

## Oxygen supersaturation mitigates the impact of the regime of contaminated sediment reworking on sea urchin fertilization process

Nunzia Limatola<sup>a</sup>, Iacopo Bertocci<sup>b,c</sup>, Jong Tai Chun<sup>d</sup>, Luigi Musco<sup>b</sup>, Marco Munari<sup>b</sup>, Davide Caramiello<sup>a</sup>, Roberto Danovaro<sup>a,e</sup>, Luigia Santella<sup>a,\*</sup>

<sup>a</sup> Research Infrastructures for Marine Biological Resources (RIMAR), Stazione Zoologica Anton Dohrn, Villa Comunale 1, Napoli, 80121, Italy

<sup>b</sup> Integrative Marine Ecology Department (EMD), Stazione Zoologica Anton Dohrn, Villa Comunale 1, Napoli, I- 80121, Italy

<sup>c</sup> Department of Biology, University of Pisa, CoNISMa, Via Derna 1, Pisa, I-56126, Italy

<sup>d</sup> Biology and Evolution of Marine Organisms Department (BEOM), Stazione Zoologica Anton Dohrn, Villa Comunale 1, Napoli, I- 80121, Italy

<sup>e</sup> Università Politecnica delle Marche, Via Brecce Bianche, Ancona, I-60131, Italy

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### ABSTRACT

Dismissed industrial plants with chronic environmental contamination globally affect all levels of biological organization in concert with other natural and anthropogenic perturbations. Assessing the impact of such perturbations and finding effective ways to mitigate them have clear ecological and societal implications. Through indoor manipulative experiments, we assessed here the effects of the temporal regime of reworking of contaminated sediment from the Bagnoli-Coroglio brownfield (Tyrrhenian Sea, Italy) on the fertilization process in *Paracentrotus lividus*. Adult sea urchins were kept for one month in tanks containing contaminated sediment that was re-suspended according to two temporal patterns of water turbulence differing in the time intervals between consecutive events of agitation (mimicking the storms naturally occurring in the study area) in seawater with natural vs. supersaturated oxygenation levels. At the end of the treatment, gametes were collected and used to test the hypothesis that the regime of contaminated sediment reworking negatively, but reversibly, affects morphological and physiological traits of the fertilized eggs. We found that aggregated events of sediment re-suspension had profound negative effects on gamete interactions and Ca<sup>2+</sup> signaling at fertilization. The same experimental condition also inflicted marked ultrastructural changes in eggs. Importantly, however, such detrimental effects were inhibited by increased oxygenation. By contrast, the regime of sediment re-working with a longer interval between consecutive turbulent events had only marginal effects. Thus, the current and predicted changes of climate-related disturbance appear to modulate the biological effects of chronic contamination in post-industrial areas, suggesting that environmental rehabilitation via restoration of habitat-forming primary producers such as seagrasses or algal canopies could alleviate the pollutants' effects on resident biota.

### 1. Introduction

During the past century, a number of industrial plants were established and operated in coastal regions all over the world due to easier access to water and the possibility of transportation of raw materials and refined products by shipping. In many cases, such industrial activities determined high local levels of contamination of terrestrial and marine sediments by a range of xenobiotics that may represent a major threat to biodiversity, ecosystem functioning and human health even after the cessation of production (e.g., Barreiro et al., 1993; Blackmore et al., 1998; Fichet et al., 1998; Steding et al., 2000; Mamindy-Pajany et al.,

2010; Leger et al., 2016). Such environmental concerns are exacerbated by the ongoing impact of global climate change and the possible interactions of current and predicted natural and anthropogenic impacts with the conditions of chronic contamination (Bishop et al., 2002; Fraschetti et al., 2006). Therefore, there is increasing scientific, societal and political pressure towards not only their decontamination, but also to the restoration of marine species and habitats degraded by past industrial activities (McGrath, 2000; Amekudzi, 2004; Alberini et al., 2005; Kaufman and Cloutier, 2006). Assessing the combined impact of local contamination and other stressors and identifying effective and feasible mitigation strategies are essential to achieve this goal.

\* Corresponding author.

E-mail address: [santella@szn.it](mailto:santella@szn.it) (L. Santella).

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In this respect, a paradigmatic case is provided by the Bagnoli-Coroglio coastal area in the Gulf of Naples (Southern Tyrrhenian Sea, Italy), where a large industrial plant for the production of various chemicals, steel, asbestos and concrete has been active for about a century, until its cessation and abandonment in the mid '90s. Such activities determined the release and accumulation of a wide range of contaminants into the local environment. High concentrations of heavy metals and polycyclic aromatic hydrocarbons (PAHs), in particular, are found in shallow subtidal sediments adjacent to the dismissed plant (Sharp and Nardi, 1987; Romano et al., 2004, 2009; Arienzo et al., 2017; Trifuoggi et al., 2017; Morroni et al., 2020). These contaminants may affect not only soft-bottom species living in the sediments, but also organisms living in nearby habitats (Bertocci et al., 2019; Pellecchia et al., 2020; Ruocco et al., 2020; Gambi et al., 2020; Tangherlini et al., 2020; Liberti et al., 2020; Milito et al., 2020; Chiarore et al., 2020; Hay Mele et al., 2020). This would occur, for example, when rough sea conditions cause sediment resuspension and transportation to adjacent areas (Sunamura and Kraus, 1984; Sherman et al., 1994; Lawes et al., 2017; Sprovieri et al., 2019), possibly promoting the release and diffusion of contaminants and their transportation to surrounding habitats, including the water column, where they can potentially cause detrimental effects on planktonic organisms and on the entire food web (Carotenuto et al., 2020; Gallo et al., 2020; Pelusi et al., 2020). In this respect, the Bagnoli-Coroglio area is naturally subject to the effects of meteorological events, such as storms, the mean intensity, frequency and temporal variance of which is predicted to increase in the near future due to ongoing global climate change (Easterling et al., 2000; Trapp et al., 2007; Burge et al., 2014; Wolff et al., 2016; Aumann et al., 2018). Thus, the pressing questions are how sediment contamination would affect local marine organisms exposed to the ongoing environmental changes via disturbances within the water column, and how to effectively mitigate its possible negative impact. For example, it has been suggested that oxygenation and bacterial degradation of contaminated sediments could be a promising tool to alleviate the toxic effects of some organic pollutants (Arzayusa and Canuela, 2004; Duran et al., 2015). Increased levels of oxygen in sediments and in the water column due to the photosynthetic activity of primary producers, including seagrasses (e.g., Duarte et al., 2004; Alagna et al., 2019, 2020), may enhance defence physiological mechanisms of other organisms, eventually providing increased resistance and resilience of marine species to harsh environmental conditions, such as those associated with ocean warming (Giomi et al., 2019). These effects may propagate across organism generations since the parental environment can strongly influence the fitness of the offspring (Braun et al., 2013; Cripps et al., 2014), and transgenerational plasticity over multiple generations to adapt to adverse conditions is sometimes observed (Shama and Wegner, 2014). Specifically, the exposure of females to different environmental stressors can induce several modifications of egg quality and number during the breeding season of animals, which consequently affects their performance at fertilization (Foo and Byrne, 2017).

