments are characterized by negative Eh values (Renzi et al., 2019). Furthermore, sediments are polluted by complex mixtures of chemicals that can be related to the type of environmental pressures (Renzi et al., 2009). Such complex mixtures can cause additive, synergistic, or antagonistic interactions that can affect the final toxicity of this matrix (Arizzi Novelli et al., 2006). Polluted sediments are a major problem in coastal areas, and the assessment of the ecotoxicity of this matrix needs to be accurately determined before dredging. On this path, several approaches have been developed in the literature to assess the associated risks (Brouwer et al., 1990).

In Italy, the risks to marine species associated with sediment pollution are assessed according to Ministerial decree No. 173/2016 (MD, 2016), which requires the determination of physicochemical and ecotoxicological variables to define sediment quality before dredging. According to this approach, the classification of sediment quality is defined using the "Weight of Evidence" approach (WOE), which is used as a broader calculation method (Del Valls et al., 2004; Broccoli et al., 2021). The final classification of risks associated with sediment dredging is the mathematical result of the integration between the ecotoxicological risk calculated based on the responses of a three-species battery and the chemical risk calculated based on a standard chemical classification of the matrix (obtained from the weighted risk associated to the measured levels of trace elements, butyl tins, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, organochlorine pesticides, and aliphatic linear hydrocarbons C > 12).

According to the Ministerial decree No. 173/2016 (MD, 2016), ecotoxicological tests are performed on the solid phase (wet sediment that must be stored at 4–6 °C and tested within 15 days after sampling) and on elutriates (testing the liquid phase that must be prepared within 10 days after sampling and analysed fresh-made or stored at -20 ± 1 °C). The Ministerial decree (MD, 2016) does not provide precise and adequate references regarding the preparation techniques of the elutriates (methods of separation of the sediment matrix from the liquid to be tested) nor the maximum storage time laps (STL) of the frozen elutriates. Ecotoxicity recorded on elutriates describes the effects of the release of contaminants from sediments into the water column and the associated risks to marine trophic webs (ASTM, 1990; Haring et al., 2012). Despite the importance of the information obtained from this type of test, elutriates can be generated by a variety of methods, i.e., by filtration (Mamindy-Pajany et al., 2010) or centrifugation (ICRAM, 2001). Also, the STL at -20 ± 1 °C of elutriates is not provided, and laboratories typically use different STL between preparation and testing. This aspect could be critical for the applicative routines of ecotoxicology according to the Ministerial decree (MD, 2016) when the number of samples that needs to be analysed in a STL shall be very high (some hundreds to thousands) and the knowledge of aspect focusing by this research became crucial. Therefore, a wide variability among laboratories occurs in terms of treatment times that can significantly affect the results. These differences could induce huge economic damages for stakeholders that have to assess and define possible sediment management strategies based on the ecotoxicological results. Indeed, sediment of low ecotoxicological risks could be managed in coastal area for beach nourishment activities; otherwise, sediments classified at high risk must be managed differently, including reclamation with deposition in the worst case, in a waterproofed reclaimed area (MD, 2016). The aim of this study was to determine if such variability can affect the ecotoxicological outcomes of tested species and WOE associated classification of tested battery (three species owning to different trophic levels) producing different options for the management of dredged sediments.

The factors tested in this study were the storage time laps (STL; for both the solid phase and the elutriates), the different methods used to prepare the elutriates (centrifugation *vs.* filtration), and the conservation method used for the elutriates (freshly prepared *vs.* freezing).

Finally, once defined the weight that tested factors of variability exert on ecotoxicological response, the other aim of this paper was to scale their extent in relation to the type of sedimentary matrix.

2. Material and methods

2.1. Experimental design and factor tested

Four different sediment types were selected to assess the ecotoxicological responses and their changes as a function of variability factors, which are of particular interest in the context of the different structure of the sediment matrix. The four sediment types were coded with a number (1–4) followed by the letters "NP". The following sediment samples were selected based on previous chemical analyses in the study area (see section 2.2.).

- 1NP - unpolluted sediment, high macronutrient levels (total organic carbon-TOC, total nitrogen-TN, total phosphorus-TP).

- 2NP - polluted sediment by chemicals, high TOC content.
- 3NP - sandy, unpolluted sediment, very low TOC content.
- 4NP - silty, low polluted sediment.

A hierarchically nested and randomly repeated experimental design was used to reduce type-I and type-II statistical errors, as described in the literature (Benedetti-Cecchi, 2004). The following experimental design is summarized in the simplified model shown in Fig. 1.

Several factors of specific interest were tested: sediment types (four levels: 1–4); storage time laps before starting the analyses (STL: T0-T4; 0, 5, 10, 15, and 30 days after sampling, respectively); three replicates (random, R1-R3). In the complex, 60 sediment samples were analysed in triplicates for the solid phase tests (n = 180).

In addition, a specific focus in this research was performed on elutriates (1:4 w/v; n = 240) to evaluate the importance of some additional factors of interest in the ecotoxicological responses. In this case, the following factors of variability were tested: different methods used to separate sediment particles from water during the preparation of elutriates (two levels): filtered at 0.45 μm, MF; and centrifuged at 1200 g for 20 min, MC); storage type (ST; two levels): testing elutriates freshly and immediately after their production (ST_I) and storing them frozen (ST_F) at $-20 \pm 1\ °C$ until analyses; replicates (three levels, random, R1-R3).

2.2. Sampling

The selection of sampling sites was based on the results of previous studies performed on sediments in Talamone (Maremma Regional Park, Italy) (unpublished data). Indeed, previous studies were useful to select different sampling sites in the study area that are characterized by different chemical pollution levels of sediments, grain-size texture, and macronutrient contents.

In July 2021, approximately 4.0 kg of each surficial 1-4NP sediment layer (0–50 cm) was sampled on the same day using a manual polycarbonate drill (10 cm inner diameter). At each sampling station, six replicates of each sediment core were collected and accurately homogenized in a glass jar using a decontaminated disposable spatula to obtain enough sediment sample to perform the whole experimental study. The homogenized samples were stored in glass jars and transported to the laboratory at $5 \pm 1\ °C$ in a thermostatic refrigerator equipped with temperature control system (Alpicool, mod. CF55).

2.3. Laboratory pre-treatment of collected samples

2.3.1. General treatment and conservation

Each glass jar containing sediment samples (1-4NP) collected in the field was homogenized under a fume hood (HEPA-II filtered) to yield two sediment aliquots of approximately 2.0 kg. Sediment aliquots were sub-fractionated to obtain the homogeneous sediment fractions to be analysed at different STL (T0-T4). Initial responses (T0) were assessed by starting analyses as far as possible as described in the next sections. The effects of STL were assessed after 5 (T1), 10 (T2), 15 (T3), and 30 (T4) days. During the experiment, sediment aliquots were stored in incubation chambers with temperature control and monitoring systems at $5 \pm 1\ °C$ (Peltier Memmet, mod. IPP55). At T0 and at defined STL, stored sediments aliquots were weighted to prepare elutriates for liquid-phase tests following the method described in section 2.3.2. (Elutriates production). Elutriates were tested both immediately after preparation and at different STL (T1-T4) preserving them at $-20 \pm 2\ °C$. Concerning frozen elutriates, thawing of aliquots to be tested occurred at $5 \pm 1\ °C$ (Peltier Memmet, mod. IPP55) for 24 h till complete dissolution of ice.
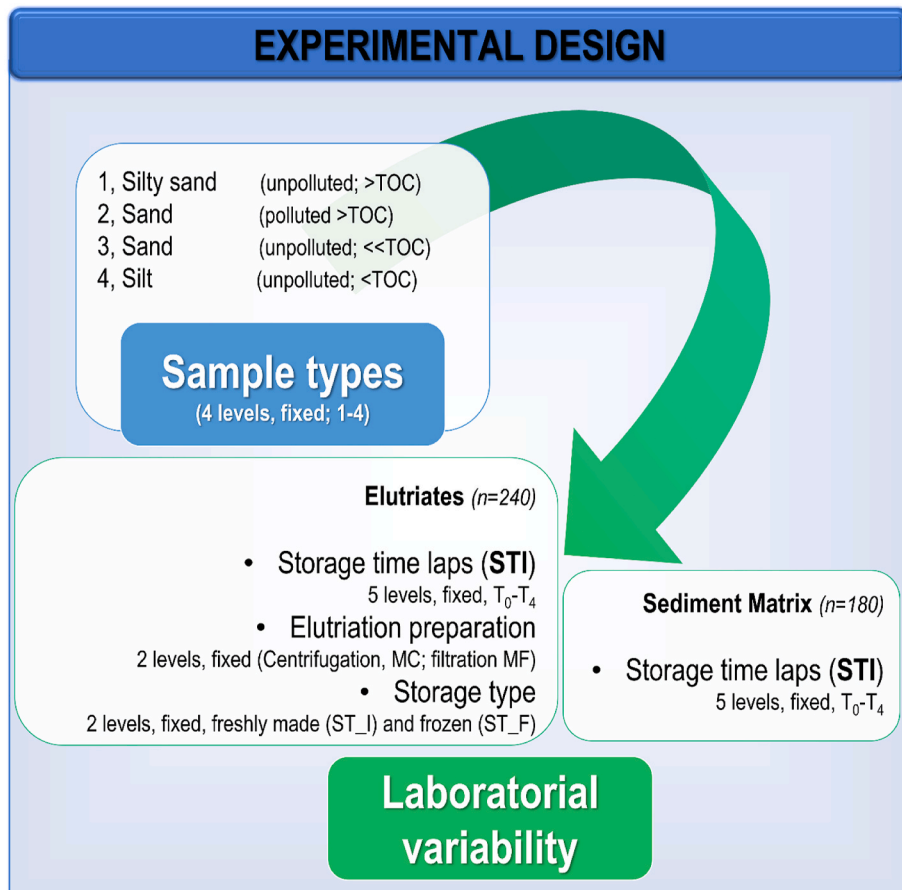
Fig. 1. Experimental design. Four sediment types characterized by a different starting grain-size and chemical compositions were tested. Ecotoxicity of sediments was evaluated immediately after sampling on both solid phase and elutriates to define starting levels of ecotoxicity. Furthermore, the effect on ecotoxicity of possible laboratory interferences such as Storage Time Laps (STL; T1-T4; 5, 10, 15, and 30 days), elutriate preparation methods (centrifugation, MC and filtration MF) and storage type of elutriates (ST; freshly made, ST_I and frozen at $-20 \pm 1\ °C$, ST_F) were also tested.

