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Research article

Unravelling the ecotoxicological impacts of gadolinium (Gd) on *Mytilus galloprovincialis* embryos and sperm in seawater: A preliminary study

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ABSTRACT

As the demand for rare earth elements (REEs) continues to surge in diverse industrial and medical domains, the ecological consequences of their ubiquitous presence have garnered heightened attention. Among the REEs, gadolinium (Gd), commonly used in medical imaging contrast agents, has emerged as a pivotal concern due to its inadvertent introduction into marine ecosystems via wastewater release. This study delves into the complex ecotoxicological implications of Gd contamination, focusing on its impact on the embryonic development and sperm functionality of *Mytilus galloprovincialis*. The findings from this study underscore the potential hazards posed by this rare element, offering a critical perspective on the ecological risks associated with Gd. Notably, this exploratory work reveals that Gd exerts a significant embryotoxic effect at elevated concentrations, with an observed half maximal effective concentration (EC50) value of 0.026 mg/L. Additionally, Gd exposure leads to a considerable reduction in sperm motility and alters sperm morfo-kinetic parameters, especially at a concentration of 5.6 mg/L. The results highlight a dose-dependent relationship between Gd exposure and the prevalence of specific malformation types in Mytilus embryos, further providing crucial insights into the potential risks imposed by this rare earth element.

1. Introduction

The world's increasing reliance on rare earth elements (REEs) to fuel technological and medical advancements has resulted in their ubiquitous presence in the environment [1,2]. However, the interaction between these elements and ecosystems has raised pressing questions regarding their ecological implications [3].

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Particularly interesting is gadolinium (Gd), a member of the lanthanide series of REEs. Due to its exceptional magnetic properties, Gd is widely employed as a contrast agent in magnetic resonance imaging (MRI) procedures [4–7]. The introduction of Gd into wastewater during medical treatments poses a significant challenge for its comprehensive removal in conventional wastewater treatment plants, leading to its subsequent entry into marine habitats [7].

The increased use of Gd has led to its detection in various ecosystems, making it an emerging micro-pollutant in aquatic environments. Concentrations have been found to range from natural levels of 1–4 ng/L to as high as 200–1100 μg/L at wastewater treatment plant (WWTP) effluent discharge points [8]. Gd concentrations in tap water have shown significant increases, such as in Berlin, where levels rose 1.5 to 11.5 times in three years [9]. Similar trends were observed in San Francisco Bay, with Gd levels increasing from about 45 pmol/L to 180 pmol/L in less than ten years [10].

Anthropogenic activities have also led to Gd anomalies in river waters in various regions, including Pennsylvania, Poland, South Korea, Australia, the Czech Republic, Italy, Brazil, France, and Germany [11–18].

The unexpected occurrence of Gd in seawater has elicited concerns regarding its potential ecotoxicological effects on marine organisms [19]. Accumulation of Gd and other REEs within organisms can occur through various pathways, necessitating a comprehensive assessment of their impacts on key species to comprehend the extent of their ecological implications [20–23].

The overall mechanism of Gd toxicity in aquatic organisms is primarily attributed to its ability to disrupt cellular processes through complex mechanisms [24]. One mechanism involves the formation of bonds between Gd cations and negatively charged chemical groups present in biological systems, such as amino acids. This interaction can lead to membrane dysfunction and interference with enzymatic activities [8]. Furthermore, the structural similarity between Gd^{3+} and Ca^{2+} enables Gd to act as an inorganic blocker of voltage-gated calcium channels, thereby disrupting calcium-dependent physiological processes crucial for organismal health [25].

Mytilus galloprovincialis, a bivalve mollusc commonly found in coastal ecosystems, is a model organism for investigating the consequences of Gd pollution.

It has been recently shown that germ cells, in particular spermatozoa, are extremely sensitive to pollutants, in mussel, in particular heavy metals cause structural alterations in head morphology and chromatin condensation [26,27]. Despite existing research exploring the consequences of Gd on diverse organisms [8,28], limited attention is confined to assessing its effects on *Mytilus galloprovincialis*.

Most of the available literature on Gd effects predominantly focus on *Mytilus* bioaccumulation and biochemical processes in mature organisms, neglecting crucial insights into the early stages of development. Trapasso et al. [29] assessed the toxicity of Gd-bioremediated water using macroalgae and *M. galloprovincialis*, considering the presence or absence of algae. Figueiredo et al. [30] explored natural REE concentrations in *M. galloprovincialis* from different locations along the Portuguese coast, considering geographic and temporal variations. Andrade et al. [31] evaluated the impacts of Lanthanum (La) and Gd on *M. galloprovincialis* under different salinities, evaluating biochemical alterations related to metabolic, oxidative, and neurotoxic pathways.

Gaining a comprehensive understanding of the potential adverse effects of Gd contamination on this species holds paramount importance for comprehending the long-term ecological consequences of such exposure in marine environments [32]. Thus, to address this gap, the study aimed to elucidate, for the first time, the spermiotoxic and embryotoxic impacts of Gd in *M. galloprovincialis*. The holistic approach undertaken in this study provides a comprehensive understanding of the toxic effects of Gd, delving into its impact on the fertilization capability of sperm, early developmental stages, and reproductive processes within *M. galloprovincialis*. This multi-faceted investigation could significantly enhance the understanding of the ecotoxicological ramifications associated with Gd contamination in marine ecosystems. By evaluating the specific embryotoxicity and spermiotoxicity exerted by Gd, this study attempts to unravel the mechanisms underlying the impact of this rare earth element on early life stages and reproductive processes.