In the present study, the above-illustrated issues were addressed by examining experimentally the combined effects of the degree of water oxygenation and patterns ('aggregated' vs. 'spaced', characterized by a different interval of time between consecutive events) of water turbulence events able to determine the re-working of contaminated sediments and their oxygenation from the Bagnoli-Coroglio brownfield on morphological, physiological and molecular aspects involved in the fertilization process of the sea urchin *Paracentrotus lividus* (Lamarck, 1816). In all animal species, fertilization is readily accompanied by rapid increases of intracellular  $Ca^{2+}$ , which plays a variety of roles toward successful egg activation (Berridge, 1993; Santella et al., 2004, 2012; Whitaker, 2006; Costache et al., 2014). In sea urchins, the sperm-induced  $Ca^{2+}$  signalling includes simultaneous  $Ca^{2+}$  influx at the periphery (named Cortical Flash, CF) and a single  $Ca^{2+}$  wave that sweeps over the egg cortex from the sperm-egg interaction to the opposite pole (Chun et al., 2014; Vasilev et al., 2019). The exquisitely

controlled  $Ca^{2+}$  signals in fertilized eggs are known to follow a precise spatiotemporal pattern, which affects patterns of gene expression and development (Ozil et al., 2006; Kim et al., 2011; Vasilev et al., 2012; Limatola et al., 2015). Thus, the alteration of the sea urchin fertilization process is an extremely useful bioassay to assess the effects of environmental conditions that occur in the same medium, i.e., seawater, where both fertilization and subsequent embryonic development take place (Limatola et al., 2019). Moreover, the spatial and temporal pattern of  $Ca^{2+}$  release induced by the fertilizing sperm is strictly dependent upon the structural organization and dynamics of the actin cytoskeleton of the egg cortex, the exquisite control of which is an essential prerequisite for the quality of the egg response to the fertilizing sperm (Limatola et al., 2015; Santella et al., 2015, 2018). Hence, modifications of the cytological morphology due to changes in environmental conditions may be another relevant bio-indicator.

Specifically, through an indoor manipulative experiment, we tested the hypotheses that patterns of water turbulence simulating climate change-related modifications of temporal occurrence of storms able to re-work contaminated sediments from the Bagnoli-Coroglio area would negatively affect the physiological state and fertilization process of *P. lividus*, and that such an impact could be mitigated or even reversed by water oxygenation. We also tested whether water turbulence *per se* could exert any effects on the examined response variables independently of the presence of contaminated sediments.

## 2. Materials and methods

### 2.1. Ethics statement

*Paracentrotus lividus* individuals were collected from a site which is not privately-owned or protected in any way, according to the Italian legislation (DPR 1639/68, 09/19/1980 confirmed on 01/10/2000). The present study did not involve protected or endangered species. All experimental procedures were carried out in accordance with the guidelines of the European Union (Directive 609/86).

### 2.2. Experimental set-up

The experiment was carried out in March 2019 in an indoor system consisting of a total of twelve independent glass tanks (about 50 L each) that was established at the facilities for the maintenance of marine organisms of Stazione Zoologica Anton Dohrn in Naples. Each tank was manually filled with natural seawater from the Gulf of Naples and included two lateral chambers containing biological and mechanical filters, an additional compartment hosting a recirculating pump (please see Ruocco et al. (2020) for a detailed description of the technical features of this experimental setting). At the beginning of the experiment, a layer about 5 cm thick of surface sediment collected from a site located within the Bagnoli-Coroglio area (UTM coordinates: North 33 429405, 23, East 33 4518298,73; 3.80 m depth of the water column above the sediment surface) was deployed in each of eight tanks chosen at random out of the total twelve available. The chemical analyses conducted *ex ante* revealed the presence of high levels of contaminants, either heavy metals or PAHs (Morroni et al., 2020). The remaining four tanks were left without sediment. Among the eight tanks with the sediment, two were randomly assigned to each combination of water turbulence patterns ('aggregated' vs. 'spaced') and oxygenation levels ('enriched' vs. 'natural'). Turbulence was generated with an electric pump (flow rate: 4,500L/h; power: 10 W) able to produce a resuspension of the sediment that mimicked the physical effect of natural storms in the field. In both patterns, two turbulent events, each lasting two days, were applied over total 30 days; in the 'aggregated' level, however, these were separated by only three days during which agitation was stopped and the sediment left to re-deposit, while in the 'spaced' pattern the interval of time between turbulent events was of 18 days. This allowed us to manipulate the temporal patterning of disturbance events while maintaining the

same their overall frequency over the course of the experiment, thus avoiding confounding the effects of temporal variance with those of the different intensity of disturbance applied by the end of the experiment (e.g., Benedetti-Cecchi, 2003; Bertocci et al., 2005, 2017; Benedetti-Cecchi et al., 2006; Vaselli et al., 2008; García-Molinos and Donohue, 2011; Maggi et al., 2012). The overall frequency of two sediment re-working events over one month was chosen based on that of 'extreme' storms naturally occurring in the study region, as derived from the analysis of available data on wind speed and direction in the Gulf of Naples, and taking into account the predicted increase of the frequency and intensity of extreme meteorological events due to climate change (Easterling et al., 2000; Trapp et al., 2007; Wolff et al., 2016; Aumann et al., 2018). A description of the experimental design, wind data nature, source and analysis is reported in great detail in Ruocco et al. (2020). Each of the same patterns of turbulence was applied in two out of the four tanks maintained without sediment to serve as a control for the possible effect of water turbulence *per se* from that of re-worked contaminated sediment. Following Viaroli and Christian (2003), each of two levels of oxygen concentration (natural and oversaturated, corresponding to ~80% and ~200% of saturation, respectively) were established in tanks allocated to each pattern of sediment re-working to mimic the oxygen-increasing effects of marine photosynthetic organisms. Oxygen supersaturation conditions are frequently observed in many aquatic systems during periods of high productivity (Jørgensen et al., 1979; Revsbech and Jørgensen, 1983). For example, the ebullition of oxygen bubbles from the surface of leaves of seagrasses has been observed *in situ* under conditions of high irradiance (Zieman, 1974; Drifmeyer, 1980; Roberts and Caperon, 1986; Wilson et al., 2012). Analogously, the release of bubbles from sediment surface was reported on sunny days as a consequence of the intense photosynthetic activity of microalgae and cyanobacteria (Hunding and Hargrave, 1973). The herein considered levels of oxygen variation corresponded to those recorded in the field in control location and a seagrass meadow in a close non-polluted area of the Gulf of Naples (see Chiarore et al., 2020 for further details). Oversaturated levels were maintained by constantly bubbling O<sub>2</sub> using an automatic control system (Touch Controller, mod. ACQ140, Aquatronica, Italy) connected to submersed oxygen probes (Dissolved Oxygen Sensor, mod. ACQ310N-O2, Aquatronica, Italy). Logistic constraints prevented us from including additional tanks allocated to each combination of levels of oxygenation and patterns of water turbulence in the absence of sediment. For the present purposes, however, such a limitation was considered relatively irrelevant since our main hypothesis was specifically related to the possible buffering effect of increased oxygenation on the negative impact of sediment contamination. Physical and chemical variables of seawater were monitored 2 or 3 times a week, respectively, using the tools and procedures described in detail by Ruocco et al. (2020). All tanks were illuminated by 18 Watt neon lamps (6500 K) and maintained under a natural cycle of alternating light and darkness periods (12 h each).