2.3.2. Elutriates production

Elutriates were prepared from wet sediments samples according to the weight/volume ratio of 1:4 as reported by the Ministerial decree n. 173/2016 (MD, 2016). The elutriates were prepared following the reference analytical methodologies for sediments (ICRAM, 2001); natural filtered seawater (0.45 μm) collected from a non-contaminated marine site in the Maremma Regional Park (Talamone) was used for the preparation of the elutriates. The filtered natural seawater was tested before being used to ensure the absence of any ecotoxicological effects. Furthermore, it was used to prepare negative controls per each batch of analyses.

Concerning the elutriate preparation, two different methods were followed to test if different techniques used by laboratories to separate the solid phase from the water could affect the ecotoxicity recorded on

Table 1

Physicochemical and ecotoxicological analyses performed in tested samples. Variables of interest and abbreviation used to refer to them are reported along with methods, measurement units, and limit of quantification. Notes: part a) is referred to methods applied to the analyses performed on sediment samples; part b) is referred to methods applied to the analyses performed on elutriates; part c) is referred to methods followed for ecotoxicological tests performed on both sediment samples (solid phase test) and elutriates (liquid phase tests). LOQ is the limit of quantification associated to the method for the specified matrix, the symbol "-" means not applicable.

Sediments	Abbr.	Method	Unit	LOQ	
H^+ content	pH	UNI EN ISO 10523:2012	pH units	0.1	
Redox activity	Eh	EPA SESDProc-113R2:2017	mV	1	
Colour	–	CIE L*a*b	L*a*b*	–	
Grain-size	–	Manuale ICRAM (2001), Scheda 3 S	%	0.1	
Water content	U	UNI EN 12880:2002	%	0.1	
Total organic carbon	TOC	UNI EN 13137:2002	%	0.3	
Total nitrogen	TN	MD 13/09/1999 Met.XIV.2	%	0.01	
Total phosphorous	TP	EPA 3050 B 1996 + EPA 6010C 2007	mg/kg	1	
Aluminium	Al	UNI EN 13657:2004 + UNI EN ISO 17294–2:2016	mg/kg	1	
Arsenic	As	UNI EN 13657:2004 + UNI EN ISO 17294–2:2017	mg/kg	1	
Cadmium	Cd	UNI EN 13657:2004 + UNI EN ISO 17294–2:2018	mg/kg	1	
Chromium	Cr	UNI EN 13657:2004 + UNI EN ISO 17294–2:2019	mg/kg	1	
Copper	Cu	UNI EN 13657:2004 + UNI EN ISO 17294–2:2020	mg/kg	1	
Mercury	Hg	UNI EN 13657:2004 + UNI EN ISO 17294–2:2021	mg/kg	0.03	
Nickel	Ni	UNI EN 13657:2004 + UNI EN ISO 17294–2:2022	mg/kg	1	
Lead	Pb	UNI EN 13657:2004 + UNI EN ISO 17294–2:2023	mg/kg	1	
Vanadium	V	UNI EN 13657:2004 + UNI EN ISO 17294–2:2024	mg/kg	1	
Zinc	Zn	UNI EN 13657:2004 + UNI EN ISO 17294–2:2025	mg/kg	1	
Polychlorinated biphenils	Σ PCB	EPA 3545 A 2007 + EPA 8270E2018	μg/kg	0.1	
Dichlorodiphenyldichloroethane	Σ DDD	EPA 3545 A 2007 + EPA 8270 E 2017	μg/kg	1	
Dichlorodiphenyltrichloroethane	Σ DDT	EPA 3545 A 2007 + EPA 8270 E 2017	μg/kg	1	
Dichlorodiphenyldichloroethylene	Σ DDE	EPA 3545 A 2007 + EPA 8270 E 2017	μg/kg	1	
Chlordan	–	EPA 3545 A 2007 + EPA 8270 E 2017	μg/kg	1	
Aldrin	–	EPA 3545 A 2007 + EPA 8270 E 2017	μg/kg	1	
Dieldrin	–	EPA 3545 A 2007 + EPA 8270 E 2017	μg/kg	1	
Endrin	–	EPA 3545 A 2007 + EPA 8270 E 2017	μg/kg	1	
α-Hexachlorocyclohexane	α-HCH	EPA 3545 A 2007 + EPA 8270 E 2017	μg/kg	1	
β- Hexachlorocyclohexane	β-HCH	EPA 3545 A 2007 + EPA 8270 E 2017	μg/kg	1	
γ-Hexachlorocyclohexan	γ-HCH	EPA 3545 A 2007 + EPA 8270 E 2017	μg/kg	1	
Eptachlor epoxid	HpE	EPA 3545 A 2007 + EPA 8270 E 2017	μg/kg	1	
Hexachlorobenzene	HCB	EPA 3545 A 2007 + EPA 8270 E 2017	μg/kg	1	
Polyciclic Aromatic Hydrocarbons	Σ PAH	EPA 3545 A 2007 + EPA 8270E2018	μg/kg	1	
Linear aliphatics light	C > 12	EPA 3550C 2007 + EPA 8015D 2003	mg/kg	5	
Linear aliphatics heavy	C < 12	US EPA 5021 A/2003 + US EPA 8260 C/06	mg/kg	0.1	
Organotins	Σ BTs	ICRAM Metodologie di riferimento (2001) – App.1	μg/kg	1	
Elutriates		Method	Units	LOQ	
H^+ content	pH	UNI EN ISO 10523:2012	pH units	0.1	
Dissolved oxygen	DO	UNI EN ISO 5814:2013	mg/L	0.1	
Salinity	S	APAT CNR IRSA 2070:2003	g/L	0.1	
Ammonium ion	NH_4^+	APAT CNR IRSA 4030:2003 met.A1	mg/L	0.01	
Ecotoxicity	Test type (Tables 2–3) All. 1 M.D. 173/2016)	Method	Endpoint	Exposure time	Meas. Unit
Aliivibrio fischeri	Type I - Solid phase	UNI EN ISO 11348–1 + ICRAM S11 (App. 2), 2001	Inhibition of bioluminescence	30 min	%
Aliivibrio fischeri	Type II - Elutriates	UNI EN ISO 11348-1	Inhibition of bioluminescence	15, 30 min	%
Phaeodactylum tricornutum	Type II - Elutriates	UNI EN ISO 10253:2017	Growth inhibition	72 h	%
Paracentrotus lividus	Type III - Elutriates (Larval stages)	EPA/600/R-95-136/S15 + ISPRA 11/17	Embryotoxicity	72 h	%

the tested elutriates. Following the literature, solid-liquid separation processes tested were filtration (MF; vacuum pump, 0.45 μm, Whatman-Philtre discs of nitrocellulose fibres) (Mamindy-Pajany et al., 2010) and centrifugation (MC; 1200 g, 15 ± 1 °C; 20 min) (ICRAM, 2001). Physicochemical parameters (section 2.4.) were measured in all elutriates to monitor their variability in relation to the production techniques. Finally, a portion of the elutriates produced at T0 were tested immediately to determine ecotoxicological responses, while an aliquot of each sample was stored at −20 ± 2 °C and tested after different STL (5, 10, 15, and 30 days) of freezing to evaluate the effect of the storage method and assess significant differences on ecotoxicological responses.

2.4. Physicochemical analyses

The physicochemical variables measured in this study, their associated methods, measurement units, and limit of quantification (LOQ) are listed in Table 1. Abbreviations related to the measured variables are also reported in Table 1.

2.4.1. Physicochemical analyses of natural sediments
Selected sediment samples (1–4 NP) were collected and analysed to determine their natural physical and chemical properties before starting the experiment. Grain-size, water content, macronutrient content (total organic carbon-TOC, total nitrogen-TN, total phosphorus-TP), and levels of chemicals of environmental interest were measured using standardized methods (Table 1). Three random replicates obtained from the same homogeneous sediment aliquot were analysed to determine means and standard deviations for each variable and sediment types.

2.4.2. Physicochemical analyses of all sediment samples
On each sediment sample studied at different temperatures and STL times, the main physicochemical parameters were assessed. pH and redox potential (Eh; Hanna device, pH/Eh-meter; HI83141 A0049215), water content (weight loss after drying in oven at 40 ± 1 °C, Peltier Memmert mod. UN55), grain-size (ASTM standard sieve ½ phi; vibrating sieve, CISA, BA200N), and colour (Eoptis, CLM-194, CieL*a *b*) were determined to evaluate the baseline level of these factors (Table 1).

2.4.3. Physicochemical analyses of the elutriates
On each elutriate, the main physicochemical parameters such as pH (Hanna Instrument, pH metre; HI83141 A0049215), salinity (Hanna Instrument, HI763100), dissolved oxygen (DO, water tester, JPB-607a), ammonium concentration (NH_4^+ spectrophotometry, Peak Instruments, c-7100 S) were measured (Table 1) and monitored under different conditions.

2.5. Ecotoxicological analyses

Three species belonging to three different trophic levels (Mariani et al., 2006) were tested to evaluate the ecotoxicological response of each species and calculate the ecotoxicological risk associated with the sediment based on the weight-of-evidence approach (WOE) reported by the Ministerial decree n. 173/2016 (MD, 2016) and by the literature (Broccoli et al., 2021) (Table 1).

Tests were performed on corrected elutriates if needed (when levels of physicochemical parameters of relevance for tested species such as salinity, pH, oxygen level resulted above the optimal range of fluctuation indicated by the followed method).

2.5.1. Ecotoxicological tests on sediments and elutriates
Concerning tests on sediments, three species owning to different trophic levels: bacteria (*Aliivibrio fischeri*, solid phase test, type-I according to All. 1, Ministerial decree 173/2016, UNI EN ISO 11348–1 + ICRAM S11, App. 2, 2001), algae (*Phaeodactylum tricornutum*, liquid phase test, type-II according to All. 1, Ministerial decree 173/2016, UNI EN ISO 10253:2017), and echinoderm (*Paracentrotus lividus*, liquid

phase test, elutriate 90%, type-III, according to All. 1, Ministerial decree 173/2016, EPA/600/R-95-136/Sect. 15 + ISPRA, 2017) were tested in replicates (R1-3; n = 3).

Results were expressed as follows.

