



# Comparative toxicities of selected rare earth elements: Sea urchin embryogenesis and fertilization damage with redox and cytogenetic effects

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## ABSTRACT

**Background:** Broad-ranging adverse effects are known for rare earth elements (REE), yet only a few studies tested the toxicity of several REE, prompting studies focusing on multi-parameter REE toxicity. **Methods:** Trichloride salts of Y, La, Ce, Nd, Sm, Eu and Gd were tested in *Paracentrotus lividus* sea urchin embryos and sperm for: (1) developmental defects in either REE-exposed larvae or in the offspring of REE-exposed sperm; (2) fertilization success; (3) mitotic anomalies in REE-exposed embryos and in the offspring of REE-exposed sperm, and (4) reactive oxygen species (ROS) formation, and malondialdehyde (MDA) and nitric oxide (NO) levels.

**Results:** REEs affected *P. lividus* larvae with concentration-related increase in developmental defects,  $10^{-6}$  to  $10^{-4}$  M, ranking as:  $Gd(III) > Y(III) > La(III) > Nd(III) \cong Eu(III) > Ce(III) \cong Sm(III)$ . Nominal concentrations of REE salts were confirmed by inductively coupled plasma mass spectrometry (ICP-MS). Significant increases in MDA levels, ROS formation, and NO levels were found in REE-exposed embryos. Sperm exposure to REEs ( $10^{-5}$  to  $10^{-4}$  M) resulted in concentration-related decrease in fertilization success along with increase in offspring damage. Decreased mitotic activity and increased aberration rates were detected in REE-exposed embryos and in the offspring of REE-exposed sperm.

**Conclusion:** REE-associated toxicity affecting embryogenesis, fertilization, cytogenetic and redox endpoints showed different activities of tested REEs. Damage to early life stages, along with redox and cytogenetic anomalies should be the focus of future REE toxicity studies.

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## 1. Introduction

Current literature on REE-associated toxicity is mostly confined to three REEs (Ce, La and Gd). Consequently, comparative information for several REEs remains relatively scarce in spite of their widespread industrial utilization and as emerging environmental contaminants (US Environmental Protection Agency, 2012; EU-OSHA, 2013; Gambogi and Cordier, 2013; Snow et al., 2014; Pagano et al., 2015a,b; González et al., 2015).

The present study aimed at providing comprehensive data on

multiple toxicity endpoints after exposure to selected REEs in sea urchin early life stages. The sea urchin assay system has been utilized extensively in the past -up to present-day studies- to address questions concerning the effects of a number of agents, including inorganics, organics, and complex mixtures, e.g. whole sediment or industrial effluents. Sea urchins have provided valuable insights on the toxicity mechanisms of many xenobiotics. This extensive body of literature is beyond the scope of this experimental report and will be reviewed in a paper currently in preparation. Multiple toxicity endpoints can be tested in sea urchin early life stages such as effects on fertilization success, embryogenesis, mitotic activity, redox balance, and other endpoints such as gene expression (Stumpp et al., 2011a,b; Evans and Watson-Wynn, 2014; Migliaccio et al., 2014). Thus, sea urchin assays can

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provide multi-parameter information on the ability of a xenobiotic to interfere with key-events in early life stages such as cell division and differentiation, fertilization and oxidative/nitrosative stress (ONS) (Korkina et al., 2000; Pagano et al., 2001a; Oral et al., 2010; Romano et al., 2011; Migliaccio et al., 2014).

With this long-established background on the use of sea urchin assays in toxicity testing, the present study's main objective included toxicity testing of selected REE trichloride salts, including Y (III), La(III), Ce(III), Nd(III), Sm(III), Eu(III) and Gd(III). Toxicity endpoints investigated included developmental defects in *Paracentrotus lividus* pluteus larvae either following exposures during embryogenesis or in the offspring of REE-exposed sperm (whose fertilization success was also tested). Furthermore, cleaving embryos, either reared in a REE medium or generated by REE-exposed sperm, were tested for REE-induced cytogenetic anomalies, including changes in mitotic activity and induction of mitotic aberrations. Finally, three ONS endpoints were tested in REE-exposed embryos or larvae, including ROS formation and MDA levels related to oxidative stress, and NO levels related to nitrosative activities. ONS is indeed known both to induce damages in biomolecules and to play key-roles in embryogenesis.