### 2.3. Gametes collection and fertilization

Adult *P. lividus* individuals were collected from the Gulf of Naples in February 2019 and kept in plastic containers containing circulating seawater (16 °C) until the beginning of the experiment. Then, a total of ten sea urchins (7 females and 3 males) were placed and maintained in each tank allocated to each experimental condition. At the end of the established period, the gametes of sea urchins from each tank were collected by surgical removal of the gonads. Eggs were released to seawater and washed several times with filtered seawater (FSW) in a small Petri dish, while spermatozoa were collected by pipetting on the male gonads and kept as 'dry sperm'. Following sperm count in hemocytometer, fertilization was performed with diluted sperm at a final concentration of  $1.84 \times 10^6$  spermatozoa/ml.

### 2.4. Microinjection and video imaging

Microinjection of sea urchin eggs was performed with an air-pressure Transjector (Eppendorf FemtoJet), following the procedure described by Chun et al. (2014). To visualize Ca<sup>2+</sup> increases in living eggs at fertilization, 500 μM Calcium Green 488 conjugated to 10 kDa dextran was mixed with 35 μM Rhodamine Red (Molecular Probes) in the injection buffer (10 mM Hepes, 0.1 M potassium aspartate, pH 7.0) and microinjected into eggs before insemination. The bright field view and the fluorescence images of cytosolic Ca<sup>2+</sup> were detected using a cooled CCD camera (MicroMax, Princeton Instruments, Inc.) mounted on a Zeiss Axiovert 135 TV microscope with a Plan-Neofluar 40x/0.75 objective, with the time resolution of about 3 s, and were analyzed with MetaMorph (Universal Imaging Corporation). The relative Ca<sup>2+</sup> signal at a given time point was normalized to the baseline fluorescence (F<sub>0</sub>) following the formula  $F_{rel} = [F - F_0]/F_0$ , where F represents the average fluorescence level of the entire egg. Thus, F<sub>rel</sub> was defined as RFU (relative fluorescence unit) for plotting Ca<sup>2+</sup> trajectories. In order to visualize the site of instantaneous Ca<sup>2+</sup> release, the incremental changes of the Ca<sup>2+</sup> rise were analyzed by applying the formula  $F_{inst} = [(F_t - F_{t-1})/F_{t-1}]$ , where F<sub>inst</sub> represents the instantaneous fluorescence obtained by subtracting the fluorescence measured in a given time point (F<sub>t</sub>) from that measured in the immediately previous moment (F<sub>t-1</sub>).

### 2.5. Scanning electron microscopy (SEM)

Sea urchin eggs were fixed in filtered seawater containing 0.5% glutaraldehyde (pH 8.1) for 1 h at room temperature and post-fixed with 1% osmium tetroxide for another hour. Specimens were dehydrated in increasing concentrations of ethanol, and subsequently treated for critical point drying with LEICA EM CP300. Samples were then coated with a thin layer of metal (gold) using a LEICA ACE200 sputter coater and observed with a JEOL 6700F scanning electron microscope.

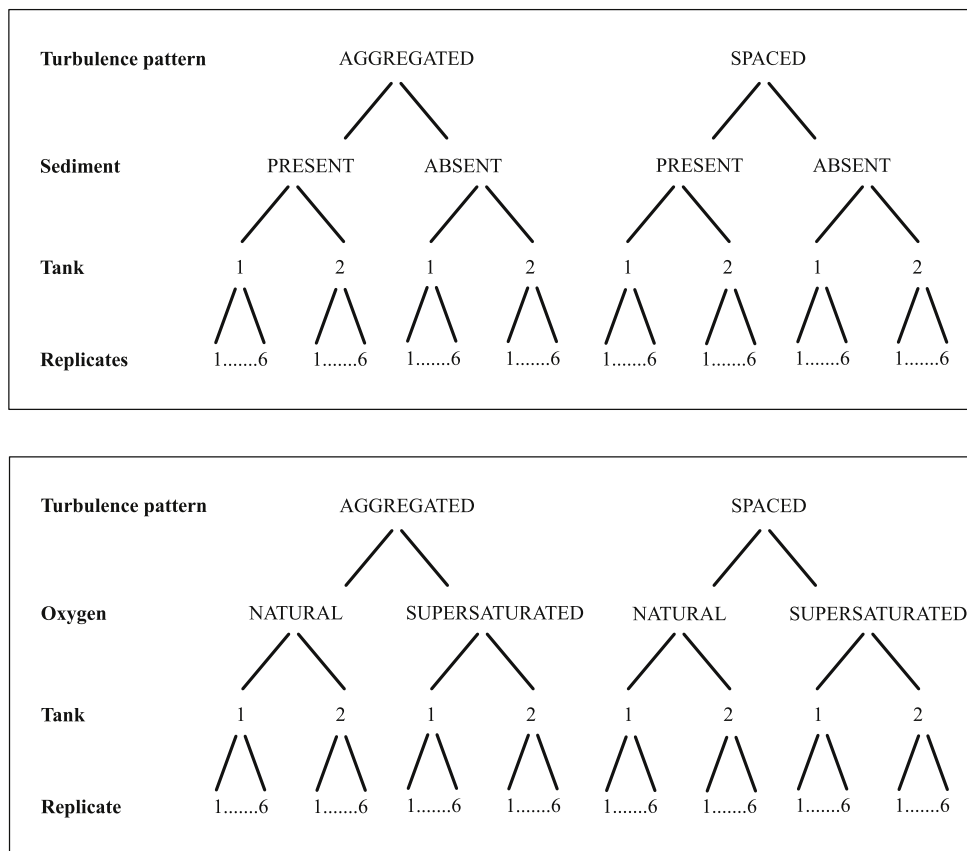
### 2.6. Transmission electron microscopy (TEM)

*P. lividus* eggs from each experimental condition were fertilized and fixed in 0.5% glutaraldehyde in natural seawater (NSW) overnight at room temperature. After six rounds of 10 min wash in NSW, the samples were post-fixed in 1% OsO<sub>4</sub> and 0.8% K<sub>3</sub>Fe(CN)<sub>6</sub> for 1 h at 4 °C. After washing again in NSW for 10 min twice, the samples were rinsed in distilled water for 10 min twice, and finally treated with 0.15% tannic acid for 1 min at room temperature. After extensive rinsing in distilled water (3 times, 10 min each), the samples were dehydrated in increasing concentrations of ethanol and subsequently embedded in EPON 12 overnight. Ultrathin sections for TEM observation (ZEISS LEO 912 AB) were stained with UAR-EMS uranyl acetate and lead citrate.