- Bacteria: percentage of inhibition of natural bioluminescence at the maximum tested concentration (I%), and natural sediment toxicity (TU_{50}) with the associated range of confidence (95%) after 30-min of exposure.
- Algae: percentage of inhibition of the growth (I%), as means and standard deviation after 72-h of exposure.
- Echinoderm: percentage of abnormal pluteus (A%) normalized for results obtained in negative controls by the Abbott's formula after 72-h of exposure (Abbott, 1987).

Data associated with reported effects were opportunely normalized to negative controls if indicated in the standardized method followed for the specific analysis. Concerning statistics and elaboration performed following Ministerial decree n.173/2016 requirement, data were expressed as required by the specific software used (Sediqualsoft®, ISPRA, Italy).

2.5.2. Ecotoxicity of NH_4^+ on tested species
Because the ammonium is a biological by-product that originates from the decomposition processes (Mackin et al., 1984), the ecotoxicity of scalar solutions of ammonium (NH_4Cl, Merk, 1.01145.0500) were tested. In this case, ecotoxicological tests were performed using the *A. fischeri* liquid phase test, Type II, M.D. 173/2016 (methods reported in Table 1).

Furthermore, the ecotoxicity of natural sediments (1–4 NP) spiked with scalar solutions of ammonium (2 μg/g; 70 μg/g; and 140 μg/g; *Aliivibrio fischeri* solid phase test, Type I, Ministerial decree 173/2016) were tested to evaluate the effects of ammonium concentrations on results of the solid phase test. Scalar dilutions for liquid phase tests (*Paracentrotus lividus*, *Phaeodactylum tricornutum*) were analysed to determine EC_{50} values and associated confidence intervals for pure chemicals and mixtures. Results were expressed as toxicity recorded for NH_4Cl for all tested type and species. Concerning *P. lividus*, to better refer results to literature, ecotoxicological responses were expressed both as NH_4^+ and NH_4Cl. EC_{50} was expressed as μg/g for the solid phase, and as mg/L for the liquid phase tests.

2.6. Quality assurance and quality control

Physicochemical determinations and ecotoxicological tests were performed under UNI EN ISO 9001:2015 and UNI EN ISO 17025:2019 guidelines. All results and laboratory parameters during experiments and tests were recorded, traced, and validated by qualified personnel. The performance of the laboratory was monitored throughout the year by staff qualification tests (one per year) and blind intercalibration exercises performed by a qualified staff. The equipment and materials used for the analyses were intended for single use or were carefully decontaminated prior to testing. Any deviation from the ranges specified in the followed methods for tested variables and species was considered acceptable, and the deviating results, if any, were replicated. Analyses were performed using calibrated equipment and qualified personnel; both blank samples and standard reference materials were analysed to control the overall quality of the analyses performed. The instruments used for data collection were calibrated by LAT (Laboratory of Calibration) and monitored using reference standard materials.

During tests, temperature during exposure and light-dark cycles were strictly maintained within the standards specified in the followed methods for each species tested and were closely monitored during the ecotoxicological tests. Parameters (i.e., pH, DO) of the samples tested were recorded and monitored. Negative and positive controls were also performed for each analytical series and were found to be acceptable.

Negative controls were performed with naturally filtered seawater (0.45 µm, Whatman). Acceptable values for negative controls were respected in all tested species. In *P. tricornutum* and *P. lividus* (72 h), the growth rate was >16 times and anomalous larvae were <20%, respectively. Positive controls were performed with the following toxicants: 3,5-dichlorophenol (*A. fischeri*, Agilent - 591-35-5), potassium dichromate (*P. tricornutum*, $K_2Cr_2O_7$, Carlo Erba - 7778-50-9) and Cu^{2+} solution (*P. lividus*, Applichem PanReac - 313178.1208).

The observed responses of the tested species to the toxicants were within the following acceptance ranges.

- *A. fischeri*: 20–80% inhibition of natural bioluminescence after 30 min exposure to 4.5 mg/L solution of 3,5-dichlorophenol.
- *P. tricornutum*: $EC_{50} = 20.1 \pm 5.3$ mg/L after exposure to $K_2Cr_2O_7$.
- *P. lividus*: $EC_{50} = 22.60$–68.34 µg/L after exposure to Cu^{2+}.

2.7. Statistical analyses

Physicochemical parameters were analysed to determine univariate (Microsoft Excel, Microsoft Corporation, USA) and multivariate relationships among variables (Primer v.7.0, University of Plymouth). Ecotoxicological test results were elaborated using Toxicity Relationship Analysis Trap® software (Trap, U.S. EPA/NHEERL/MED) to determine EC_{50} values for positive controls, pure chemicals, and chemical mixtures tested in this study for each species considered. Univariate statistics (test-T Student, $p < 0.05$) and Spearman's correlation matrix were applied. Statistical significance was set at $p < 0.05$.

Results from this study were also analysed using multivariate statistical analysis (Primer v.7.0, Plymouth University) to determine the significance of the factors tested for the observed ecotoxicological responses. Data were pre-treated with square root and normalized before calculating the Euclidean matrix of distances between samples. Principal component analysis (PCA) was performed on the whole dataset to evaluate similarities among sediment types. Non-metric multidimensional scaling (*nm*-MDS) and ANOSIM tests (one-way test, 9999 permutations; Pairwise test for couple-distance significant) were then performed to examine the significance of the observed distribution by the tested factors of potential variability.

2.8. Classification of ecotoxicological sediment risk according WOE approach

Hazard Quotients (HQ) of each species and tested battery associated with each sample examined in this study were calculated using the weight of evidence approach (WOE) (Broccoli et al., 2021; Piccardo et al., 2021) and, as reported in Ministerial decree No. 173/2016 App.1 (MD, 2016), calculations were performed using Sediqualsoft® v. 109. x online release (license, 020, issued by the College of Marche; last running at 10th September 2022).

Hazard Quotients were calculated under each tested conditions to evaluate as the final ecotoxicological risk could be affected by tested factors. The tests on *P. lividus* used for the calculations of HQ were performed with 1:4 elutriates (90%) as indicated in the literature for the specific test (ISPRA, 2017). The Sediqualsoft® routine was performed for ecotoxicological effects recorded on samples without normalization to negative controls as reported for the application of this software.

To obtain the sediment quality class reported in the Ministerial decree No. 173/2016 (MD, 2016), the processing of the chemical data started with the comparison of the concentrations measured in the sediments with two limit values (L1 and L2); for each chemical parameter analysed, the deviation from the limit value was calculated and corrected according to the "weight" of the pollutant to obtain a value and highlight the importance of the deviations observed for the most hazardous pollutants. For our purposes, the chemical risks were not calculated, but only characterized to have a clear idea of how the ecotoxicological analyses can be affected at T0 and under different treatments tested depending on the sediment matrix (i.e., granulometry, pH, redox condition), the presence of organic matter and the chemical pollutants.

Formulae applied for the calculation of the HQ of the tested battery and ecotoxicological risk associated to the sediment type tested are widely described and reported in the Ministerial Decree No. 173/2016 App. 1 (MD, 2016) and in literature (Broccoli et al., 2021; Piccardo et al., 2021).

3. Results

3.1. Characteristics of the natural sediments

3.1.1. Physical features

The results of physicochemical analyses performed are summarized in Table 2. The main granulometric fractions considered in this study were the three indicated in ICRAM S3 – Determination of the grain-size of sediments (ICRAM, 2001): gravel (x > 2 mm), sand (0.063 < x < 2 mm) and silt (mud + clay; x < 0.063 mm). Comparing the four sediment types sampled in this study, the 1NP sediment consists of about 1/3 of the total mass of silt and a large proportion (more than 50% dry weight, d. w.) of bioclasts larger than 2 mm consisting of bivalves and calcareous fragments of biological origin classified as gravel (bioclasts). 2NP consists mainly of sands (about 80% d. w.) with a silty component of less than 20%. 3NP is a sandy sediment with a very small amount of silt (about 3% d. w.). Sediment sample 4NP is a silty sediment (90% silt, 10% sand d. w.).

In sediments, pH varied between 7.6 (2NP) and 8.2 (4NP). The redox potential (Eh) ranged from an oxidized (+53.0 mV; 3NP) to a reduced (−205.5 mV; 4NP) level. The Spearman's correlation matrix between the tested variables were also calculated (Supplementary material, Table S1). Values of pH, and Eh were negatively correlated ($\rho S = -0.666$; $p < 0.01$; see Spearman's correlation matrix, Suppl. Mat. Table S1). Eh was also negatively correlated with silt content in sediments ($\rho S = -0.997$; $p < 0.01$), and with macronutrients (TOC, $\rho S = -0.762$; $p < 0.01$). The redox potential was related to sediment colour, which was: brownish grey for 4NP; blackish brown for 1NP, while the other two sediments were both brown.

3.1.2. Chemical features

The Ministerial decree No. 173/2016 (MD, 2016) defines chemical features that must be determined in dredged sediments and others that shall be determined by technical reasons due to the presence of presumed pollution sources. Tested chemicals were compared to threshold values of hazard named L1 and L2. The detected levels of macronutrients and chemicals of ecological and ecotoxicological interest according to the Ministerial decree No. 173/2016 (MD, 2016) are shown in Table 2 for samples 1–4 NP; L1 and L2 limits (when defined) were also reported for the chemicals tested. Chemicals detected in the tested samples 1-4NP that exceeded the L2 threshold are highlighted in red.

