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Antioxidant and immune response of the sea urchin *Paracentrotus lividus* to different re-suspension patterns of highly polluted marine sediments

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ABSTRACT

Marine pollution due to disused industrial activities is a major threat to ecosystems and human health, for example through the effects of re-suspension of toxic substances that are present in contaminated sediments. Here, we examined the effects of different re-suspension patterns of polluted sediments from the site of national interest Bagnoli-Coroglio, on the immune system of the sea urchin *Paracentrotus lividus*. An indoor experiment was set up exposing sea urchins for 34 days to such sediments and evaluating the effects of two patterns of water turbulence, mimicking natural storms at sea. One group of animals experienced an "aggregated" pattern of turbulence, consisting in two events, each lasting 2 days, separated by only 3 calm days, while a second group experienced two events of turbulence separated by 17 calm days (spaced pattern). At different times from the beginning of the experiment, coelomic fluid was collected from the animals and immune cells were examined for cell count and morphology, oxidative stress variables, and expression of genes involved in metal detoxification, stress response and inflammation. Our results highlighted that the aggregated pattern of turbulence was more noxious for sea urchins. Indeed, their immune system was altered, over the exposure time, as indicated by the increase of red amoebocytes number. Moreover, despite of an increase of the antioxidant power, animals from this group displayed a very significant ROS over-production at the end of the experiment. Conversely, animals in the spaced condition activated a different immune response, mainly having phagocytes as actors, and were able to partially recover from the received stress at the end of the experiment. No changes in the expression of genes related to antioxidant and anti-inflammatory responses were observed in both groups. By contrast, a down-regulation of various metallothioneins (4, 6, 7 and 8) in the group subjected to aggregated pattern was observed, while metallothionein 8 was up-regulated in the animals from the group exposed to the spaced pattern of turbulence. This work provides the first evidence of how sea urchins can respond to different re-suspension patterns of polluted sediments by modulating their immune system functions. The present data are relevant in relation to the possible environmental restoration of the study site, whose priorities include the assessment of the effects of marine pollution on local organisms, among which *P. lividus* represents a key benthic species.

1. Introduction

Disused industrial plants can lead to the accumulation of pollutants in the marine environment with deleterious impacts on ecosystem functioning and human health. Policies of environmental restoration allow the reuse of the sites and the development of new activities. Important steps in this process are the definition of the state of the site contamination, the evaluation of the effects of polluted sediments on selected key marine species, and the development of the methodologies necessary for the restoration. In this context, Bagnoli-Coroglio Site of National Interest (SNI) represents a paradigmatic case study in southern Italy. Since the early 1900s large industrial plants have been settled in the area, dealing with steel industry, production of cement and asbestos. Although a phase of divestment of the entire industrial district has begun since the mid-1980s, the area has remained highly polluted, especially by heavy metals and large quantities of hydrocarbons (Albanese et al.,

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2010).

In 2017, the Ministry of Education, University and Research funded a project named ABBaCo "Environmental Restoration and Bathing of Bagnoli-Coroglio SNI" coordinated by the Stazione Zoologica Anton Dohrn and carried out in collaboration with other Campania and Italian excellences in the field of environmental research. This project aims at identifying and testing innovative actions with the final goal of the environmental rehabilitation of this area (Morroni et al., 2020). As part of the ABBaCo Project, there is an effort to attain an exhaustive cognitive framework of current environmental status, evaluating the effects of polluted sediments on local species. Indeed, the accumulation of toxic substances in the sediments and their re-suspension by natural (e.g. waves) or artificial (e.g. dredging) turbulence events, can cause severe impairment of the physiology and behaviour of local populations (Arienzo et al., 2019; Bertocci et al., 2019; Hay Mele et al., 2020), with potential effects on the entire ecosystem. For example, the chronic pollution of Bagnoli-Coroglio SNI has greatly impacted benthic meio- and macrofauna (Gambi et al., 2020), as well as prokaryotic assemblages (Tangherlini et al., 2020). On the other hand, plankton communities seem to be relatively unaffected, probably due to their fast turnover and high spatial dynamics (Margiotta et al., 2020).

Besides their key role in structuring benthic communities and whole marine ecosystems through their grazing activity (e.g., Bulleri et al., 1999), sea urchins are common model organisms in ecotoxicology. Indeed, they are important bio-indicators of marine pollution for their sensitivity to pollutants and subsequent ability to reflect the health of the surrounding environment (Lukyanova et al., 2017; Samuel et al., 2017; Soualili et al., 2008). Specifically, the role of sea urchins as bio-indicators is mainly ascribed to the high sensitivity of reproduction processes and embryonic development to environmental pollutants (Migliaccio et al., 2014, 2015; Lister et al., 2017; Lukyanova et al., 2017) and to the presence in adult animals of a complex immune system involving both humoral and cellular components (Chia and Xing, 1996; Smith et al., 2010). The cell-mediated immune response of sea urchins is due to the presence of a heterogeneous population of cells, coelomocytes, present in the coelomic fluid, including phagocytes, vibratile cells, white and red amoebocytes (Matranga et al., 2006; Smith et al., 2010, 2018). Phagocytes represent the most abundant (80–85%) cell type in the coelomic fluid, followed by amoebocytes (15%) and vibratile cells (5–6%). Coelomocytes can be activated by different kinds of physical and chemical stimuli and are therefore considered key sentinels of environmental stress (Matranga et al., 2000; Pinsino and Matranga, 2015; Pinsino et al., 2008, 2015; Rast et al., 2006; Smith et al., 2018). For example, when sea urchins are exposed to zinc, stressed phagocytes undergo changes in shape, i.e. from a petaloid to a philopodial-like morphology, concurrently the relative proportion of red amoebocytes increases (Pagliara and Stabili, 2012). The increase of red amoebocytes, together with a higher expression of the heat shock protein 70 (hsp70), has also been reported in coelomocytes from injured animals or collected from contaminated sites (Matranga et al., 2000; Pinsino et al., 2008). Genes and protein families responsible for the flexible and highly protective features of the sea urchin immune system have been increasingly studied (Matranga et al., 2005; Smith, 2012; Smith and Lun, 2017). Specific pathways are selectively activated in coelomocytes, depending on the stress agent. For example, titanium dioxide nanoparticles have been reported to stimulate immune cell phagocytic and antioxidant metabolic activities as well as to suppress the expression of genes encoding for proteins involved in immune response and apoptosis (Alijagic et al., 2020; Pinsino et al., 2015). The effects of marine pollution, using both polluted sediments or pore water/elutriates, have been widely assessed on sea urchin embryos/larvae (Cesar et al., 2009; Chiarore et al., 2020; Geffard et al., 2001; Limatola et al., 2020; Pagano et al., 2017; Ruocco et al., 2020). By contrast, only few studies deal with the pollution-induced response in adult sea urchins, mainly assessing the concentration of heavy metals in various tissues and compartments of specimen collected from different impacted regions (Pancucci et al., 1993; Soualili et al., 2008; Warnau et al., 1998). Recently, the effect of various environmental factors has been investigated on *Paracentrotus lividus* adults assessing reproduction ability, progeny fitness, and immune system response (Migliaccio et al., 2015, 2016, 2019).