2. Materials and methods

Thirty mussels of the species *Mytilus galloprovincialis* were collected in the Bay of Naples (Italy, 40° 51′ 22.72″ N; 14° 14′ 47.08″ E), during the breeding season, in April. After cleaning from epibiont fauna, the mussels were placed awaiting gamete retrieval in 25 L polycarbonate tanks containing aerated and synthetic seawater (16 ± 1.5 m), maintained in a natural light/dark photoperiod. All individuals were processed within a few hours after the collection. The embryotoxicity assessment followed the procedures outlined in ASTM (2021) and Libralato et al. [33]. To induce spawning in adults, thermal stimulation cycles were alternated between 18 ± 1 °C and 28 ± 1 °C.

The ASTM artificial seawater, characterized by a salinity of 34 psμ, was employed for gametes collection and embryo testing. Each test involved gametes from three males and three females, which were separately filtered at 32 μm (sperm cells) and 100 μm (eggs) to remove impurities.

The experimental design involved nominal concentrations of 0.01, 0.1, 1, and 10 mg/L, based on the study conducted by Mestre et al. (2019) [34], which investigated the mussel embryotoxic effects of other REEs such as Yttrium and Lanthanum). Nevertheless, the measured values obtained from the experiment were 0.01, 0.04, 0.2, and 5.6 mg/L. To investigate the impact of Gd on the early stages of mussel development, three types of toxicity tests were carried out: spermiotoxicity before and after fertilization, and embryotoxicity. The experiments were conducted in triplicate. Tests were performed on sperm exposed to various concentrations of Gd, evaluating boththe shape and the sperm motility. The exposed sperm was then used to assess its fertilization capability in unexposed eggs. Finally, an embryotoxicity test was conducted, exposing eggs and sperm to different Gd concentrations, and the embryonic development was evaluated.

2.1. Chemical analysis

Gd concentrations were assessed using inductively coupled plasma mass spectrometry (ICP-MS) with an Aurora M90 instrument from Bruker Daltonics Inc. High-purity water with a resistivity of 18.2 MΩ cm was sourced from a Milli-Q unit (Millipore, United States). Nitric acid (HNO_3) at a concentration of 69 % v/v, classified as Ultratrace@ ppb-trace analysis grade, was supplied by Scharlau (Barcelona, Spain). For ICP-MS analysis, all samples were prepared in a 2 % v/v HNO3 solution, and the analysis was conducted in Normal Sensitivity mode. Calibration curves for rare earth elements (REEs) determination spanned from 0.5 to 1000 μg/L and were freshly prepared each day before analysis using standard solutions. The internal standard used for both the calibration curve and sample analysis was ^{115}In.

2.2. Histological analysis of spermatozoa

The general morphology of the spermatozoa was studied by means of toluidine blue (TB) staining of the sperm chromatin. This staining was performed according to Monachesi et al. (2019) [35], with some modifications specifically adapted to the seminal fluid smear of mussels [26]. In detail, the spermatozoa of each experimental group were smear-stained on a slide, air-dried, and fixed with 96 % ethanol and acetone 1:1 for 30 min at 4 °C. Subsequently, staining was performed using a 0.05 % diluted TB working solution for 10 min at room temperature. The smears were then washed with distilled water and mounted with aqueous mounting medium. At least 200 spermatozoa were evaluated for each experimental spot. The images were acquired using an Axiocam MRc5 camera (Carl Zeiss) and Axiovision 4.7 software (Carl Zeiss) [36].

2.3. Sperm motility and viability

Mussel spermatozoa motility and kinetics after Gd treatments was assessed using the following methodology. Briefly, mussel spermatozoa were incubated with Gd-contaminated solutions for 30 or 60 min at 33 °C. For each sample, three independent Makler chamber on a pre-heated stage at 33 °C was loaded with 10 ml of sperm suspension, opportunely diluted, and observed under a Nikon Eclipse TE 2000 inverted microscope (Nikon, Amstelveen, Netherlands) connected to a Basler Vision Technology A312 FC camera with a positive phase contrast 20X objective. At least 100 cells and randomly chosen fields (8–10 fields/sample) were acquired and analyzed for each semen sample. The Sperm Class Analyzer (SCA) (Microptic S.L. Barcelona, Spain) software was used to evaluate spermatozoa progressive motility and kinetics by adjusting the set-up for Mytilus species using the following settings: 25 frames/s, 10 frames/object, 10 μm/s velocity limit for slow spermatozoa, 15 μm/s velocity limit for moderately motile spermatozoa, 35 μm/s velocity limit for fast spermatozoa, 50 % minimal linearity, 80 % straightness for progressive fast spermatozoa. Videos were recorded with 60 Hz frame rate.

The control group consisted of sperm suspension that was not exposed to the Gd. The following parameters were, then, measured: the percentage of motile spermatozoa (total motility, %); the percentage of progressive and non-progressive motile spermatozoa (progressive (P)/non-progressive (NP) motility, %); the average path velocity (VAP, μm/s), calculated as the velocity of a sperm head along its average path; the straight-line rectilinear velocity (VSL, μm/s), calculated as the velocity of the sperm head along the trajectory between the first and the last spotted position; the curvilinear velocity (VCL, μm/s), calculated as the velocity of a sperm head along its curvilinear path; the beat-cross frequency (BCF, Hz), the average rate at which the curvilinear path crosses the average path; the amplitude of the lateral head displacement (ALH, μm). Furthermore, sperm velocity distribution was analyzed by identifying four sperm cell movement subcategories based on fish-specific VAP cut-off, as reported in the SCA system v.3.4.0: percentage of rapid cells (fraction of cells moving with VAP $>$50 μm/s; rapid, %); percentage of medium speed cells (fraction of cells moving with VAP values ranging from 20 to 50 μm/s; medium, %); percentage of slow cells (fraction of cells with VAP $<$20 μm/s; slow, %) and percentage of static cells (fraction of cells not moving at all; static, %). Spermatozoa motility at time zero was used as control.