Regarding the measured concentrations of macronutrients of particular interest, the collected samples showed significant differences. Based on the measured values, 1NP is unpolluted, TOC-enriched silty-gravelly sediment characterized by a high TOC content (1.8 ± 0.2%) associated with a high content of TN (0.43 ± 0.22%) and TP (220.0 ± 50.5 mg/kg). 2NP is a naturally contaminated sediment with a sandy prevalence and a high TOC content (1.9 ± 0.3%). 3NP is a non-contaminated sandy sediment with a very low content of TOC (0.3 ± 0.1%), TN (0.18 ± 0.03%), and TP (106.3 ± 5.5 mg/kg). Sediment sample 4NP is a silty sediment characterized by a high TOC content (2.1 ± 0.3%) associated with a high content of TN (0.30±<0.01%), and TP (187.7 ± 124.9 mg/kg). Macronutrients were reciprocally positively correlated (ρS TOC *vs.* TN = 0.578; ρS TN *vs.* TP = 0.929; ρS TP *vs.* TOC = 0.665). In addition, there was a positive correlation with water content, and silt content ($\rho S = 0.604$), while there was a negative correlation with Eh value ($\rho S = -0.762$ TOC; $\rho S = -0.611$ TP) and sand content

Table 2

Physicochemical characteristics of sediment. In this table, principal features of the four different sediment types tested in this study are reported and summarized. Mean values (*n* = 3) and standard deviations (SD) are reported for 1-4NP sediments collected (Regional Park of Maremma, Talamone site). Data reported in this table are values recorded performing analyses immediately after sampling (value at T0). In section "a", grain-size, water content, pH, Eh, and colour are reported. In section "b", chemical composition (mean and standard deviation) is summarized concerning macronutrients, metals, and organic pollutants. L1 and L2 thresholds are also reported (MD, 2016). For the abbreviation, see Table 1. ΣBTs = sum of measured levels of mono-butyltin, dibutyltin, tributyltin forms; ΣPCB = sum of congeners: 28, 52, 77, 81, 101, 118, 126, 128, 138, 153, 156, 169, 180; ΣDDD = 2,4′-DDD+4,4′-DDD; ΣDDE = 2,4′-DDE+4,4′-DDE; ΣDDT = 2,4′-DDT+4,4′-DDT; ΣPAH = sum of levels of fluoranthene, naphthalene, anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo (g,h,i)perylene, Inden (1,2,3-cd)pyrene, acenaphthene, fluorene, phenanthrene, Pyrene, Benzo(a)anthracene, Chrysene, Dibenzo (a, h)anthracene, Acenaphthylene. LOQ = limit of quantification; NC = incalculable. Cells highlighted in yellow means values > L1, red cells mean values > L2.

a – Physical features		Gravel (%)		Sand (%)		Silt (% Mud + Clay)		Water content (%)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
1NP	Unpolluted TOC enriched	51.0	1.2	14.9	0.1	34.0	1.2	43.6	2.5
2NP	Polluted TOC enriched	2.8	0.4	78.5	2.4	18.8	2.1	48.2	4.3
3NP	Sandy sediment	3.9	0.3	93.0	1.2	3.1	1.5	20.6	1.5
4NP	Silty sediment	0.0	0.0	9.8	0.6	90.2	0.6	37.5	0.3

	Sample description	pH		Eh (mV)		Colour CIE L*a*b* coordinates		
		Mean	SD	Mean	SD	L*	a*	b*
1NP	Unpolluted TOC enriched	7.8	0.1	−39.0	2.8	45,37	0.36	10.28
2NP	Polluted TOC enriched	7.6	0.1	+24.0	4.2	44.59	1.79	12.03
3NP	Sandy sediment	8.0	0.1	+53.0	4.2	51.56	−0.03	11.20
4NP	Silty sediment	8.2	0.1	−205.5	43.1	43.28	0.35	7.05

b – Chemical features		M.D. 173/16		1NP		2NP		3NP		4NP	
		L1	L2	Mean	SD	Mean	SD	Mean	SD	Mean	SD
TOC	%	–	–	1.8	0.2	1.9	0.3	0.3	0.1	2.1	0.3
TN	%	–	–	0.43	0.22	0.30	<0.01	0.18	0.03	0.30	<0.01
TP	mg/kg	–	–	220.0	50.5	142.9	63.8	106.3	5.5	187.7	124.9
Al	mg/kg	–	–	30,489	6531	4011	865	2897	202	10,032	8881
As	mg/kg	12	20	17.5	2.7	35.4	7.2	3.9	0.4	19.9	11.8
Cd	mg/kg	0.3	0.8	0.043	0.021	<LOQ	NC	0.025	0.021	<LOQ	NC
Cr	mg/kg	50	150	68.9	13.9	17.6	4.7	16.3	1.8	33.4	26.3
Cu	mg/kg	40	52	51.4	32.1	13.2	0.4	12.2	11.7	21.9	19.8
Hg	mg/kg	0.3	0.8	0.79	0.10	0.71	0.16	<LOQ	NC	0.69	0.40
Ni	mg/kg	30	75	54.4	11.8	15.6	1.4	15.5	1.6	28.9	24.3
Pb	mg/kg	30	70	27.5	6.9	11.4	0.8	2.4	0.4	10.2	6.5
V	mg/kg	–	–	95.6	16.3	44.3	1.5	10.1	0.8	51.4	37.3
Zn	mg/kg	100	150	131.1	30.1	34.8	6.4	18.4	3.1	54.1	42.0
Σ BTs	µg/kg	5	72	1.00	<0.01	<LOQ	NC	<LOQ	NC	<LOQ	NC
Σ PCB	µg/kg	8	60	10.4	0.2	5.4	4.2	<LOQ	NC	4.3	1.0
Σ DDD	µg/kg	0.8	7.8	<LOQ	NC	<LOQ	NC	<LOQ	NC	<LOQ	NC
Σ DDT	µg/kg	1.0	4.8	0.86	NC	<LOQ	NC	<LOQ	NC	<LOQ	NC
Σ DDE	µg/kg	1.8	3.7	1.01	0.20	0.79	0.48	<LOQ	NC	0.63	0.22
Chlordan	µg/kg	2.3	4.8	<LOQ	NC	<LOQ	NC	<LOQ	NC	<LOQ	NC
Aldrin	µg/kg	0.2	10	<LOQ	NC	<LOQ	NC	<LOQ	NC	<LOQ	NC
Dieldrin	µg/kg	0.7	4.3	<LOQ	NC	<LOQ	NC	<LOQ	NC	<LOQ	NC
Endrin	µg/kg	2.7	10	<LOQ	NC	<LOQ	NC	<LOQ	NC	<LOQ	NC
a-HCH	µg/kg	0.2	10	<LOQ	NC	<LOQ	NC	<LOQ	NC	<LOQ	NC
b-HCH	µg/kg	0.2	10	<LOQ	NC	<LOQ	NC	<LOQ	NC	<LOQ	NC
g-HCH	µg/kg	0.2	1	<LOQ	NC	<LOQ	NC	<LOQ	NC	<LOQ	NC
Eptachlor epoxid	µg/kg	0.6	2.7	<LOQ	NC	<LOQ	NC	<LOQ	NC	<LOQ	NC
HCB	µg/kg	0.4	50	<LOQ	NC	0.36	0.37	<LOQ	NC	<LOQ	NC
Σ PAH	µg/kg	900	4000	96.8	48.7	5339.5	3842.6	<LOQ	NC	147.4	27.8
C > 12	mg/kg	–	50	11.5	0.7	<LOQ	NC	14.5	6.5	<LOQ	NC
C < 12	mg/kg	–	–	<LOQ	NC	<LOQ	NC	<LOQ	NC	<LOQ	NC

(ρS = −0.704) as reported in Supplementary material (Table S1).

Univariate relationships (see Supplementary Table S1) showed that metal and metalloid pollution (excluding As) were closely related to each other but not to silt content with the only exception of Hg (ρS = 0.533), while organic chemicals, excepted ΣPAHs, were almost exclusively related to macronutrient concentrations.

Concerning chemicals higher than L1 and L2 in tested sediments, the following relationships were observed.

- Arsenic (As) showed a strong positive correlation with ΣDDE (ρS = 0.689), TOC (ρS = 0.719), Hg (ρS = 0.746), HCB (ρS = 0.838), ΣPAH (ρS = 0.850), As-a* (ρS = 0.889), water content (ρS = 0.902). A negative correlation was recorded with Cd (ρS = −0.559), Eh (ρS = −0.601), L* (ρS = −0.693), and C > 12 (ρS = −0.817).
- ΣPAH showed a positive correlation with water content (ρS = 0.607), a* (ρS = 0.982), and HCB (ρS = 1.000). Contrarily, ΣPAH were

negatively correlated with Cd (ρS = −0.549), C > 12 (ρS = −0.586), and pH (ρS = −0.769).

- ΣPCB levels were positively correlated to almost all tested chemical with the only exception of As and Cd.
- Cr, Cu, Ni, Zn were neither correlated to TOC nor to As, HCB, ΣPAH, C > 12, silt, water content, pH, Eh, and colour components. On the contrary, they were negatively correlated to the sand content (ρS value ranged from −0.663 to −0.787) and positively correlated to the gravel content (ρS value ranged from 0.907 to 0.954), and TP (ρS = 0.800).
- Hg was positively correlated to As, TOC, silt, and almost all other tested elements except for Cd, BTs, DDT, HCB, ΣPAH, gravel, pH, Eh, and colour components. Hg resulted negatively correlated to sand (ρS = −0.705), and C > 12 (ρS = −0.605).

3.1.3. Statistical relationships among variables

Physicochemical variables of sediment samples were used to perform the PCA. PCA showed that the first three axes accounted for 100% (51.2%, 30.7%, and 18.1%, respectively) of the variability of the sampling sites. Distribution of variables determining sediment similarity was represented in Fig. 2. Major similarities were observed among 4NP and 2NP sediment types while 1NP and 3NP differed each other and with 2NP and 4NP.

Factors that contributed to determine differences among sediment types are represented, for the first axis by Al (−0.243), Cr (−0.240), Cu (−0.237), Hg (−0.216), Ni (−0.238), Pb (−0.252), V (−0.257), Zn (−0.252), BTs (−0.208) PCB (−0.242), DDT (−0.208), DDE (−0.225) PAH, HCB for chemical pollutants, TN (−0.255), TP (−0.255) for macronutrients, and sand (0.214) for physical descriptors.

The second axis is negatively correlated to As (−0.279), HCB (−0.247), PAH (−0.268), for chemical pollutants; TOC (−0.240) for macronutrients; water content (−0.205), and a* (−0.311) for physical descriptors. It is also positively correlated to Cd (0.319), C > 12 (0.327), for chemical pollutants, gravel (0.202), L* (0.316) for physical descriptors.

3.1.4. Ecotoxicity of natural sediments (T0)

Ecotoxicological risks calculated based on the WOE approach using Sediqualsoft® of 1-4NP sediment types (T0) are reported in Table 3. Results are also shown in Fig. 3a (1-4NP, T0; ST_I elutriate). Sediment 1NP (unpolluted, >TOC), 2NP (polluted, >TOC), and 3NP (unpolluted sand, ≪TOC) showed the lowest HQ battery (respectively 0.79, 0.77, and 0.01) associated with ecotoxicological risk "Absent". 4NP (unpolluted silty < TOC) showed low HQ for tree species battery (1.04) associated with ecotoxicological risk "Low".

