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Morphological and molecular responses of the sea urchin *Paracentrotus lividus* to highly contaminated marine sediments: The case study of Bagnoli-Coroglio brownfield (Mediterranean Sea)

Nadia Ruocco[a], Iacopo Bertocci[b,c], Marco Munari[c], Luigi Musco[c], Davide Caramiello[d], Roberto Danovaro[e,f], Valerio Zupo[a,**], Maria Costantini[a,*]

[a] *Department of Marine Biotechnology, Stazione Zoologica Anton Dohrn, Villa Comunale, 80121, Naples, Italy*
[b] *Department of Biology, University of Pisa, CoNISMa, Via Derna 1, 56126, Pisa, Italy*
[c] *Department of Integrative Marine Ecology, Stazione Zoologica Anton Dohrn,Villa Comunale, 80121, Naples, Italy*
[d] *Unit Marine Resources for Research, Stazione Zoologica Anton Dohrn, 80121, Naples, Italy*
[e] *Stazione Zoologica Anton Dohrn, Villa Comunale, 80121, Naples, Italy*
[f] *Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona, 60131, Italy*

ABSTRACT

Marine sediments store complex mixtures of compounds, including heavy metals, organotins and a large array of other contaminants. Sediment quality monitoring, characterization and management are priorities, due to potential impacts of the above compounds on coastal waters and their biota, especially in cases of pollutants released during dredging activities. Harbours and marinas, as well as estuaries and bays, where limited exchanges of water occurr, the accumulation of toxic compounds poses major concerns for human and environmental health.

Here we report the effects of highly contaminated sediments from the site of national interest Bagnoli-Coroglio (Tyrrhenian Sea, Western Mediterranean) on the sea urchin *Paracentrotus lividus*, considered a good model for ecotoxicological studies. Adult sea urchins were reared one month in aquaria in the presence of contaminated sediment that was experimentally subject to different patterns of re-suspension events (mimicking the effect of natural storms occurring in the field), crossed with O_2 enrichment versus natural gas exchanges in the water. The development of embryos deriving from adult urchins exposed to such experimental conditions was followed until the pluteus stage, checking the power of contaminated sediment to induce morphological malformations and its eventual buffering by high oxygenation. *Real-Time qPCR* analysis revealed that the expression of several genes (among the fifty analyzed, involved in different functional processes) was targeted by contaminated sediments more than those exposed in oxygen-enriched condition. Our findings have biological and ecological relevance in terms of assessing the actual impact on local organisms of chronic environmental contamination by heavy metals and polycyclic aromatic hydrocarbons affecting the Bagnoli-Coroglio area, and of exploring enhanced sediment and water oxygenation as a promising tool to mitigate the effects of contamination in future environmental restoration actions.

1. Introduction

Chemical pollution in marine coastal areas is a major ecological threat, due to the variety of toxic substances discharged and accumulated in sediments that act both as sink and source of pollution (Arizzi Novelli et al., 2006). Polluted sediments exhibit potential detrimental effects on marine ecosystems, especially in harbours and marinas, as well as in embayments and off coastal areas subjected to high and diverse human pressures, including commercial and industrial port activities, human settlements and tourism; moreover, sediment pollution may be deleterious to human health, especially in presence of recreational and mariculture activities, but also in nearby areas (Mamindy-Pajany et al., 2010). In fact, natural (*i.e.*, bioturbation and waves) or artificial (i.e., dredging) perturbative events can determine the re-suspension of sediments, the release of accumulated contaminants, such as polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB) and organotin compounds (OTCs), and their diffusion to surrounding habitats both within the water column and along the coasts, where they can cause acute stress to populations and assemblages. Indeed, marine ecosystems are globally influenced by human activities exerting direct and indirect negative effects on biodiversity, functioning and ultimately on human health (Myers and Worm, 2003;

* Corresponding author. Tel.: +39 081 583 3285; fax: +39 081 764 1355.
** Corresponding author. Tel.: +39 081 5833503.
E-mail addresses: vzupo@szn.it (V. Zupo), maria.costantini@szn.it (M. Costantini).

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Lotze et al., 2006; Halpern et al., 2008).

Dismissed post-industrial plants located along the coast are often a source of xenobiotic compounds (Krinke, 2001; Loures, 2015). A notable example is represented by the cessation and complete abandonment of the Bagnoli-Coroglio industrial plant (Southern Tyrrhenian Sea, Italy) in the mid-1990's, after about one century of production of chemicals, steel, concrete and asbestos. Due to high levels of pollution deriving from such activities, in 2014 the Bagnoli-Coroglio area was designated as a Site of National Interest (SIN), following the Italian national rules for the management of brownfields. In this part of the Gulf of Naples, marine sediments resulted severely contaminated by heavy metals, PAHs and PCBs (Sharp and Nardi, 1987; De Vivo and Lima, 2008; Albanese et al., 2010; Arienzo et al., 2017; Trifuoggi et al., 2017). Resident biota is thus potentially exposed to a mixture of contaminants when associated to the sediment (e.g., Bertocci et al., 2019), but also when living in nearby habitats that can be in contact with contaminated sediment, when it is subjected to highly turbulent events (i.e., storms) that may cause resuspension of material down to considerable depth (e.g., Arienzo et al., 2019; Sunamura and Kraus, 1984; Sherman et al., 1994). After sediment resuspension, contaminants can be transferred to the water column and dissolved substances can be absorbed by organisms from this compartment. This is of great concern as both the intensity and frequency of storms are expected to increase in the near future (Easterling et al., 2000; Trapp et al., 2007; Wolff et al., 2016; Aumann et al., 2018). Extreme meteorological events associated with climate change, may directly or indirectly increase the potential treats, including larger exposure of human and other populations to abiotic and biological noxious agents (Burge et al., 2014). Therefore, understanding the ecological effects of different anthropogenic disturbances associated with changes of meteorological patterns and identifying effective mitigation tools are key and urgent requirements of current fundamental and applied research. In this context, a promising tool suggested for mitigating or even solving environmental problems (Arzayus and Canuel, 2005; Duran et al., 2015), may be the oxygenation and further bacterial degradation of contaminants present in sediments, for example through the restoration of seagrass meadows, able to increase the penetration of oxygen in the sediments, through their roots (Duarte et al., 2004).

In the present study, we established a mesocosm setting, suited to expose target organisms related to environmental cues, i.e., individuals of the sea urchin *Paracentrotus lividus* (Sartori et al., 2017) to crossed experimental combinations of O_2-enriched vs. naturally oxygenated sea water with different patterns of temporally aggregated vs. more spaced events of re-suspension of contaminated sediments from the Bagnoli-Coroglio post-industrial area. The study area is located within the Gulf of Naples (southern Tyrrhenian Sea), and included in the municipality of Naples in its western part, at the south-eastern portion of Pozzuoli Bay, about 10 km west of the city of Naples (Fig. 1).