Sperm viability was evaluated (after 4 h of incubation) under a bright field light microscope Nikon Eclipse Ci (Nikon H550S, Amstelveen, Netherlands) with the Eosin Test assay (Sigma Aldrich E4009) according to the manufacturer's instructions. Briefly, 10 μL of 0.5 % EosinY in 0.9 % NaCl was added directly to a slide containing 10 μL of every collected sample. The slide was sealed with a glass coverslip and sperms were observed at 40x or 100x magnification with an immersion objective. Under such conditions, viable spermatozoa appear colorless, while dead spermatozoa appear red.

2.4. Toxicity tests

For spermiotoxicity tests, spermatozoa aliquots were incubated for 30 min in Gd-contaminated solutions and subsequently used to fertilize untreated oocytes from three animals. After fertilization, the development of the resulting embryos was monitored [37]. Microscope observation was conducted at regular intervals to assess any morphological abnormalities or developmental delays in the embryos.

In embryotoxicity test, according to Ref. [38] fertilization was carried out by injecting sperm suspension into 500 mL artificial seawater containing eggs, resulting in a ratio of approximately 1:1 $\times$ 10^6 egg/sperm in the final mixture. Fertilization success was qualitatively assessed using optical microscopy. The density of fertilized eggs was determined by counting four subsamples of a known volume.

Then, zygotes (obtained by combining sperm and eggs suspensions) were added to the test REE solutions to achieve a density of around 70 eggs/mL within a 3 mL final volume, were then incubated for 48 h at 18 $\pm$ 1 °C. During this exposure period, the embryos were maintained in controlled laboratory conditions. After the exposure, samples were fixed with buffered formalin (4 %), 100 larvae

were counted for each replicate suspension differentiating between normal larvae (D-shell stage) and abnormalities, which encompassed malformed larvae (characterized by a concave, malformed, or damaged shell, and a protruding mantle) as well as pre-D stages (trochophore larvae or earlier stages). Samples analysis were conducted using Cell Imaging System (Juli Stage microscope, Nano Entek), equipped with an image acquisition toolbox, enabling the capture of representative light microscopic pictures.

2.5. Data analysis

All tests were performed in triplicate to ensure the reliability of the results. Negative controls (untreated gametes and embryos) and positive controls (treatments known to induce toxicity) were included to validate the experimental setup.

Statistical analyses were conducted to assess the significance of the observed effects on embryonic development in the presence of different Gd concentrations. The data were analyzed using appropriate statistical software (GraphPad Software Inc.; San Diego, CA, USA), and results were presented as mean ± standard deviation (SD) or mean ± standard error of the mean, and when possible, as EC50 calculated using nonlinear regression (least squares regression with 95 % confidence). Sperm motility and kinetic were reported as cumulative percentages. The different endpoints among treatments were compared though one-way analysis of variance (ANOVA), followed by *post-hoc* Tukey's HSD tests ($p < 0.05$).

3. Results

3.1. Spermatozoa morphology

The morphological results yielded by spermatozoa collected from unexposed and exposed mussels (Fig. 1) in all experimental conditions stained with toluidine blue (TB). TB staining not only highlights the head and tail of the spermatozoa, but also allows the degree of chromatin condensation of the spermatozoa to be assessed. Specifically, in spermatozoa with decondensed chromatin, the dye binding to the DNA produces a dark blue-purple metachromatic stain, corresponding to aggregates of TB-interacting nucleotides and negative nucleotide phosphates. Spermatozoa with condensed chromatin stain blue (orthochromatic staining) due to the involvement of far fewer TB molecules that bind as monomers to DNA by insertion or ionic bonding. In detail, the spermatozoa of control and mussels exposed at 0.01 mg/L of Gd (Fig. 1A, we show a single picture since the controls and the treatment with 0.01 Gd showed the same result) showed a light blue colour and do not exhibited signs of aggregation; they appeared as isolated. In contrast, mussels treated with concentrations of 0.04 and 0.2 mg/L (shown in the same figure) of Gd already showed initial aggregation at the level of the heads (Fig. 1B). This aggregation becomes particularly evident in spermatozoa exposed to 5.6 mg/L of Gd, where dark blue-stained chromatin was also evident (Fig. 1C).

3.2. Evaluation of sperm motility after Gd treatments

SCA system was used to assess the total motility percentage, considering both progressive and non-progressive sperm movements, in mussels sperm suspension exposed to different concentrations of Gd (0.01, 0.04, 0.2, and 5.6 mg/L) in comparison to untreated control groups (CTL). After 30 min of Gd exposure, sperm motility remained unaffected at specific concentrations (0.01, 0.04, 0.2 mg/L). However, at a concentration of 5.6 mg/L, there was a reduction in progressive motility, leading to an increase in non-progressive motility parameters (7.7 %) compared to CTL. Similarly, after 60 min, total motility closely resembled CTL at concentrations of 0.01, 0.04 and 0.2 mg/L, but notably decreased at 5.6 mg/L (12.7 %) (Fig. 2).