In this work, we investigated the effects of different re-suspension patterns of contaminated sediments from Bagnoli-Coroglio SNI on the sea urchin *P. lividus*. We set-up an indoor experiment in which animals were maintained in tanks containing polluted sediments for 34 days in total and were exposed to different patterns of water turbulence. Water turbulence, caused in nature for example by intense storms, may determine the re-suspension of sediments (e. g., Arienzo et al., 2019; Sherman et al., 1994; Sunamura and Kraus, 1984) and the possible release of contaminants that would potentially affect also nearby habitats (Lawes et al., 2017; Pellecchia et al., 2020). This environmental problem is likely to be exacerbated by the ongoing and predicted increase of the intensity and frequency of extreme meteorological events due to climate change (Aumann et al., 2018; Easterling et al., 2000; Trapp et al., 2007; Wolff et al., 2016). Indeed, assessing the biological and ecological impact of disturbance associated with climate change is a main goal of current ecological research and of increasing concern at the societal and policy level. Among the most important factors influencing the response of individual species to disturbance, is its temporal variation, which can affect the mortality (or emigration) of local species, but also their colonization (or immigration) for the increase of available fresh resources (Bertocci et al., 2005). Moreover, temporal variation associated with changes in time interval between two disturbance events can differentially affect the recovery of organisms with different life-traits (Bertocci et al., 2005, 2017). In this context, a gap of knowledge exists on how such disturbance factors can influence sea urchin populations, whose immune system can be negatively affected, finally leading to cell death of the individuals. The present study is intended to assess the effects of a combined stress due to polluted sediments and different temporal regimes of disturbance on sea urchin immune system. Specifically, through a manipulative experiment, we exposed adult sea urchins to treatments where the same total number of turbulence events, i.e. two, were established at different intervals of time ("aggregated" *vs* "spaced" pattern of contaminated sediment re-suspension), and tested the general hypothesis that changes in the regime of such a disturbance cause different immune responses in sea urchins. Moreover, we tested for the possible effect of water turbulence *per se*, independently of the presence of contaminated sediment in the tanks. After the collection of coelomic fluid and the isolation of immune cells from animals exposed to each experimental condition, such responses were examined at multiple level, including morphological observations, oxidative status-related biochemical assays, and the expression of selected genes associated with metal-detoxification, antioxidant and anti-inflammatory processes.

2. Materials and methods

2.1. Ethic statement

Paracentrotus lividus individuals were collected at Ischia island (Gulf of Naples), from a location that is not privately owned nor protected in any way, according to the authorization of Marina Mercantile (DPR 1639/68, 09/19/1980, confirmed by D. Lgs. January 9, 2012 n.4). The sampling did not involve endangered or protected species. All animal procedures were in compliance with the guidelines of the European Union (directive 2010/63 and following D. Lgs. 4/03/2014 n.26).

2.2. Experimental strategy

Collected sea urchins were transported in an insulated box to the laboratory within 1 h after collection and maintained in tanks with circulating sea water. The animals were acclimated for a minimum of 10 days before the experiment and they were fed with fresh macroalgae (*Ulva* spp). A set of total 8 glass tanks was set-up at the facility for the

maintenance of marine organisms of Stazione Zoologica Anton Dohrn. Each tank (47 × 31 × 35 cm, 35 L) was filled with filtered (0.22 μm) sea water. On the bottom of four out of these eight tanks, a layer of 5 cm of sediment collected from a sampling station within the Bagnoli-Coroglio SNI (UTM coordinates: N 33 429405,23; E 33 4518298,73; water depth: 3.80 m) was deployed at the beginning of the experiment. This sediment was characterized by coarse sand >63 μm (93%) and fine sand <63 μm (7%) (Morroni et al., 2020) and was manually homogenized prior to the deployment to guarantee the same sediment condition in all tanks. The remaining tanks were left without sediment to serve as control for the effect of water turbulence *per se*. Ten animals were transferred into each tank and kept for 34 days in a closed flow-through system. During the experiment the tanks were continuously aerated with a Micraplus recirculation pump. Two out of the four tanks with sediment and two out of the four without sediment were randomly assigned to each of two turbulence patterns. In the "aggregated" pattern, two turbulence events (the first starting the 22nd day since the beginning of the experiment), each lasting two days, were separated by three days during which turbulence was stopped. In the "spaced" pattern, two turbulence events of the same two-day duration each (the first starting the 8th day since the beginning of the experiment) were separated by 17 'calm' days. Such an experimental setting was suited to vary the temporal patterning of turbulence events while maintaining the same overall frequency and intensity of disturbance over the study period, as illustrated by Benedetti-Cecchi (2003) and in several manipulative studies (e.g., Benedetti-Cecchi et al., 2006; Bertocci et al., 2005, 2007, 2017; García-Molinos and Donohue, 2011; Maggi et al., 2012; Vaselli et al., 2008). Turbulence was produced by Voyager movement pumps able to generate re-suspension of the sediment, where present, analogously to natural storms in the field.

The duration (2 days) of each turbulence event and their frequency over the period of the experiment (2 events over 34 days) were established based on meteorological data of extreme storm events recorded as part of ongoing monitoring programs that are carried out at Stazione Zoologica Anton Dohrn, and taking into account the predicted increase in the frequency of extreme meteorological events due to climate change (for details, please see Ruocco et al., 2020).

The no-sediment tanks provided a control for the effect of turbulence pattern itself, a source of disturbance that can modulate a range of physiological and behavioural responses of several invertebrates, including sea urchins (e.g., Fuchs et al., 2017; Gaylord et al., 2013; Kregting et al., 2013; Yamaguchi and Hayami, 2018).

The coelomic fluid was collected at days 3, 10, 24 and 34 since the start of the experiment from 3 animals in each tank (6 animals/condition) as described below. The structure of the experiment, including the occurrence of the sampling times, is illustrated in Fig. 1.

2.3. Coelomic fluid sampling and immune cell counting

1 ml of the coelomic fluid was withdrawn through a puncture in the peristomial membrane of the animals by a needle (26 gauge) coupled to a sterile syringe (5 ml), previously filled with 1 ml of ice-cold 2 × anticoagulant solution/coelomocyte culture medium (CCM) (1 M NaCl, 10 mM $MgCl_2$, 40 mM Hepes, 2 mM EGTA pH 7.2) (Pinsino et al., 2008). Aliquots of 10 μl subsamples were loaded into Fast-Read 102® counting chamber and total coelomocytes and qualitative composition of heterogeneous cell populations were assessed under optical microscope (Zeiss Axioscope). Based on cell concentration, each sample was divided in aliquots for all the different analyses as follows: 1.5 × 10^6 cells/animal for ROS, 1.0 × 10^6 cells/animal for RNA extraction and the remaining volume for total antioxidant capacity analysis. Samples were differently processed for each analysis as described below.

