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Comparison of different ecotoxicological batteries with WOE approach for the environmental quality evaluation of harbour sediments

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ABSTRACT

This study was conducted under the Italian Ministerial Decree D.M. 173/2016 which regulates the assessment of the *Sediment Class Quality* in Italy using ecotoxicological bioassay and chemical analysis (Weight-Of-Evidence model). The aim of this work was to evaluate the real classification obtained by the theoretically equivalent responses of nine different combinations of batteries based on six different species: *Aliivibrio fischeri* (inhibition of bioluminescence), *Phaeodactylum tricornutum, Skeletonema costatum, Dunaliella tertiolecta* (inhibition of algal growth), *Paracentrotus lividus and Crassostrea gigas* (embryotoxicity).

Bioassays, in many cases, showed a non-bioavailability effect of the pollutants; these one highly revealed by the chemical analyses. Algal species showed responses very similar from each other. Otherwise, species used for embryotoxicity produced wide responses, consequently modifying the quality class of sediments and the handling management (i.e. landfill confinement or beach nourishment) allowed by the Law.

1. Introduction

Harbour areas represent a strategic point due to their economic importance, but also a potential source of marine pollution due to the presence of environmental contaminants (Renzi et al., 2009). Indeed, a wide range of persistent inorganic and organic pollutants can be released into the water column by human activities, with a subsequent absorption into the underlying sediment (Montero et al., 2013). In this regard, dredging activities, which are regularly needed to primarily maintain accessibility and vessel depths, provoke the disruption of the natural equilibrium between sediment and water, leading to the remobilization of chemicals from the sediment (Eggleton and Thomas, 2004). Contaminants can thus become bioavailable and may have negative effects on aquatic organisms (Davoren et al., 2005; D'Alessandro et al., 2020). For this reason, dredging activities require a proper assessment and management of contaminated sediments (Bocchetti et al., 2008). A growing number of studies apply a multidisciplinary approach to assess the environmental quality of harbour sediments (DelValls et al., 2004; Benedetti et al., 2012; Bebianno et al., 2015). Multidisciplinary studies based on the integration of chemical and biological measurements, represent an added value to monitoring and management protocols and their use is encouraged by European Directives and various international agencies (e.g., OSPAR, HELCOM, MEDPOL, ICES), through the integration of different quality indicators (Benedetti et al., 2012; Regoli et al., 2019). Indeed, chemical analyzes alone don't provide information on real bioavailability and biological risk of measured pollutants and basing management decisions only on the results of chemical analyzes can lead to an overestimation of the risk and consequent increase in management costs (Bradham et al., 2006). Ecotoxicological bioassays are increasingly being used to quantify the potential biological hazard caused by bioavailable multifactorial contamination, thus providing a more environmentally relevant response that is integrated and not restricted to the quantification of a predetermined list of contaminants (Volpi Ghirardini et al., 2005). It is a common opinion that single bioassays cannot provide a relevant environment quality, ind fact the use of batteries is recommended, testing different matrices and exposing different test species with different phylogenetic position, trophic level, incubation time, endpoint (Filipic, 1995; Dell'Orto et al., 1997;

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Bierkens et al., 1998; Pandard et al., 2006; Baudo et al., 2011).

In recent years the multidisciplinary Weight of Evidence (WOE) approach has been applied to evaluate the environmental quality of dredging sediments. The use of the WOE integration, which combines and weights different kinds of data and analyzes (i.e. sediment chemistry, ecotoxicological bioassays), or Lines of Evidence (LOEs), allows to better discriminate the presence of contaminants and their short and/or long-term environmental consequences (Regoli et al., 2019). The importance of the application of WOE models is particularly evident in complex environmental scenarios where seemingly conflicting results are provided by various LOEs (Morroni et al., 2020). The WOE approach has been summarixed in a quantitative user-friendly model (SediqualSoft®, free software), that has been validated in several case studies for environmental risk assessment related to polluted sediments, harbour areas, or complex natural and anthropic impacts on the marine environment (Piva et al., 2011; Benedetti et al., 2012, 2014; Regoli et al., 2014, 2019; Bebianno et al., 2015;; Pittura et al., 2018).

In SediqualSoft® different LOEs are elaborated independently, using specific criteria for each data, weighting the typology of chemical pollutants and the toxicological relevance of the measured endpoints (Morroni et al., 2020). Synthetic and quantitative hazard indices are calculated for each LOE, before their overall inclusion in the WOE assessment: the calculated risk level is assigned with a value between class 1 and 5 which classifies the risk from absent to serious (Piva et al., 2011; Regoli et al., 2019). The risk classification thus obtained is linked to the possible management options of the classified material. Weighted criteria for elaboration of chemical data and ecotoxicological bioassays were incorporated in the last Italian law for determining quality class and management options for dredged marine sediments, based on the weighted elaboration and integration of their chemical and ecotoxicological characteristics (D.M. 173/2016).

The aim of this study was first of all to evaluate the real classification obtained by nine different combinations of batteries based on six different species: *Aliivibrio fischeri* (inhibition of bioluminescence), *Phaeodactylum tricornutum, Skeletonema costatum, Dunaliella tertiolecta* (inhibition of algal growth), *Paracentrotus lividus* and *Crassostrea gigas* (embryotoxicity); the selected battery combinations are considered theoretically equal. Furthermore, this study wants to demonstrate the practical efficacy of the WOE approach with the powerful use of the SediqualSoft® tool for investigate the environmental quality.

To achieve this goal six batteries of bioassays were compared, assigning a different weight to each LOE to compare the real influence on one with the other. Each tested battery tested consisted of three species. Species were selected according to the battery rule established by ISPRA (2016), based on the matrix and the type of endpoint tested: the first chosen to assess acute toxicity on a solid phase matrix, the second and the third species were selected to evaluate acute (or algal responses) and chronic/sublethal toxicity, respectively, on the liquid matrix (elutriate of sediments). In particular, bioassays were carried out, analyzing harbour sediment of differently polluted areas, following the procedures suggested by the Technical Annex of DM 173/2016. The knowledge of the real influence of the combination of test species in WOE integration could be an important tool to facilitate site-oriented and since-based management options for dredged sediments in harbour areas.

2. Materials and methods

2.1. Sediment sampling and elutriate preparation

Sediments were collected between November 2018 and April 2019, in two harbour areas: Piombino and Olbia (Italy). Twenty sampling points (15 from Piombino and 5 from Olbia) were selected on the basis of the previously monitoring campaigns.

Sediments collected from Piombino were denoted with “P”, while the others from Olbia with “OL”.