The western part of the coast is generally low and sandy, although often protected by artificial rocky reefs, while the eastern part of the cost is mainly rocky due to the presence of an ancient volcanic cone (the Nisida Island) (Bertocci et al., 2019). Marine sediments are mainly represented by coarse sand and sandy silt on the littoral shelf, fine sand at the margin of the Gulf (Cocco et al., 1988) and silty-clay particles in the central basin (De Pippo et al., 1988). In particular, the sediment used in this study was characterized by about 93% of coarse sand (>63 μicron) and about 7% of fine sand (<63 μicron) (see Morroni et al., 2020).

The Bagnoli Coroglio brownfield is a post-industrial site where the first plant, farming chemical products, was built in 1854. In early 1900s, the area was identified as a key site for the industrialization of Italy, which led, in the following decades, to the opening of several other plants with diversified activities, from the steel industry to the production of cement and asbestos (Eternit, Cementir, Italsider then Ilva). Beginning in the mid-1980s, the environmental risk represented by industrial production in the Bagnoli area became evident and a phase of

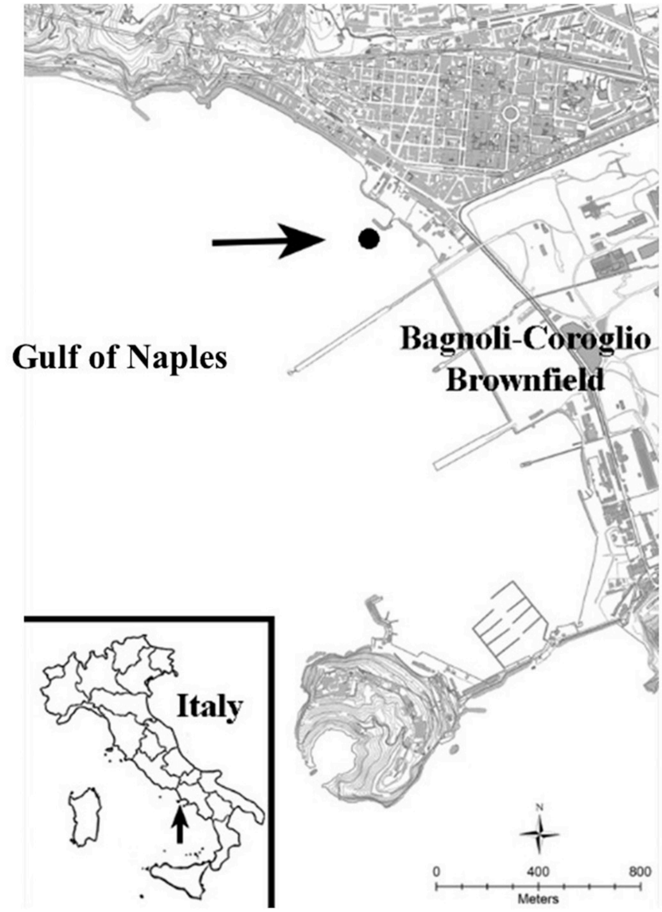

Fig. 1. Map of Bagnoli-Coroglio, the study-case area. A black dot highlights the site of sediment collection: Nord Utm 33 429405,23; Est Utm 33 4518298,73; depth 3,80 m.

dismantling of the entire industrial district began, which was completed in the middle of the 1990s. Subsequently, however, environmental investigations have shown that the area was polluted by heavy metals such as lead, zinc, tin, manganese, iron and large quantities of hydrocarbons (Sharp and Nardi, 1987; Romano et al., 2004, 2009; Arienzo et al., 2017; Trifuoggi et al., 2017; Pieretti et al., 2020). This high level of contamination resulted as a strong impact on meiofaunal assemblages (Gambi et al., 2020; Mele et al., 2020), on the turnover diversity of benthic prokaryotic assemblages (Tangherlini et al., 2020), on the biodiversity of macrozoobenthic assemblages (Guglielmo et al., 2020).

Specifically, we tested the hypotheses that (i) different patterns of occurrence of sediment reworking had different effects on the morphological development of fertilized embryos obtained from exposed adult urchins and on the expression level of several genes, spanning a range of functional classes, involved in natural responses to stress, and that (ii) the negative effects (i.e., induced malformations) of contaminated sediment could be mitigated or even cancelled by increased oxygenation.

We adopted the sea urchin *P. lividus* as a model for our experiments, because this species represents a bioindicator for detecting environmental perturbations (Dinnel et al., 1988; Sconzo et al., 1995; Morale et al., 1998; Matranga et al., 2000). Among the echinoderms, the sea urchin *P. lividus* is considered suitable to study the ecotoxicological response of marine invertebrates to environmental pollutants (Zito et al., 2005; Kobayashi and Okamura, 2005; Bellas, 2008; Bošnjak et al., 2010; Pinsino et al., 2010; Bonaventura et al., 2011; Pagano et al., 2017). Moreover, it is an important component of coastal marine communities in the Mediterranean Sea and the Atlantic Ocean (Zupi and Fresi, 1984;

Boudouresque and Verlaque, 2007; Agnetta et al., 2015); it has a long reproductive period (from October to May); extraction and maintenance of gametes are easy; its embryos grow rapidly and synchronously (pluteus stage is reached 48 h post fertilization); the embryos are transparent and therefore suitable for microscopic detection of sub-lethal effects of pollutants on development (Zito et al., 2005; Kobayashi and Okamura, 2005; Bellas, 2008; Bošnjak et al., 2010; Pinsino et al., 2010; Bonaventura et al., 2011).

2. Materials and methods

2.1. Ethics statement

Adult *Paracentrotus lividus* (Lamarck) were collected off San Pancrazio in the Ischia island (Bay of Naples), which is not privately owned or protected in any way, according to the Italian laws (DPR 1639/68, 09/19/1980 confirmed on 01/10/2000). Field studies did not include endangered or protected species. All experimental procedures on animals were in compliance with the guidelines of the European Union (Directive 609/86).

2.2. Experimental mesocosms

A set of microcosms consisting of twelve independent, closed recirculation tanks (~50 L each) was devised in the facilities for the maintenance of marine organisms of the Stazione Zoologica Anton Dohrn.

Each tank was manually filled with natural seawater collected from the Gulf of Naples and included two lateral chambers hosting mechanical and biological substrates and an additional compartment for recirculating pumps (pump flow rate: 600L/h; pump power: 6.5 W; see Fig. 2 for details).

To balance the evaporation, sea water level was daily manually adjusted in each tank by adding distilled water and checking the salinity by a refractometer, to reach back the initial values (see Supplementary Table S1).