2.4. Intracellular levels of reactive oxygen species (ROS)

Intracellular levels of ROS were determined using the specific

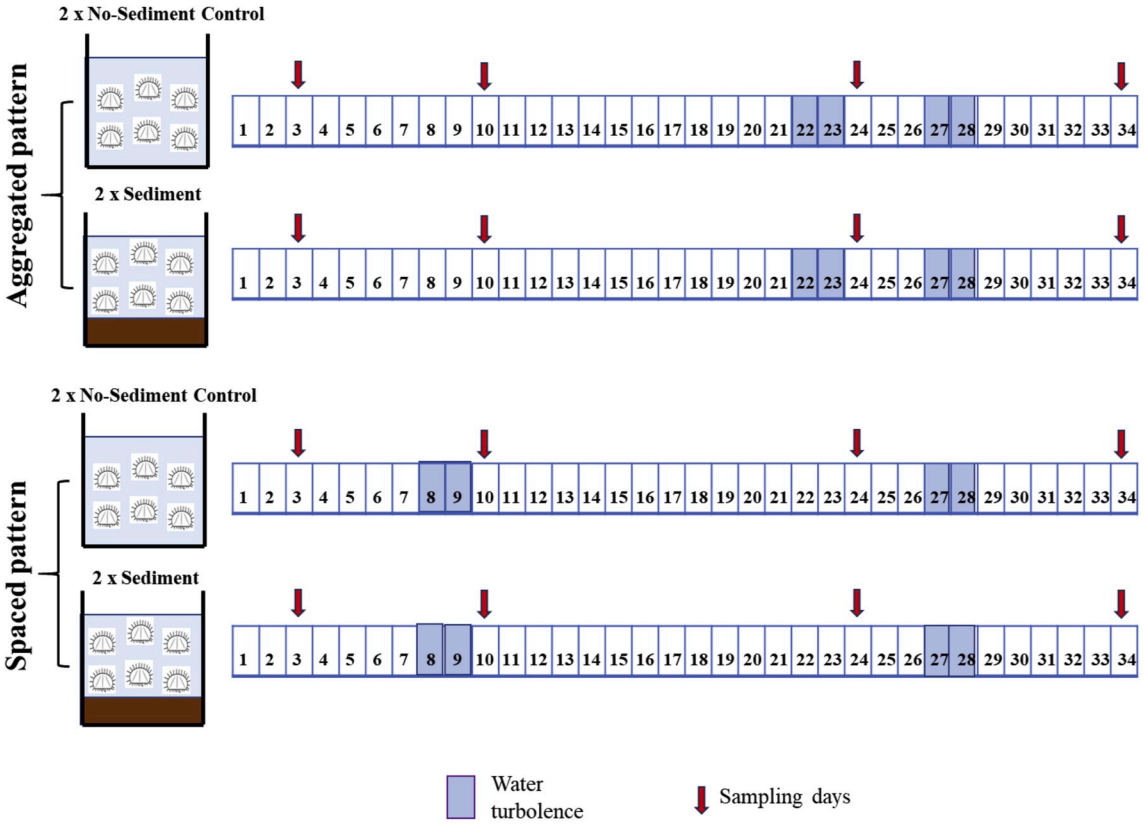

Fig. 1. Schematic representation of the experimental design.

fluorescent probe H_2DCF-DA (2′,7′-dichlorohydro-fluorescein diacetate) according to McCaughey and Bodnar (2012), with some modifications. Briefly, 1.5×10^6 cells/animal, were incubated with 20 μM H_2DCF-DA (stock solution in DMSO, DMSO not exceeding 0.1% in the incubation mixture) for 1 h in the dark at room temperature. In control samples, the same amount of coelomocytes was incubated with DMSO. After incubation, cells were collected by centrifugation at 8000 rcf for 10 min at +4 °C, briefly rinsed twice in CCM 1*x* and stored at −80 °C until use. Frozen cells were resuspended in 0.5 ml Tris–HCl buffer 40 mM, pH 7.0, vortexed for 1 min, and finally centrifuged for 10 min at 8000 rcf at +4 °C. The supernatant was collected and the fluorescence was measured using spectrofluorometer (Tecan Infinite M1000 Pro Microplate Reader) at an excitation and emission wavelengths of 488 nm and 525 nm, respectively. Fluorescence values were normalized by subtracting the auto-fluorescence of DMSO pre-incubated control samples. All samples were analyzed in triplicate and results were expressed as arbitrary units (a.u.) of fluorescence intensity.

2.5. Total antioxidant capacity

The total antioxidant capacity (TAC) of immune cells was determined according to Migliaccio et al. (2019), evaluating the ability of hydrogen-donating antioxidants in the samples to decolorize the pre-formed radical cation of 2,2′-azinobis-(3-ethylbenzothiazoline-6--sulfonic acid) (ABTS), generated by oxidation of ABTS with hydrogen peroxide in the presence of horseradish peroxidase. Aliquots of immune cells, collected in CCM as described above, were centrifuged at 8000 rcf for 10 min at +4 °C, briefly rinsed twice in CCM and stored at −80 °C. Frozen cells were resuspended in PBS 1*x* (1:2 w:v), centrifuged at 14000 rcf for 30 min at +4 °C and the supernatant was used for the assay. ABTS radical scavenging activity of immune cells was determined spectrophotometrically at 730 nm (Agilent spectrophotometer). A standard curve with fixed concentrations of ascorbic acid, ranging from 1 to 15 μM, was performed. All samples were analyzed in triplicate and results were expressed as ascorbic acid equivalents normalized by protein content, measured by Bradford assay (Bradford, 1976).

2.6. RNA extraction and retrotranscription

1.0×10^6 coelomocytes/animal in CCM were collected in a sterile tube, centrifuged at 8000 rcf for 10 min at +4 °C. Pellet was rinsed twice in PBS 1*x*, resuspended in 100 μl lysis solution (RNAqueous™-Micro Total RNA Isolation Kit, Invitrogen, AM 1931), deep-frozen in liquid nitrogen and stored at −80 °C until use. Samples were homogenized using a tissue lyser for 1 min at 20 Hz (2 cycles with a 1 min break on ice). Total RNA was extracted according to the kit's instructions, including DNase treatment. RNA samples were quantified by measuring the absorbance at 260 nm (ND-1000 Spectrophotometer; NanoDrop Technologies, Wilmington, DE, USA) and then checked for integrity by agarose gel electrophoresis. 500 ng from each RNA sample was retrotranscribed with iScriptTM cDNA Synthesis kit (Biorad) and the T100 Thermal cycler (Bio-Rad), following the manufacturer's instructions.

2.7. Reverse transcription-quantitative PCR (RT-qPCR) experiments

The expression levels of selected genes were analyzed by RT-qPCR using specific primers previously reported in literature (Supplementary Table S1). RT-qPCR was performed in MicroAmp Optical 384-Well reaction plate with Optical Adhesive Covers (Applied Biosystems) in a Viia7 Real Time PCR System (Applied Biosystem). Serial dilutions of cDNA were used to generate the standard curves and calculate reaction efficiency ($E = 10^{-1/slope}$) and correlation factor R^2 for each primers pair (Supplementary Table S1). The reaction was carried out in 10 μL for each sample, including 5 μL of Fast Start SYBR Green Master Mix (Roche), 1 μL of cDNA template (1:10 dilution) and 0.7 pmol/μL for each primer. The RT-qPCR thermal profile was obtained using the following amplification protocol: 95 °C for 20 s, 40 cycles of 95 °C for 1 s and 60 °C for 20 s. The melting curve of each amplicon was revealed by the program from 60 °C to 95 °C, reading every 0.5 °C. The presence of single peaks confirmed the gene-specific amplification and the absence of primer-dimers. All RT-qPCR reactions were carried out in triplicate and each assay included three no template negative controls for each primer pair. Expression levels of target genes were normalized using three reference genes (18S, Z12 and RPL17) whose stability in RT-qPCR was assessed using three different algorithms: BestKeeper (Pfaffl et al., 2004); NormFinder (Andersen et al., 2004) and geNorm (Vandesompele et al., 2002). Data were analyzed through the REST tool (Relative Expression Software Tool, Pfaffl et al., 2002) using data derived from animals kept in tanks without the polluted sediment as control condition.

2.8. Chemical analyses of sea water and particulate matter

Chemical analyses of sea water and particulate matter were performed by BIOSCIENCE RESEARCH CENTER SRL, Orbetello, Grosseto. Sea water samples were collected at the second day of the turbulence events and filtered through 0.2 μm filter. The volumes of the samples and the weight of the sediments were recorded. The extraction and the analyses were performed following the standardized methods EPA 3050B 1996 and EPA 6020B 2014 for metals and EPA 3545A 2007 and EPA 8270E 2017 for polycyclic aromatic hydrocarbons.