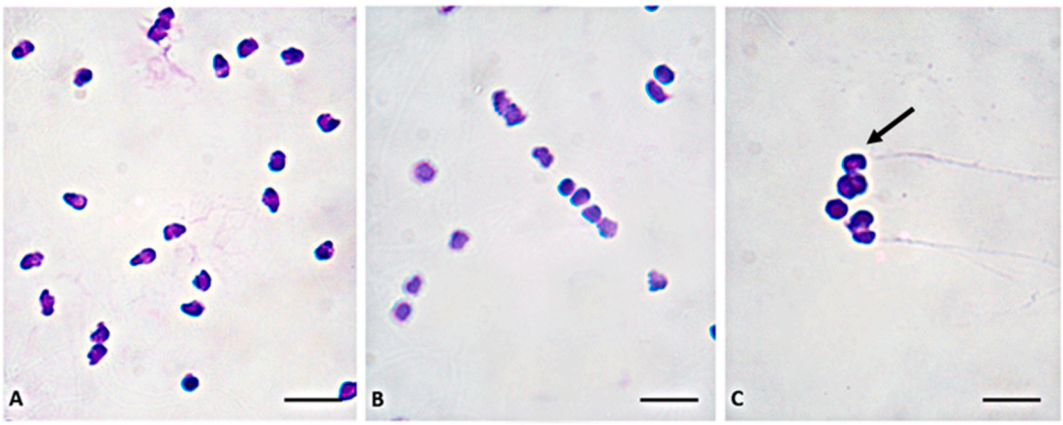

Fig. 1. Spermatozoa of *M. galloprovincialis* from control and exposed specimens with different concentrations of Gd stained with toluidine blue. (A) Spermatozoa from control animals and those exposed to 0.01 mg/L. (B) Spermatozoa from animals treated with Gd at concentrations of 0.04 and 0.2 mg/L. (C) Spermatozoa from animals treated with 5.6 mg/L of Gd. The scale bars correspond to 5 μm.

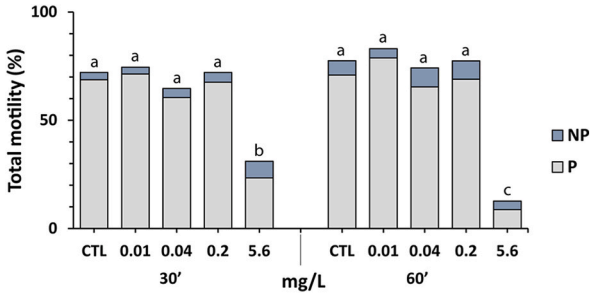

Fig. 2. Effect of Gd exposure on spermatozoa motility. Total spermatozoa motility after 30 and 60 min of Gd exposure at different concentrations (0.01, 0.04, 0.2, and 5.6 mg/L). Total motility (whole bar), progressive motility (P, light grey) and non-progressive motility (NP, dark blue) of spermatozoa with control medium (CTL) or Gd-containing medium. NP = non-progressive motile spermatozoa, P = progressive motile spermatozoa. The data labelled with different letters (a–c) are significantly different (Tukey *post hoc*, $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2.1. *Effect of Gd on sperm kinetic*

A comprehensive study was conducted to assess the impact of Gd exposure on the kinetic parameters of mussel sperm using SCA system. Representative images (Fig. 3) of mussel sperm trajectories on untreated and treated samples at Gd concentrations of 0.01, 0.04, and 0.2 mg/L showed that after 30 min, a significant proportion of mussel sperms exhibited large circular trajectories and a low percentage of straight trajectories. Sperm exposed to Gd for 30 min at a concentration of 5.6 mg/L displayed tightly coiled circles and a high number of type c and d sperm (immotile spermatozoa). This observation remained consistent after 60 min of Gd exposure, where more than 80 % of sperm appeared immotile. Moreover, by examining the subcategories of progressive sperm movements (rapid, medium, slow, and static), a decrease in the rate of rapid and medium sperm cells at 5.6 mg/L, and a significant increase of static cells (68.9 %) was observed. This effect was more pronounced at the concentration of 5.6 mg/L after 60 min of Gd exposure (87.3 %) (Table 1).

Furthermore, the sperm velocity sub-parameters (VAP, VSL, VCL) were not affected after short term Gd exposure (30 min). Low VSL values (<30 %) indicated that spermatozoa exhibited circular trajectory. Notably, after 60 min of Gd exposure, all velocity sub-parameters were preserved at concentrations of 0.01, 0.04, and 0.2 mg/L, but fell down significantly at the 5.6 mg/L (Fig. S1).

Additionally, BCF was not significantly altered at 30 min of Gd exposure at 0.01 and 0.2 mg/L. A slight reduction was found at 0.04 mg/L, while at the highest concentration (5.6 mg/L) of Gd the flagellar movement was extremely reduced. BCF was significantly affected from 0.04 to 5.6 mg/L concentrations after 60 min of Gd exposure. Furthermore, the sperm head movement was affected by the presence of Gd at 0.04 and 5.6 mg/L after 30 min of Gd incubation. An increase in both Gd exposure time and concentration resulted in a decrease of head displacement, leading to alterations in spermatozoa movement (Fig. S2).

Lastly, sperm viability was assessed following the Gd treatments using the Eosin assay. The results showed a 20 % decrease in sperm viability at concentrations of 0.1, 0.04, and 0.2 mg/L after 4 h of treatment, while a significant drop of 22 % was observed at the highest concentration of 5.6 mg/L (Fig. 4).

3.3. *Spermiotoxicity after gadolinium exposure*

When *M. galloprovincialis* sperm were suspended in Gd at various concentrations for 30 min, fertilization success was inhibited to varying degrees by Gd exposure (Fig. 5A). The initial concentration of 0.01 mg/L demonstrated a moderate effect on sperm function and viability of 9.25 %. As the Gd concentration increased to 0.04 mg/L, the spermiotoxic effect significantly increased to 63 %. Further raising the concentration to 0.2 mg/L resulted in a spermiotoxicity of 100 %, characterized by the compromised functionality and viability of spermatozoa, and this condition was maintained even at the highest concentration of 5.6 mg/L (Fig. 5A). The concentration-dependent nature of these spermiotoxic effects underscores the progressive impact of Gd exposure on spermatozoa, with the EC50 value for Gd-induced spermiotoxicity determined to be 0.030 mg/L and a 95 % confidence interval ranging from 0.029 to 0.033 mg/L (Table S1).