The single ecotoxicological responses obtained when testing the species battery consisting of *A. fischeri* (solid phase), *P. tricornutum* (liquid phase, elutriate), and *P. lividus* (liquid phase, elutriate) on tested sediment and elutriates 1-4NP samples (T0) listed in the supplemental materials (Table S2) with results obtained on negative controls (Table S2) and positive controls (Table S3). Effects recorded on tested species (T0 samples) were lower than 20% for *P. tricornutum* and *P. lividus* in all tested sediment types. Furthermore, solid phase test on *V. fischeri* showed TU$_{50}$ values that resulted very low compared to

Table 3

Classification of ecotoxicological risks of tested sediment types. In this table, HQ obtained by the application of the WOE approach using Sediqualsoft® on collected data testing natural sediment types are reported and associated to the qualitative classification of risk. Results are reported for samples MC (Centrifugated), ST_I (freshly made analysed), and for all different STL tested. Results referred to STL T0 are considered in this study as representative of the "real" toxicity of sediment type tested and data associate to these samples are used to performed comparisons with other factor of variability. Colour legend for toxicity classification: Absence (green), Low (pink), Medium (yellow), High (red), Very High (dark red).

Samples MC, ST_I	1NP	2NP	3NP	4NP
STL T0	0.79	0.77	0.01	1.04
STL T1	0.21	0.29	0.37	0.68
STL T2	0.73	0.44	0.74	1.72
STL T3	4.05	4.23	0.63	3.82
STL T4	1.49	6.63	0.96	1.97

natural toxicity of the pelitic fraction measured in tested sediments (ICRAM, 2001).

As shown in Fig. 3c, the ecotoxicological effects in the 1-4NP test at T0 were primarily due to the elutriates (100% of the contribution to the observed effects).

3.2. Ecotoxicity of NH_4^+

3.2.1. Solid phase tests type-I (A. fischeri)

Ecotoxicity was found to slightly increase based on ammonium concentration (inhibition at the maximum dose tested of 94.6% for 2 μg/g, 97.0% for 70 μg/g; and 98.6% for 140 μg/g). As for the measured toxicity (TU), recorded levels varied from 243.5 TU (range 95% LCL-UCL = 239.8–247.2 TU), measured in not spiked sediment sample (negative controls) to 242.5 TU (range 95% LCL-UCL = 217.4–270.3) after the addition of 2 μg/g NH$_4$Cl. The sediment ecotoxicity index (STI) calculated for the negative controls and for 2 μg/g NH$_4$Cl did not change and was 1.2 STI in both cases. An amount of 70 μg/g NH$_4$Cl added to the sediment resulted in values of 217.1 TU (range 95% LCL-UCL = 185.3–254.4 TU) and a much lower STI (1.1 STI). Only the addition of 140 μg/g to sediments resulted in a TU increase of 291.6 (range 95%
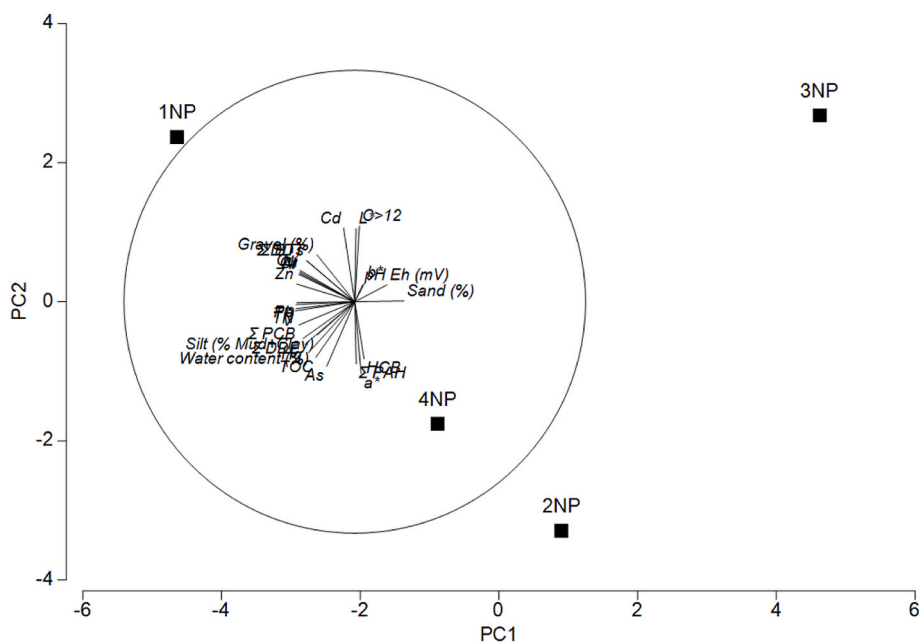

Fig. 2. Principal component analysis performed on variables measured in natural sediments. Two-dimensional PCA is represented to highlight similarity among different 1-4NP sediment types tested.
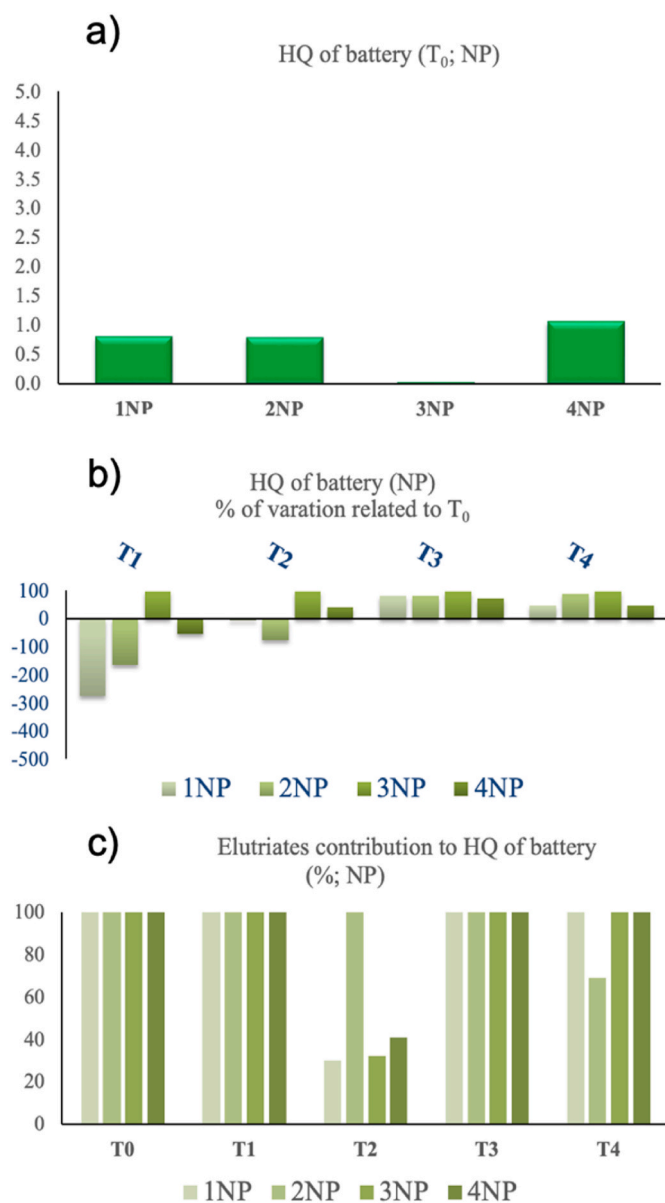
Fig. 3. Ecotoxicological responses of tested battery. In figure, HQ values obtained by the application of the WOE approach using Sediqualsoft® and percentages of contribution to the total HQ values by the toxicity of elutriates are reported. In details, figures reports: a) HQ of the three species battery tested; b) percentage of variation of the HQ of tested battery according to the factor STL (T1-T4); c) percentage of contribution to recorded HQ due to elutriates for all STL tested including T0. In figure b) positive values mean a recorded HQ value higher than the value recorded for the same sample at T0. Otherwise, negative values mean a decrease of HQ value compared to T0.

LCL-UCL = 224.2–379.2) for the same species, which was associated with an increase in STI from 1.2 to 1.5 (corresponding to +19.8%).

Liquid phase tests type-II (*A. fischeri*). Bacteria were also tested in the liquid phase (type-II, elutriate) by exposing this species to a 1:2 scalar dilution ranging from 6.25 to 200 mg/L NH$_4$Cl. A hormetic response was observed in this species at the lowest dilutions (weak biostimulation; I of $-1.10 \pm 0.60\%$). The first significant effect (I of $7.97 \pm 0.13\%$) was observed at an exposure of 100 mg/L. The maximum effect recorded in this species was $11.5 \pm 2.4\%$ after 30 min of exposure at 200 mg/L but the EC$_{50}$ was not calculable within the tested interval of NH$_4$Cl dilutions (EC$_{50} > 200$ mg/L).

Liquid phase tests type-II (*P. tricornutum*). Tested algae showed any

inhibition associated with the tested concentrations: $-1.42 \pm 1.20\%$ (2.5 mg/L NH$_4$Cl), $-4.35 \pm 1.50\%$ (5.0 mg/L NH$_4$Cl), and $-8.49 \pm 2.12\%$ (10.0 mg/L NH$_4$Cl), while a similar effect was observed at lower concentrations ($-1.55 \pm 3.86\%$; for 0.6–1.3 mg/L). Again, the EC$_{50}$ was not calculable within the tested interval of NH$_4$Cl dilutions (0.6–20.0 mg/L, EC$_{50} > 20$ mg/L).

3.2.2. Liquid phase tests type-III (P. lividus)

Inhibition of larval development tested on *P. lividus* proved to be the most sensitive endpoint to NH$_4^+$ tested in this study. After 72 h of exposure, an EC$_{50}$ value of 2.22 mg/L (6.58 mg/L expressed as NH$_4$Cl) with a range 95% LCL-UCL of 1.92–2.51 mg/L (5.69–7.30 mg/L expressed as NH$_4$Cl) was measured. Significant effects above those of the negative controls ($4.0 \pm 0.8\%$) were observed exceeding 0.5 mg/L of NH$_4^+$ (1.48 mg/L expressed as NH$_4$Cl; $9.0 \pm 1.7\%$, corrected to 5.2% by the Abbott formula).