2.9. Statistical analyses

Univariate analysis of variance (ANOVA) was used to test for the effect of the factor Sediment (Se) (2 levels: sediment/no sediment) on the analyzed variables at intermediate sampling times (3, 10 and 24 days). This analysis was based on a two-way model, including Se as a fixed factor, and Tank (Ta) as a random factor nested in Se, for each "turbulence group" (aggregated *vs* spaced). The structure of the experiment was such that, at each of the intermediate times of sampling, the two levels of turbulence patterns differed for the number of disturbance events occurred in the preceding period and for the time elapsed since the last disturbance event, which would have led to crucially confound any intended effect of turbulence patterns if tested at such times. At the end of the experiment (34 days), when all sample groups were subjected to the same number of turbulence events (two), a three-way ANOVA was performed, including Se and Turbulence (Tu) as crossed fixed factors, and Ta as a random factor, nested in Se x Tu. Student–Newman–Keuls (SNK) pairwise test was used in case of Se x Tu interaction significant effects. Shapiro-Wilk and Cochran's C tests were used before each analysis to assess the normal distribution of data and homogeneity of variance, respectively. Percent data were arcsin (radq) transformed before the analysis according to Ahrens et al. (1990). When parametric assumptions were not met, the significance level was lowered to 0.01 to reduce the chance of Type I error. All ANOVAs were performed using the statistical package STATISTICA (StatSoft, Inc. v. 10). The multivariate dataset including different cell types (phagocytes, vibratile cells, white and red amoebocytes, ROS, TAC) was analyzed at the end of the experiment (34 days) by means of Permutational Multivariate Analysis of Variance (PERMANOVA) based on the same three-way model as that previously described for ANOVA ($n = 4$). The PERMDISP procedure was used to test for homogeneity of multivariate dispersion among groups (Anderson et al., 2008). All analyses were conducted with the Primer 6 v.6.1.12 & PERMANOVA + v.1.0.2 software package (PRIMER-E Ltd) (Clarke and Gorley, 2006). Data on the most affected variables (red cells, ROS and TAC) obtained at the end of the experiment (34 days) were recorded in a matrix along with the contents of heavy metals and PAHs both in water and in the particulate matter, obtained at first and second turbulence event for both experimental groups (aggregated *vs* spaced). This matrix was submitted to Cluster Analysis (paired groups, Euclidean distance) using PAST software package, version 3.14 (Hammer et al.,

2001). RT-qPCR data are presented as mean ± standard error and statistics were performed using the Pair Wise Fixed Reallocation Randomization test by REST (Pfaffl et al., 2002). Gene expression ratios ≥ 2 and with p value ≤ 0.05 were considered significant. Graphs were built using GraphPad Prism.

3. Results

To evaluate the impact of the re-suspension of sediments from Bagnoli-Coroglio site by different temporal turbulence patterns on sea urchin, we examined the status of the *P. lividus* immune system.

Coelomocytes were collected from *P. lividus* at different times of exposure to polluted sediment (days 3, 10, 24 and 34) and results were compared to no-sediment control for each analysis performed. According to the experimental plan (see materials and methods *2.2.*, Fig. 1), the sampling at day 3 represents the zero time before any turbulence; day 10 represents the day just after the first turbulence event for the animals in the "spaced pattern" condition; day 24 represents the first turbulence event for animals in the "aggregated pattern" condition; day 34 represents the end of the experiment after the second turbulence event for both experimental groups (Fig. 1).

3.1. Immune cell count and morphology

Counting of circulating total immune cells showed no changes in sediment-exposed animals from the "aggregated pattern" group at any experimental time, except for a slight decreasing trend at day 10 (Fig. 2; Supplementary Tables S2, S3, S4). On the other hand, a significant increase of total coelomocytes was detected in sediment-exposed animals in the "spaced pattern" condition at day 10, representing the first turbulence event for this group (Fig. 2; Table S3B).

The percentage of each immune cell type respect to the total coelomocytes was also determined. Results showed no variations at day 3 in both groups (Fig. 3A; Table S2). By contrast, at day 10 of exposure to sediment, animals from the aggregated group showed a trend in increasing white amoebocytes and vibratile cells, and decreasing phagocytes (Fig. 3B; Table S3A), while those from the spaced group only increased the percentage of phagocytes compared to no-sediment control animals (Fig. 3B; Table S3B). At 24 days, the sediment-exposed animals subjected to the aggregated pattern condition showed the same trend in decreasing phagocytes and significantly increased their content of red amoebocytes, maintained at high levels also at 34 days (Fig. 3C and D, Fig. 4; Tables 1 and S4). No differences were detected in the spaced pattern group at these experimental times (Fig. 3C and D; Tables 1 and S4).

3.2. Oxidative stress in coelomocytes

The intracellular redox status of immune cells was analyzed by measuring the levels of reactive oxygen species (ROS) and the total antioxidant capacity (TAC). Results showed no variations in ROS content up to day 24, except for a decreasing trend at 3 days in sediment-exposed animals from both turbulence groups, obtained also at 24 days for the spaced group (Fig. 5; Tables S2 and S4). Conversely, a significant ROS increase at day 34 in sediment-exposed animals compared to no-sediment control was detected for both groups (Fig. 5; Table 1).

The TAC of immune cells significantly increased in animals from the aggregated pattern group at day 3 and 34 of exposure to sediment compared to no-sediment control animals, whereas those from the spaced pattern group showed an increasing trend at day 3 and a significant increase at day 24 (Fig. 6; Tables 1, S2, S4).

3.3. Evaluation of chemical and physical disturbance at the end of the experiment

At 34 days of exposure to sediment, both the red cells content and TAC were affected by the 'Se x Tu' interaction, while the presence of sediment affected the ROS content independently of changes in the turbulence pattern, although the 'Se x Tu' interaction was just slightly above the significance limit ($p = 0.06$; Table 1). Post-hoc tests revealed that both red cells and TAC significantly increased in the sediment-exposed animals from the aggregated group compared to no-sediment controls. Moreover, TAC levels of the sediment-exposed animals from the aggregated group, were significantly higher compared to the spaced group (Table 1). No significant effects were detected for all other variables (Table 1). The multivariate analysis on the whole dataset detected only a significant main effect of the presence *vs* absence of sediment (Table 2). PERMIDISP test for the factors sediment, turbulence and tank, showed no significant deviations from centroids ($P_{(perm)} = 0.36$, $P_{(perm)} = 0.20$, and $P_{(perm)} = 0.85$, respectively).

Moreover, we evaluated the possible association of the observed increase in red cells, ROS and TAC at t = 34 days with heavy metals and polycyclic aromatic hydrocarbons (PAH) content, assessed both at first and second turbulence event for both turbulence groups (Table S5). When we considered chemical analysis obtained at first turbulence event, cluster analyses revealed that all heavy metals in water were correlated with the measured variables, especially Ni with red cells and TAC and As with ROS (Fig. S1A). Also, cluster analyses performed using heavy metals and PAH content in particulate matter showed a strong correlation of red cells and TAC with all metals and PAH, except with Cu and acenaphthene, which instead were associated with ROS changes (Figs. S1B and C). Similar results were obtained with chemical analyses at second turbulence event (Fig. S2).

3.4. Gene expression pattern

The expression levels of a series of genes related to detoxification,

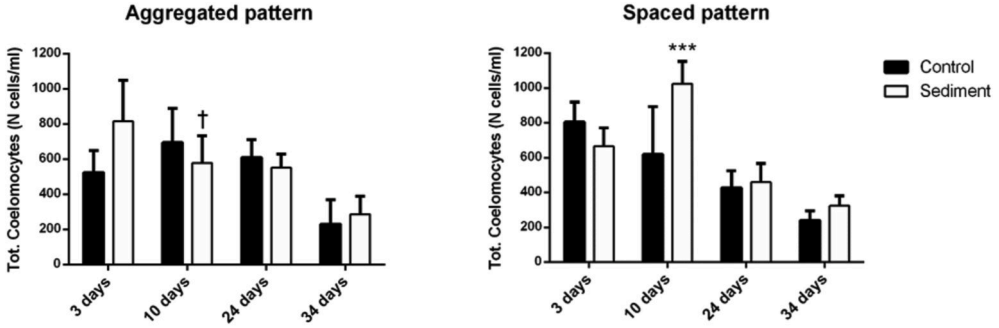

Fig. 2. Total immune cells from coelomic fluid of *P. lividus* exposed to polluted sediment in aggregated and spaced pattern conditions. Total coelomocytes were counted and reported as number of cells/ml for control and sediment conditions along all the experimental times (3, 10, 24, 34 days). Data are reported as means ± SD. ***$p \leq 0.001$ represent significance compared to no-sediment control. Effects at significance limits ($p \leq 0.1$) are indicated by † simbols.