3.4. *Embryotoxicity after gadolinium exposure*

Rearing *M. galloprovincialis* embryos in seawater with Gd concentrations ranging from 0.1 to 5.6 mg/L revealed a nuanced relationship between Gd concentration and developmental defects (Fig. 5B). At 0.01 mg/L, a relatively low embryotoxicity effect was observed, with an average effect of 23.25 % across replicates, whereas at 0.04 mg/L, the embryotoxicity effect notably increased to 62.54 %. Surprisingly, as the Gd concentration increased to 0.2 mg/L, embryo developmental defects resulted in an average of 19.54 %. Possible factors, such as the complexity of biological responses, potential adaptive mechanisms, or biological variability among replicates, could have contributed to the observed variation. Nevertheless, at the highest concentration of 5.6 mg/L, the effect reached 100 %, indicating severe adverse impacts with no successful embryo development. The EC50 value for Gd was determined to be 0.026 mg/L, with a 95 % confidence interval ranging from 0.020 to 0.035 mg/L (Table S1). This value was found to be lower but comparable

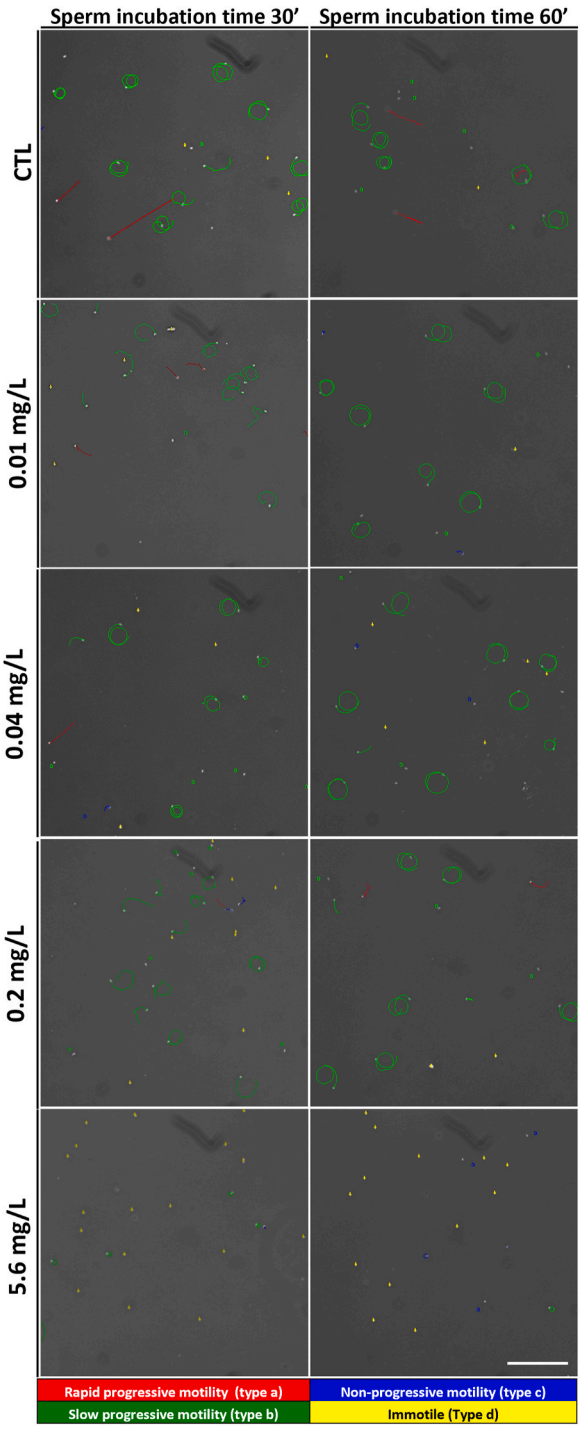

Fig. 3. Sperm trajectories after Gd exposure. SCA representative micrograph showed the differences between sperm trajectories after Gd exposure at different concentrations (0.01, 0.04, 0.2 and 5.6 mg/L) and time (30 min and 60 min). Scale bar 100 mm.

to the reported EC50 values for sea urchin embryos, including *Sphaerechinus granularis* (1.45 mg/L, 95 % CI: 0.61–2.00), *Arbacia lixula* (0.41 mg/L, 95 % CI: 0.35–0.46), and *Paracentrotus lividus* (0.52 mg/L, 95 % CI: 0.01–0.36) [39]. The results of the study, as shown in Fig. 6, revealed different percentages of malformation types in *Mytilus* embryos at different concentrations of Gd, denoted as 0.01, 0.04, and 0.2 mg/L. A larva was considered normal when the shell was D-shaped (straight hinge) and the mantle did not protrude out of the shell [40]. Notably, the percentage of normally developing mussels larvae (Type a) displayed fluctuations across concentrations, with the highest incidence at 0.01 mg/L (44.5 %). In contrast, the prevalence of malformed concave larvae displaying mantle

Table 1
Subcategories in percentages of progressive sperm movements after Gd exposure. The data are expressed as median value (%) ± Standard deviation.

	Sperm movements	CTL	0.01	0.04	0.2	5.6
Gd incubation time 30 min	**Rapid**	40.3 ± 0.1	47.2 ± 1.7	37.4 ± 2.4	50.3 ± 2.7	13.1 ± 0.9
	Medium	31.3 ± 0.4	25.5 ± 1.4	28.6 ± 1.8	19.6 ± 1.5	12.6 ± 0.8
	Slow	10.4 ± 0.8	1.9 ± 0.1	6.8 ± 0.2	2.2 ± 0.1	5.4 ± 0.2
	Static	17.9 ± 0.7	25.5 ± 2,1	27.2 ± 1.9	27.9 ± 1.1	68.9 ± 2.9
Gd incubation time 60 min	**Rapid**	48.4 ± 1.4	48.4 ± 1.8	39.7 ± 0.1	41.3 ± 1.9	2.3 ± 0.1
	Medium	26.9 ± 1.2	33.7 ± 1.7	30.1 ± 1.7	29.7 ± 2.8	5.8 ± 0.2
	Slow	2.2 ± 0.1	1.1 ± 0.1	4.4 ± 0.2	6.5 ± 0.3	4.6 ± 0.3
	Static	22.5 ± 1.8	16.8 ± 0.5	25.7 ± 1.9	22.6 ± 2.1	87.3 ± 2.9