3.3. Factors affecting ecotoxicity of elutriates

3.3.1. Separation treatment (T0)

In elutriates, oxygenation levels measured were always higher than 85% of saturation without any significant recorded among tested factors. Otherwise, pH and salinity ranged wider. Mean results obtained on pH and salinity are reported in Table S4 (a; T0, MC *vs.* MF) and Table S4 (b; MC, STL, ST_I *vs.* ST_F); Table S4 (c; MF, STL, ST_I *vs.* ST_F).

In general, the separation treatment did not significantly change the pH and salinity of the samples, yet some little differences were reported. In the centrifuge elutriates, pH ranged from 7.6 (2NP) to 8. (3NP) with a mean of 7.8 ± 0.1, while salinity ranged from 27.9 g/L (3NP) to 31.1 g/L (4NP) with a mean of 30.2 ± 1.0 g/L. In the filtered samples, pH values showed similar values (7.9 ± 0.1), while salinity showed a greater variation (from 25.3 g/L in 1NP to 31.1 g/L in 4 P), with a mean of 30.0 ± 1.9 g/L. A T-test performed for these variables showed that the variations were not significant ($p > 0.05$; Supplemental material, Table S4).

The ecotoxicological results on T0 samples (Supplementary material, Table S5), showed that centrifugation can produce a significantly different response compared with filtration.

Comparison of treatments (MC *vs.* MF) in *P. tricornutum* recorded that inhibition of growth significantly changed ($>\pm30\%$ at the highest concentration tested) in 1NP, 2NP, 4NP. Sediment type 3NP showed a decrease of effect included within $\pm10\%$. In *P. lividus*, recorded anomalous larvae significantly changed ($>\pm30\%$ at the highest concentration tested) in 2NP and 3NP, while others remained constant.

Fig. 4 shows the multivariate statistics for the centrifuged and filtered natural sediment types. Filtration reduced the natural variability of the physicochemical and ecotoxicological effects maintained by centrifuged sediments. In addition, the statistics showed a clear separation between the responses obtained with the different separation methods (MC *vs.* MF) tested.

3.3.2. Storage of elutriates (ST_I versus ST_F)

The results of univariate statistics and T-test performed on physicochemical data are shown in Table S4. The centrifuged elutriates were analysed both freshly made (ST_I) and frozen (ST_F) to detect significant differences in the main physicochemical parameters and ecotoxicity recorded. The differences between ST_I and ST_F were significant ($p < 0.05$).

3.3.3. Storage time laps (STL)

Concerning the solid phase, during the storage a 5 ± 1 °C, natural sediments showed an increase of the Eh value over the time and a decrease of pH. Also, colour changed for almost all sediment, from dark-based to brownish colours. Sediment 2NP and 4NP changes towards brownish red. Recorded changes of Eh, and pH resulted significant ($p < 0.01$) starting from T1 in almost all tested sediment excepted 3NP. Colour changed significantly starting from T2 in almost all samples ($p <$
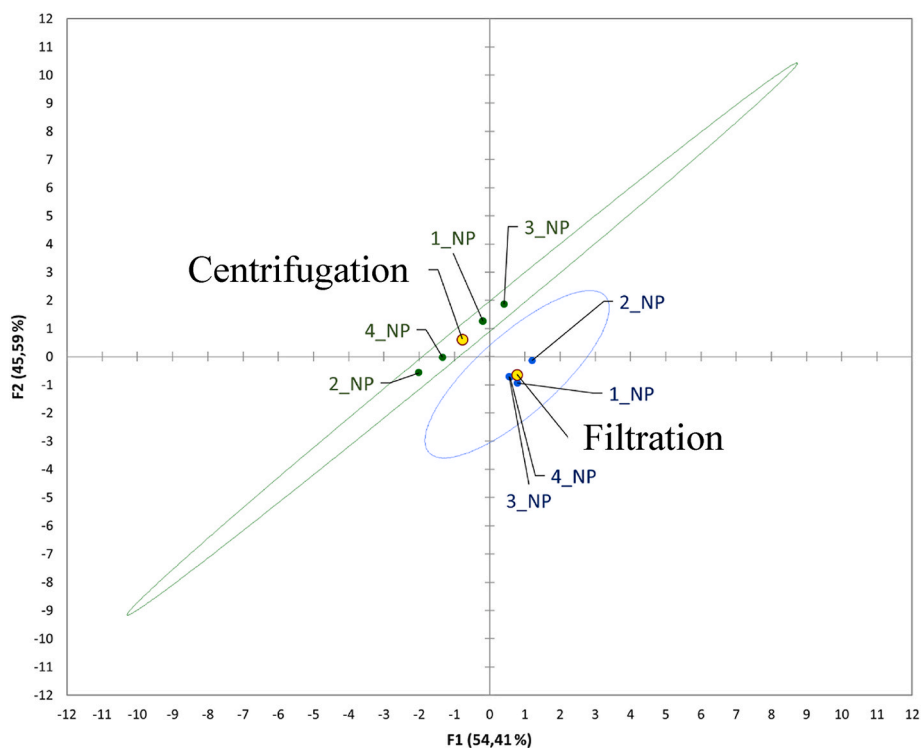
Fig. 4. Multivariate statistics on elutriates (MC *versus* MF). Multivariate statistics were performed to determine centroids and distances between the two different elutriate treatments tested on sediment types. Centroids are represented using a yellow dot while samples are green dots.

0.05). The only exception of the observed trend was represented by 3NP that remained similar to T0 over the holding time till T4 according to colour, pH, and redox potential.

The centrifuged elutriates were analysed both freshly made (ST_I) and frozen (ST_F) at different storage time laps (STL; T0, T1, T2, T4) to detect significant differences in the main physicochemical parameters and ecotoxicity. Mean values of salinity ranged according STL within 24.5–30.8 g/L with minimum values of 24.5 (T3) and 30.8 (T0); significant differences according to couples of times were recorded at any STL. Concerning pH, mean values ranged within 7.6–7.8 with minimum values of 7.5 (T2) and maximum of 7.8 (T4). The differences between ST_I and ST_F were significant at T1 (p = 0.017); at T2 significant increased (p = 0.002) while at T4 a significant change was not recorded. The results of univariate statistics and T-test performed on physicochemical data are also shown in Table S4.

Considering the ecotoxicological effects recorded on each of the tested species (Table 5S), results, highlighted that, for *A. fischeri*, 3NP sample changed within the 15% of the effect and could be considered stable according to all tested STL. Otherwise 1NP and 4NP changed starting from T2 higher than 30% of the effect recorded at T0. Sediment type 2NP showed a trend of variation that remained constant (0–20%) till T4 (21–40%).

In *P. tricornutum* (Table S5), Comparing ST_I with the same SLT of ST_F (MC samples), in general ST_F were much more effective than ST_I ones; differences were not significant till T4 for all tested sediment types. At T4 results highlighted a significant increase of the effect recorded, for sample 2NP.

In *P. lividus* (Table S5), effects recorded at T0 were lower than 20% in almost all tested. Significant changes were recorded in ST_I at T2 with effects always higher than 24%. At T4 in ST_I sample, mean significant effects higher than 45% were recorded; only sample 3NP showed effects <30% till T4. An increase till 100% of the effect at the maximum concentration dose was recorded in 2NP. ST_F elutriates remained stable.

ANOSIM test performed on factor STL highlighted significant (Global Test Sample Statistic R of 0.508, significance level of sample statistic

0.01%; number of permuted statistics greater than or equal to R: 0).

The ecotoxicological risks calculated based on the WOE approach using Sediqualsoft® are shown in Fig. 3 (1-4NP). The deviation of the calculated HQ values at each time point compared to the HQ values at T0 is shown in percent. Negative values indicate a decrease in HQ -value, while positive values indicate an increase in ecotoxicity. It is interesting to note that STL can determine significant changes in HQ -value, with a significant increase of more than 100% at T3 for almost all tested sediment types with the only exception of 3NP. As shown in Fig. 3e, the ecotoxicological effects in the 1–4 NP test at T0 were primarily due to elutriate toxicity for all sediments tested (100%). This trend remained during STL only for the sample 3NP while others showed an increase of ecotoxicity of the solid phase test.

3.4. Factors affecting NH_4^+ levels in elutriates

Levels of NH_4^+ measured at T0 in 1-4NP elutriate of sediments evidenced similar levels ranging within 5.1–5.3 μM of NH_4^+ (92.18–95.19 μg/L). In Table S6 (Supplementary materials) measured levels of NH_4^+ in elutriates performed from 1 to 4 NP sediments at different STL (T0-T4) and conservation methods (ST_I versus ST_F) are reported. A comparison among different elutriate production technique (MC; MF, elutriates made T0) is, also reported.

Recorded levels are low compared to values reported in marine sediments. Univariate statistics performed to explore the variance between means of different groups of interest (T-test, p-value) are reported in Table 4. Treatment of elutriate (MC *vs* MF) resulted not able to significantly change NH_4^+ levels in tested samples. Freeze (SI_F) elutriates showed lower levels compared to T0 in all tested 1–4 samples. A slight decreasing trend of NH_4^+ levels was recorded during the time with minimum levels recorded for all T4 samples. Concerning elutriates made from sediment stored at +5 ± 1 °C for different STL (T1-T4), NH_4^+ levels measured at T4 are significantly higher than T0 for all tested samples. It is interesting to note that NH_4^+ levels at T1 are significantly higher than T0 for 1-2NP samples. On the contrary, 3-4NP samples showed levels

Table 4

Univariate statistics (T-test) on NH_4^+ levels in elutriates. P-values recorded for couples of data (in bold values < 0.01; * means p < 0.05) are reported. T0 refers to values determined in ST_I.

MC; ST_F, T0 elutriates tested at different STL (T1-T4)			
	T0 *vs* T1	**T0 *vs* T2**	**T0 *vs* T4**
NP	0.145	0.164	**0.001**
	T0 (ST_I)		
MC *versus* MF	0.633		
MC; ST_I elutriates made and tested at different STL (T0-T4)			
	T0 *vs* T2	**T0 *vs* T3**	**T0 *vs* T4**
NP	0.208	**0.002**	**<0.001**

that decreased from T0 to T3 highlighting a major stability of this sediment types till T1. Comparing ST_I with ST_F at different STL it is evident the increase of NH_4^+ in ST_I elutriates relating to the STL (p < 0.001; T0 *vs*. T4).