### 2.7. Statistical analyses

Different sets of analyses of variance (ANOVA) were used to test for the possible effect of water turbulence independently of the presence of contaminated sediments, and for the combined effect of patterns of contaminated sediment re-working and oxygenation level on physiological events associated with the fertilization process of *P. lividus*, namely the latency of the Ca<sup>2+</sup> response, i.e. the latency to the initiation of the Ca<sup>2+</sup> response following insemination; the CF amplitude, i.e. the amplitude of the first Ca<sup>2+</sup> increase that occurs simultaneously at the egg cortex (cortical flash, CF); the latent period, i.e. the time lapse between the CF and the onset of the Ca<sup>2+</sup> wave; the Ca<sup>2+</sup> peak amplitude, i.e. the amplitude of the Ca<sup>2+</sup> wave that follows the CF; the time to peak, i.e. the time at which the intracellular Ca<sup>2+</sup> increase reaches its maximum; and the traverse time, i.e. the time of propagation of the Ca<sup>2+</sup> wave from the point of its initiation to the opposite one.

The effect of water turbulence *per se* was tested using data from tanks with and without sediment allocated to each turbulence pattern,



**Fig. 1.** Diagrammatic representation of the experimental design used for examining the effect of water turbulence *per se* (upper panel) and the combined effect of water turbulence and oxygenation (lower panel) on different variables involved in the fertilization process of sea urchins.

excluding the oxygen oversaturated tanks from the analysis. This was based on the logical hypothesis that, if the pattern of turbulence had an effect only when combined with sediment re-working, significant differences in the response variables between the 'aggregated' and the 'spaced' pattern should have been observed in tanks where sediment was present, but not in tanks containing just water. This hypothesis was tested with a three-way ANOVA model including the crossed factors 'Turbulence pattern' and 'Sediment' (both fixed, with two levels each: aggregated vs. spaced, and present vs. absent, respectively), and the random factor 'Tank' (two levels) nested within their interaction. Six measurements of each response variable provided the replicates for this analysis (Fig. 1, upper panel).

The combined effect of patterns of sediment re-working and oxygenation was tested with an analogous three-way ANOVA model applied to data from tanks where sediment was present, subject to each turbulence pattern. In this case, the 'Sediment' factor was replaced by 'Oxygenation' (two levels, fixed, Fig. 1, lower panel).

Before each ANOVA, the assumption of homogeneity of variances was checked with Cochran's C test, and data were log-transformed when necessary. When no transformation could stabilize significantly heterogeneous variances, the analysis was performed on untransformed data and the subsequent results were considered robust when not significant (since the probability of type II error is not affected by heteroscedasticity), or when significant at  $p < 0.01$ , instead of  $p < 0.05$ , (to compensate for increased probability of type I error) (Underwood, 1997).

Three and one replicates were lost from tanks used for the first and the second set of analyses, respectively. In those cases, missing data were replaced by the average of the remaining five replicates in that combination of factor levels and degrees of freedom were corrected in the analyses as needed. In some cases, the 'Tank' term was eliminated from

the model to obtain a more powerful test of the other sources of variation. This procedure was done following the logic described in Winer et al. (1991) and Underwood (1997).

When relevant, Student-Newman-Keuls (SNK) tests were used for post-hoc comparisons of means.

### 3. Results

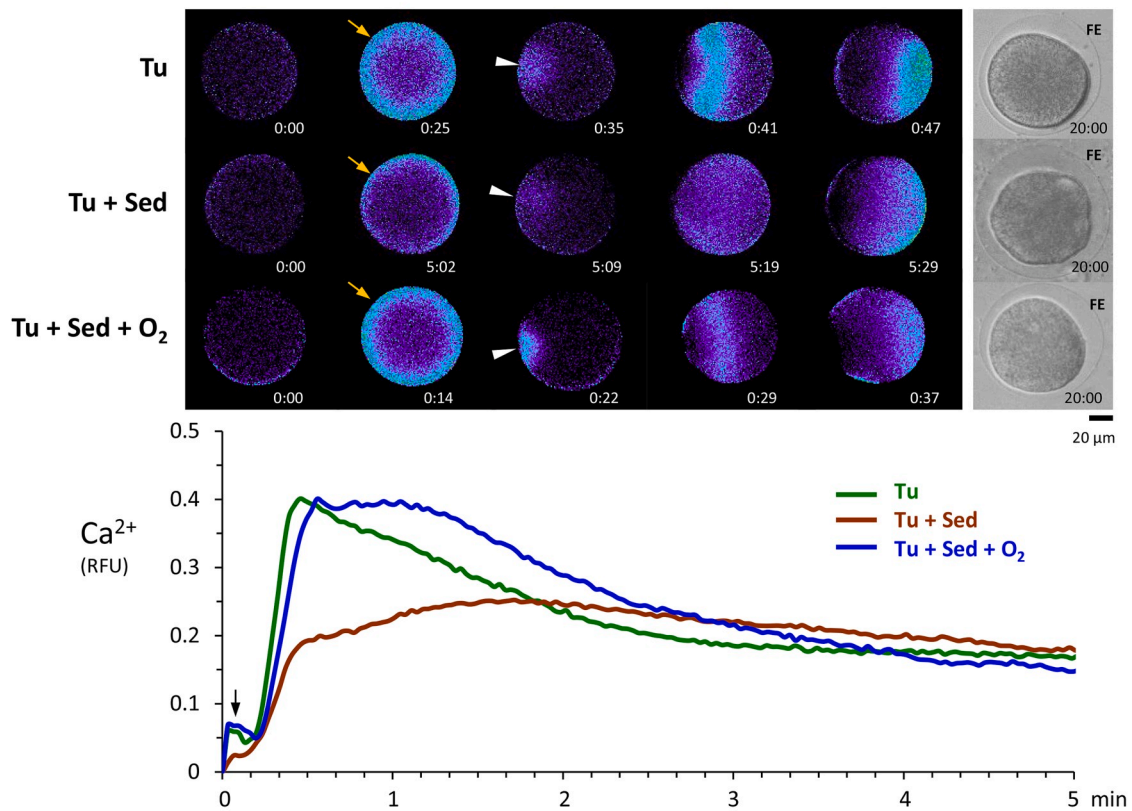
#### 3.1. Effects on $Ca^{2+}$ signaling in the fertilized eggs of sea urchin