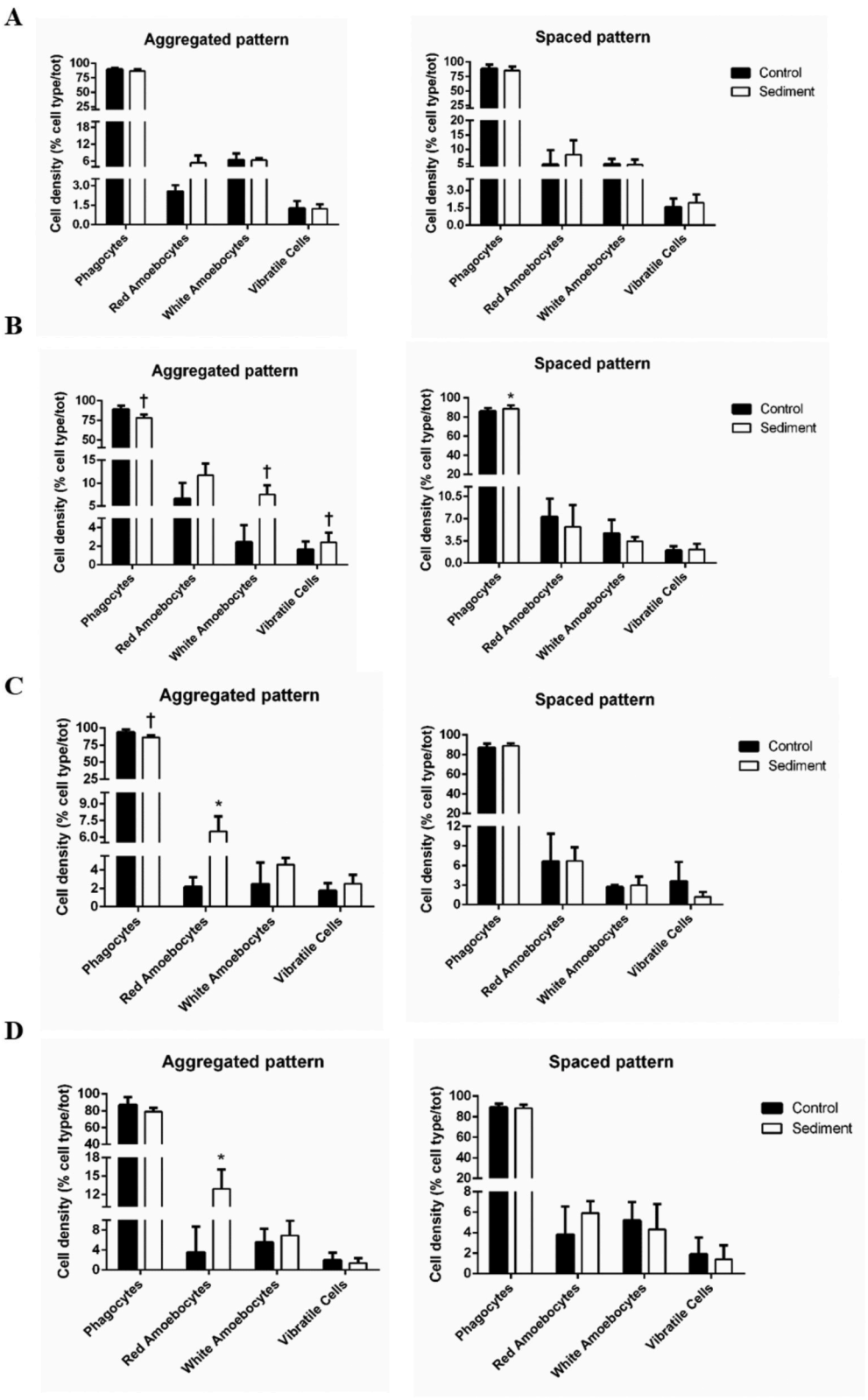

Fig. 3. Differential coelomocytes concentration in *P. lividus* exposed to polluted sediment in aggregated and spaced pattern conditions. Phagocytes, red and white amoebocytes, and vibratile cells were counted and reported as % cell type/total coelomocytes for control and sediment conditions in samples collected at days 3 **(A)**, 10 **(B)**, 24 **(C)** and 34 **(D)**. Data are reported as means ± SD. *p ≤ 0.05 represents significance compared to no-sediment control. Effects at significance limits (p ≤ 0.1) are indicated by † simbols.

oxidative stress and inflammatory processes were followed in the immune cells. The genes encoding the metallothioneins MT4, MT5, MT6, MT7 and MT8 were investigated as genes involved in metal detoxification, whereas the genes encoding the heat shock proteins hsp70, hsp60 and hsp56 were examined as stress genes. We also measured the expression of OvoA, the enzyme involved in the biosynthesis of ovothiol, a powerful antioxidant produced by sea urchins (Palumbo et al., 2018; Castellano and Seebeck, 2018), known to be involved in the response to environmental stress at least in embryos and larvae (Castellano et al., 2016); and the genes encoding for proteins/transcription factors associated with inflammation including Toll-like receptor 4-like (TLR4-like), nuclear factor kappa B (NF-kB), the Jun transcription factor (Jun) and