3.5. Relevance of considered factors on WOE classifications

Ecotoxicological risks calculated based on the WOE approach using Sediqualsoft® of natural sediment types are reported in Table 5. All

Table 5

Effects on tested species and associated WOE ecotoxicological risks. In this table, percentage of changes of recorded effects according to tested factors of variability are reported grouping them per species considered and per discrete classes. In particular, the percentage of variation between tested couples are reported as follow: 0–20% (green colour), 21–40% (pink colour), 41–60% (violet colour), 61–80 (orange colour), 81–100 (dark red colour). HQ obtained by the application of the WOE approach using Sediqualsoft® on collected data testing sediment types are reported and associated to the qualitative classification of risk. HQ for tested battery is calculated under optimal conditions (T0, MC) and under different factor of variability tested (STL, ST_I, ST_F). In this case colours are associated to the class of risk: Absent (green), Low (Pink), Medium (Orange), High (Red), Very High (Black). MC (Centrifugated), MF (Filtered), ST_I (freshly made analysed), ST_F (Frozen, T0 elutriates).

Aliivibrio fischeri				
	1NP	2NP	3NP	4NP
T0	21–40	0–20	0–20	41–60
T1	0–20	0–20	0–20	0–20
T2	81–100	0–20	0–20	81–100
T3	21–40	0–20	0–20	41–60
T4	21–40	21–40	0–20	21–40
Phaeodactylum tricornutum				
	1NP	2NP	3NP	4NP
T0	0–20	0–20	0–20	0–20
T1	0–20	0–20	0–20	0–20
T2	0–20	0–20	0–20	0–20
T3	0–20	0–20	0–20	0–20
T4	0–20	0–20	0–20	0–20
Paracentrotus lividus				
	1NP	2NP	3NP	4NP
T0	0–20	0–20	0–20	0–20
T1	0–20	0–20	0–20	0–20
T2	21–40	21–40	21–40	21–40
T3	21–40	21–40	21–40	21–40
T4	41–60	41–60	21–40	41–60
HQ				
ST_I (MC) T0	Absent	Absent	Absent	Low
ST_I (MC) T1	Absent	Absent	Absent	Absent
ST_I (MC) T2	Absent	Absent	Absent	Medium
ST_I (MC) T3	High	High	Absent	High
ST_I (MC) T4	Low	Very High	Absent	Medium
ST_F (MC) T1	Absent	Absent	Absent	Absent
ST_F (MC) T2	Absent	Absent	Absent	Low
ST_F (MC) T4	Low	Medium	High	Low
ST_F (MC) T3/T4	Low	High	Absent	Absent
ST_F (MF) T1	Absent	Absent	Absent	Absent
ST_F (MF) T2	Absent	Absent	Absent	Low
ST_F (MF) T4	Absent	Medium	Absent	Low
ST_F (MF) T3/T4	Absent	Low	Absent	Low

described variations of recorded effects on the battery of tested species were weighted according to the applied approach to obtain the class of risk concerning ecotoxicological responses. Results for MC, ST_I, T0 tested samples are considered as reference to define when reported variation of effect due to different tested factors could be considered acceptable.

At T0, tested natural sediment types were classified in a risk "Absent" (1-3NP) and "Low" (4NP). Concerning ST_I (MC) sample, at T1 all natural sediment types resulted "Absent" showing that original classifications were maintained for almost all sediment types. Sample 4NP showed a decreasing of the classification of 1 class. Concerning 3NP, sediment classification was "Absent" (HQ ranged within 0.01–0.96) and was maintained till T4. 1-2NP maintained their classification till T2 while at T3 a significant increase was observed (+3 class of risk, final classification "High") and 2NP increased again till T4 (+4 class of risk; final classification "Very High"). 4NP showed a fluctuating behaviour progressively increasing class of risk from T2.

Concerning ST_F (MC) results on the risk assessment (HQ), starting from T2, showed significant changes of classification that resulted particularly critical for 2NP. On the contrary, ST_F (MF) resulted significant different for 2NP.

4. Discussion

According to M.D. 173/2016 (MD, 2016), to correctly evaluate the quality class of the sediment and whether it can be used as a resource of materials for coastal replenishments, a complete physicochemical and ecotoxicological characterization of the sediment material must be carried out, using obtained results to calculate the specific risk associated to sediment dredging. The structural heterogeneity of the sediments (i.e., granulometric, organic, oxidative, etc.) can lead to anomalous results related not only to the different nature of the matrix, but also to the timing and methods of treatment of the sample used by the laboratory that performed analyses.

To properly assess the dynamics associated with sediment ecotoxicity, it is critical to account for this variability as much as possible.

4.1. Physicochemical features of natural sediments

Sediments collected at the 1-4NP sampling sites in this study are characterized by variable composition and structure in terms of grain size, organic material and biological detritus, silt composition, and chemical contamination. The presence of large amounts of organic material (TOC) results in a negative redox potential indicating a reducing state (−205.5 mV; 4NP, silty sediment, 90% silt), while sandy sediments poor in organic material are oxidized (+53.0 mV; 3NP, sandy sediment, <5% silt). Much of the 1NP sediment is composed of bioclasts larger than 2 mm, consisting of bivalves and calcareous fragments of biological origin classified in the "gravel" class (bioclasts). Small sediment particles <20 μm (silt and clay), reduced and enriched in organic matter represent the principal component of harbor sediments.

In general, Italian harbours are characterized by soft sediment composed mainly of small silt and clay particles (<2–20 Ø), although sandy soils are also represented (Renzi et al., 2011). The samples collected in this study represent well both structural conditions in the harbor, including factors responsible of the largest variability of this experimental results. This aspect is crucial for the evaluation of the results and their significance in this study; in fact, some treatments could be more suitable for sandy sediments not enriched in organic matter than for sediments enriched in bioclasts, or for silty and TOC-enriched sediments. Based on this, STL, treatment types, and other factors tested must be optimized based on the specific characteristics of the tested sediment.

The sediments sampled in this study exhibit different types of chemical contamination at the 1-4NP sampling sites here examined. In terms of chemicals of ecotoxicological concern, the sediments of 1NP

and 3NP were not contaminated. In 3NP, the levels of almost all chemicals were very low, except for C < 12 hydrocarbons, which were 14.5 ± 6.5 mg/kg, below the specific chemical risk limits. 2NP, on the other hand, was contaminated by As and PAHs which exceed the L2 risk limits for the tested chemicals reported in M.D. 173/16 (All.1, PAH = 4000 μg/kg). In addition, in this sample, almost all measured metals were higher than the L1 values reported in M.D. 173/2016 (L1 values were As = 12 mg/kg, Cd = 0.3 mg/kg, Cr = 50 mg/kg, Cu = 40 mg/kg, Hg = 0.3 mg/kg, Ni = 30 mg/kg, Pb = 30 mg/kg, Zn = 100 mg/kg), and the sum of polychlorinated biphenyls was above L1 (8 μg/kg d. w.). Sediment sample 4NP was a silty sediment that showed natural enrichment of metals and other chemicals due to their affinity for the silty fraction of the matrix (Renzi et al., 2009) and 4NP is naturally enriched in metals due to the high proportion of silt fraction. Nevertheless, the chemical concentrations detected in this sample were very low compared to the specific L1 and L2 limits reported in M.D. 173/16 (MD, 2016).

In sediment samples considered in this study, high levels of trace elements recorded are probably due to the presence of high percentage of silt fractions that tend to accumulate trace elements, iron, and manganese oxides (Gadd, 2008). The crystal structure of sediments can determine high levels of arsenic and other phyllosilicates linked compounds (Tessier et al., 1979). Furthermore, the Elba-Argentario Basin, including Talamone sampling site, is settled inside the natural geochemical anomaly for trace elements associated to its volcanic origin (Leoni and Sartori, 1997). A strong correlation among As, Hg, Zn and other metals associated to the volcanic-originated mineralization was reported by the literature for geological anomalous areas and for the Elba-Argentario, in particular (Bigongiari et al., 2015). It is interesting to observe a positive correlation of Cr, Cu, Ni, Zn with bioclasts (gravel), rather than silt and other trace elements, that is also associated to a positive correlation with TP (1NP). These relationships suggest the occurrence of dynamics involving bioclasts in trapping trace elements. In contrast, the levels of PAH (2NP) are principally associated with the use or distribution of gasoline and the burning of organic matter (Renzi et al., 2009, 2011, 2019).

Results on studied sediment types supported that collected samples are representative of the large variability attended to test different matrix features and the influence exerted by the matrix variability on ecotoxicity according to factors of interest tested in this study.

4.2. Ecotoxicity of NH_4^+

Results obtained on tested chemicals and chemical mixtures highlighted that *P. lividus* test on larval stage is the more sensitive among the battery of species tested in this study while *P. tricornutum* was the less sensitive of all of them. Any data are available on tested mixture; nevertheless, some records are available on the toxicity of its components. This chemical represents a critical aspect to be monitored in ecotoxicological study because of it is toxic for marine species and larval stages (Saco-Alvarez et al., 2010). Despite that, harbor sediments are usually enriched in NH_4^+ as reported by ISPRA (2021) showing that average values of NH_4^+ in interstitial water of dredged sediment could range within <0.002–40 mg/L.

Aliivibrio fischeri and *P. tricornutum* resulted low affected by tested ammonium levels. Our results are in accordance with the literature (ISPRA, 2021). For the species *A. fischeri*, Onorati et al. (2007) reported an EC_{50} value of 1194 mg/L; while for *P. tricornutum*, a value of 20.0 mg/L was reported (ISPRA, 2021).

On the contrary, *P. lividus* resulted significantly affected by ammonium levels in water solution. The literature suggest that 2 mg/L represent the maximum level of tolerance of this species to be used in ecotoxicological tests for the application of M.D. 173/2016 (ISPRA, 2021), higher than those recorded in this study (mean value of 2.22 mg/L). Nevertheless, values lower than EC_{10} (9.0 $\pm$ 1.7%) were recorded for levels of NH_4^+ of 0.5 mg/L.

Our results confirmed that NH_4^+ could significantly affect *P. lividus* development; values lower than 0.5 mg/L are attended to produce an effect < EC_{10} and shall be considered as an optimal threshold to reduce NH_4^+ interference on tested species.