## 2. Methods

### 2.1. Sea urchins

Sea urchins (*P. lividus*) were collected in the Bay of Naples by the staff of the Stazione Zoologica Anton Dohrn. Gametes were obtained and embryos were reared as reported previously (Pagano et al., 2001b). Controls consisted of embryos reared in natural filtered seawater (FSW) run as triplicate blanks. The embryos were reared in trichloride salt solutions of Y(III), La(III), Ce(III), Nd(III), Sm(III), Eu(III) and Gd(III) that were diluted from a  $10^{-1}$  M stock solution stored refrigerated at pH 3 (by HCl addition). The correspondence of nominal vs. analytical concentrations was determined by a set of ICP-MS analyses using an Aurora M90 Brucker apparatus. Embryo exposures to REE trichloride salts at concentrations in the order of  $10^{-8}$  to  $10^{-4}$  M were performed throughout embryogenesis, starting from zygote (10 min post-fertilization) up to the pluteus larval stage (72 h post-fertilization). Embryos were incubated in FSW at  $18 \pm 1$  °C in Falcon™ Tissue Culture Plates (6 wells, 10 ml/well). Experiments were run with a total of 4–12 replicates. A series of experiments was performed on *P. lividus* sperm, by suspending a 50- $\mu$ l sperm pellet for 1 h in 30 ml FSW containing REE salts,  $10^{-5}$  to  $10^{-4}$  M; thereafter, 50- $\mu$ l of sperm suspension were used to inseminate 10 ml of untreated eggs (~50 eggs/ml).

### 2.2. Embryological analysis

Embryological analysis was performed on living plutei immobilized in  $10^{-4}$  M chromium sulfate 10 min prior to observation, approx. 72 h after fertilization. In each treatment schedule, the first 100 plutei were scored for the percentages of: (1) normal larvae (N); (2) retarded larvae (R, size < 1/2N); (3) malformed larvae (P1), mostly observed through damaged skeletal differentiation; (4) embryos/larvae unable to attain the pluteus stage i.e. abnormal blastulae or gastrulae (P2), and (5) dead (D) embryos or larvae. Total developmental defects (DD) were scored as (P1 + P2).

### 2.3. Cytogenetic analysis

Cytogenetic analysis was carried out on 30 cleaving embryos from four cultures in each treatment schedule (either embryo

exposure or following sperm exposure), and triplicate controls (each in quadruplicate cultures) amounted to a total of 12 control cultures. The embryos were fixed in Carnoy's fluid (60% ethanol, 30% chloroform and 10% glacial acetic acid) 5 h after fertilization, and stained by acetic carmine (Pagano et al., 2001b). The cytogenetic endpoints both allowed for measurements of quantitative and morphological abnormalities. Quantitative parameters included: (a) mean number of mitoses per embryo (MPE), and (b) percent interphase embryos (IE). The frequencies of morphologic abnormalities were scored as: (a) anaphase bridges; (b) lagging chromosomes; (c) acentric fragments; (d) scattered chromosomes; (e) multipolar spindles; (f) total mitotic aberrations per embryo and (g) percent embryos having  $\geq 1$  mitotic aberrations [E(Ab+)].

### 2.4. Sperm bioassays

Following a 1-h sperm pretreatment, fertilization success was measured as percent fertilized eggs also expressed as fertilization rate (FR) on live cleaving embryos 1–3 h post-fertilization. Thereafter, the embryos were cultured up to pluteus stage and scored for developmental defects as described above in order to evaluate the effects, if any, of sperm exposure on offspring health status. Each observation was carried out blind on randomly-tagged specimens.

### 2.5. Redox endpoints – ROS measurement

ROS production was determined by measuring the oxidation of 2',7'-dichlorohydro-fluorescein diacetate (DCFH-DA, D6883, Sigma-Aldrich). Sea urchin embryos (about 5000) were incubated in 5  $\mu$ M DCFH-DA in phosphate buffer saline (PBS) for 1 h under darkness. After incubation, embryos were collected by centrifugation at  $1800 \times g$  for 10 min at +4 °C, briefly rinsed in PBS without DCFH-DA and frozen in liquid nitrogen. Frozen embryos were resuspended in 0.2 ml Tris-HCl buffer 40 mM, pH 7.0, vortexed for 1 min, and finally centrifuged for 10 min at  $13,500 \times g$  at +4 °C. The supernatant was collected and examined for protein concentration using a Bradford assay. Fluorescence was measured using spectrofluorometer (Shimadzu RF-5301 PC) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Fluorescence values were corrected by subtracting the autofluorescence of unlabeled extracts. A standard curve was prepared using dichlorofluorescein (DCF, 35848, Sigma-Aldrich), diluted in the same Tris-HCl buffer. Fluorescence from DCF was linear in the range of 0–50 nM.