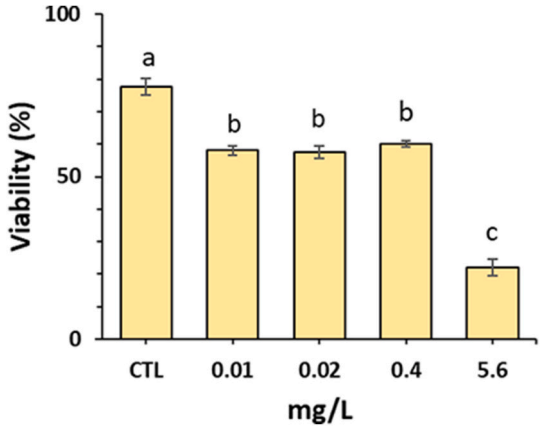

Fig. 4. Viability assay of mussel spermatozoa after Gd treatments. The graph showed the results of the Eosin assay conducted to assess sperm viability after exposure to different concentrations of Gd (0.1, 0.04, 0.2, and 5.6 mg/L) at two distinct time intervals (30 min and 60 min). The data labelled with different letters (a–c) are significantly different (Tukey *post hoc*, $p < 0.05$).

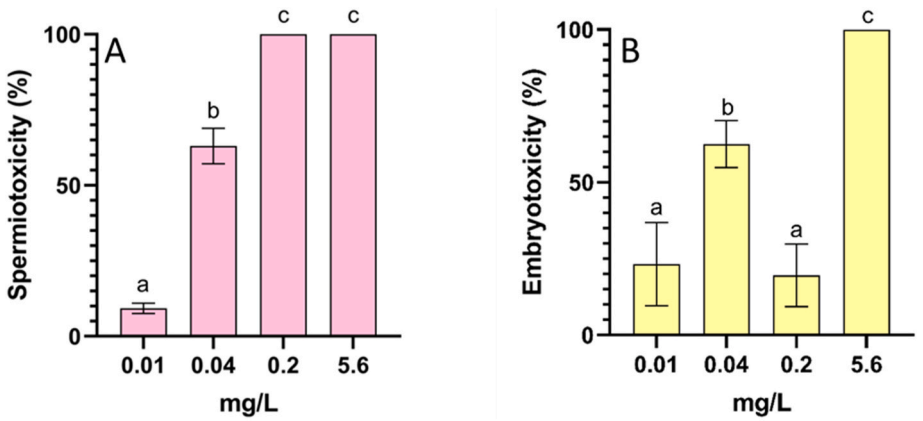

Fig. 5. (A) Spermiotoxicity assay on mussel sperm after Gd exposure. Different concentrations of Gd (0.1, 0.04, 0.2, and 5.6 mg/L) inhibited sperm fertilization success. The data labelled with different letters (a–c) are significantly different (Tukey *post hoc*, $p < 0.05$). **(B) Embryotoxicity evaluation after Gd exposure.** Different concentrations of Gd (0.01, 0.04, 0.2, and 5.6 mg/L) induced developmental defects in *M. galloprovincialis* larvae reared 48 h of exposure. The data labelled with different letters (a–c) are significantly different (Tukey *post hoc*, $p < 0.05$).

hypertrophy and shell deformity (Type b) kept a lower and steadier presence, ranging from 3.5 % to 7.25 %. Malformations involving hypertrophy of the mantle and shell deformity (Type c) exhibited variability, with the highest occurrence at 0.04 mg/L (24 %). On the other hand, trochophore larvae (Type d) increased with higher Gd concentrations, ranging from 22.5 % at 0.01 mg/L to 34 % at 0.2 mg/L. Finally, pre-D stages (earlier stage) (Type e) showed little differences across all Gd concentrations, ranging from 4 % to 7 %. These findings suggested a potential dose-dependent relationship between Gd exposure and the prevalence of specific malformation types in *Mytilus* embryos, underscoring the need for further statistical analysis to ascertain the significance of these observations and