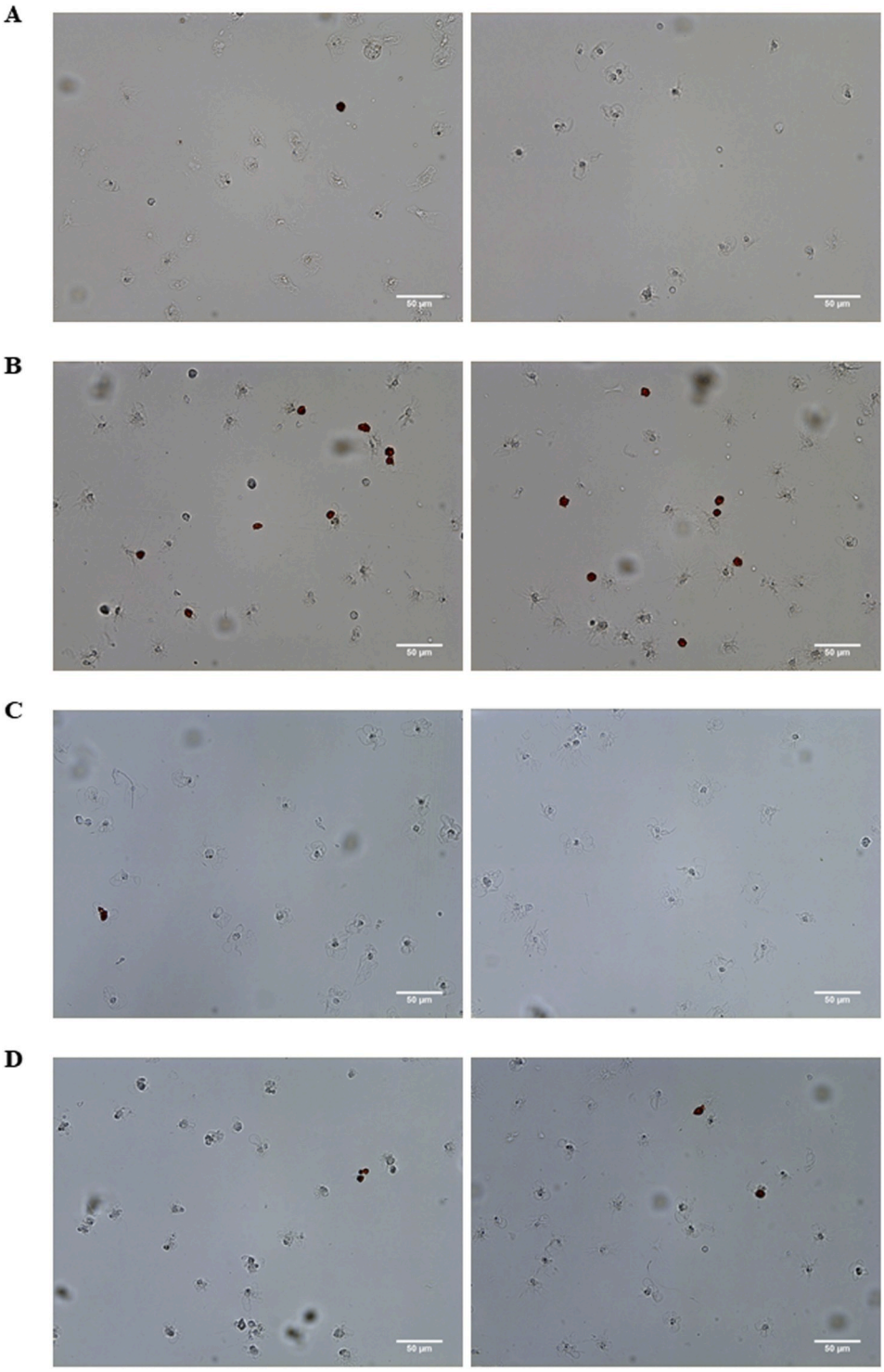

Fig. 4. *P. lividus* coelomocytes morphology at 34 days. Immune cells from *P. lividus* were observed under the microscope: **(A)** Control animals from aggregated pattern group; **(B)** Sediment-exposed animals from aggregated pattern group; **(C)** Control animals from spaced pattern group; **(D)** Sediment-exposed animals from spaced pattern group. Pictures were taken at microscope (Zeiss Axio Imager M1) at 10 x magnification.

the allograft inflammatory factor 1 (AIF-1). RT-qPCR experiments showed that most of variations occurred for the aggregated pattern group compared to the spaced pattern group (Fig. 7). Indeed, in the first group, MT genes were significantly down-regulated in sediment-exposed animals compared to controls, at all the experimental times. In detail, at day 3 of exposure MT7 and MT8 were down-regulated (Fig. 7A), while at day 10 MT4 was significantly down-regulated and MT6 decreased its expression levels reaching the threshold value 2, although it was not statistically significant ($p < 0.2$) (Fig. 7B). At the exposure day 24, all MTs significantly decreased their gene expression, except for MT5 which reached the threshold value 2 but it was only approaching the statistical significance ($p < 0.1$) (Fig. 7C). Despite MT5 and MT6 expression levels returned to basal levels at day 34, MT4 and MT8 remained significantly down-regulated and a trend was also visible for MT7 although only approaching the significance ($p < 0.1$). As regarding the spaced pattern group, no significant variations were

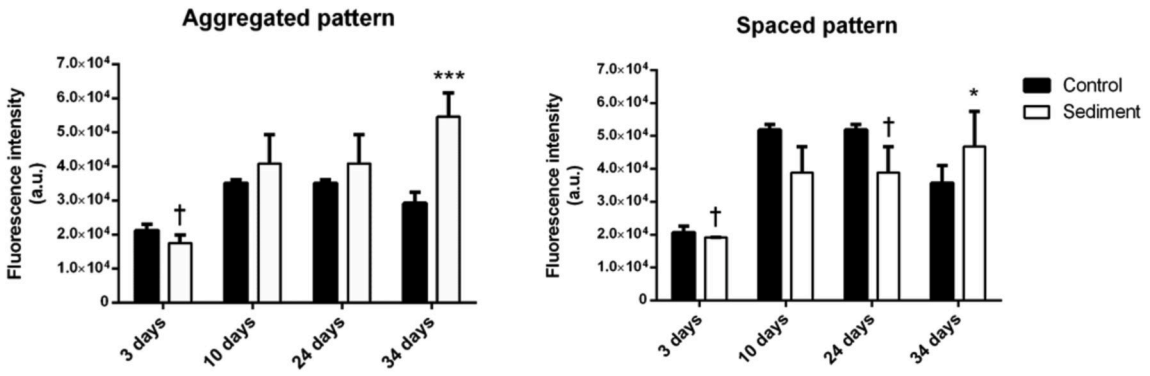

Fig. 5. Intracellular levels of reactive oxygen species (ROS) in coelomocytes of *P. lividus* exposed to polluted sediment in aggregated and spaced pattern conditions. ROS content was determined in coelomocytes and reported as arbitrary units (a.u.) of fluorescence intensity. Data are reported as means ± SD. **p ≤ 0.01 and ***p ≤ 0.001 represent significance compared to no-sediment control. Effects at significance limits (p ≤ 0.1) are indicated by † simbols.

Table 1
Three-way ANOVA on the univariate datasets at the end of the experiment (34 days). Significant effects ($p < 0.05$) are in bold ($n = 4$) and post hoc tests for Sediment*Turbulence interaction significant effects are reported. Effects at significance limits ($p \leq 0.1$) are underlined ($n = 4$).

Three-way ANOVA t=34 days						
Effect	df	SS	MS	F	p	SNK pairwise tests
Total coelomocytes						
Sediment	1	1.95E+04	1.95E+04	2.81	0.17	
Turbulence	1	2.86E+03	2.86E+03	0.41	0.55	
Sediment*Turbulence	1	9.41E+02	9.41E+02	0.14	0.73	
Tank(Sediment*Turbulence)	4	2.76E+04	6.90E+03	0.90	0.49	
Error	12	9.19E+04	7.66E+03			
Phagocytes						
Sediment	1	0.02	0.02	2.34	0.20	
Turbulence	1	0.02	0.02	3.26	0.15	
Sediment*Turbulence	1	0.01	0.01	1.57	0.28	
Tank(Sediment*Turbulence)	4	0.03	0.01	1.34	0.34	
Error	8	0.04	0.01			
Red cells						
Sediment	1	0.09	0.09	27.71	**0.01**	
Turbulence	1	3.95E-03	3.95E-03	1.27	0.32	
Sediment*Turbulence	1	0.03	0.03	10.51	**0.03**	Aggregated: Sediment > no-Sediment
Tank(Sediment*Turbulence)	4	0.01	3.10E-03	0.28	0.88	
Error	8	0.09	0.01			
White cells						
Sediment	1	6.00E-05	6.00E-05	0.02	0.91	
Turbulence	1	4.16E-03	4.16E-03	1.12	0.35	
Sediment*Turbulence	1	2.95E-03	2.95E-03	0.79	0.42	
Tank(Sediment*Turbulence)	4	0.01	3.72E-03	1.29	0.35	
Error	8	0.02	2.89E-03			
Vibratile cells						
Sediment	1	6.34E-04	6.34E-04	0.11	0.75	
Turbulence	1	7.00E-06	7.00E-06	1.24E-03	0.97	
Sediment*Turbulence	1	4.60E-05	4.60E-05	0.01	0.93	
Tank(Sediment*Turbulence)	4	0.02	0.01	1.56	0.27	
Error	8	0.03	3.57E-03			
ROS						
Sediment	1	1.98E+09	1.98E+09	52.59	**1.75E-03**	
Turbulence	1	3.79E+05	3.79E+05	0.01	0.92	
Sediment*Turbulence	1	2.52E+08	2.52E+08	6.70	0.06	Aggregated=spaced: Sediment > no-Sediment
Tank(Sediment*Turbulence)	4	1.50E+08	3.75E+07	0.76	0.57	
Error	15	7.43E+08	4.95E+07			
TAC						
Sediment	1	5.34	5.34	111.58	**3.03E-04**	
Turbulence	1	6.59	6.59	137.76	**1.95E-04**	
Sediment*Turbulence	1	4.42	4.42	92.41	**4.49E-04**	Aggregated: Sediment > no-Sediment; Sediment: Aggregated > Spaced
Tank(Sediment*Turbulence)	4	0.19	0.05	0.21	0.93	
Error	10	2.18	0.22			