Sub-samples of sediments, useful for granulometric and chemical analysis were stored at 4 °C and the ecotoxicological solid-phase test was conducted within 15 days from the collection day (pursuant to D.M. 173/2016). Within ten days from sampling, elutriates were prepared in accordance with USEPA (1991) guidelines and literature (Volpi Ghirardini et al., 2005). Sediment samples were mixed in a 1:4 (v/v) ratio of sediment to filtered sea water (FSW) and placed on a rotary shaker table for 1 h, at a speed of 300 rpm, at room temperature. The dilutions were made up with 0.45 μm Filtered Sea Water (FSW) collected in a long-term monitored reference site (Fortullino, Livorno, Italy) located far from sources of pollution from human activities. The salinity was adjusted on the basis of the bioassay procedures followed. After mixing, the samples were centrifuged (Thermo Scientific SL 16R, Rodano, Italy) for 20 min at 3000 rpm (4 °C) and the aqueous fractions (elutriate samples) were collected and stored at -20 °C until the use to perform biotests (Morroni et al., 2016).

2.2. Granulometric and chemical analysis

In accordance with SNPA (2018), sediment samples were treated with H_2O_2, for 48 h at room temperature, to promote the degradation of organic matter and facilitate the disaggregation of sediments particles. Afterwards, samples were wet sieved at 63 μm and the two obtained fractions were oven-dried at 105 °C; then, the > 63 μm fraction were dry sieved from 4 mm to 63 μm sieving plates and collected fractions on each sieve were weighted.

Chemical analyses included about 63 analyzes and the group parameters investigated were metals and metalloids (MP), organostannic compounds (BTs), Linear aliphatic hydrocarbons (C>12), polycyclic aromatic hydrocarbons (PAH), organochlorine pesticides, and polychlorinated biphenyls (PCB); in details:

- MP = Al, Ar, Cd, Cr, Fe, Hg, Ni, Pb, Cu, V, Zn;
- BTs = TBT; BTs Total;
- PAH = Acenaphthene, Acenaphthylene, Anthracene, Benzo [A] anthracene, Benzo[A]pyrene, Benzo[B]fluoranthene, Benzo[G,H,I] perylene, Benzo[K]fluoranthene, Chrysene, Dibenzo[A,H]anthracene, Phenanthrene, Fluoranthene, Fluorene, Indeno[1,2,3-CD] pyrene, Naphthalene, Pyrene, PAH Total;
- Organochlorine pesticides = Aldrin, Chlordane, DDD, DDE, DDT, Dieldrin, Endrin, Heptachlor epoxide, HCB, α-HCH, β-HCH, γ-HCH;
- PCB = congenus 28, 52, 77, 81, 101, 105, 114, 118, 123, 126, 128, 138, 153, 156, 157, 167, 169, 180, 189, PCB Total.

The followed procedures were EPA 3051A, EPA 6010D 2018, EPA 7473 2007, UNI EN 15,192:2007 and APHA Standard Methods for Examination of Water and Wastewater 23rd ed. 2017 3125 for metal and metalloids; while for the organic compounds were followed UNI EN ISO 16,703:2011, EPA 3545 2007, EPA 8270E 2018, EPA 1668C 2010, UNI EN ISO 15,662 2009, EPA 3535A 2007 and EPA 8270E 2018.

Granulometric analyzes and single chemical parameters were summarized in **Supplementary Material**.

Furthermore, additional parameters as salinity (Hanna Edge Instruments with HI2020 probe), pH (Hanna PHmeter Instruments HI 83,141), dissolved oxygen (Hanna Edge Instruments with HI2040 probe), nitrite and ammonia concentrations were measured on the elutriates (Aqualytic Photometer System AL450); results were summarized in **Supplementary Material**.

2.3. Ecotoxicological bioassays

2.3.1. Inibithion of bacterial bioluminescence

The Microtox® Solid-Phase test (MSPT) with *Aliivibrio fischeri* was conducted in accordance with Azur Environmental (1998) and Onorati & Mecozzi (2004). In particular, 7.0 g of wet sediment was resuspended in 35 mL of FSW (salinity: 37 PSU) by magnetic stirring for 10 min.

Subsamples of this suspension were serial diluted in 3 mL and after 15 min of equilibration, 20 μL of bacteria were mixed to the suspension and incubated for 20 min at 15 °C, and further separated from sediment particles by filtration. A subsample of the liquid phase was equilibrated for five minutes and light emission was recorded after 5 and 15 min with Microtox® M500 instrument; output data were analyzed with MicrotoxOmni software (Azur Environmental). Results were expressed as EC_{50} (g/L) and TU_{50} ($1/EC_{50}$); than the Toxicity Sediment Index (STI) was estimated previously pelitic component conversion (Onorati et al., 1999).

2.3.2. Algal growth inhibition

The algal bioassays were conducted using three different species: *Phaeodactylum tricornutum, Skeletonema costatum,* and *Dunaliella tertiolecta* following a standardized method (ISO, 2006). After exponential growth of the algal cultures the cell density was adjusted to 10^6 cells/mL, for *P. tricornutum* and *S. costatum*, and to 2×10^5 cells/mL, for *D. tertiolecta*. To perform tests, 0.1 mL of algal cultures and 0.165 mL of nutrient solutions (in accordance with ISO 2006) were added to 10 mL of elutriate; then, 2.5 mL of the subcultures were spitted into 24-well plates. After 72 h of incubation, under 10.000 lux of continuous illumination, at 20 ± 2 °C, algal cells were counted using an inverted microscope (Olympus, Milan, Italy) and a Thoma's counter chamber. Result was expressed as percentage of inhibition, as reported on standardized protocol ISO, 2006.

2.3.3. Embryo-larval bioassays

Larval growth bioassays were conducted with sea urchin *Paracentrotus lividus* (ASTM, 2012; ISPRA, 2017; Volpi Ghirardini et al., 2005), and oyster *Crassostrea gigas* (USEPA, 1995; His et al., 1997; ARPA, 2006). Adult of *P. lividus* were collected during the breeding season by free divers along the southern coast of Livorno (Italy) while the oysters were bought from Guernsey Sea Farms (Guernsey Island). Spawning was induced by osmotic shock in sea urchins, while in oysters' gametes were directly removed from gonads. After gametes collection the quality of eggs and sperm were checked. In particular, female animals with eggs not round, immature forms or debris, and males with low sperm motility were discarded. Correct density eggs suspensions were prepared (10^3 cells/mL for sea urchins and 3×10^3 cells/mL for oysters) and fertilization with sperm/egg ratio 10:1 (His et al., 1997) was induced. A period of 20 min of incubation at 18 ± 2 °C was allowed before starting the incubation with elutriate solutions, then, 1 and 0.15 mL of fertilized suspensions was mixed in 9 and 9.5 mL of elutriate, respectively, for sea urchin and oysters.

Only for the sea urchin, belong the recommendation of ISPRA (2016) to avoid any confounding factors in this bioassay, the elutriates were also diluted at 50%.

Sea urchins were incubated for 72 h, at 18 ± 2 °C, while oysters for 24 h, at 24 ± 1 °C, both in a dark room. At last, morphological evaluation was performed and the results were expressed as percentage of abnormal embryos, using the Abbott's correction (Abbott, 1975).