The effects of water turbulence *per se* were detected only for the latency of the  $Ca^{2+}$  response and the traverse time (Supplemental Table 1). The latency of the  $Ca^{2+}$  response, however, was affected mainly by turbulence pattern, although as expected, (as a consequence of the aggregated compared to the spaced pattern), a longer latency was much more evident in the presence than in the absence of sediment (Supplemental Fig. 1). Traverse time, on the other hand, was larger under spaced compared to aggregated turbulence in the absence of sediment, but this pattern of difference was reversed in the presence of sediment (Supplemental Fig. 1). All other variables did not show any effect of water turbulence irrespective of sediment since either nonsignificant interactive and main effects of each factor were found, or, when a significant interaction was found, this corresponded to relatively larger values of that variable under the spaced pattern where sediment was present, but no differences between patterns where sediment was absent (Supplemental Table 1 and Supplemental Fig. 1).

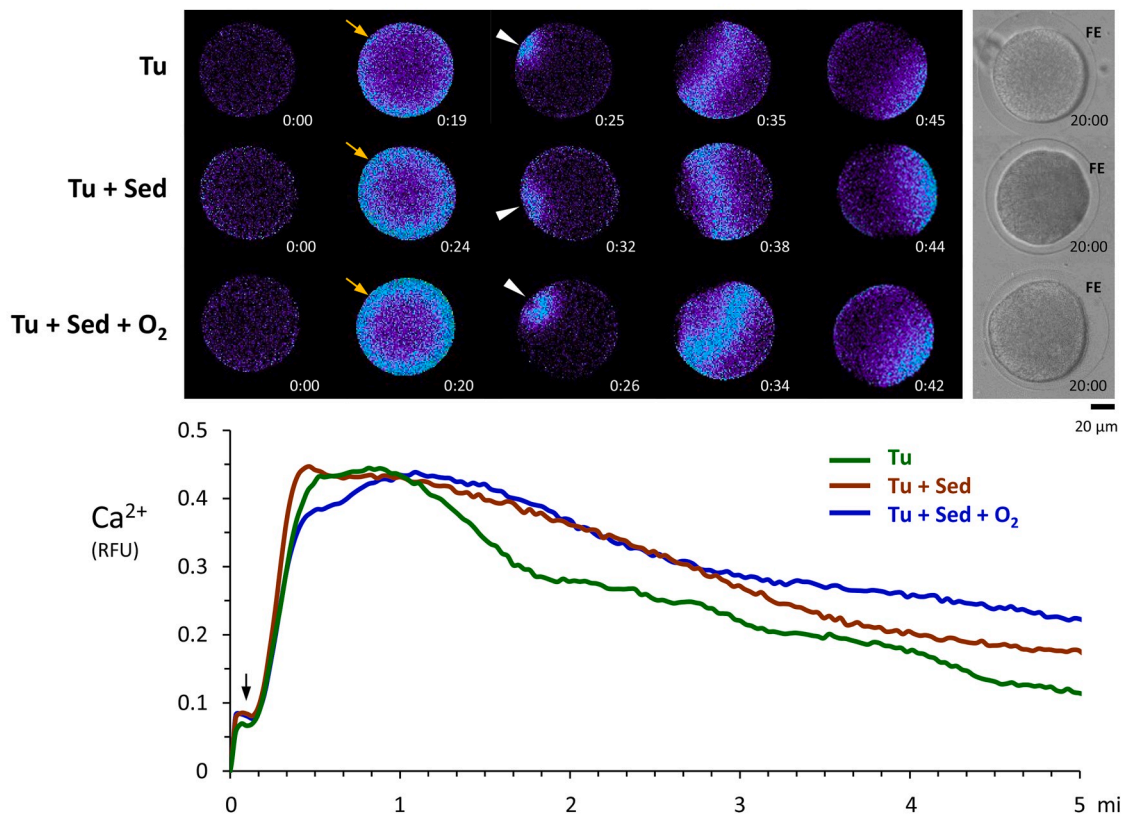
Eggs taken from sea urchins exposed to contaminated sediments were able to produce the expected  $Ca^{2+}$  increases at fertilization, which also led to elevation of the fertilization envelope (FE) (Fig. 2A and B). In the case of the aggregated pattern, however, these eggs required significantly longer incubation time with the sperm before developing



**A**



**B**



(caption on next page)

**Fig. 2.** The pattern of disturbance and oxygenation enrichment have profound effects on  $\text{Ca}^{2+}$  signaling in the fertilized eggs of sea urchin. *P. lividus* eggs collected from animals kept at different environmental conditions were microinjected with calcium dyes and fertilized, and the consequent changes of the intracellular  $\text{Ca}^{2+}$  levels were monitored with a CCD camera in (A) aggregated pattern of water turbulence or (B) spaced pattern of turbulence. A) On the top panel, the pseudo-colored relative fluorescence images representing the sites of momentary  $\text{Ca}^{2+}$  increases at the key time points (min:sec). Tu: eggs from the female sea urchins subjected to aggregated turbulence but without sediments. Tu + Sed: eggs from the female animals subjected to aggregated turbulence in the presence of sediments from Bagnoli-Coroglio area. Tu + Sed +  $\text{O}_2$ : eggs with same treatment as Tu + Sed, but with the supplement of oversaturating oxygenation of the seawater. The cortical flash (CF) and the site of sperm-egg interaction are indicated by yellow arrows and white arrowheads, respectively. Bright field view images of the same zygotes are located on the right hand side of the  $\text{Ca}^{2+}$  images, showing the elevated fertilization envelope (FE). On the bottom panel,  $\text{Ca}^{2+}$  trajectories showing the pattern of  $\text{Ca}^{2+}$  increase in the fertilized eggs of Tu (green curve), Tu + Sed (red curve), and with Tu + Sed +  $\text{O}_2$  conditions (blue curve). The time frame immediately before the first detectable  $\text{Ca}^{2+}$  signal was set to  $t = 0$ . Vertical arrow: CF. B) On the top panel, pseudo color images of  $\text{Ca}^{2+}$  signals at key moments. Tu: eggs from the female sea urchins subjected to spaced turbulence but without sediments. Tu + Sed: eggs from the female animals subjected to spaced turbulence in the presence of sediments from Bagnoli-Coroglio area. Tu + Sed +  $\text{O}_2$ : eggs with same treatment as Tu + Sed, but with the supplement of oversaturating oxygenation of the seawater. On the bottom panel,  $\text{Ca}^{2+}$  trajectories in the spaced turbulence. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the  $\text{Ca}^{2+}$  response. Indeed, the onset of their intracellular  $\text{Ca}^{2+}$  increase occurred, on average,  $269.1 \pm 61.9$  s (mean  $\pm$  standard error,  $n = 12$ ) after the addition of sperm, as opposed to a mean time lag in the absence of sediment of  $41.5 \pm 11.4$  s (Supplemental Fig. 1, see “Latency of  $\text{Ca}^{2+}$  response”).