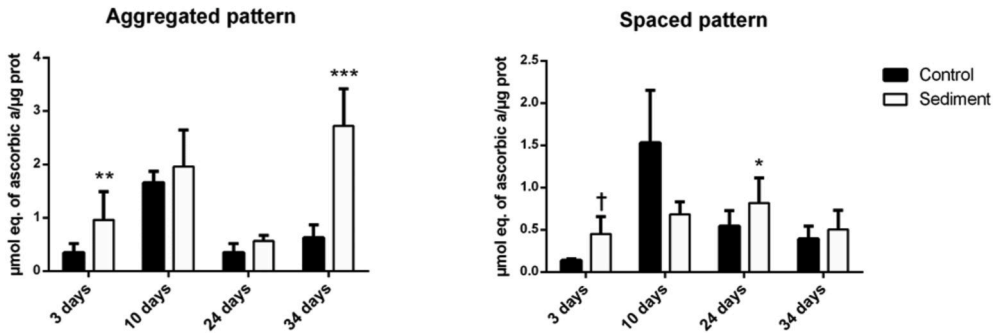

Fig. 6. Total intracellular antioxidant capacity (TAC) in coelomocytes of *P. lividus* exposed to polluted sediment in aggregated and spaced pattern conditions. TAC was determined and reported as µmol equivalent of ascorbic acid/µg protein. Data are reported as means ± SD. *p ≤ 0.05 and ***p ≤ 0.001 represent significance compared to no-sediment control. Effects at significance limits (p ≤ 0.1) are indicated by † simbols.

Table 2
Three-way PERMANOVA on the multivariate dataset at the end of the experiment (34 days). Significant effects ($P_{(perm)} \leq 0.05$) are reported in bold ($n = 4$).

Three-way PERMANOVA						
	df	SS	MS	Pseudo-F	$P_{(perm)}$	Unique perms
Sediment (Se)	1	1.2255E9	1.2255E9	27.711	**0.0292**	270
Turbulence (Tu)	1	67803	67803	1.5331E-3	0.9316	268
Se×Tu	1	1.8855E8	1.8855E8	4.2635	0.1587	270
Tank (Se×Tu)	4	1.769E8	4.4225E7	0.58355	0.6925	9970
Res	8	6.063E8	7.5787E7			
Total	15	2.1973E9				

assessed except for MT8 which was significantly up-regulated at days 24 and 34, despite a high variability among replicates (Fig. 7C and D). No changes were found in the expression levels of the other target genes investigated (Fig. 7).

4. Discussion

Sea urchins are a widely used model system for ecotoxicology studies. Indeed, as key species dominating both planktonic and benthic environments at larval and adult stages, respectively, and being highly sensitive to external conditions, sea urchins can be used to evaluate the effects of marine pollution on local species. The impact of polluted seawater and sediments on sea urchin health at several stages of their life, i.e. larval and adult, can be considered as a sentinel of stress for the marine ecosystem. In this regard, most of the studies have been carried out on sea urchin early life stages testing the effects of sediments or pore water or elutriates (Chiarore et al., 2020; Pagano et al., 2017; Ruocco et al., 2020), whereas very little information is available on the adult response. Moreover, together with the chemical disturbance, i.e. presence of toxic molecules in the sea, physical disturbance (water turbulence), mainly caused by natural storms, can negatively affect the fitness of local species. In this context, temporal variation of disturbance events can strongly affect the recovery of marine organisms depending on their life-traits (Bertocci et al., 2005, 2017).

In this study, we evaluated the antioxidant and immune response of *P. lividus* to polluted sediments from the Bagnoli-Coroglio SNI, testing the interactive effects of sediment-dependent stress with physical disturbance caused by the application of two different re-suspension patterns of water turbulence (aggregated *vs* spaced). In particular, we set-up an indoor experiment with the aim to reproduce sediment re-working activity associated to natural storm events whose intensity and frequency are predicted to increase in the future due to climate changes (Aumann et al., 2018). The sea urchin cell-mediated immune system is dominated by different types of coelomocytes: phagocytes, red and white amoebocytes and vibratile cells. The function of each cell type is not fully understood, although it is known that phagocytes are among the main players in the immune response, being involved in many processes like encapsulation, aggregation and phagocytosis (Matranga et al., 2000), as well as reactive oxygen (ROS) and nitrogen species (RNS) production (Beck et al., 2001; Coteur et al., 2002; Ito et al., 1992), inflammation, complement synthesis and cytotoxic activity (Gross et al., 1999; Haug et al., 2002; Lin et al., 2001; Smith et al., 2010). Moreover, red amoebocytes increase in animals under stressful environmental conditions, suggesting a crucial role in immune response of sea urchins (Matranga et al., 2000; Pinsino et al., 2008). We evaluated the impact of polluted sediments under different re-suspension patterns on the total number of coelomocytes and percentage of each cell type at different times of exposure, as well as their intracellular redox status and the expression of genes, mainly related to antioxidant and inflammatory response, as well as to metal detoxification. Overall our data highlighted a different immune response to the two different temporal patterns of turbulence, with the "aggregated pattern" resulting to be the most stressful for the animals. To measure an early response to the contaminated sediments, apart from the physical disturbance, we performed a sampling at 3 days (before any turbulence event for both groups). Thus, we revealed an early increase of total antioxidant capacity at this time point in animals from both experimental groups, which were able to efficiently counteract the oxidative stress. Indeed, not only ROS did not increase at 3 days in sediment-exposed animals from both turbulence groups but the levels were even lower compared to no-sediment controls. Yet, it is interesting to observe that, while animals in the spaced pattern condition responded by increasing total number of coelomocytes, mainly phagocytes, at first turbulence event, animals in the aggregated pattern condition responded in a different way. Indeed, they modulated their immune system by increasing red amoebocytes at the expense of phagocytes, after the first turbulence event (day 24) and at day 34, as result of the two aggregated events, separated only by 3 calm days. It is interesting to note that red amoebocytes increase at 24 days followed the increasing trend of their precursors at 10 days, i.e. white cells. These results suggest that temporal variation of water turbulence is a key factor to stimulate different responses in sea urchins, with the aggregated pattern being more stressful for the animals compared to the spaced one. This explanation is supported by the fact that, in spite of an increase in antioxidant capacity at the end of the experiment (34 days) in sediment-exposed animals in the aggregated condition compared to controls, it was not enough to balance the ROS levels, which were instead still high at this final time point. On the other hand, animals in the spaced condition activated the antioxidant response at earlier time (day 24), when the second turbulence event was applied, leading to concomitant lower ROS levels. These results, together with the lower ROS increase at the end of the experiment, indicates that the application of two temporally distant turbulence events is less stressful for the