A layer of about 5 cm of sediment collected from a site located within the Bagnoli-Coroglio area (UTM coordinates: North 33 429405,23, East 33 4518298,73; depth: 3.80 m; Fig. 1) was then deployed in each of eight, randomly chosen tanks at the beginning of the experiment, while the remaining four tanks were left without sediment and used as control for the possible effect of water turbulence, independent of the presence of sediment. Two out of the eight tanks with sediment were assigned, randomly, to each combination of two levels of turbulence events ('aggregated' vs. 'spaced', with total two events, each lasting two days, over the time of the experiment) and gas addition (enriched with Oxygen vs. natural aeration) over a total period of 30 days. Water turbulence was simulated by means of a centrifugal pump (capacity 4500L/h) able to produce a re-suspension of the sediment in the tank simulating the effects of natural storms. In the 'aggregated' pattern, the first event of sediment re-suspension was established twenty-two days after the beginning of the experiment, and it was separated by three 'calm' days only from the subsequent event. In the 'spaced' pattern, the first turbulent event occurred eight days after the beginning of the experiment and it was followed by eighteen 'calm' days until the second event (Fig. 3). This guaranteed for the application of the same overall frequency and intensity of disturbance, but different temporal patterns of events, according to the theory developed by Benedetti-Cecchi (2003) and then applied in several manipulative studies (e.g., Bertocci et al., 2005, 2007; 2017; Benedetti-Cecchi et al., 2006; Vaselli et al., 2008; García-Molinos and Donohue, 2011; Maggi et al., 2012). Benedetti-Cecchi (2003) reported that temporal variability in studies of disturbance is generally expressed in terms of event frequency. A possible solution to the problem involves experimental designs in which intensity and variability are chosen independently over explicit spatial or temporal scales and treated as fixed, orthogonal factors. This novel approach has important implications for understanding variability in a wide range of ecological contexts and for predicting the response of assemblages to increased environmental fluctuations.

Duration and frequency of experimental turbulent events were established based on calculations of the duration and frequency of natural storm events in the study area. These were estimated according to meteorological data collected within a monitoring programs of Stazione Zoologica Anton Dohrn. Specifically, wind data recorded daily from an oceanographic buoy located about two miles offshore in the Gulf of Naples were available for the period November 20th, 2015 until November 4th, 2016. This time series was first analyzed for identifying

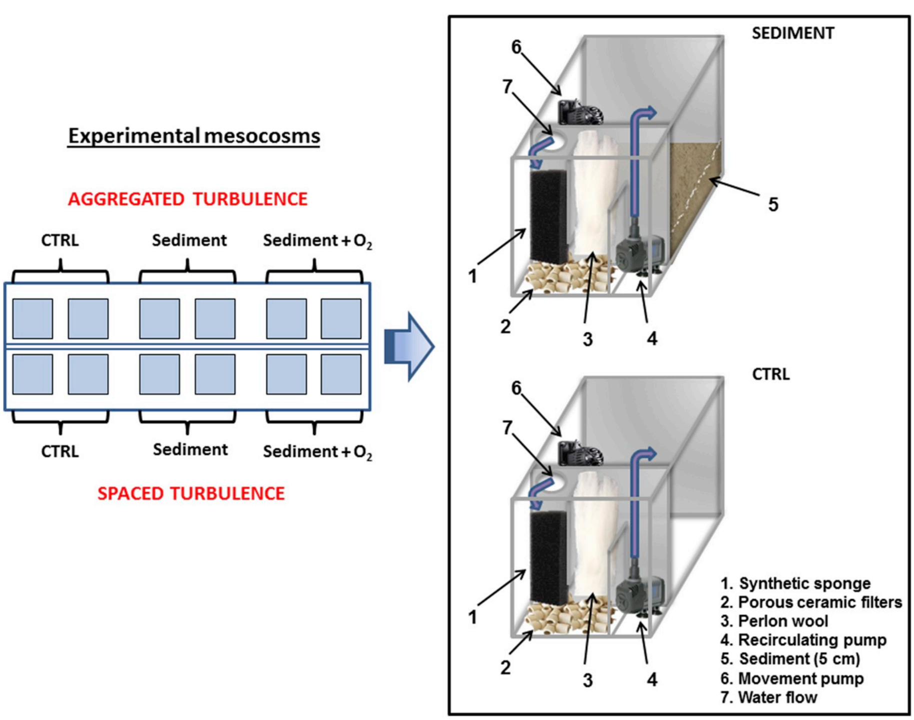

Fig. 2. Schematic overview of set of mesocosms (on the left), consisting of twelve experimental tanks, six of which were used for "aggregate turbulence" and six for "spaced turbulence". The sets of tanks in each condition consisted of two control tanks (indicated with CTRL) containing seawater without sediment, two tanks (indicated with "Sediment") containing seawater and sediment with normal aeration and two tanks (indicated with "Sediment + O_2") containing sediment with gaseous O_2 supply. On the right an enlargement of the frontal view of a tank with the description of all the elements: a mechanical filtration was applied using a synthetic sponge (1) and perlon wool (3) able to physically trap flowing particulate matter. Porous ceramic rings (2) were added too, to allow biological filtration, since they favour the growth of nitrifying bacteria that oxidize the ammonia into nitrites and then to nitrates. Water recirculation was guaranteed by pumps (4) and a circular hole (6) that connects the tank to filters compartment. Turbulence was generated by movement pumps (5).

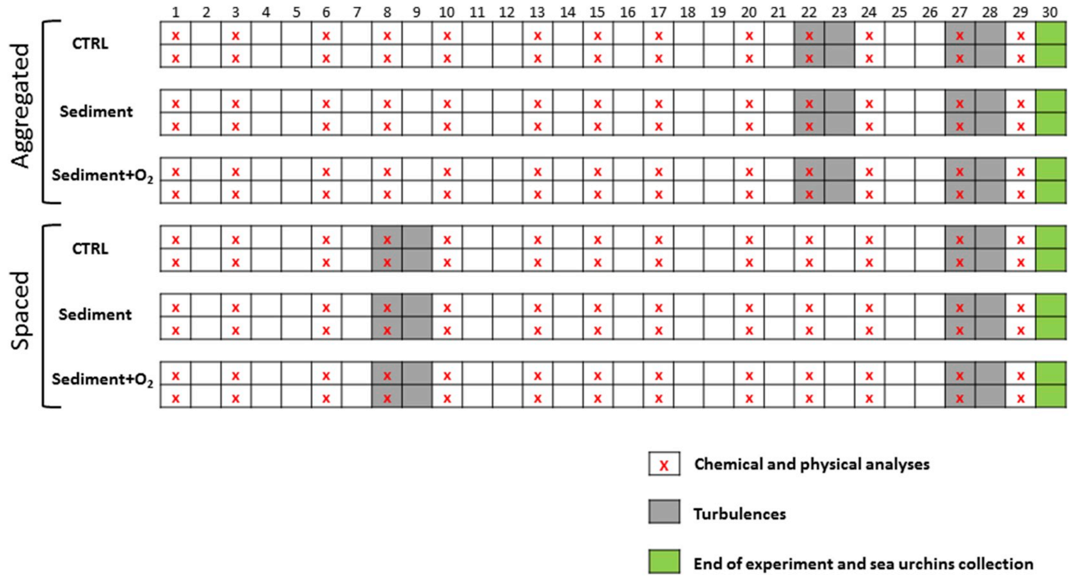

Fig. 3. Experimental design. Grey color indicates when turbulent events were applied for “aggregated” and “spaced” conditions, whereas X-shaped red crosses represent the days in which chemical and physical analyses were performed in each experimental aquarium. Sea urchins were collected and sacrificed after 30 days of experiment (green color). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the maximum wind speed recorded, and the number and duration of windy periods considered as ‘extreme’ events likely associated with large waves impacting the Bagnoli-Coroglio coast. Based on the coastline orientation, these were assumed to be those characterized by wind direction in the range 180–270° and intensity equalling or exceeding 80% of the maximum wind speed recorded during the examined period. Results indicated a threshold wind speed of 16.2 m/s, corresponding to 80% of the maximum recorded speed of 20.2 m/s, and total 3 extreme events, each lasting on average 1.3 days, over eleven months and a half (i.e., one event every 3–4 months). Based on such an evidence, the duration of experimental turbulent events was set to two days, while their frequency over the period of the experiment was set to two over 30 days. These experimental settings were considered realistic in terms of the natural occurrence of extreme storm events in the study area, and under the aim of reproducing their predicted increase in both intensity and frequency due to climate changes. Similar ‘aggregate’ and ‘spaced’ patterns of turbulence were also simulated in the experimental tanks (two for each pattern; for more details see also the legend to Fig. 2) kept without sediment representing the control tanks, to test possible effects of water turbulence *per se* from the effect of re-worked contaminated sediment on the sea urchins.