Several other aspects of the intracellular  $\text{Ca}^{2+}$  signal were altered by the exposure to sediment subjected to aggregated turbulence. Specifically, both the amplitude of the CF (Fig. 2A, arrow) and the  $\text{Ca}^{2+}$  wave were reduced in these eggs (red curve in Fig. 2A, and Fig. 3B and D). Furthermore, once started, the  $\text{Ca}^{2+}$  wave also propagated much slower, as indicated by the time required for the wave to reach the antipode (Fig. 3F, “Traverse time”). Remarkably, however, these negative trends were almost all reversed when the same sediment was re-worked in the presence of supersaturating oxygen (Table 1 and Fig. 3A). Indeed, the latency of the  $\text{Ca}^{2+}$  response in these eggs ( $43.1 \pm 15.6$  s) was virtually the same as in the absence of sediment ( $41.5 \pm 11.4$  s) (Figs. 2A and 3A). Analogously, the amplitude of both CF and  $\text{Ca}^{2+}$  wave were restored to that of the control (no sediment) eggs by the addition of  $\text{O}_2$  (Figs. 2A, 3B and D). This remarkable alleviation of the effects of sediments effects on  $\text{Ca}^{2+}$  signaling by the supply of  $\text{O}_2$  was also evident in the dynamics of the  $\text{Ca}^{2+}$  rise expressed by the ‘traverse time’ ( $21.6 \pm 0.9$  s vs.  $17.9 \pm 1.4$  s in the natural oxygenation vs. supersaturated condition, respectively: Fig. 3E and F) and the ‘time to peak’ ( $148.7 \pm 15.1$  s vs.  $64.8 \pm 11.7$  s in the same conditions, Fig. 3E).

By contrast to the aggregated turbulence pattern, the exposure of female urchins to sediment re-working events separated by a longer interval of time (spaced perturbation) had only modest effects on the  $\text{Ca}^{2+}$  signaling at fertilization when assayed on the 30th day (Table 1 and Fig. 2B). The latency of the  $\text{Ca}^{2+}$  response and the amplitude of the CF and the  $\text{Ca}^{2+}$  wave were all unaffected by this pattern of turbulence. Nonetheless, eggs from sea urchins subject to this experimental condition displayed faster  $\text{Ca}^{2+}$  wave propagation at fertilization (traverse time of  $16.6 \pm 0.4$  s vs.  $19.3 \pm 0.6$  s in the presence vs. the absence of sediment, respectively; Fig. 3F). Similarly, the peak amplitude of the  $\text{Ca}^{2+}$  wave was attained much faster in these eggs ( $33.9 \pm 2.5$  s after onset of the  $\text{Ca}^{2+}$  response) than in the condition without sediment ( $84.9 \pm 11.6$  s), although the amplitude of the  $\text{Ca}^{2+}$  peaks was virtually the same (Supplemental Fig. 1D). Once again, however, these changes in  $\text{Ca}^{2+}$  dynamics were buffered by oversaturating oxygenation (Figs. 2B and 3, Table 1).

### 3.2. Effects on ultrastructure of the egg cortex

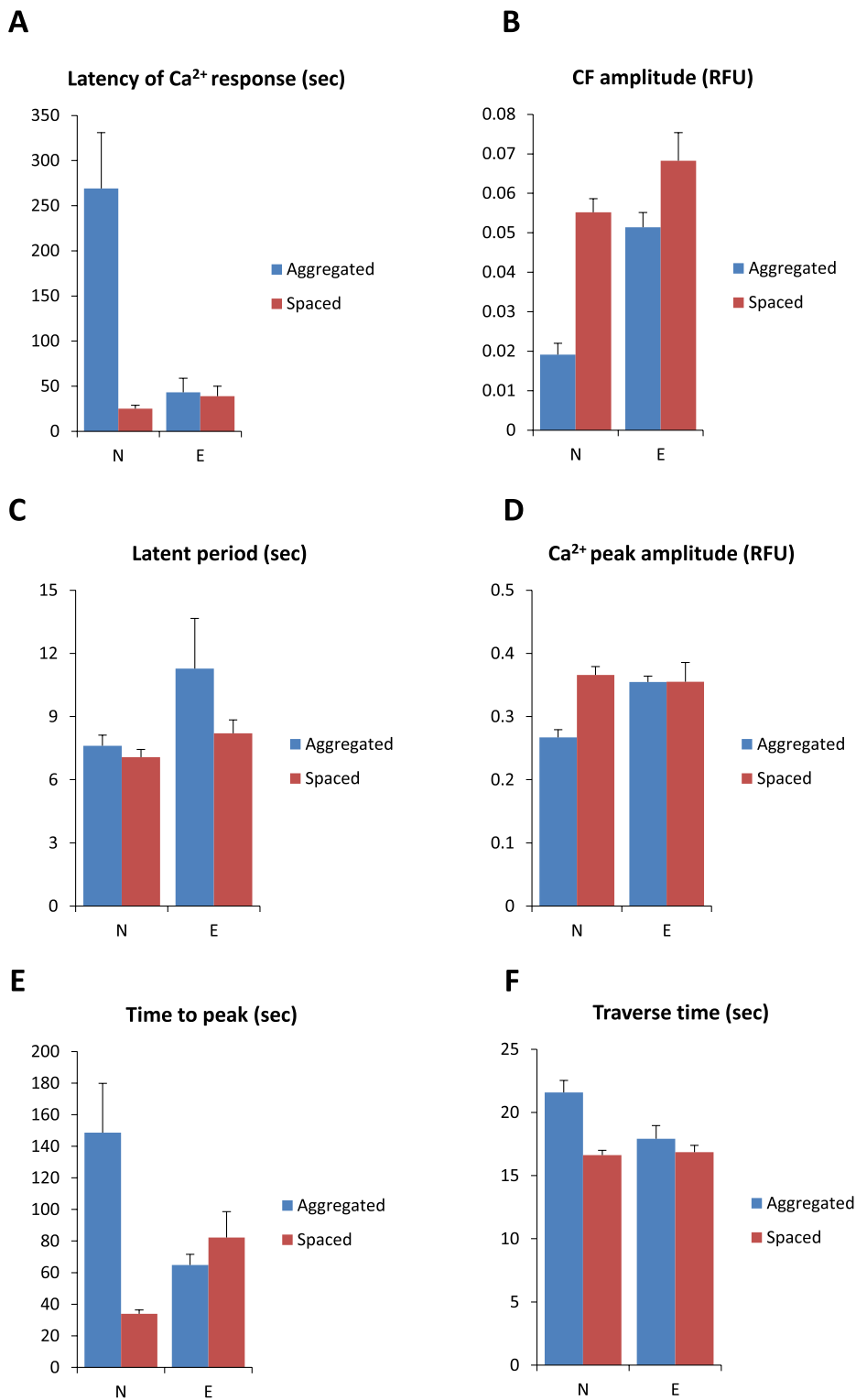
The observed physiological responses of sea urchin eggs to contaminated sediment and oxygenation were associated with evident modifications at the egg surface, i.e., the cell portion where fertilization occurs. As shown in Fig. 4A, cortical granules (CG) in the eggs from animals exposed to sediment under aggregated turbulence were slightly disarrayed and dislodged from the plasma membrane, whereas the CG in the eggs from the animals subjected to turbulence without sediment were, as expected, intimately apposed to the plasma membrane. The