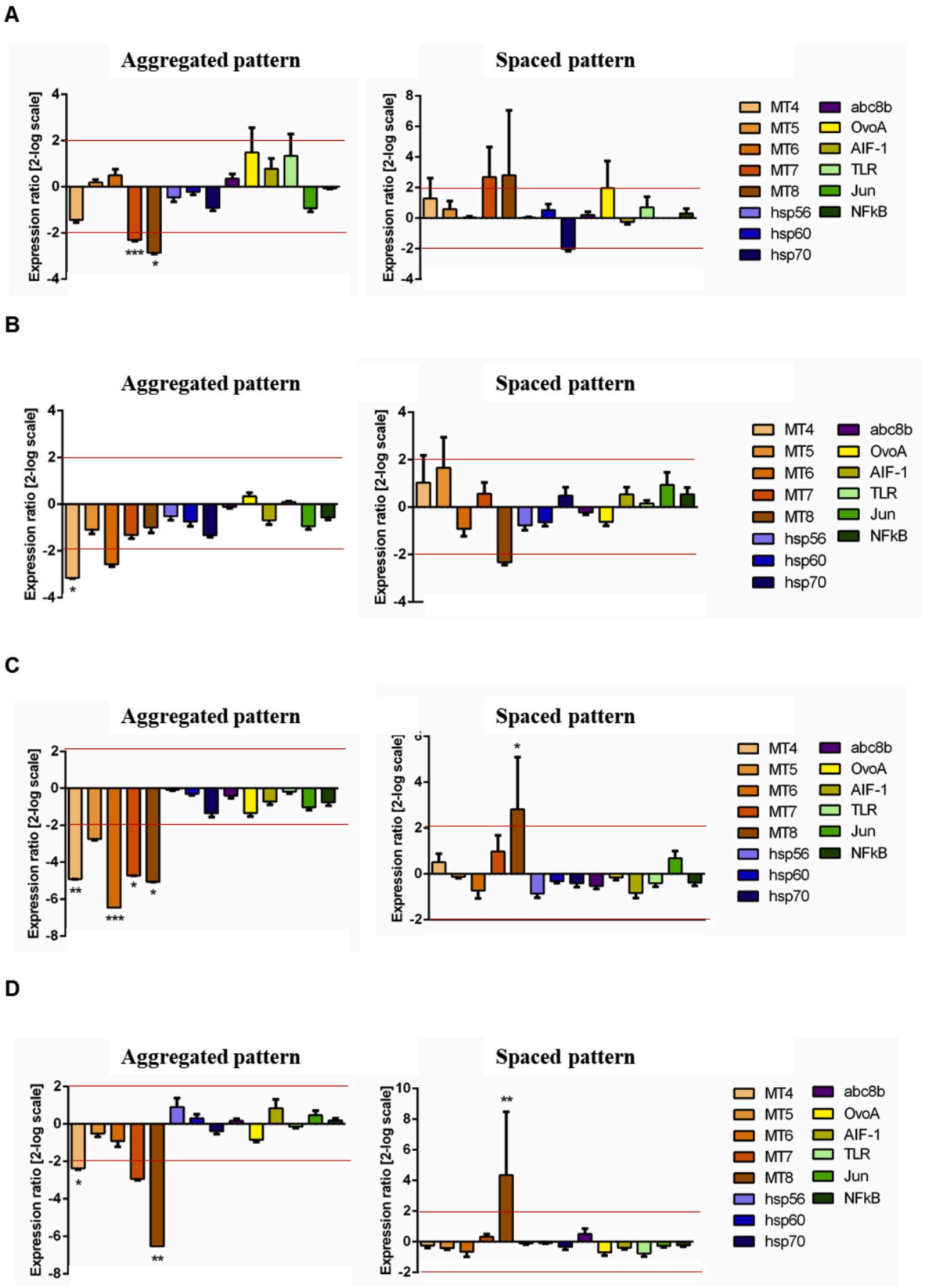

Fig. 7. Gene expression analyses in coelomocytes of *P. lividus* exposed to polluted sediment in aggregated and spaced pattern conditions. The relative expression of the indicated target genes has been assessed after 3 **(A)**, 10 **(B)**, 24 **(C)** and 34 days **(D)** of exposure to polluted sediments. Control condition (baseline) is represented by no-sediment control animals for each experimental group. *$p \leq 0.05$; **$p \leq 0.01$ and ***$p \leq 0.001$ represent significance compared to control condition. Data are presented as means ± standard error.

animals, which were partially able to recover from the received physical disturbance.

Since water turbulence can cause the re-suspension of toxic molecules present in contaminated sediments enhancing their dangerous effect, we can speculate that such responses could be due to the increase of re-suspension of heavy metals and hydrocarbons, mainly present in these sediments. Indeed, cluster analyses revealed that the most affected measured variables (red cells, ROS and TAC) are associated with most of the heavy metals and hydrocarbons measured both at first and second turbulence events.

Moreover, the use of no-sediment controls, still experiencing the two conditions of turbulence patterns, allowed us to ensure that the effects were not simply due to the turbulence *per se* but to the association of the two different temporal patterns with the sediment. Indeed, the formal analyses performed at the end of the experiment allowed us to assess that the interaction of such factors is crucial for the observed variations, especially in terms of red amoebocytes and antioxidant capacity.

To investigate the molecular response of *P. lividus* immune cells we evaluated the gene expression of a selected panel of genes related to metal detoxification, oxidative stress and inflammation processes, mostly studied in *P. lividus* embryos (Migliaccio et al., 2014, 2015, 2016; Ruocco et al., 2020), while their involvement in the immune response is still obscure except for few case studies in other sea urchin species (González-Aravena et al., 2015, 2018). The results indicated the absence of activation of genes involved in the stress and inflammatory responses, whereas we observed variations in gene expression of metallothioneins (MTs), cysteine-rich proteins which bind metal ions like Zn, Cu, Cd and Ni, and are well characterized in *P. lividus* (Ragusa et al., 2013, 2017).

In particular, we found a strong down-regulation of MTs gene expression in sediment-exposed animals from the aggregated group at all the experimental times, while this was not found in animals in the spaced condition, instead showing an up-regulation of MT8 at days 24 and 34 of exposure. The observed down-regulation of MTs in the aggregated pattern group was probably induced by a negative feedback mechanism due to a higher concentration of metals, re-suspended by the two close turbulence events. Present findings suggest that the aggregated pattern of turbulence is more stressful for sea urchins, due to the short time (3 days) occurring between the two turbulence events, which does not allow the animals to recover from the stress received, leading to strong MTs down-regulation. By contrast animals exposed to the spaced turbulence pattern are able to recover from the first disturbance event, and finally up-regulate MT8, the major player in the observed molecular response.

Interestingly and in support of our findings, similar indoor studies reported that the exposure of adult *P. lividus* to an aggregated re-suspension pattern of polluted sediments, leads to negative effects on gamete interaction and Ca^{2+} signaling at fertilization, and to a higher percentage of abnormal development of the progeny compared to the spaced condition (Limatola et al., 2020; Ruocco et al., 2020). Yet, no transgenerational buffer effects were found when progeny from pre-exposed parents was in turn left to develop in presence of elutriates (Chiarore et al., 2020).

5. Conclusions

In conclusion, data from this study indicate that sediments from the Bagnoli-Coroglio SNI may affect the health of the adult *P. lividus* with possible consequences for the whole ecosystem. Indeed, the dangerous effect on *P. lividus* immune system is much stronger when re-suspension of toxic substances present in the sediments, mainly heavy metals and polycyclic aromatic hydrocarbons, is caused by an aggregated temporal pattern of water turbulence events. Thus, considering that this scenario is intended to mimick the increase of natural storms due to climate change, we predict that, if no action will be taken, the dangerous effects of marine pollution on local species will considerably worsen over time. This study, together with other scientific outcomes from ABBaCo Project, may contribute to the assessment of the current environmental status of the site, necessary for a restoration policy. Yet, here we provide the first case study, to the best of our knowledge, aimed at understanding the effects of marine sediments from a polluted area, in interaction with physical disturbance, on adult sea urchins and in particular on the immune system response. The outcomes of this study may contribute to unravel the immune system functioning in sea urchin and how these animals can counteract marine pollution.

Declaration of competing interest

The authors declare no conflicts of interest.

CRediT authorship contribution statement

Alfonsina Milito: Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft, Writing - review & editing. **Carola Murano:** Investigation, Methodology, Visualization, Writing - review & editing. **Immacolata Castellano:** Conceptualization, Funding acquisition, Writing - review & editing, Supervision. **Giovanna Romano:** Conceptualization, Funding acquisition, Writing - review & editing, Supervision. **Anna Palumbo:** Conceptualization, Funding acquisition, Project administration, Resources, Writing - original draft, Writing - review & editing, Supervision.

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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.2020.104978.

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