In order to mock oxygen production by photosynthetic organisms, two levels of oxygen saturation were tested, natural (~80% of saturation) and oversaturated (200%), both in the presence and in the absence of contaminated sediments (Viaroli and Christian, 2003). During the exposure, oversaturated levels were obtained by bubbling O_2 using an automatic control system (Touch Controller, mod. ACQ140, Aquatronica, Italy) connected to submersed oxygen probes (Dissolved Oxygen Sensor, mod. ACQ310N-O2, Aquatronica, Italy). A flux of atmospheric air was applied to tanks assigned to natural condition of aeration to distinguish the effect of forced oxygenation from that of the aeration system.

Physical and chemical characteristics of water were repeatedly checked during the experiment (see Fig. 3). In particular, physical and chemical variables were monitored twice a week: temperature and dissolved oxygen were checked by a multi-parameter probe (YSI 85); redox (REDuction-OXidation) state and pH were measured using WTW 197-S (SenTix® 41) electrodes (Supplementary Table S1). Water salinity was also recorded by a refractometer (Sper Scientific) and adjusted with distilled water if necessary (Supplementary Table S1). Analyses of nutrients, performed three times a week, detected the concentration of nitrates (NO_3^-), nitrites (NO_2^-), phosphates (PO_4^{3-}) and ammonia (NH_3^-) using a quick colorimetric assay (reagents by HACH Company; Supplementary Table S2). The absorbance was measured by a HACH Odyssey DR/2500 spectrophotometer.

Water and suspended particles were collected on the second day following each event of turbulence (days 9, 23 and 28) and analyzed for heavy metals and polycyclic aromatic hydrocarbons (PAHs) content. Chemical analyses were performed by Bioscience Research Center SRL (Orbetello, Italy) (Supplementary Tables S3–S6). For both water and suspended matter two standardized methods were applied: EPA 3050B 1996 + EPA 6020B 2014 (for metals in particles), EPA 6020B 2014 (for metals in water) and EPA 3545A 2007 + EPA 8270E 2017 (for IPA in particles).

2.3. *Sea urchin collection, their incubation in microcosms, gamete collection and developmental studies*

Adult *Paracentrotus lividus* were collected during the reproductive season (February) by scuba-divers in the Gulf of Naples and immediately transported to the laboratory, using a thermically insulated box. In the laboratory, they were transferred to open-cycle fiberglass tanks until the start of the experiment. Ten individuals (7 females and 3 males) were reared in each of twelve experimental tanks, under a 12h:12h light:dark photoperiod (light emitted by 18 W neon lamps, daylight, 6500 K).

After one month of exposure to each experimental condition, gametes were collected from reared adults. Sea urchins were injected 1 mL of 0.5M KCl into the coelom to stimulate the contraction of gonads. They were vigorously shacked and females were placed with their mouths up, over a 50 mL beaker until the gametes were released into filtered (0.22 μm Millipore) seawater, to facilitate the collection of eggs. Eggs from each female were washed three times with filtered seawater, whereas concentrated ‘dry’ sperm was collected and kept undiluted at +4 °C until use. Eggs were fertilized utilizing sperm-to-egg ratios of 100:1. Fertilization success and first cleavage were checked as soon as fertilization occurred. Embryos were grown at 20 ± 1 °C in a thermostatic chamber with a 12h:12h light:dark cycle. At the reaching of pluteus stage (~48 h post-fertilization; hpf), morphological observations were performed on 100 embryos from each female (fixed in 0.5% glutaraldehyde) using a light microscope (ZeissAxiovert 135 TV, Carl Zeiss, Jena, Germany), and the percentage of normal and malformed and/or delayed embryos for each experimental condition to which adults had been subject was calculated.

2.4. Embryo collection, RNA extraction and Real Time qPCR

About 5000 sea urchin plutei (48 hpf), deriving from four females exposed to each experimental condition were collected by centrifugation at 1800 relative centrifugal force (rcf) for 10 min in a swing out rotor centrifuge at 4 °C. Embryos were placed in at least 10 vol of the RNA*later*®, an RNA Stabilization Reagent (Qiagen, Hilden, Germany), and then frozen in liquid nitrogen and kept at −80 °C until use.

Total RNA was extracted using Aurum™ Total RNA Mini kit (BioRad; Ruocco et al., 2017a) from four biological replicates for each condition. The amount of total RNA extracted was estimated by measuring absorbance at 260 nm and the purity by 260/280 and 260/230 nm ratios, by a NanoDrop spectrophotometer (ND-1000 UV–Vis Spectrophotometer; NanoDrop Technologies, Wilmington, DE, USA). The integrity of RNA was evaluated by agarose gel electrophoresis. About 1 μg of RNA was used for cDNAs synthesis by an iScript™ cDNA Synthesis kit (Bio-Rad, Milan, Italy), following the manufacturer's instructions. Variation of expression levels of fifty genes involved in stress response, development and differentiation, skeletogenesis and detoxification processes (Marrone et al., 2012; Varrella et al., 2014; Ruocco et al., 2016, 2017b) were analyzed by *Real-Time qPCR*. Undiluted cDNA was used as a template for PCR reactions. The expression of each gene was analyzed and normalized against the housekeeping genes *Ubiquitin* and *18S* rRNA (Romano et al., 2011; Ragusa et al., 2013) using REST software (Relative Expression Software Tool, Weihenstephan, Germany) based on the Pfaffl method (Pfaffl, 2001; Pfaffl et al., 2002). Relative expression ratios greater than ±1.5 were considered significant. Each Real Time qPCR plate was repeated at least twice.

2.5. Statistical analyses

Data-sets were analyzed by means of a Shapiro-Wilk normality test to check that normality was not violated (at p-value>0.05). To check the homogeneity of variance across different groups (at p-value>0.05), Bartlett's test was applied. Three fixed factors (presence vs. absence of contaminated sediment, aggregated vs. spaced pattern of turbulence and enriched vs. environmental oxygen exposure) were crossed by a three-way analysis of variance (ANOVA). When relevant, post-hoc comparisons of significant factors were performed by Tukey's test. Statistical analyses were performed using GraphPad Prism Software (version 7.00 for Windows, GraphPad Software, La Jolla, California, USA, www.graphpad.com).