2.4. WOE elaboration

All results, for various typologies of data, have been elaborated within the quantitative WOE, SediqualSoft® model, summarizing specific hazard indices for individual LOEs, before their overall integration in the final WOE assessment (Piva et al., 2011, Benedetti et al., 2012, Regoli et al., 2019, Morroni et al., 2020). Logical flow charts, based on expert judgment and legislative constraints, were converted into algorithms for weighted elaboration of data from sediment chemistry and ecotoxicological effects measured at organism level (laboratory bioassays): the individual LOEs have been finally integrated for the WOE evaluation (see below).

2.4.1. LOE 1: chemical characterization of sediments

The evaluation of chemical hazards in sediments is based on the initial calculation, for each pollutant, of the Ratio to Reference (RTR), a parameter calculated by the ratio between concentration measured in sediments and threshold indicated by European Directive 2013/39/UE; in this case, between the thresholds L1 (minimum toxicant limit) and L2 (major toxicant limit). From the ratio to reference, a RTRw is obtained by the application of a correction factor (w) which, depending to the typology of chemicals, ranges from 1 to 1.3, (i.e., $w = 1$ for "non-priority", $w = 1.1$ for "priority" and $w = 1.3$ for "priority and hazardous" pollutants), according to EC Directive 2008/105. In the calculation of the Chemistry Hazard Quotient (HQ_c), an average RTR_w is obtained for all of the parameters with RTR <1, while for those with RTR >1, the RTR_w are individually added into the summation.

$$HQ_C = \frac{\sum_{j=1}^{N} RTR_W(j)_{RTR(j)\leq 1}}{N} + \sum_{k=1}^{M} RTR_W(k)_{RTR(k)>1}$$

Based on expert judgment, the values of HQ_c (ranging from 0 to >13) are assigned to six classes of chemical hazard (from absent to severe; i.e. 3 value= slight, while 15 value=severe) depending on the number, typology, and severity of exceeding chemicals (Piva et al., 2011; Regoli et al., 2019).

2.4.2. LOE 2: ecotoxicological bioassays

Weighted criteria to elaborate results from standardized ecotoxicological bioassays (LOE-2) are based on specific thresholds and weights assigned to each bioassay depending on the biological endpoint, tested matrix, time of exposure, and the possibility of hormetic responses. In particular, the results of each bioassay, with Abbot's correction, are weighted on the basis of the significance of differences with control, using specific thresholds and the z factor ($Effect_w$).

$$Effect_w = Effect \cdot \frac{z}{threshold}$$

The cumulative Battery Hazard Quotient (HQ_b) is obtained by the summation of the weighted effects ($Effect_w$), on the single bioassays, multiply by W_2; last parameter depends on the biological relevance of the endpoint (i.e., larval development = 1.9, algal growth = 2.1, bioluminescence = 2.4) the tested matrix (i.e., sediment = 1.0, elutriate = 0.7) and the test period exposure (i.e., acute = 1.0 and chronic = 0.7) (Piva et al., 2011; Regoli et al., 2019).

$$HQ_b = \sum\nolimits_{k=1}^{N} Effect_w(k) \cdot w_2$$

The HQ_b is normalized to a scale range from 0 to 10, where 1 is the battery threshold (when all the measured bioassays exhibit an effect equal to the threshold), and 10 when all the assays exhibit 100% of effect. Afterwards, on the base of HQ_b values one of five classes of hazard (from Absent to Severe) were assigned (i.e. 1.4 value = Slight, while 6.5 value = Severe).

2.4.3. WOE integration

The huge datasets of results elaborated from the 2 LOEs have been finally integrated through a WOE approach based on the quantitative model SediqualSoft®. The quantitative hazard quotients (HQ_s) obtained for each LOEs are normalized to a common scale and given a different weight according to previously validated procedures (Regoli et al., 2019). An overall WOE level of risk is thus calculated and assigned to 1 of 5 classes of risk from Absent (*Sediment Class Quality* = *A*) to Severe (*Sediment Class Quality* = *E*), with related management option referring the D.M. 173/2016:

- A class: sand nourishment, immersion in non-costal marine environment (above 3 nautical miles) and immersion in confined marine environment;

- B class: immersion in non-costal marine environment (above 3 nautical miles) and immersion in confined harbour environment;
- C class: immersion in confined harbour environment;
- D class: immersion in waterproof confined harbour environment;
- E class: removal from the marine environment.

Each possible combination of battery, obtained by mixture of the single bioassays composing the batteries, were evaluated and integrated with the chemical analyses, recording data from six different scenarios.

2.5. Statistical analyses of bioassays batteries results

Multivariate analyses were conducted on the bioassays results obtained from WOE elaborations. In particular, *Agglomerative Hierarchical Clustering* were performed with Xlstat 2020.4.1 by Addinsoft software. Bioassays with bacterium *A. fischeri* were neglected in the elaboration due to their presence in each battery combination. Starting from a data frame based with specific settlement (i.e., Proximity type = Dissimilarity Euclidean distance, Agglomeration method = Ward's method, Truncation = Automatic-Entropy), a dendrogram, showing the real distances in terms of dissimilarity between the different combinations of the ecotoxicological batteries were plotted.

3. Results

3.1. Granulometric and chemical results

Granulometric characterization of the sediments showed a predominant composition by pelitic compounds in 75% of the samples (P01, P02, P04, P05, P06, P07, P84, P87, P88, P90; P91, OL1, OL3, OL13 and OL18); whereas, the remaining samples (P08, P85, P86, P89, and OL22) showed, mostly, prevalence of sandy compound (data showed in the **Supplementary materials**).

The measured individual values of the chemical analyses are fully reported in **Supplementary materials**. Concentrations of chemicals reveal critical values in most of the sediments from Piombino harbour; all the trace metals (except Cr VI for all samples and Cu for P02, P04, P06, P08), hydrocarbons $C>12$, PAH (except few analytes for P02, P08, P85), all congeners of PCB, TBT (except P02) and organostannic compounds in few samples (P84, P85, P86, P87, P88, P89, P90, P91), showed higher values than their respective L1 and L2 Italian thresholds. For example, Zn in P84 shows a value (3611 mg/kg d.w.) much higher than their respective L1 and L2 threshold (100 and 150 mg/kg d.w., respectively), also hydrocarbons >12 for P87 shows a value (3.2×10^6 mg/kg d.w.) much higher than L2 (5.0×10^4 mg/kg d.w., L1 limit is not assigned). The HQ_c results (Fig. 1) show Severe level (of pollution) for almost all sediments, except for P02, P05 and P08, which show Slight or Moderate levels. To be underline that samples with Sever hazard level exceed the limit ($HQ_{c\geq}$ 13) with values very higher (i.e., P84; HQ_c = 116.2).