eggs from females subject to the same sediment and turbulence pattern, but under oversaturating oxygenation of seawater, displayed a remarkably similar ultrastructure of the egg surface (Fig. 4A: panels on top, unfertilized eggs). Furthermore, shortly after fertilization (i.e., about 1 min after insemination), the unusually distributed CG in eggs from females exposed to sediments re-worked by aggregated turbulence showed an abnormal pattern on exocytosis, as indicated by their apparent difficulties in extruding the contents of the CG to the perivitelline space (Fig. 4A and B, red arrows), which is thought to be more than just delayed exocytosis following the belated  $\text{Ca}^{2+}$  response in these eggs.

## 4. Discussion

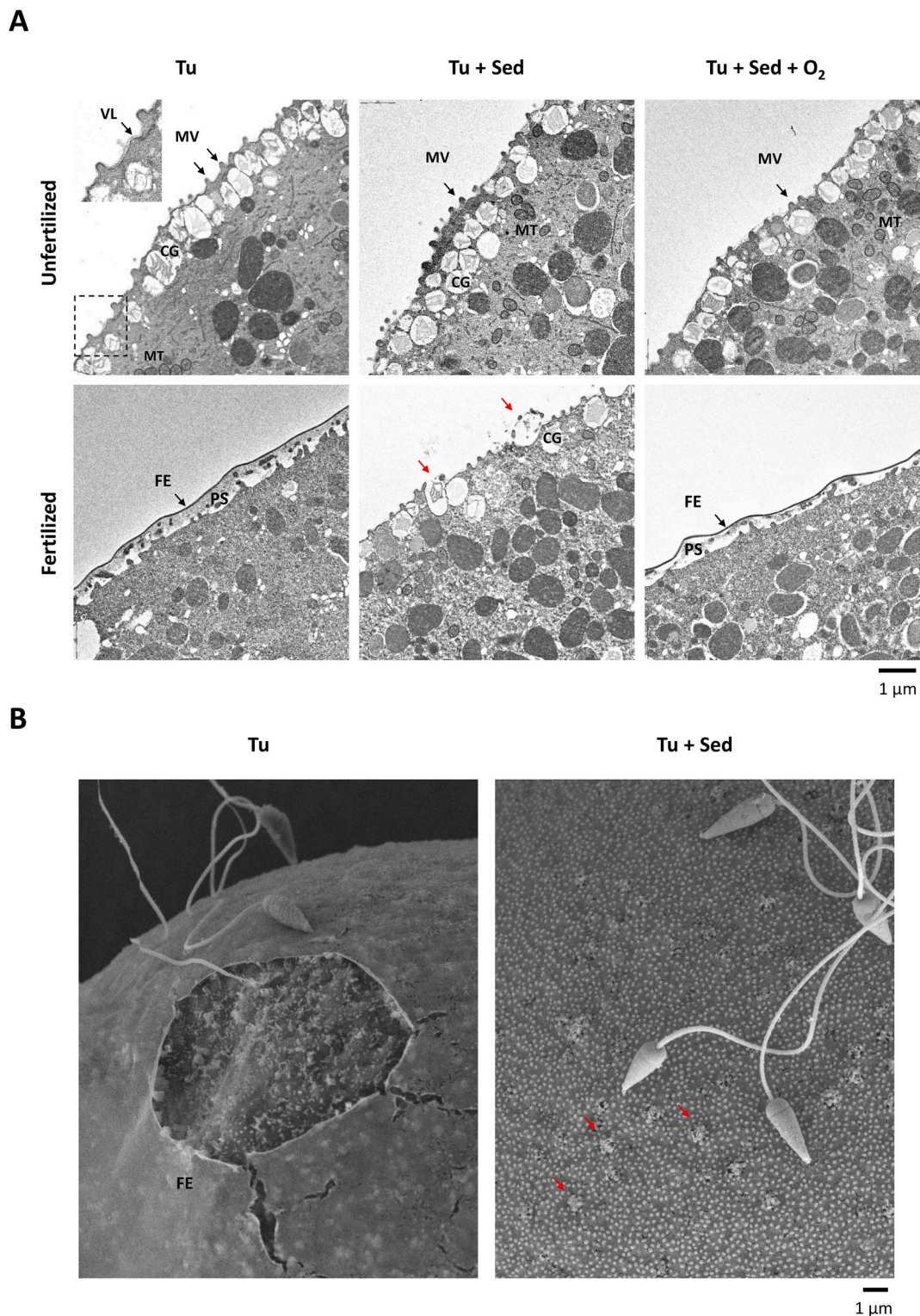
Fertilization is one of the most vulnerable stages in the life of marine species, which can be profoundly affected by environmental stressors especially when reproduction depends on external fertilization. Indeed, the interaction between the male and female gametes of sea urchin is a highly delicate and species-specific process that can be modulated by a range of environmental changes (Hardt and Safina, 2010). Our findings indicated that the most profound effect on  $\text{Ca}^{2+}$  signaling in fertilized eggs occurred when the females were exposed to contaminated sediments from the Bagnoli-Coroglio brownfield, and these were re-worked by aggregated events of water turbulence. The  $\text{Ca}^{2+}$  response came out with over 4–5 min delay in the eggs obtained from animals maintained in this condition compared to those exposed to more temporally spaced events of turbulence. This suggests that, even though the eggs were inside the female gonad within the sea urchin body, they were negatively affected by external turbulence showing a functional deficit in responding to the fertilizing sperm with  $\text{Ca}^{2+}$  signals. In other words, it seems that animals subject to aggregated turbulence operating on contaminated sediments exhibited eggs of lowered competence in interacting with the gametes of the other sex. This response was also accompanied by reduced amplitude of  $\text{Ca}^{2+}$  signals in terms of both CF and wave, as well as by much slower propagation of the wave. Most importantly and interestingly, however, such detrimental effects on the fertilization process were reversed by the oversaturation of seawater with oxygen, inspiring hope about the possibility that habitat remediation through the restoration of photosynthesizing organisms may contribute to alleviate the environmental problem affecting the Bagnoli-Coroglio area.