Data on larval malformations were recorded in a matrix along with the contents of heavy metals and PAHs in aggregated and spaced turbulence patterns, both in the water and in the suspended matter. This matrix was submitted to Cluster Analysis (single linkage, Euclidean distance) and Correspondence Analysis (Benzécri et al., 1973). The significance of ordinations was tested using the Broken Stick model (Frontier, 1976). All statistical analyses were performed using Statistica version 10 data analysis software system (StatSoft StatSoft, Inc. (2011). www.statsoft.com).

3. Results

3.1. Morphological observations

Gametes were collected after exposure of adult sea urchins for one month to polluted sediment, with and without oxygen supply. The percentages of fertilization success and first mitotic cleavage didn't show significant differences between exposed sea urchins (in both turbulence conditions and with natural aeration or with oxygen supply) and control individuals (p-value > 0.05). Morphological observations at the pluteus stage (48 hpf) showed an increase of malformed embryos collected from sea urchins exposed to contaminated sediments (Fig. 4).

No significant interactions among the three experimental factors were detected (Table 1).

The development of sea urchin embryos, in contrast, was affected by the presence of sediment interacting either with the pattern of turbulence or the oxygenation (Table 1). Specifically, post-hoc comparisons showed that sediment exposure under both patterns of turbulence induced an increase of malformed plutei compared to any control condition where sediment was absent (Fig. 4). Furthermore, under both conditions of turbulence, the percentage of malformed plutei was significantly (~20–30%) higher in samples deriving from sea urchins that had been exposed to polluted sediment at normal aeration level as compared to those where the same patterns of sediment re-working had been established under increased oxygen supply. Most notably, under such a condition, the small percentage of malformed plutei was quite similar to that observed in the no-sediment controls (Fig. 4). The sea urchin *P. lividus* has a natural level of developmental anomalies of about 10% in embryos (Varrella et al., 2016b). Finally, the aggregated patterns of turbulent vent also induced a developmental delay, under both oxygen conditions, compared to the control conditions, with ~15–20% of embryos still being at an early pluteus stage at the time of observation (Fig. 4). Malformations observed in the embryos at the pluteus stage mainly affected the arms, spicules and apex, in comparison with control embryos (Supplementary Fig. S1A). More in details, some embryos showed a poorly-formed apex (Supplementary Fig. S1B) or the entire

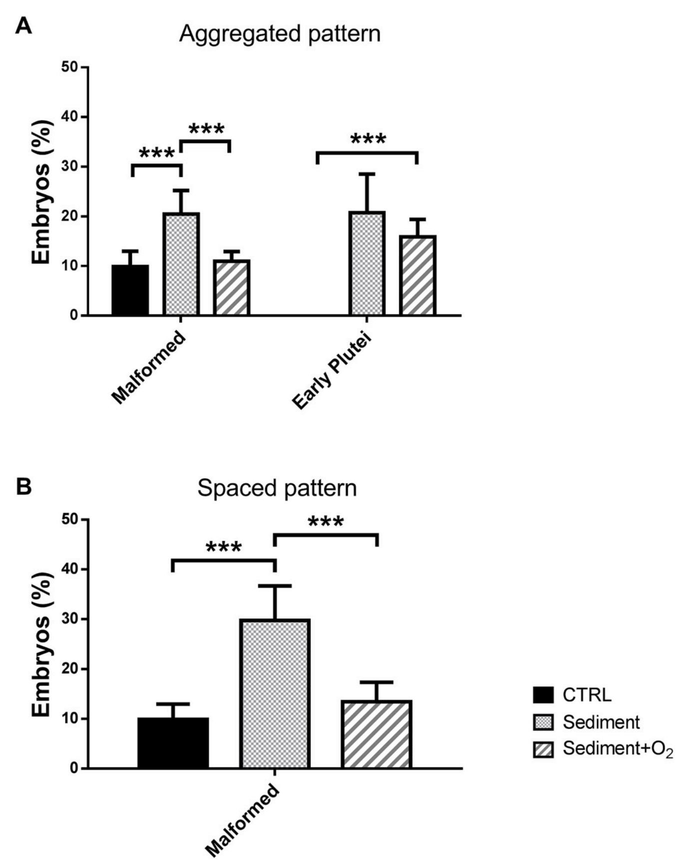

Fig. 4. Percentage of malformed embryos and early plutei from sea urchins in (A) aggregated and (B) spaced pattern of turbulence. Morphological observations were done on embryos from adults exposed to control condition (CTRL, without sediment) and contaminated sediments with oxygen supply (Sediment + O_2) and with natural aeration (Sediment). Data are reported as mean ± standard deviation (M±SD). Three-way ANOVA followed by Tukey's test for multiple comparisons (*** p-value < 0.001, ** p-value < 0.01, * p-value < 0.05).

Table 1
Three-way ANOVA analysis, reporting the degrees of freedom (DF), mean squares (MS), F-test (F) and p-value in different experimental conditions.

ANOVA Table	DF	MS	F (DFn, DFd)	P value
Oxygen	1	482	F (1,1) = 31.4	P < 0.0001
Sediment	1	890.6	F (1,1) = 58.03	P < 0.0001
Turbolence	1	64.19	F (1,1) = 4.182	P < 0.05
Oxygen x Sediment	1	482	F (1,1) = 31.4	P < 0.0001
Oxygen x Turbolence	1	14.09	F (1,1) = 0.9177	P > 0.3
Sediment x Turbolence	1	64.19	F (1,1) = 4.182	P < 0.05
Oxygen x Sediment x Turbolence	1	14.09	F (1,1) = 0.9177	P > 0.3
Residual	40	15.35		

body plan of the plutei was strongly damaged and malformed with a slight reduction of body length (Supplementary Figs. S1C–D).

A statistical analysis correlated these biological results with chemical analysis (Supplementary Tables S3–S6). Cluster Analysis suggested that all metals are associated with toxicity effects, with special reference to Ni, Cu, Cd when suspended matter is considered in both turbulence patterns and in the two turbulence events (Supplementary Figs. S2A–B and S3A-B). Similarly, for both first and second turbulence events all heavy metals present in water seem to be associated with the malformation process, besides Zn (Supplementary Figs. S4A and S5A). However, Correspondence Analysis suggested that malformations are mainly linked to the dissolved heavy metals in the water in both turbulence patterns without oxygen supply (reported as SPS and AGS for the first turbulence, and SS2 and AS2 for the second turbulence in the Supplementary Figs. S4B and S5B). Furthermore, the main metals contributing to this pattern seem to be Zn and Ni, besides Cr in the first turbulence and Pb in the second turbulence. As for PAHs, both in suspended matter and in the water, the Cluster Analysis suggested that all are associated with the toxicity effect on embryos with the exception of Fluoranthene during both turbulence events (Supplementary Figs. S6A, S7A, S8A and S9A). Furthermore, Correspondence Analysis also suggested that malformations are related mainly to the spaced pattern without oxygen supply (Supplementary Figs. S6B, S7B, S8B and S9B). In both turbulence conditions, a remarkably identical picture is drawn suggesting that there are no specific PAHs (both in suspended matter and in the water) associated with the process but all IPAs could be especially effective in the conditions of space pattern without oxygen supply.