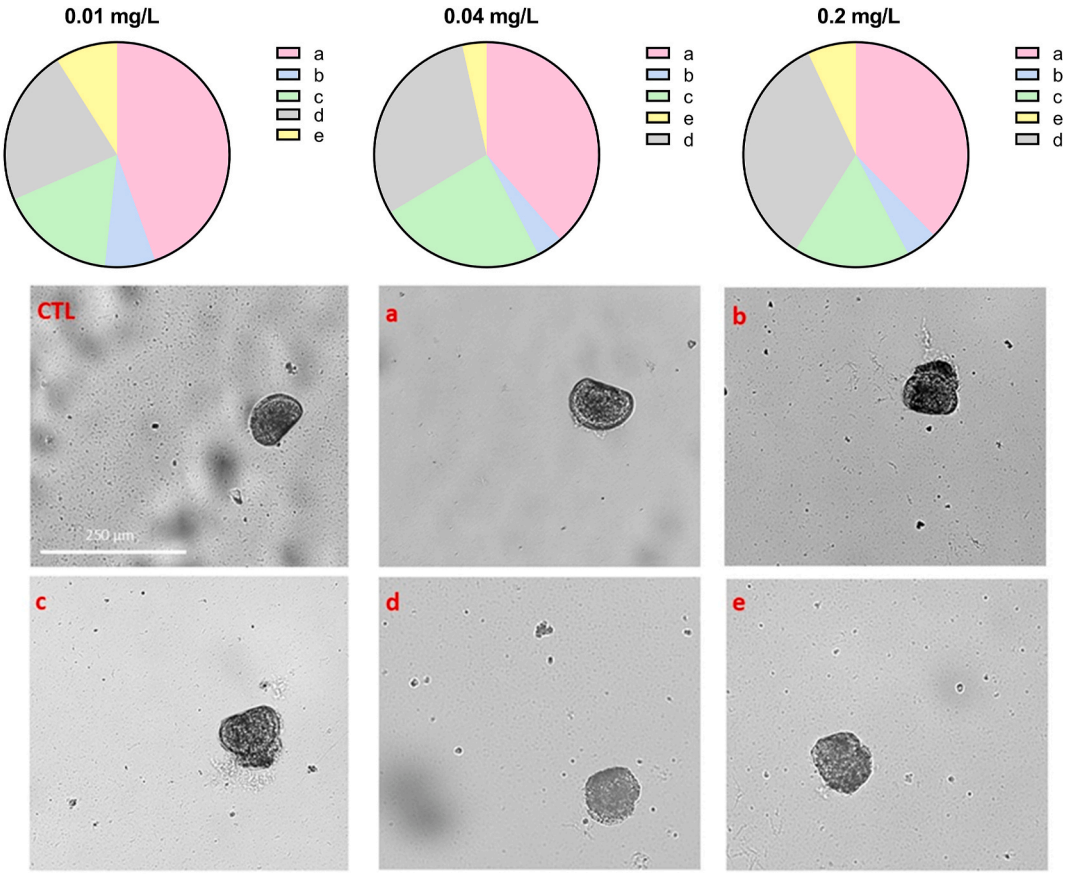

Fig. 6. Analysis and morphological classification of mussel larvae exposed to 0.01, 0.04 and 0.2 mg/L Gd. CTL) normal late D-stage/veliger in control a) normal D-stage/veliger; b) malformed concave larvae with mantle hypertrofy and shell deformity); c) malformed larvae with mantle hypertrophy and shell deformity); d) pre-D stages (trochophore larvae); d) pre-D stages (earlier stages). The graphs show a dose-dependent increase in morphologies b, c and d compared to controls.

their ecological implications.

4. Discussions

Pollution, along with climate change and over-exploitation, is one of the most severe consequences of the Anthropocene for both marine and freshwater ecosystems [41]. Among the harmful substances affecting aquatic organisms, Gd emerged as growing environmental concern due to its widespread introduction through routine use in MRI, resulting in continuous exposure for all organisms inhabiting surface and coastal waters [42]. In this context, the exploratory study aimed to investigate the effects of Gd on spermatozoa and embryos of *M. galloprovincialis*, an edible invertebrate widely used in biomonitoring studies, shedding light on potential mechanisms underlying Gd toxicity [43]. The findings suggested that Gd toxicity may be attributed to its role as a calcium (Ca^{2+}) channel blocker, as previously suggested by the similarity in ionic radius between Gd and divalent calcium ions [44].

The inhibition of calcium channels by Gd could significantly impact both sperm motility and embryonic development. Spermiotoxicity, arising from compromised calcium signalling, could disrupt the normal motility of spermatozoa, a critical factor in the fertilization process, since it is essential for reaching and penetrating the egg [45]. In external fertilization, such as that of mollusks, the interaction between signal molecules released by the oocyte and the receptor on the spermatozoa membrane leads to a progressive increase in calcium ion levels. This increase is associated with the sperm motility, which is essential for reaching the egg [46]. The reduction in sperm motility could also be attributed to cellular aggregation, as demonstrated by morphological investigations. This aggregation could resulted from the charged elements distribution on the plasma membrane surface, as previously demonstrated for cadmium in the tropical fish *Gymnotus carapo* [47]. Another aspect to consider was the kinetic pattern of mussel sperm, which have yet to be investigated in the context of Gd exposure. Mussel exhibit circular swimming patterns, an evolutionary adaptation that prevents straying in turbulent water conditions [48]. These mechanisms finely guide spermatozoa, ensuring their successful journey toward the egg. In our study, the kinetic parameters of mussel sperm resulted unaltered after 30 min of Gd contact, while a strong reduction in all parameters occurred after 60 min at highest Gd concentration. This reduction affected sperm motility and altered sperm kinetic patterns. VSL values consistently remain low, aligning with the circular motion of mussel sperm [49]. Analysis of sperm trajectories

revealed that the majority exhibited a circular trajectory after exposure to low concentrations of Gd, with only a few following a straight trajectory, in line with the literature [50,51]. Interestingly, with increasing exposure to Gd concentrations, a variation in trajectories with the production of smaller circles, leading to a complete loss of motility after 60 min at the highest Gd concentrations was observed. These data likely indicated a reduction in sperm energy reserves at low Gd exposures for short time, though leading to a loss of motility and reduced viability, resulting in inefficiency in fertiliation capability at high Gd concentrations. As previously reported [48,50] slow and tight circular movements of the sperm are thought to be a strategic response to chemical signalling (chemoattractants) from the oocyte. In our study, the gradual reduction in the circularity of sperm trajectories in the absence of chemoattractants suggested a transition to an immobile state rather than a mechanism for sperm to maintain their fertilization capacity.

Moreover, the observed fluctuation of the flagellar beating values, particularly at higher concentrations of Gd exposure, indicated a link with the progressive motility reduction and trajectory variation. Likewise, the head displacement alteration, highlighted Gd interference with head movement, indicating potential impairment in sperm trajectory.