Instead, the sediments from Olbia harbour not revealed critical chemical characterization; only Cr (from 48 to 63 mg/kg d.w.), Cu (from 48 to 61 mg/kg d.w.) and Zn (from 66 to 206 mg/kg d.w.) as trace metals, TBT (from 32 to 70 μg/kg d.w.) and organostannic compounds (from 78 to 151 μg/kg d.w.) showed values few major than thresholds (mostly only for L1 limits). The HQ_c results show Absent level for OL18, Negligible for OL13, and Moderate for OL1, OL3 and OL22.

3.2. Ecotoxicological results

3.2.1. Single bioassays

Inhibition of bacterial bioluminescence. Sediments from Piombino harbour showed a significant toxicity level compared to those from Olbia (Fig. 2). In accordance with the toxicity levels (toxicity ranging 0–1 S.T.*I* = absent, 1.01–3 S.T.*I* = slight; 3.01–6 S.T.*I* = moderate, 6.01–12 S.T.*I* = toxic, >12 S.T.*I* = higher) established by Onorati (1999), four samples showed a high toxicity (P87 = 9,2; P84 = 8,3; P04 = 7,8; P90 = 7,2), six samples showed moderate toxicity (P05 = 5,9; P89 = 4,0; P86 = 3,6; P88 = 3,5; P91 = 3,5; P01 = 3,3), and all the others collected from Piombino reported, as all those of Olbia, slight or absent toxicity.

Algal growth inhibition. None of the samples showed relevant inhibition effects with algal species tested (Fig. 3). A major biostimulation were recorded by *P. tricornutum* bioassays while a slight inhibition effect (*I* >15%) is obtained in P86 and OL3 by *S. costatum* (29% and 16%, respectively). Almost always, the effects were included between the basal threshold (10%) and the biostimulation threshold (-40%), with reference to the DM 173/2016.

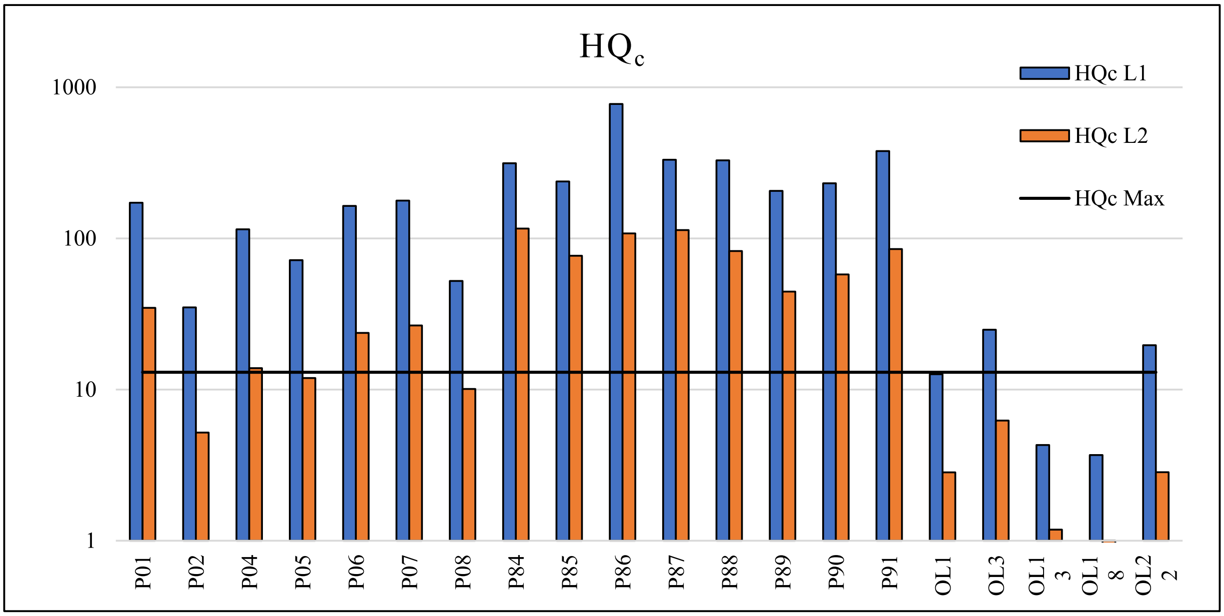

Fig. 1. At the top are showed the HQc (Chemical Hazard Quotient) results (logarithm scale) based on the italian L1 (lower) and L2 (higher) thresholds pursuant to the DM 173/2016 (the black line represent the limit value of 13); At the bottom are showed the HQc values based on the L2 threshold. The colours of the cells indicate the pollution of the sediment: white = absent, green = negligible, yellow = slight, red = moderate, black = severe.

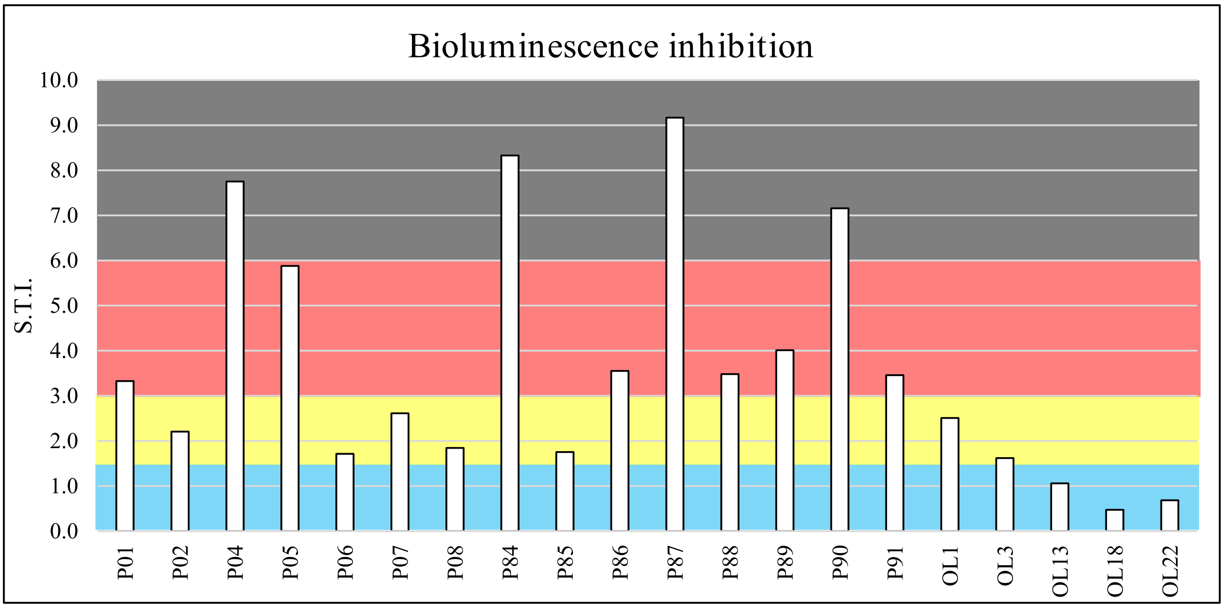

Fig. 2. Sediment Toxicity Index (effect) carried out from the bioluminescence inhibition bioassay with bacterium A. fischeri on the sediment samples. Black = effect between 6 and 10; Red = effect between 3,1–6; Yellow = effect between 1,6–3; Light blue = effect between 0 and 1,5.