3.2. Gene expression by Real Time qPCR

Several genes involved in stress response, development and differentiation, skeletogenesis and detoxification processes were affected in plutei deriving from adult sea urchins exposed to contaminated sediment of Bagnoli-Coroglio area (Figs. 5-6 and Supplementary Table S7). Differences have been observed in the "aggregated" and "spaced" patterns of turbulence and with and without oxygenation.

- Genes affected by "aggregated" pattern of turbulence (Fig. 5)

The highest number of altered genes found in treatments without oxygen supply were involved in development and differentiation processes, such as, *hat* (−5.9), *BP10* (1.5), *Blimp* (−7.9), *Alix* (1.8), *tcf4* (3.4), *Foxo* (2.0), *TAK1* (−4.9), *JNK* (2.5). Among these genes almost all were not affected in conditions of oxygen oversaturation, except for *TAK1* and *tcf4* genes, which resulted down-regulated (−3.0 and −1.6, respectively) in comparison to control. The expression of other five

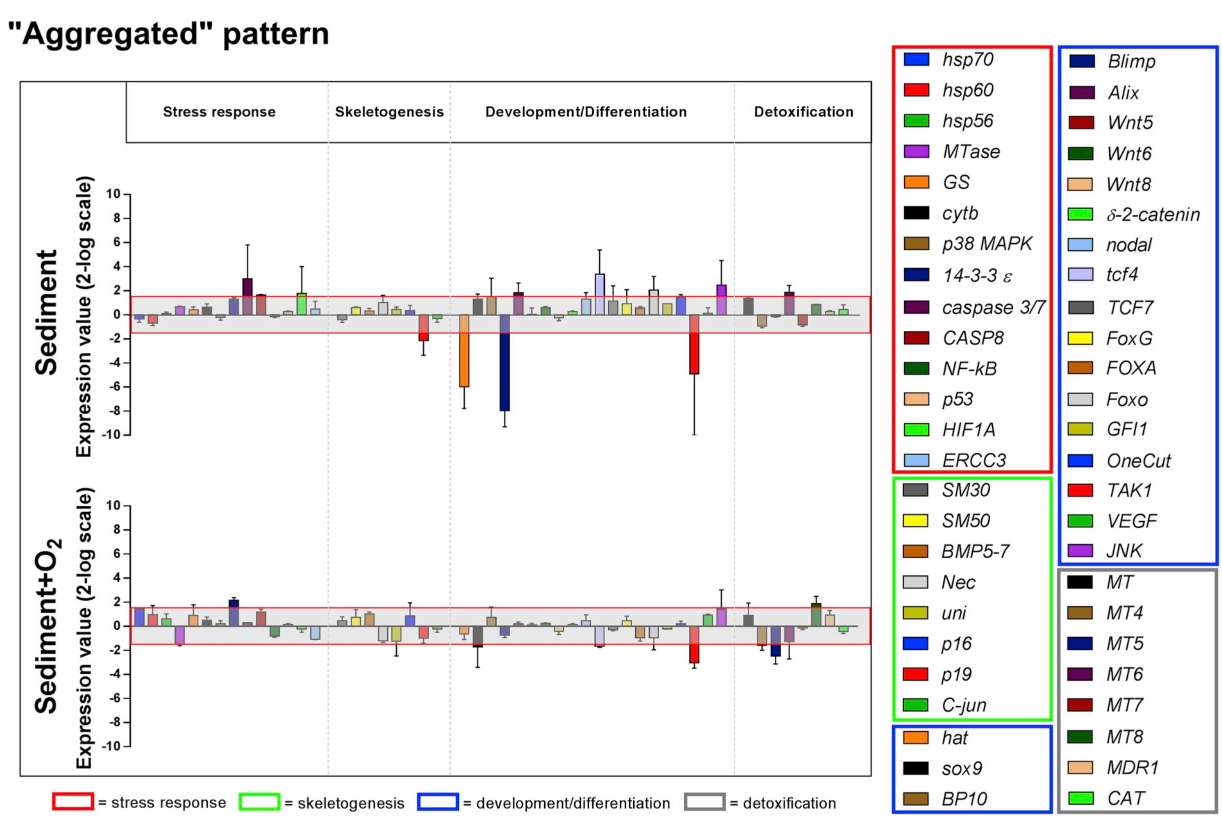

Fig. 5. Real-Time qPCR of sea urchin plutei (48 hpf) from sea urchins exposed to aggregated pattern. Histograms show the differences in expression levels of fifty genes involved in stress response (genes in the red box), skeletogenesis (genes in the green box), development/differentiation (genes in the blue box) and detoxification (genes in the grey box) in plutei deriving from adults exposed for one month to polluted sediment with natural O_2 (indicated with "Sediment) and with O_2 supply (indicated with "Sediment + O_2"). Data are reported as a fold difference (mean ± SD) compared with control (CTRL) embryos. Fold differences greater than ±2 (see red horizontal guidelines at values of +1.5 and −1.5) were considered significant (For further details, see Supplementary Table S7). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