There is evidence that the perturbation of egg  $\text{Ca}^{2+}$  dynamics may be due to the alteration of egg structure, which inevitably compromises its capacity to respond properly to the fertilizing sperm (Chun et al., 2014; Vasilev et al., 2019; Limatola et al., 2019). In the present study, this happened through ultrastructural changes of eggs induced by aggregated turbulence in the presence of contaminated sediment, but the underlying morphological and physiological mechanisms are yet to be elucidated, as are those responsible for the buffering effect of increased oxygenation. We can speculate that metal contaminants in sediments somehow induced these negative responses, but that their toxic activity



**Fig. 3. Comparison of the Ca<sup>2+</sup> responses in the fertilized eggs in the presence of polluted sediment with different oxygenation levels.** *P. lividus* eggs collected from the animals in the presence of sediments from Bagnoli-Coroglio area and a natural (N) or enriched (E) oxygenation supply. The eggs microinjected with calcium dyes were fertilized, and the changes of the intracellular Ca<sup>2+</sup> levels were monitored. Values are reported as “mean ± standard error” in a total of 12 replicates for each condition (n = 12). Data from two tanks in each combination of levels of experimental condition were averaged for ease of visualization. A) Latency of Ca<sup>2+</sup> response; B) CF amplitude; C) Latent period; D) Ca<sup>2+</sup> peak amplitude; E) Time to peak; F) Traverse time.





**Fig. 4.** Sediments with ‘aggregated turbulence’ drastically alter the ultrastructure of the egg cortex, which is reversible with oversaturating oxygenation of the seawater. **A)** The ultrastructure of the *P. lividus* egg cortex viewed by transmission electron microscopy (TEM). Top panels: unfertilized eggs. Lower panels: after insemination (1 min). The definition of the eggs entitled ‘Tu,’ ‘Tu + Sed’ and ‘Tu + Sed + O<sub>2</sub>’ were provided in the text and in the legend of Fig. 1. Abbreviations: VL (vitelline layer), MV (microvillus), CG (cortical granules), MT (mitochondria), FE (fertilization envelope), PS (perivitelline space). **B)** Scanning electron micrographs (SEM) of the eggs surface 1 min after insemination. Left: A fertilized egg from female sea urchin subjected to aggregated turbulence without sediment. Right: an egg from the female sea urchin subjected to aggregated turbulence with sediments sand in the presence of polluted sediment (right panel). Microvilli are visible beneath the FE that was open during fixation. Also note the bumps on the surface of the ‘Tu + Sed’ eggs that were presumably created by the hampered exocytosis of individual cortical granules (red arrows). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)



**Table 1**

Results of ANOVA testing for the effect of patterns of water turbulence ('Aggregated' = Agg vs. 'Spaced' = Spa) reworking contaminated sediment and oxygenation (Natural = N vs. Enriched = E) on six Ca<sup>2+</sup> response variables involved in the fertilization process of *Paracentrotus lividus*. Residual degrees of freedom corrected for missing data. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, ns = not significant.

Source of variation	df	Latency Ca <sup>2+</sup> response		CF amplitude		Latent period		Ca <sup>2+</sup> peak		Time to peak		Traverse time	
		MS	F	MS	F	MS	F	MS	F	MS	F	MS	F
Turbulence = Tu	1	13.11	9.11*	0.008	3.69	39.0	1.04	0.037	4.63	28415	6.92 <sup>ab</sup>	108.6	14.17 <sup>ab***</sup>
Oxygenation = Ox	1	9.61	6.68	0.006	2.73	69.3	1.85	0.013	1.61	3766	0.92 <sup>b</sup>	35.2	4.60 <sup>b</sup>
Tank(Tu x Ox)	4	1.44	1.88	0.002	3.56*	37.5	2.07	0.032	2.13	2519	0.61	6.2	0.81
Tu x Ox	1	11.57	8.04*	0.001	0.48	19.2	0.51	0.023	2.85	52419	12.76 <sup>ab***</sup>	45.3	5.92 <sup>ab*</sup>
Residual	39	29.91		0.001		18.1		0.004		4108		7.7	
Cochran's test		C = 0.265, ns		C = 0.733**		C = 0.836**		C = 0.600**		C = 0.743**		C = 0.331, ns	
Transformation		Ln(x+1)		None		None		None		None		None	
SNK test		Tu x Ox (SE = 0.346)										Tu x Ox (SE = 0.789)	
		N: Agg > Spa										N: Agg > Spa	
		E: Agg = Spa										E: Agg = Spa	
		Agg: N > E										Agg: N > E	
		Spa: E = N										Spa: E = N	

<sup>a</sup> Tested over the Residual MS after elimination of the Tank(TuxOx) term that was not significant at p > 0.25.

was inactivated by oxidation. Alternatively, cytological changes could have been stimulated by hypoxic conditions associated with sediments in tanks, while they logically could be inhibited by oxygenation (Viaroli and Christian, 2003). An intriguing finding of this study, however, was that the same sediment, when re-worked with a different pattern of turbulence (i.e., spaced events), did not induce drastic changes in Ca<sup>2+</sup> signaling of eggs at fertilization. This may be at least in part explained by the ability of the sea urchins to recover their competency to reproduce during the prolonged time intervals when turbulence was stopped.

Irrespective of the specific mechanisms involved, the present findings suggest that building a structural system suited to prevent the contaminated sediment from mixing during storms, possibly associated with the restoration of local meadows of primary producers with proper methods (e.g. Alagna et al., 2019, 2020), might help to lessen the ecological threats caused by the chronic contamination of the Bagnoli-Coroglio brownfield and possibly other similar post-industrial areas that are naturally exposed to current and predicted climate change. Indeed, besides producing oxygen, seagrasses may further contribute to stabilize sediments against local hydrodynamics through different mechanisms, such as the attenuation of currents and waves and the subsequent reduction of resuspension (Bouma et al., 2005; Hemminga and Duarte, 2000; Madsen et al., 2001), and the compaction of the substrate by the rhizome system (Barbier et al., 2011). Provided the removal of conditions that potentially prevent their survival, this reinforces the restoration of seagrass meadows as an optimal option for environmental rehabilitation.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### CRediT authorship contribution statement

**Nunzia Limatola:** Investigation, Formal analysis, Validation, Visualization, Writing - original draft, Writing - review & editing. **Iacopo Bertocci:** Formal analysis, Validation, Conceptualization, Methodology, Writing - original draft, Writing - review & editing. **Jong Tai Chun:** Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Luigi Musco:** Resources, Methodology, Writing - review & editing. **Marco Munari:** Methodology, Writing - review & editing. **Davide Caramiello:** Methodology. **Roberto Danovaro:** Funding acquisition, Writing - review & editing. **Luigia Santella:** Conceptualization, Supervision, Project administration, 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.2020.104951>.

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