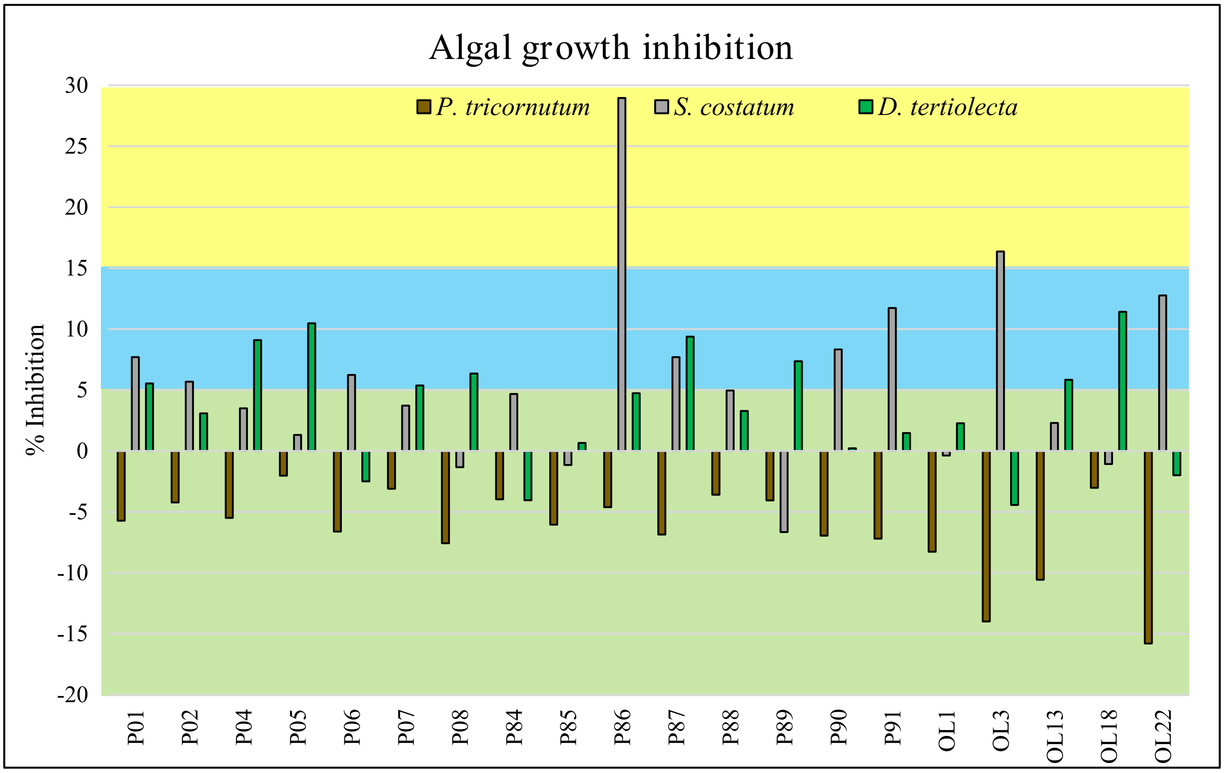

Fig. 3. Percentage inhibition of algal growth in P. tricornutum, S. costastum and D. tertiolecta observed on elutriates. Yellow = effect between 16 and 30%; Light blue = effect between 0 and 15%; Green: effect of biostimulation.

Embryo-larval bioassays. Fig. 4 shows the embryotoxicity effects in sea-urchin and oyster fertilized eggs exposed to sediment elutriates from the sites sampled. The results of the sea-urchin bioassays tested on elutriated not diluted show a severe toxicity effect (ranging effect between 80 and 100%) in samples P04, P07, OL1, OL13, OL18, a moderate effect (ranging effect between 50 and 79%) in P02, P05, P08, P85, P86, OL22, low effect (ranging effect between 10 and 49%) in P84, P87, P88, P89, P90, P91, OL3, and absent toxicity (ranging effect between 0 and 9%) in P01 and P06; results on elutriates tested at 50% of dilution show severe effect in P07 and OL13 (64% and 86%, respectively), moderate effect in P04 and P86 (average 32% both), low effect in P08, P85, P87, P89 and OL3, and absent toxicity in P01, P02, P06, P84, P87, P88, P90, P91, OL1, OL18 and OL2. Lastly, results of oyster bioassays show an absent-low toxicity effect in all elutriates tested (all samples with effect <15%), except for OL3 that shows a severe toxicity (91%).

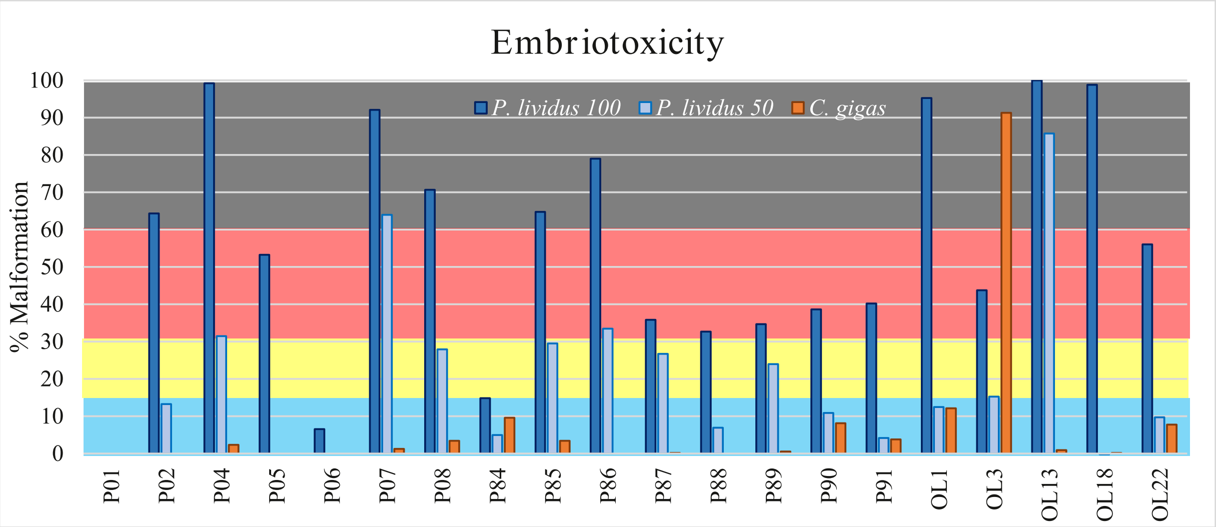

Fig. 4. Effects (% embryos malformation) in embriotoxicity bioassays with sea urchin P. lividus and oyster C. gigas tested on elutriates; only for P. lividus the elutriates are tested also with dilutions at 50% (P. lividus 50). Black = effect between 61 and 100%; Red = effect between 31 and 60%; Yellow = effect between 16 and 30%; Light blue = effect between 0 and 15%.