### 2.6. Lipid peroxidation determination

Lipid peroxidation was measured by the thiobarbituric acid method assay (TBA test), which is based on the reactivity of the end product of lipid peroxidation, malondialdehyde (MDA) with TBA to produce a red adduct. Sea urchin developing embryos (about 15,000) were collected at the pluteus stage by centrifugation at  $1800 \times g$  for 10 min at +4 °C. The pellet was homogenized in Tris-HCl containing 0.1 mM EDTA and 0.2% triton X-100 (1:2 W/V) and centrifuged (at  $14,000 \times g$  for 30 min, +4 °C). The supernatant (200  $\mu$ l) was added to 1 ml of the reaction buffer containing 15% trichloroacetic acid (TCA), 0.375% TBA and 0.1% butylated hydroxytoluene (BHT). The buffer was prepared by adding TBA (previously dissolved in 1–2 ml of 12 M HCl) to the TCA solution while stirring on a hot plate. After incubation at +95 °C for 15 min, the reaction mixture was centrifuged and its absorbance was read at 532 nm. A standard curve for MDA was prepared using 1,1,3,3-tetraethoxypropane as reported by Esterbauer and Cheeseman (1990).

## 2.7. Nitric oxide (NO) determination

The endogenous NO levels were measured by monitoring nitrite formation by Griess reaction (Migliaccio et al., 2014). Sea urchin developing embryos (about 15,000) were collected at the pluteus stage by centrifugation at  $1800 \times g$  for 10 min in a swing-out rotor at  $+4^\circ\text{C}$ . The pellet was washed with PBS, frozen in liquid nitrogen and kept at  $-80^\circ\text{C}$  until use. Samples were homogenized in PBS (1:2 w/v), centrifuged ( $12,000 \times g$  for 30 min at  $+4^\circ\text{C}$ ) and the supernatants were analyzed for nitrite content (Migliaccio et al., 2014).

## 2.8. Statistical analysis

Results are given as mean  $\pm$  standard error, or with 95% confidence interval (CI). The half maximal effective concentrations (EC50s) were calculated by using a non-linear regression analysis with CIs. Statistical assumptions were verified at the onset of each analysis, and a square-root data transform was applied when underlying statistical assumptions were violated. Differences between control groups were determined through an unpaired two-tailed Student's *t*-test or with One-way Anovas with Dunnett's multiple comparison test as a post-hoc analysis. Those variables that were unsuitable for a parametric approach (from cytogenetic analysis) were evaluated with nonparametric tests:  $\chi^2$  test and Mann–Whitney *U* test. To carry out several simultaneous comparisons, Tukey's and Bonferroni's methods were used. Differences were considered significant when  $p < 0.05$ .

## 3. Results

### 3.1. Nominal REE concentrations

In order to verify the correspondence of nominal concentrations, a set of ICP-MS analyses (shown in Table 1) were conducted on seawater solutions of  $\text{YCl}_3$ ,  $\text{CeCl}_3$  and  $\text{GdCl}_3$  at nominal concentrations ranging from 1 to 100  $\mu\text{M}$ ; the ICP-MS determinations provided nominal/analytical ratios close to 2, except for 100  $\mu\text{M}$   $\text{GdCl}_3$ , with a nominal/analytical ratio = 6.88. These nominal/analytical ratios were confirmed by using algal culture medium, with the same pH (8) yet a much lower ionic strength compared to seawater; the  $\text{GdCl}_3$  apparent anomaly was much less expressed, with a nominal/analytical ratio confined to 2.87. Altogether, these analyses corroborated the plausible closeness of nominal REE concentrations vs. analytical determinations.

**Table 1**

Comparisons of nominal vs. analytical concentrations of  $\text{YCl}_3$ ,  $\text{CeCl}_3$  and  $\text{GdCl}_3$  in seawater. Analysis was carried out by inductively coupled plasma mass spectrometry (ICP-MS).