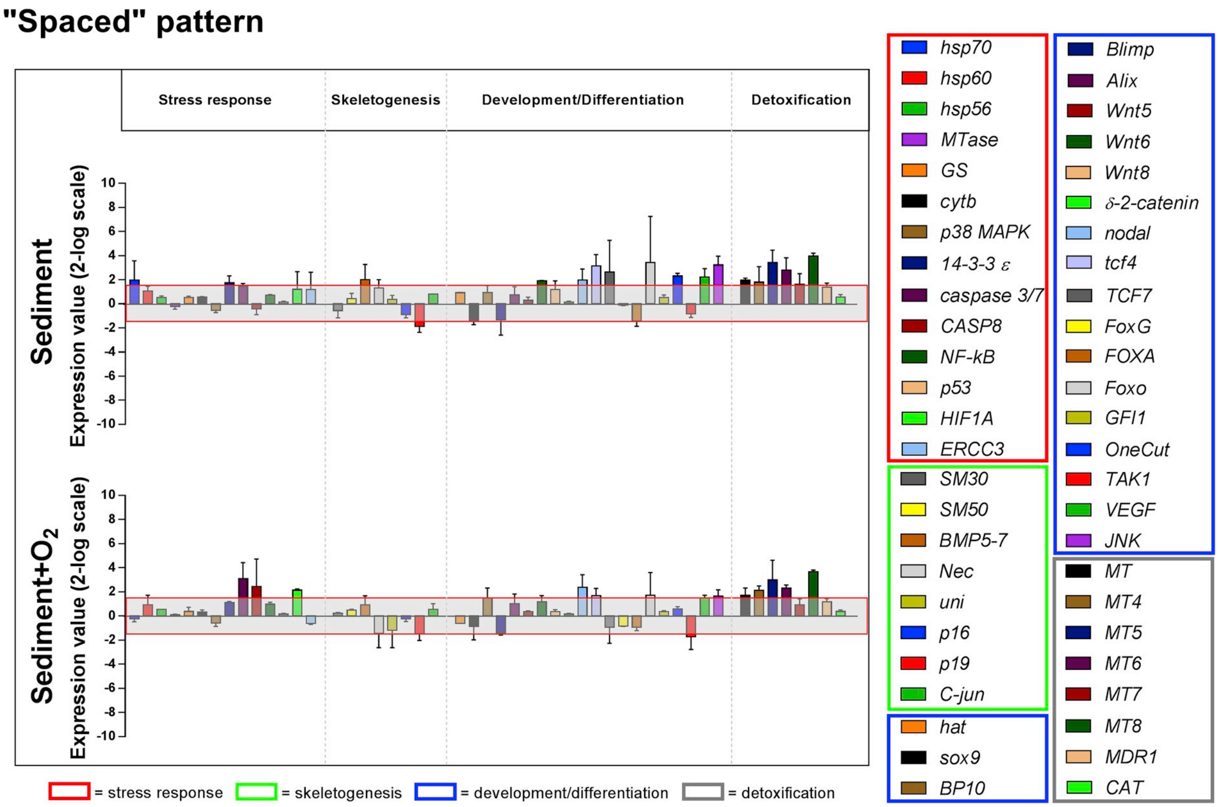

Fig. 6. Real-Time qPCR of sea urchin plutei (48 hpf) from sea urchins exposed to spaced pattern. Histograms show the differences in expression levels of fifty genes involved in stress response (genes in the red box), skeletogenesis (genes in the green box), development/differentiation (genes in the blue box) and detoxification (genes in the grey box) in plutei deriving from adults exposed for one month to polluted sediment with natural aeration (indicated with "Sediment) and with O_2 supply (indicated with "Sediment + O_2"). Data are reported as a fold difference (mean ± SD) compared with control (CTRL) embryos. Fold differences greater than ±1.5 (see red horizontal guidelines at values of +1.5 and −1.5) were considered significant (For further details, see Supplementary Table S3). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

genes was targeted without oxygen overasaturation: the stress genes *caspase 3/7* (3.0), *CASP8* (1.6) and *HIF1A* (1.8) showed up-regulation, whereas the skeletogenic gene *p19* (−2.1) was down-regulated and the metallothionein *MT6* (1.9) increased its expression. In this turbulence condition combined with the oversaturation of oxygen the following genes varied their expression levels: *MTase* (−1.5), *14-3-3ε* (2.2), *sox9* (−1.7), *MT4* (−1.6), *MT5* (−2.5) and *MT8* (1.9).

- Genes affected by "spaced" pattern of turbulences (Fig. 6)

Eight genes involved in developmental and differentiation processes were switched on by sediment exposure in conditions of normal aeration: *Wnt6* (1.9), *nodal* (2.0), *tcf4* (3.1), *TCF7* (2.6), *Foxo* (3.4), *OneCut* (2.3), *VEGF* (2.2) and *JNK* (3.2).

Two skeletogenic genes were also altered: *BMP5-7* (2.0) and *p19* (−1.8), which showed a decrease of its down-regulation (−1.5) with oxygen addition. Moreover, all metallothioneins were up-regulated in both conditions (natural aeration and O_2 supply for oversaturation), except for *MT7* that showed an increase of its expression level only in the case of natural aeration and with no variation in the condition with oxygen oversaturation. Two stress genes were also targeted by this turbulence condition *hsp70* (2.0) and *14-3-3ε* (1.8) On the contrary, five genes were targeted only with oxygen oversaturation: *caspase 3/7* (3.1), *CASP8* (2.4), *HIF1A* (2.1), *BP10* (1.5) and *TAK1* (−1.7).

4. Discussion

The discharge of contaminants, nutrients and sediments, deriving from increased urban and industrial development, is responsible for habitat alteration, especially in coastal areas (Vitousek et al., 1997; Agardy et al., 2005; Spalding et al., 2014; Neumann et al., 2015). In fact, harbor areas represent highly contaminated environments, where organisms are exposed to mixtures of pollutants, whose effects are difficult to interpret and predict exclusively from chemical analyses (Regoli et al., 2004). For these reasons, different biological tests have been recently developed to evaluate biological responses to environmental pollution at cellular and organismic levels (McCarthy and Shugart, 1990; Shugart et al., 1992; Viarengo et al., 1997; Kerambrun et al., 2012). Kobayashi (1971) can be considered the pioneer on the evaluation of water quality, using sediment analyses in the assessment of the adverse effects of marine pollution on resident biota in Seto Bay (Japan). In particular, in these studies fertilized sea urchin eggs were used as indicators for marine pollution bioassay. Further, sea urchin embryo and sperm bioassays have been used in a great number of investigations on sediment toxicity by testing pore water, elutriates or whole sediment (see Pagano et al., 2017 for a review; Chiarore et al., 2020). Moreover, as reported in Zhadan et al. (2017) sea urchins are characterized by high levels of phenotypic plasticity. In fact, their morphological and physiological characteristics, such as growth rates, maximum sizes of body and gonads, and morphology of the body, are prone to changes during the adaptation to selected environmental conditions. This adaptation occurs in sea urchins but it is a long-term exposure adaptation. For example, the former authors reported the results of long-term studies (2003–2015) of the reproductive biology of the sea urchin *Strongylocentrotus intermedius* in populations inhabiting anthropogenically polluted areas. The adaptation represents a long processes, also because a potential for adaptation intrinsically requires the presence of genetic variation leading to gene variants more suitable for altered environmental conditions, as reported very recently by Uthicke et al. (2019). In our experiments the exposure of one month to contaminants is too short to lead to adaptation; thus, the effects found on sea urchin embryos are due to the contaminants released by the sediments.

To our knowledge, this research is among the first on marine sediment toxicity in mesocosms suited to produce hydrodynamics effects and sediment re-working mimicking those associated to natural storm events. Using the Bagnoli-Coroglio post-industrial area as a typical and notable case of a contaminated marine site, we experimentally tested and provided novel insights into the biological impacts of multiple stressors, and the possibility to mitigate them through increased oxygenation. In fact, chemical analyses on both water and suspended matter showed high concentrations of several heavy metals, including As, Cr, Pb, Cu and Zn, and PAHs, such as acenaphthylene, benzoanthracene, fluoranthene, anthracene, benzopyrene, benzo(b)fluoranthene and benzo(k)fluorantene. A severe contamination of these elements in this area caused significant variations of cellular markers in fish lysosomal membrane destabilization in mussels, genotoxic effects both in fish and molluscs (Morroni et al., 2020). All of these elements and compounds exhibit demonstrated genotoxic activities, and may contribute to DNA damage (Williams, 2014). Under natural conditions, however, their ability to get mobilized and transferred from subtidal sediments to surrounding habitats and organisms is crucially dependent on the intensity and temporal patterns of occurrence of storms which may determine sediment re-working. Such traits of meteorological events are expected to increase in association with current and predicted climate changes (Lotze et al., 2006; Halpern et al., 2008; Ummenhofer and Meehl, 2017). Our results suggested that among the heavy metals Zn, Ni, Cr and Cd could have the major influence on sea urchin embryos. On the contrary there are no specific PAHs associated with the observed biological effects.