3.2.2. *Combinations of bioassays batteries*

Table 1 shows the HQ_b obtained by the SediqualSoft® elaborations concerning the six possible combinations of the species, with the order battery rule established by ISPRA (2016) where the 1° species is choose for the solid phase bioassay, the 2° for liquid phase bioassay observing acute effect or algal effect and the 3° for liquid phase bioassay observing chronic/sub-chronic/sublethal or long term effects. Most of samples from Piombino show a hazard level range between Moderate and Major; instead, most of Olbia's samples show a hazard level range between Absent and Moderate. The *Agglomerative Hierarchical Grouping* made it possible to better describe the HQ_b results by drawing a dendrogram (Fig. 5): the three batteries composed by *P. lividus* (P100), used as the 3rd species tested on complete elutriates, showed the major dissimilarity from the others six. At a lower level of the dendrogram (less dissimilarity) is showed a relevant dissimilarity from batteries composed by algae *S. costatum* to the others four. At least, the lowest dissimilarity (hugest similarity) is recorded between batteries composed by *P. tricornutum* or *D. tertiolecta* as 2nd species combined with *P. lividus*, tested on 50% diluted elutriates, or on *C. gigas* as 3rd species (in both cases, *A. fischeri* as 1° species tested on the solid phase).

3.3. *SediqualSoft® integration*

Chemical and ecotoxicological integration performed on tested samples is showed in Table 2, where single cells correspond at specific battery test exposed on a single sample. Vertical scrolling of the cells changes the samples, while horizontal scrolling changes the tested battery. Using the different batteries the samples from Piombino showed a mainly attributions of worst classes (D and E) at most of them:

Table 1

HQ_b (Batteries Hazard Quotient) of the 9 possible combinations of batteries, concerning the species rules established by DM 173/2016 (A. fischeri, P. tricornutum, S. costatum, D. tertiolecta, P. lividus 100 tested on elutriates not diluted, P. lividus 50 tested on elutriates diluted, C. gigas). The colours of the cells indicate the cumulative biological effect, by the three species composing the batteries, observed on the samples: white = absent, green = negligible, yellow = medium, red = high, black = very high.

Sample	Aliivibrio fischeri								
	Crassostrea gigas	Paracentrotus lividus 50	Paracentrotus lividus 100						
	D. tertiolecta	P. tricornutum	S. costatum	D. tertiolecta	P. tricornutum	S. costatum	D. tertiolecta	P. tricornutum	S. costatum
P01	1,99	1,73	2,23	1,99	1,73	2,23	1,99	1,73	2,23
P02	1,39	1,23	1,72	1,75	1,59	2,08	3,16	3,00	3,49
P04	2,92	2,29	2,48	3,75	3,12	3,31	5,62	4,99	5,19
P05	2,93	2,14	2,18	2,93	2,14	2,18	4,39	3,61	3,65
P06	0,88	0,88	1,90	0,88	0,88	1,90	0,93	0,93	1,97
P07	1,76	1,47	1,66	3,52	3,23	3,42	4,29	4,00	4,18
P08	1,28	0,99	0,99	2,00	1,71	1,71	3,19	2,90	2,90
P84	2,43	2,43	3,28	2,35	2,35	3,20	2,71	2,71	3,56
P85	0,94	0,93	0,93	1,71	1,68	1,68	2,67	2,64	2,64
P86	2,04	1,78	4,96	2,96	2,70	5,88	4,21	3,96	7,13
P87	2,92	2,33	3,61	3,64	3,06	4,33	3,90	3,32	4,59
P88	1,91	1,77	2,05	1,98	1,83	2,11	2,80	2,66	2,94
P89	2,36	1,89	2,46	3,01	2,54	3,11	3,30	2,84	3,41
P90	2,36	2,32	2,94	2,38	2,35	2,96	3,33	3,30	3,91
P91	0,59	0,56	2,06	0,59	0,56	2,06	1,45	1,41	3,13
OL1	1,86	2,31	1,74	1,86	2,31	1,74	4,15	4,60	4,03
OL3	3,23	3,80	5,49	1,14	1,71	3,40	1,92	2,49	4,18
OL13	0,26	0,42	0,11	2,25	2,48	2,02	2,64	2,87	2,41
OL18	0,48	0,00	0,00	0,48	0,00	0,00	2,94	2,21	2,21
OL22	0,07	0,52	1,51	0,17	0,63	1,67	1,03	1,72	2,94