Element	Nominal concentration ( $\mu\text{M}$ )	Analytical concentration 2 h	Analytical concentration 48 h	Mean $\pm$ SD 2 h + 48 h	Nominal/analytical concentration ratio
$\text{YCl}_3$	0	< 5	< 5		
	89 (1)	55	44	$49.5 \pm 3.9$	1.80
	889 (10)	557	449	$503 \pm 38.2$	1.77
	8891 (100)	5795	4711	$5253 \pm 383.3$	1.69
$\text{CeCl}_3$	0	< 5	< 5		
	140 (1)	96	100	$98 \pm 1.4$	1.43
	1401 (10)	549	776	$662.5 \pm 80.3$	2.11
	14,012 (100)	9656	7204	$8430 \pm 866.9$	1.66
$\text{GdCl}_3$	0	< 5	< 5		
	157 (1)	59	108	$83.5 \pm 17.3$	1.88
	1572 (10)	549	928	$738.5 \pm 134.0$	2.13
	15,725 (100)	1958	2611	$2284.5 \pm 230.9$	6.88

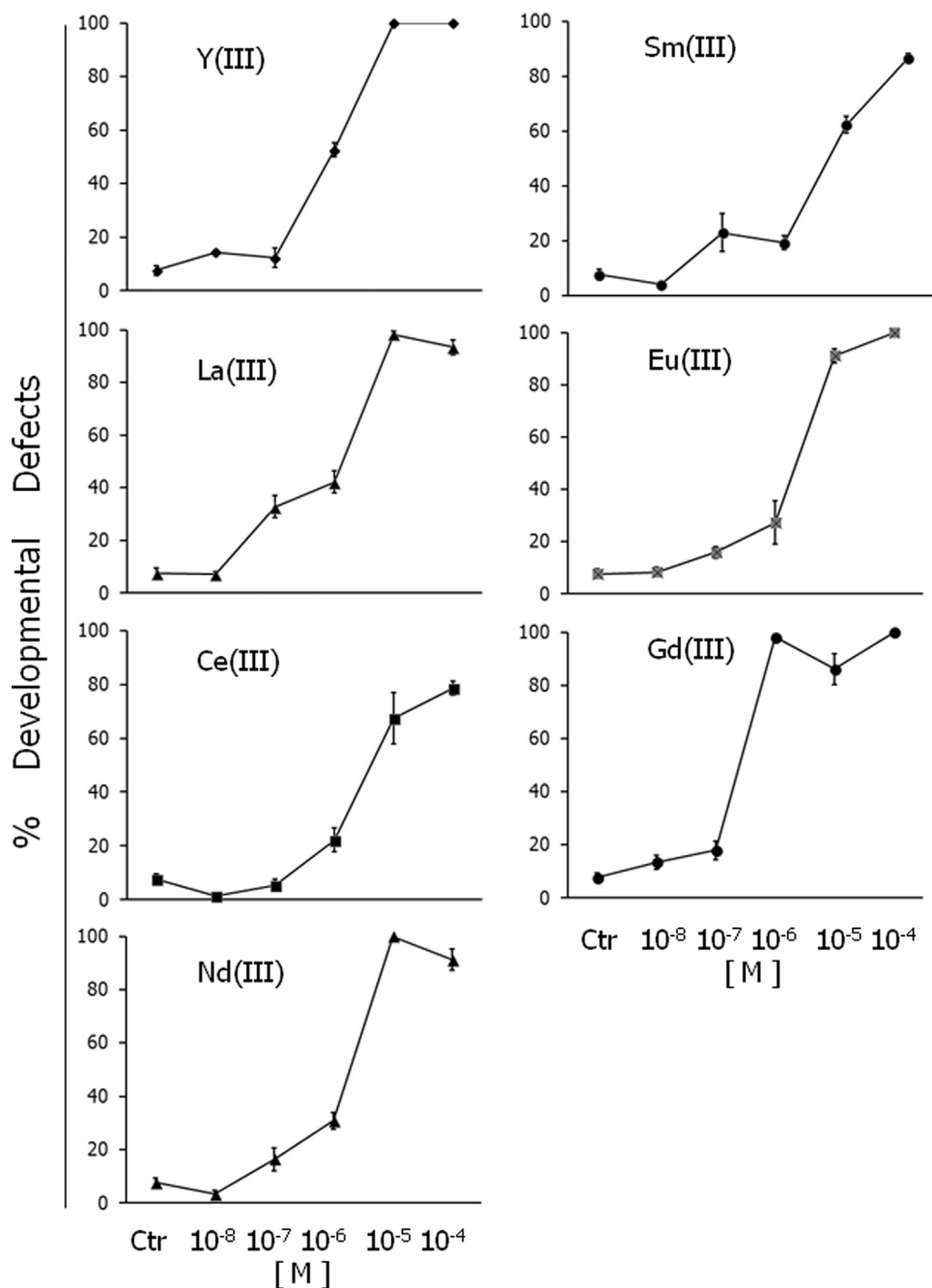
## 3.2. Embryo bioassays: developmental and cytogenetic toxicity