4.3. General features affecting ecotoxicity of sediments

4.3.1. Ecotoxicity of sediment types

Results obtained in this study highlighted that immediately after sample (T0), 1-4NP sediments resulted HQ-values within 0.01–1.04 (classification of risk "Absent" 1-3NP – "Low", 4NP). The recorded effects on total toxicity are associated at 100% to results obtained by the elutriate phase test highlighting that the effect of the solid phase resulted not significant. This fact is consistent to the presence and correlation of metals and organic pollutants in solid phase with TOC and silt levels supporting that sediment acted as a chelator for chemicals in the solid phase. Release of chemicals in elutriates is reduced and consequently, ecotoxicity recorded at T0 is low. Sediment 4NP showed a risk classification "Low" due to the higher effect of *P. lividus* in this sample compared to others. Nevertheless, the shift of a single class of risk must be considered accurately as well as this occurrence could be meaningless by an ecological point of view. In fact, when results are borderline, a little change of recorded effect could induce the class shift even effects could be considered within the natural variability of the assay. This is supported by the HQ recorded in 4NP that resulted only 0.04 over 1.00 that is the upper threshold level for the class "Absent". For the exposed reasons, such type of changes shall be considered not significant for the evaluation of ecotoxicological risk and during the decision-making process concerning management of dredged sediment. Given that this value represents a legal threshold (M.D. 173/16) it might be helpful to include other consideration to support decisions concerning borderline samples (i.e., TOC levels, silt percentages, NH_4^+ concentration, etc.).

4.3.2. Effect of different methods for the elutriate production

Literature reported a wide range of application of both tested methods (EPA 600; ISPRA, 2021). Based on results obtained in this study, centrifugation at T0 resulted the better method to maintain differences recorded in physicochemical features of tested 1-4NP sediment types. Despite that, when differences were explored during tested STL, in ST_F, HQ are similar to T0 in both MC and MF samples till T2 (10 days from sampling). These results are due to the variation contained in the ecotoxicological responses in the tested species which, although present and significant, is such as not to determine a significant variation in the risk class.

4.3.3. Effect of storage type and holding time (ST_I versus ST_F)

Oxidation is a critical factor that can affect the rate of mineralization of organic matter in sediments (Wainright and Hopkinson, 1997) and cause the release of nutrients from sediments into the water column (Fanning et al., 1982; Blackburn, 1997) and, in this case, into elutriates. Samples with high organic matter content (1NP, 2NP, 4NP) can be affected by air-induced oxidation during laboratory storage STL(T1-T4), which can result in the release of macronutrients and organic matter degradation products such as ammonium (NH_4^+) and hydrogen sulphates (Fanning et al., 1982; Blackburn, 1997; ISPRA, 2017). The increase in redox potential in T0 reduced sediments due to air oxidation processes could also lead to the release of chemical contaminants from the silty sediments (1NP and 4NP), which could explain a different ecotoxicological response of the tested species during the storage time laps in freshly prepared elutriates compared to the T0 responses. In fact, in a slightly basic, highly anaerobic environment, metals and organic chemicals are immobilized; in contrast, they are desorbed with increasing redox potential, oxidation, and acidification (Gambrell et al., 1991). It is known by the literature that an oxidized sandy sediment has different stability compared to a muddy sediment rich in organic matter in which reduced conditions are prevalent. Decomposition of organic

matter showed to be significantly affected by oxygenation of sediments due to its relationships with microbiota activity (Pepi et al., 2020). Oxygen level in sediments determine biogeochemical cycle of trace elements due to the presence of ferrous and ferric ions which oxides and immobilized H_2S producing FeS and pyrite FeS_2 (Berner, 1984; Luther, 1991; Rickard and Luther, 1997; Theberge and Luther, 1997; Rozan et al., 2002).

When elutriates are prepared from stored sediment, if oxidation occur during STL, the release of macronutrients, acid by-products, and chemicals from silts into the elutriation water, can modify the recorded ecotoxicity of samples. It is well-known by the literature, in fact, that metals can significantly affect ecotoxicological responses of target species of this study (ISPRA, 2021). Nitrification of organic matter in stored sediments is inhibited by reduced conditions as low oxygenation levels (<1 mg/L) reduces *Nitrobacter* spp. activity (Wetzel, 2001). Under oxygenated conditions, phosphates are bounded to ferric oxides and hydroxides (Golterman, 1995, 2001) and to carbonates present in clays and bioclasts (Dodge et al., 1984; De Jonge and Villerius, 1989) reducing their release into elutriates. Anoxic conditions produce high denitrification rates (Herbert and Nedwell, 1990) increasing nitrate release from sediments while, oxidation accelerates the nitrification processes producing nitrates predominance against the presence of reduced forms (Revsbech et al., 1980).

Literature reported high levels of ammonium in lagoon sediments and some coastal areas of Adriatic Sea (Losso et al., 2004; ISPRA, 2021) are associated to high content of organic matter. NH_4^+ is not considered a pollutant in dredged sediments because of its rapid oxidation during dredging procedures (ISPRA, 2021). Nevertheless, results obtained in this study showed a significant increase of NH_4^+ in elutriates according to STL showing that longer storage time could affect the production and release of such chemicals probably to the occurring of decomposition under reduced conditions (Pepi et al., 2020). Our test on elutriates and sediments added with NH_4^+, showed that the species that could significantly be affected by the release of NH_4^+ during the storage time is *P. lividus* (type-III). Even if tested elutriates in this study did not showed levels higher than 0.2 mg/L of NH_4^+, a clear increasing trend according to STL was recorded.

For the exposed reasons, to standardize elutriate production, T1 (5 days) shall represent the maximum temporal deadline to perform elutriates from stored sediments. In fact, almost all stored sediments explored in this study showed oxidation and significant changes of pH, redox potential, and colour starting from T1 (day 5). The only exception was represented by sands (3NP) that resulted stable concerning redox potential till T4. It is interested to observed that Eh, and pH changes in elutriates become significant early highlighting the occurrence of organic matter decomposition. This could be associated to an early release of chemicals and organic by-products of decomposition in elutriates and to a consequently change of ecotoxicity. For this reason, tests shall be performed within T1 but when it is impossible due to the large number of samples to be managed, elutriates shall be performed and frozen within the first 0–5 days from sampling to prevent an excessive alteration. T3 and T4 are to be considered excessive as also shown by the significant change of colours. It is interesting to observe that sands (3NP) samples, showed not significant changes longer than T4. This could open to the chance to evaluate in the future a longer holding time for sands than those that are fixed by the Law (10 for the elutriates and 15 days for solid phase test, M.D. 173/2016).

4.4. Weighting the importance of tested factors

The application of WOE approach allows to perform a synthetic evaluation of the ecotoxicological risk that better allow comparisons by weighing differences among effects recoded that could apparently and statistically be considered significant but that are of any relevance for the evaluation of risk (Table 6).

To elaborate practical indication of some interest in laboratories

Table 6

Weighted importance of tested factors. This table summarized changes recorded on the classification of risk of different sediment types tested under different interference of factor of variability of specific interest. Differences between the optimal classification (ST_I, T0 tested, MC) obtained for sediments, are compared to classifications obtained using effects recorded under different conditions (STL, ST_F) to determine the wide of recorded changes and to define a scale of importance of observed variation as reported: optimal (zero changes of class of risk, green coloured); tolerable (±1 changes of class of risk, yellow coloured); critical (±2 changes of class of risk, orange coloured); inacceptable (more than ±3 changes of class of risk, red coloured).

Factor	1NP	2NP	3NP	4NP
ST_I (MC) T0 Reference classification of risk	Absent	Absent	Absent	Low
ST_I (MC) T1	0	0	0	−1
ST_I (MC) T2	0	0	0	+1
ST_I (MC) T3	+3	+3	0	+2
ST_I (MC) T4	+1	+4	0	+1
ST_F (MC) T1	0	0	0	−1
ST_F (MC) T2	0	0	0	0
ST_F (MC) T4	+1	+2	+3	0
ST_F (MC) T3/T4	+1	+3	0	0
ST_F (MF) T1	0	0	0	−1
ST_F (MF) T2	0	0	0	0
ST_F (MF) T4	0	+2	0	0
ST_F (MF) T3/T4	0	+1	0	0

activity, a scale of importance of observed variation was reported and classified as optimal (zero changes of class of risk, green coloured); tolerable (±1 changes of class of risk, yellow coloured), critical (±2 changes of class of risk, orange coloured), inacceptable (more than ±3 changes of class of risk, red coloured). Based on the results obtained in this study, natural sediment types showed an acceptable ecotoxicological classification of risk till T2 for almost all sediment samples tested. Sediment type 4NP resulted the more critical showing changes in sediment classification till T2 within 1 class of risk. It is to notice that at T3 silty sediment 1-2NP and 4NP become unacceptable while sandy ones (3NP) resulted stable till T4 and over the maximum STL allowed by the M.D. 173/2016 for the solid phase test and the production of elutriates (respectively 15 days and 10 days from sampling). For these reasons working on solid phase tests and ST_I elutriate shall be limited to T2 (10 days). Even if *P. tricornutum* showed a significant increase of ecotoxicity at T4, in frozen elutriates, those resulted to produce acceptable classification of risks (0 and ± 1 changes of class of risk) till T4 (1NP and 3NP, 4NP). On the contrary, 2NP resulted acceptable till T2.

5. Conclusions

Findings from this study are of great importance for environmental management as they show how ecotoxicological tests and related responses require more standardization of aspects that can significantly affect the classification of ecotoxicological risk obtained. First, each type of sediment (sandy, silty, nutrient enriched, or naturally polluted) shows its own behaviour with a different severity of interference with ecotoxicological response by the factors tested in this study. This requires a strict control of the risk factors regardless of the nature of the sediment. As for the preparation of the elutriates, filtration at 0.45 μm produced less toxic and more homogeneous results reducing differences among the matrix types compared to centrifugation. For this reason, centrifugation is to be preferred to filtration to preserve a better representation of sediment heterogeneity. Freezing of elutriates does not seem to show any significant effect on the toxicity found, while the STL (storage time laps) of the sediment sample from sampling to the time of occurring both solid phase analysis and elutriate formation is able to significantly affects the results. This effect is matrix dependent, and sands resulted much more stable than TOC enriched silty sediments. Optimal results are obtained if analysis is performed as soon as possible after sampling to

avoid deterioration of sediments and classification changes. The best response obtained in this study refers to tests performed immediately with centrifuged elutriates. Nevertheless, if this is not possible, acceptable classifications are reported till T2 in ST_I elutriate and till T4 in ST_F of sands (3NP) and unpolluted TOC enriched sediments (1NP, 4NP). Nevertheless, T4 resulted not acceptable for polluted sediments (2NP, ST_F).

Credit author statement

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.

Acknowledgments

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