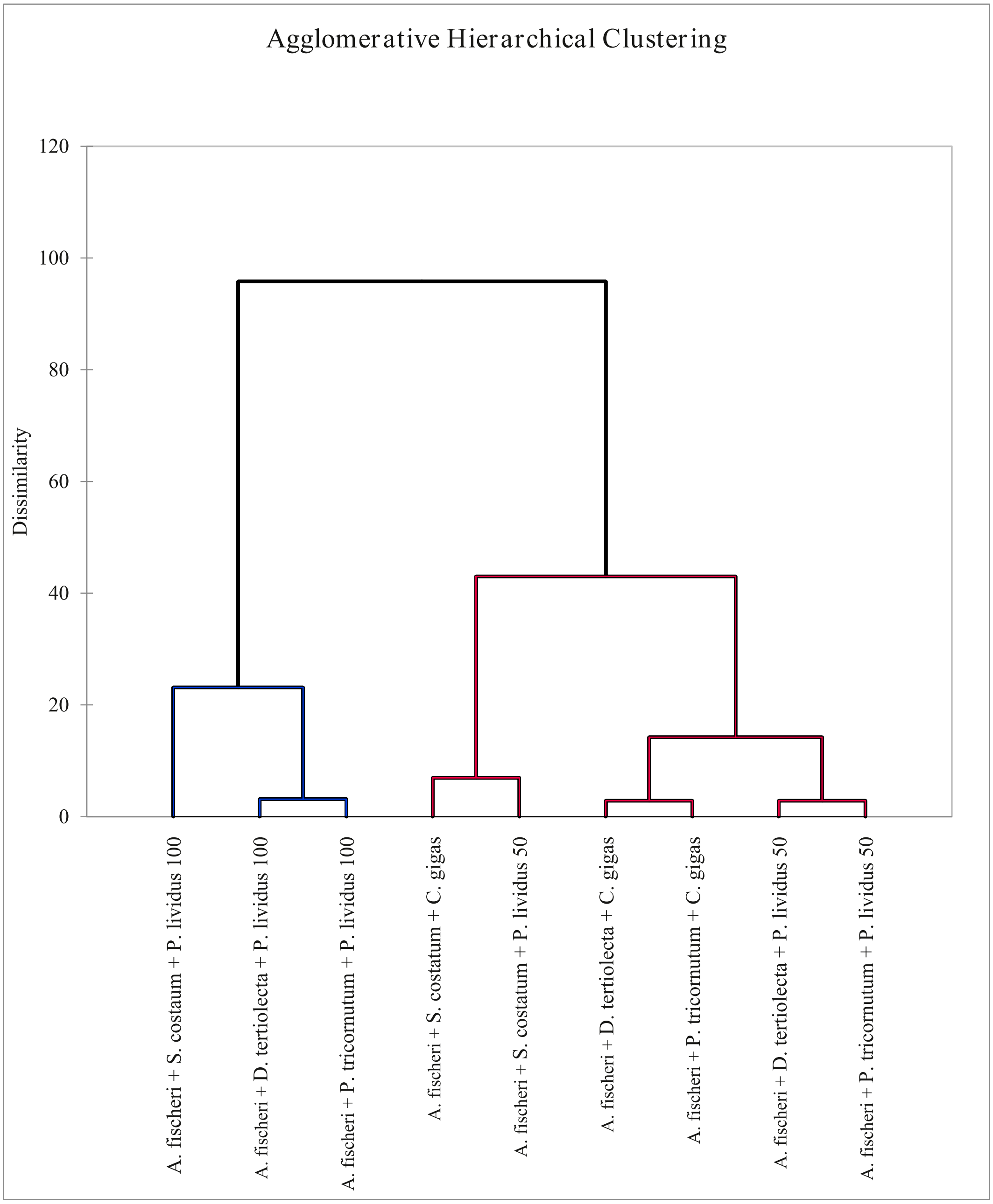

Fig. 5. Agglomerative Hierarchical Clustering carried out from the various combinations of batteries (HQ_b). The figure shows the dissimilarity between the different battery combinations.

- 29% of “E classes” (63% of total attributed by *A. fischeri* – *P. tricornutum* or *D. tertiolecta* or *S. costatum* – *P. lividus* 100% batteries);
- 64% of “D classes” (42% of total attributed by *A. fischeri* – *P. tricornutum* or *D. tertiolecta* or *S. costatum* – *C.gigas* batteries);
- 7% of “C classes” (100% of total attributed by *A. fischeri* – *P. tricornutum* or *D. tertiolecta* or *S. costatum* – *C.gigas* batteries).

While Olbia’s samples showed a wide range classes attribution:

- 26% of “E classes” (50% of total attributed by A. fischeri – P. tricornutum or D. tertiolecta or S. costatum – P. lividus 100% batteries);
- 30% of “D classes” (50% of total attributed by A. fischeri – P. tricornutum or D. tertiolecta or S. costatum – P. lividus 100% batteries);
- 26% of “C classes” (64% of total attributed by A. fischeri – P. tricornutum or D. tertiolecta or S. costatum – P. lividus 100% batteries);
- 7% of “B classes” (50% of total attributed by A. fischeri – P. tricornutum or D. tertiolecta or S. costatum – C.gigas batteries and the other 50% attributed by substitution of the third species with P. lividus 50% batteries);
- 11% of “A classes” (67% of total attributed by A. fischeri – P. tricornutum or D. tertiolecta or S. costatum – C.gigas batteries).

4. Discussion

A global panorama now confirms the importance of a multidisciplinary approach that integrates traditional chemical analyzes of abiotic matrices with those that show the biotic onset effects ranging between different levels organization, from bioaccumulation processes and

Table 2
Attribution of the integrated HQs (Sediment Class Quality) pursuant to the DM 173/2016 (A. fischeri, P. tricornutum, S. costatum, D. tertiolecta, P. lividus 100 tested on elutriates not diluted, P. lividus 50 tested on elutriates diluted, C. gigas). On the bottom, the legend indicates the possible risk of the sediment samples.

Sample	Aliivibrio fischeri								
	Crassostrea gigas	Paracentrotus lividus 50	Paracentrotus lividus 100						
	D. @@@@tertiolecta	P.@@@@tricornutum	S.@@@@costatum	D. @@@@tertiolecta	P. @@@@tricornutum	S. @@@@costatum	D. @@@@tertiolecta	P. @@@@tricornutum	S. @@@@costatum
P01	D	D	D	D	D	D	D	D	D
P02	C	C	C	D	D	D	E	E	E
P04	D	D	D	E	E	E	E	E	E
P05	D	D	D	D	D	D	E	E	E
P06	D	D	D	D	D	D	D	D	D
P07	D	D	D	E	E	E	E	E	E
P08	C	C	C	D	D	D	E	D	D
P84	D	D	E	D	D	E	D	D	E
P85	D	D	D	D	D	D	D	D	D
P86	D	D	E	D	D	E	E	E	E
P87	D	D	D	E	E	E	E	E	E
P88	D	D	D	D	D	D	D	D	D
P89	D	D	D	E	D	E	E	D	D
P90	D	D	D	D	D	D	E	E	E
P91	D	D	D	D	D	D	D	D	D
OL1	D	D	D	D	D	D	E	E	E
OL3	E	E	E	C	D	E	D	D	E
OL13	A	A	A	C	C	C	C	C	C
OL18	A	A	A	A	A	A	C	C	C
OL22	B	B	D	B	B	D	C	D	D
Class Quality *A* = Absent									
Class Quality *B* = Slight									
Class Quality *C* = Moderate									
Class Quality *D* = Major									
Class Quality *E* = Severe									

molecular alterations, up to population and communities' structures (Moore et al., 2004; Hylland et al., 2006; Chapman, 2007; Viarengo et al., 2007). Combining chemical and biological analyzes adds value to monitoring and management protocols, in line with the recent European Directives, which recommend the use of multiple quality indicators for aquatic ecosystems (Morroni et al., 2020; Lyons et al., 2010).

The WOE approach, integrating single lines of evidence through qualitative or quantitative methods, is widely used in ecological and risk assessments to draw conclusions and justify the selection of regulatory benchmarks (Linkov et al., 2009, 2015). It is well known that the application of quantitative weighted criteria to process and integrate large amounts of heterogeneous data from different LOEs allows complex scientific information to be summarized for easier interpretation by environmental managers and policy makers (Morroni et al., 2020; Piva et al., 2011; Borja et al., 2017; Regoli et al., 2019). For their acceptance in a decision-making process, within a normative procedure, the LOEs used must be must quantitative and transparent. Several studies have provided scientific elements to formaliz various WOE methods in different fields, such as those proposed by the USA and by European Food Safety Authority (Linkov et al., 2009, 2011, 2015; Suter et al., 2017). In recent years, this approach has also been developed also in the environmental risk assessment in the marine environment, related to pollutants (Piva et al., 2011, Benedetti et al., 2012, 2014, Bebianno et al., 2015). Based on these studies a quantitative model (Sediqualsoft) was developed and validated, to be afterwards included in the last Italian law on management of dredged sediments (DM 173/2016), in line also with European Directives which recommend the use of multiple quality indicators for aquatic ecosystems (Lyons et al., 2010).