In partial support to our initial hypotheses, we first showed that, independently of the level of oxygenation, sea urchins were sensitive to the re-worked contaminated sediment, which exerted negative effects on embryos. The "aggregated" patterns of turbulence, in particular, induced a relatively stronger effect on sea urchin embryonic development, consisting in an increase of abnormal (malformed plutei) embryos and of delayed embryos still at the early pluteus stage, compared to the "spaced" patterns. This result could be explained considering that in the "aggregated" condition adult sea urchins were subjected to a strong turbulence-induced stress over the short period of time between the two experimental events of sediment re-suspension. This could have negatively affected adult sea urchins and the 'quality' of their gametes not only mechanically, but also through increased and more diffused concentrations of contaminants released into the water column during such close turbulent events. Under the "spaced" pattern, in contrast, although being eventually subject to the same intensity of mechanical disturbance over the period of the experiment, sea urchins may have been able to recover effectively between one turbulent event and the other, which were separated by eighteen days.

Second and most interestingly, we detected an interactive effect of contaminated sediment and oxygenation. Specifically, the harmful effects (embryonic malformations) of contaminated sediment were clearly mitigated by increased oxygenation, with the number of malformed embryos from adults exposed to the contaminated sediment in the presence of oxygen supply, which was drastically reduced compared to the normally oxygenated condition and was comparable to that observed in the control (no-sediment) condition. Such a mitigation by oxygenation of the negative effects of contaminated sediments generally occurred under both patterns of turbulence. The only partial exception was the number of delayed plutei associated with the "aggregated" pattern, which was still lower in the oxygen-enriched compared to the naturally oxygenated condition, but without equaling the number observed in embryos deriving from control urchins.

Furthermore, our results showed a possible relationship between water oxygenation and pH increase. In fact, we observed a pH increase in oxygen-enriched tanks. In marine environments characterized by low levels of O_2, hypercapnia (high level of CO_2) is normally observed, and therefore reduction of pH, due to organism's respiration not adequately balanced by the supply of oxygen (Howarth et al., 2011; Brewer and Peltzer, 2009). In our experimental conditions it is not surprising that oxygenation produced an increase in pH, being tanks with natural aeration not in hypoxic conditions. The treatment with O_2 supply counterbalanced the effect of respiration (CO_2 production), which can be more important in tanks with no oxygen oversaturation.

It is also worth noting that our research represents a first attempt to assess marine sediment toxicity through a molecular approach applied to the sea urchin *P. lividus*. To the best of our knowledge, similarities with our approach can be found only in the study of Luna et al. (2012), who, however, used *Real Time qPCR* for the identification and quantification of fecal bacteria (*Escherichia coli*, *Enterococcus* spp. and *Salmonella* spp.) in contaminated harbour sediments. A second study examined the effects of contaminated estuarine sediments on flounder fish exposed in mesocosms for seven months, showing, through transcriptomic analysis of liver tissues, that the immune response and apoptotic pathways were those with the greatest number of differentially expressed transcripts (Williams, 2014). Our morphological results indicated that the majority of malformations affected the skeleton and the development plan of sea urchin embryos, and this is consistent with molecular results. Specifically, the expression levels of several genes spanning a range of functions, including the canonical stress response, skeletogenesis, developmental/differentiation and detoxification processes, were switched-on by contaminated sediments. The two turbulence patterns had very few shared molecular targets (*p19*, *tcf4*, *Foxo*, *JNK* and *MT6*), suggesting that they affected different molecular pathways and thereby explaining the different morphological effects.

A similar approach has been used to test antioxidant and immune response of the sea urchin *P. lividus* (Milito et al., 2020) and the ascidian *Ciona robusta*, revealing an alteration of its immune function and microbiome by dysregulation of different genes (Liberti et al., 2020). Studies has been also performed testing elutriates from contaminated coastal sediment of Bagnoli-Coroglio industrial area on three planktonic diatoms, demonstrating an impairment of their growth, sexual reproduction and spore germination (Pelusi, 2020). Also copepods revealed different sensitivity to elutriates from these contaminated sediments (Carotenuto et al., 2020).

Most interestingly, our molecular results were quite supportive of the positive effect of oxygen supply. Actually, genes targeted under both turbulence conditions were completely switched-off or showed lower gene expression variation in the O_2-enriched vs. natural condition. Previous microcosm studies reported the ability of indigenous microbial communities, in sediments from two sites in South Carolina, to degrade, under aerobic conditions, Tert-butyl alcohol, a suspected human carcinogenic substance typically present in groundwater (Bradley et al., 1999; North et al., 2012). Very recently, Giomi et al. (2019) showed that oxygen oversaturation resulting from photosynthesis was able to extend the survival to extreme temperatures of six common marine species living in mangroves, seagrass meadows and coral reefs.

On a broader perspective, the present study indicates that, thanks to the development of molecular/omics approaches, it is possible to study through an experimental manipulative approach how environmental stress induced by contaminated marine sediments can influence gene-gene interactions and gene-environment interactions. These data are essential for a deeper understanding of how species adapt to marine environmental stress under present conditions and future perspectives (Masel, 2006; Loraine, 2009; Orlando et al., 2009; Sreenivasulu et al., 2010; Runcie et al., 2012; Varrella et al., 2016a; Ruocco et al., 2017b). Furthermore, it is worthy that this impacted area is inserted and surrounded by areas, which host a diversified zoobenthic fauna and different coastal habitats, providing populations of some ecologically relevant species (such as sponges and seagrasses) to attempt and implement restoration (Gaglioti et al., 2020; Alagna et al., 2020).

In addition, our findings strongly suggest an increase in oxygenation, for example through the restoration of seagrass meadows such as *Posidonia oceanica*, as a promising tool for environmental restoration, but its application on a large scale requires other *ad hoc* studies.

Author contribution

Conceptualization: MC, IB, VZ, RD. Data curation: NR, MC, IB, VZ. Formal analysis: NR, MC. Funding acquisition: LM, MC, RD. Investigation: MC, NR, DC. Methodology: MC, NR, IB, VZ, MM, DC. Project administration: MC, LM. Resources: MC, LM. Supervision: MC, IB, VZ. Validation: MC, IB, VZ. Writing original draft: NR, MC. Writing review & editing: all the authors.

CRediT authorship contribution statement

Nadia Ruocco: Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Iacopo Bertocci:** Conceptualization, Data curation, Methodology, Supervision, Validation, Writing - review & editing. **Marco Munari:** Methodology, Writing - review & editing. **Luigi Musco:** Funding acquisition, Project administration, Writing - review & editing, Resources. **Davide Caramiello:** Investigation, Methodology, Writing - review & editing. **Roberto Danovaro:** Conceptualization, Funding acquisition, Writing - review & editing. **Valerio Zupo:** Conceptualization, Data curation, Methodology, Supervision, Validation, Writing - review & editing. **Maria Costantini:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2019.104865.

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