It is commonly assumed, across various aquatic species, that relatively faster-swimming sperm are more successful at fertilizing eggs due to the physics of sperm−egg collision rates [52,53]. By measuring the subcategories of progressive sperm movements (rapid, medium, slow, and static), a decrease in the rapid and medium sperm cells rate was observed at high Gd concentrations, accompanied by a significant increase in static cells. This effect was particularly pronounced at the concentration of 5.6 mg/L after 60 min of Gd exposure. Similarly, some reports demonstrated that the exposure of marine invertebrate sperm, such as those from short-spine sea urchin (*A. crassispina)* and the green-lipped mussel (*P. viridis*) to metals lead to impaired sperm motility [54,55], resulting in ultra-structural damage and acrosome reaction inhibition [56]. Furthermore, other research revealed the adverse effect of Cd on sperm motility in the tropical sea urchin (*Anthocidaris crassispina),* demonstrating a reduction in sperm velocities and the presence of enlarged sperm midpiece with disorganized mitochondrial membranes [57]. Likewise, blue mussel sperm (*Mytilus trossulus*) exhibited decreased velocities and reduced fertilization success when exposed to high concentration of Cu (100 μg L^{-1}), which was attributed to Cu-induced interference with mitochondrial activity [55].It is also noted that 100 % spermiotoxicity was recorded, especially in sperm exposed to 0.2 and 5.6 mg/L of Gd that could also be determined by the triggering of apoptotic process, as recently demonstrated in human keratinocytes [58]. Exposure to Gd has been shown to increase the levels and activity of a pro-apoptotic factor such as casapase 3 in HaCat cells. Additionally, these cells also showed alterations in chromatin composition [58]. This result is in line with our morphological investigation using toluidine blue, where a reduction in chromatin condensation was recorded in smears of mussel sperm exposed to high concentrations of Gd, as well as previously demonstrated in *M. galloprovincialis* for heavy metals [26]. Regarding embryo, the interference with calcium channels could have prevented the regulation of critical processes for embryonic development that depend on calcium signalling. Specifically, Ca^{2+} is involved in shell growth in bivalves, including mussels; the larvae of these animals absorb Ca^{2+} from the environment and transport it to the shell-forming area. Whereby Gd acting on calcium channels blocks the growth of this essential structure for successful mussels development [59,60].

This outcome could be heightened by considering the synergistic impact of seawater temperature and acidification, which results in reduced availability of calcium carbonate, a crucial requirement for biomineralization in organisms such as molluscs [61]. However, the loss of structural integrity due to Gd exposure may affect shell strength and reduce protection against predators and environmental changes in individuals that undergo successful development, as evidenced by the observed malformations and incidence of congenital defects in *Mytilus* embryos.

Recent research in sea urchin species has shown that aberrant and asymmetrical spicule production during the late blastula stage of skeletal formation may be one of the processes that Gd modifies through a dose-dependent mechanism [62]. Finally, Gd could also act on the oocyte again altering Ca^{2+} pathways impairing both the polysperm blockage process (calcium-dependent mechanics [63]) and post-fertilisation metabolic activation. In *Mytilus edulis,* a species extremely similar to *M. galloprovincialis*, Ca^{2+} plays a significant role in oocyte metabolic activation. Specifically, the post fertilization extracellular Ca^{2+} entry triggers a transient increase in this second messenger within the cytoplasm, subsequently followed by a Ca^{2+} release mediated by the IP3 receptor. These signals are relevant to the completion of the first meiotic division of oocytes blocked in metaphase I [64].

Embryonic development represents a pivotal phase in the life cycle of marine organisms, and perturbations at this stage can have implications for population dynamics [65]. Impairments in sperm motility and kinetics raised concerns regarding the reproductive fitness of marine species under Gd exposure scenarios [66].

Reduced reproductive success resulting to Gd exposure has the potential to promote declines in population abundance, effects in genetic diversity and shifts in community composition within marine ecosystems [67], influencing the resilience of ecosystems to environmental stressors [68,69].

Given the role of these processes in shaping population dynamics and maintaining ecosystem resilience, the implications of Gd toxicity extend far beyond individual organisms to encompass entire marine communities.

In the context of global marine pollution, our study emphasized the importance of adopting a proactive approach to address emerging contaminants like Gd. By integrating comprehensive risk assessments and predictive modelling techniques, regulatory authorities can anticipate and mitigate the environmental impacts of Gd contamination, alongside enhancing wastewater treatment protocols to remove this type of contaminants [8].

5. Conclusions

This study provided key insights into the mechanisms underlying the embryotoxic and spermiotoxic effects of Gd in *Mytilus galloprovincialis*. The observed disruptions in signalling pathways could have broad-ranging consequences, affecting both sperm function

and embryonic development. Alterations in sperm kinetic patterns, reduced motility, and increased static cells at high Gd concentrations suggested potential inefficiency in fertilization, raising concerns about the ecological consequences of Gd contamination. The recorded spermiotoxicity, apoptotic processes, and disruptions in chromatin composition further highlight the detrimental effects of Gd exposure. This work highlighted the relationship between sperm motility, kinetics, and environmental factors in *M. galloprovincialis*. Therefore, future research should consider these factors, with specific attention to investigating the roles of chromatin dynamics, apoptosis mechanisms, and calcium signaling pathways.

The findings of this study hold implications for environmental policymakers, wastewater treatment practices, and the preservation of marine organisms. Enhanced monitoring and regulation of REEs in aquatic ecosystems are essential for effective management strategies to mitigate Gd contamination risks.

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CRediT authorship contribution statement

Marisa Spampinato: Writing – original draft. **Antonietta Siciliano:** Writing – original draft, Supervision, Conceptualization, Writing – review & editing. **Angela Travaglione:** Methodology, Data curation. **Teresa Chianese:** Formal analysis, Data curation. **Aldo Mileo:** Methodology, Data curation. **Giovanni Libralato:** Validation, Data curation, Writing – review & editing. **Marco Guida:** Supervision, Funding acquisition, Conceptualization. **Marco Trifuoggi:** Formal analysis. **Vincenza De Gregorio:** Writing – original draft, Supervision, Methodology, Writing – review & editing. **Luigi Rosati:** Writing – original draft, Supervision, Conceptualization, Writing – review & editing.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31087.

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