The Italian law (DM 173/2016), based on the weighted elaboration and integration of chemical and ecotoxicological properties of dredged marine sediments, is aimed to determine a quality class. The latter is useful to decide whether the environmental quality of the sediment is acceptable for a sand nourishment (class "A"), or if it is highly polluted and the only way to manage it is to remove from the marine-harbour environment (class "E"). The Italian Law, to obtain an ecotoxicological result useful for the output of the class quality, admit to perform batteries of bioassay composed by three species (belonging to different phylogenetic position or trophic level), each one selected by a wide range of species (Technical Attachment of the D.M. 173/2016) which the Law considers equivalent.

In this study, we wanted to focus on the species selection and on the comparability of the follow-up batteries, in order to improve their practical use in the assessment of the environmental quality of harbor sediments. In our study, the chemical analysis and the HQ_c elaborations reveal different pollution situation between the sediments sampled from the two harbor areas.

Harbour of Piombino showed a severe toxicity in all sites considered, caused by the enormos concentration of pollutants (i.e., MP, $C>12$, PAH, and PCBs) probably poured off in the coastal environment by the intensive steel industries located in that area, while Olbia showed a lower toxicity, due to the lesser influences of anthropic activities.

Concerning the ecotoxicological studies, the *A. fischeri* bioassay showed a significant toxicity effect in sediments from Piombino, in contrast to those of Olbia. The three algal species tested in this study showed an equivalent response among themselves. Only *S. costatum* showed a less relevant effect (observed in only three samples) compared to the responses of *P. tricornutum* and *D. tertiolecta*.

In the embryotoxicity bioassays, the oyster species showed a slight-absent toxicity in almost all samples tested, while the sea urchin showed a more relevant toxicity in both harbour areas. To avoid possible eventually effects due to any confounding factors additional parameters such as pH, RedOx, salinity, nitrite, and ammonia concentrations were measured. The results obtained did not show any influence by these factors.

Concerning the single bioassays is important to underline that the *A. fischeri* seems to be more aligned with the chemical characterization: this test showed a major effect of toxicity in sediments from Piombino than the other ones of Olbia. In literature is noted that the sediment compartment, respect the derived elutriates, keeps more pollutants, due to the negative charges of the pelitic fraction and the more hydrophobic affinity of some chemical species to be trapped between the sediment grains, indeed following the water (US EPA, 1978).

The HQ_b SediqualSoft® elaboration, based with the nine possible combinations, showed that: a more sensibility and variability was leaded primarily by the choice of the 3rd battery species, rather than the 2nd species (a little sensibility is improved by the choice of *S. costatum* as 2nd species). Anyway, *Agglomerative Hierarchical Clustering* showed that relevant differences between the batteries were influenced by choosing sea urchin *P. lividus*, tested on complete elutriates, and algae *S. costatum.* At least, more equivalence batteries were recorded by using *P. tricornutum* or *D. tertiolecta* as 2nd species combined with *P. lividus*, tested on 50% diluted elutriates, or *C. gigas* on undiluted elutriates as 3rd species (*A. fischeri* as 1° species).

The ecotoxicological and the chemical integration performed by SediqualSoft® software lead the attribution of the sediment class quality (**Table 1**). The samples from Piombino showed a worst class quality attribution (meaningful of a sediment removal from the marine environment) than the other ones of Olbia (i.e. sediment useful for a sand nourishment or an immersion in marine environment), with a shift of one or two worst classes. Generally, the differences on the ecotoxicological battery effects, between Piombino and Olbia, are influenced by metals, PAH, Hydrocarbons $C>12$ and PCBs.

Worst classes are mainly attributed by the using of batteries composed by *P. lividus* as 3rd species, and a little shift is attributed choosing *S. costatum* as 2nd species.

No strong correlations were observed between the battery effects to specific (single or type) chemical pollutants cause to the homogeneous kind of pollutants inside all the samples of Piombino and the other ones of Olbia. Only on the batteries composed by *S. costatum* as 2nd species the higher ecotoxicological effects are probably attributed by the influence of the PAH, in-fact the samples P86 and P91, with a marked algal effect, are those with a higher level of these pollutants. Furthermore, is noted that a low TBT pollution (i.e. OL13 and OL18) generate a low inhibition biolumiscence effect on *A. fischeri* with a consequent higher attribution of the sediment class quality.

Generally, on the same sample is noted a shift of the *Sediment Class Quality* (HQ_s) attributed by choosing different battery combinations: apart the chemical characterization the variety of effects are probably do by an intrinsic responses of the species test. This feature (re)marks the importance of revising the weight of the combinations (and bioassays) considered theoretically equal. From our results we can suggest, for example, to promote the using of *P. tricornutum* and *D. tertiolecta* species, instead *S. costatum* as 2nd species (algae); on the other hand for the 3rd species we suggest to performing bioassays with *P. lividus* tested on elutriates diluted at 50% or *C. gigas*. Another option could be the creation of a mathematical corrective factor based on the specific bioassay used to reduce eventually comparability errors.

Furthermore, in this study we confirm the importance of a multidisciplinary approach to assess the marine environmental quality. In fact, the severe amounts of pollutants present in the sediments from Piombino should have induced a severe ecotoxicological response on the organism species (and batteries) tested but this hypothesis didn't occur. This situation confirmed the lack of the chemical analysis in revealing the real bioavailability of the chemical species measured in sediments and remarked the importance of a Weight-Of-Evidence approach to the environmental risk assessment evaluation.

5. Conclusion

Weighted criteria for elaboration of chemical data and ecotoxicological batteries were incorporated in the Italian law (D.M. 173/2016) for determining quality class and management options for dredged marine sediments with the SediqualSoft® software. The multidisciplinary approaches, with the application of a Weight of Evidence model, were confirmed an added value to the use of individual LOEs, indeed the ecotoxicological batteries can reveal the real pollutants bioavailability respect the chemical analyzes.The selection of the species used for embryotoxicity produced not equivalent results and it modifies the attribution of the class quality sediments with significant consequences on the possible management option allowed by the Law (in particular *P. lividus*, tested on the whole elutriates, generate a high malformation effect, in contrast of *C. gigas*). On the contrary, algal species resulted quite equivalent (little exception for *S. costatum*) and less sensitive to pollution levels.

In a context of worldwide panorama where ecotoxicological laboratories daily perform bioassays, our results suggest the need to re-evaluate the equivalence of the battery combinations (and single bioassays) to standardize the responses, for example promoting the using of specific species or differently weighting the various species-test.

CRediT authorship contribution statement

Andrea Broccoli: Investigation, Software, Writing – original draft. **Lorenzo Morroni:** Conceptualization, Supervision, Writing – review & editing. **Andrea Valentini:** Writing – review & editing. **Valentina Vitiello:** Supervision, Writing – review & editing. **Monia Renzi:** Funding acquisition, Resources, Writing – review & editing. **Caterina Nuccio:** Writing – review & editing. **David Pellegrini:** Conceptualization, Supervision, Funding acquisition, Resources.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Supplementary materials

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