By rearing *P. lividus* embryos in FSW containing REEs of various concentrations ( $10^{-8}$  to  $10^{-4}$  M), increases in developmental defects (DD) were observed in a dose-dependent fashion for individual REEs. As shown in Fig. 1, exposures to REEs  $\geq 10^{-7}$  M resulted in dose-dependent increases in DD for all tested REEs. In order to compare relative toxicities of individual REEs, Fig. 2 reports the DD values of pluteus larvae reared in  $10^{-6}$  M REE. The strongest toxicity was exerted by  $10^{-6}$  M  $\text{Gd(III)}$  with DD close to 100% ( $98.2 \pm 2.6$ ), vs.  $7.7\% \pm 1.9$  in control larvae. Lower, yet significant ( $p < 0.001$ ) increases in DD were observed in larvae reared in  $\text{Y(III)}$  ( $52.7\% \pm 2.7$ ),  $\text{La(III)}$  ( $42.2\% \pm 4.2$ ) and  $\text{Nd(III)}$  ( $30.8 \pm 3.3$ ). The corresponding EC50s for the individual REEs confirmed the strongest embryotoxicity from  $\text{Gd(III)}$  exposure with a corresponding  $\text{EC50} = 1.97 \times 10^{-7}$  M (CI:  $0.28\text{--}13.76 \times 10^{-7}$  M).  $\text{La(III)}$  with an  $\text{EC50} = 6.66 \times 10^{-7}$  M (CI:  $0.97\text{--}45.44 \times 10^{-7}$  M), and  $\text{Y(III)}$  with an  $\text{EC50} = 7.98 \times 10^{-7}$  M (CI:  $2.87\text{--}22.18 \times 10^{-7}$  M) were the other most embryotoxic elements. The other tested REEs had EC50's in the micromolar range.

Cytogenetic analysis of embryos exposed to  $\text{La(III)}$ ,  $\text{Ce(III)}$ ,  $\text{Sm(III)}$  or  $\text{Gd(III)}$ , at concentrations ranging from  $10^{-6}$  to  $10^{-4}$  M, showed concentration-related inhibition of mitotic activity, as decreasing numbers of mitoses per embryo (MPE), and increasing percent embryos lacking active mitoses, or "interphase embryos" (IE) (Fig. 3). The same held true for mitotic aberrations in REE-exposed embryos, expressed as percent embryos with  $\geq 1$  aberrations [E(Ab+)]. A less sensitive endpoint when compared to DD, mitotoxicity (evaluated through MPE and IE measures) was more clearly affected at higher REE levels (or at  $10^{-5}$  and  $10^{-4}$  M). At these levels, significant mitotoxic effects were exerted by  $\text{Sm(III)}$  and  $\text{Ce(III)}$  exposure followed by  $\text{La(III)}$  and  $\text{Gd(III)}$  (Fig. 3). Similar to DD, mitotic aberrations showed the greatest dose-dependent increase after exposure to  $\text{Gd(III)}$  ( $p < 0.01$ ).

## 3.3. Sperm bioassays: effects of fertilization rate and offspring quality

When *P. lividus* sperm were suspended for 1 h in a set of trivalent REE salts at  $10^{-5}$  and  $10^{-4}$  M, fertilization success was inhibited to a different extent by REE exposure (Fig. 4). At  $10^{-4}$  M  $\text{Eu(III)}$  and  $\text{Y(III)}$  exhibited the highest degree of spermiotoxic effect ( $p < 0.001$ ), while the other tested REEs inhibited fertilization to similar extents [except for  $\text{Ce(III)}$  exposure that resulted in non-significant inhibition of fertilization success]. Sperm exposure to REEs resulted in significant damage to offspring embryogenesis



**Fig. 1.** Concentration-related % Developmental Defects in *P. lividus* larvae reared 72 h in seven REE solutions at levels ranging from  $10^{-8}$  to  $10^{-4}$  M.

determined through an increase in DD rates (Fig. 5). From most toxic to least toxic, DD rates in the offspring were influenced by sperm exposures to:

$Y(III) \cong La(III) > Ce(III) \cong Nd(III) \cong Sm(III) \cong Eu(III) \cong Gd(III)$ .

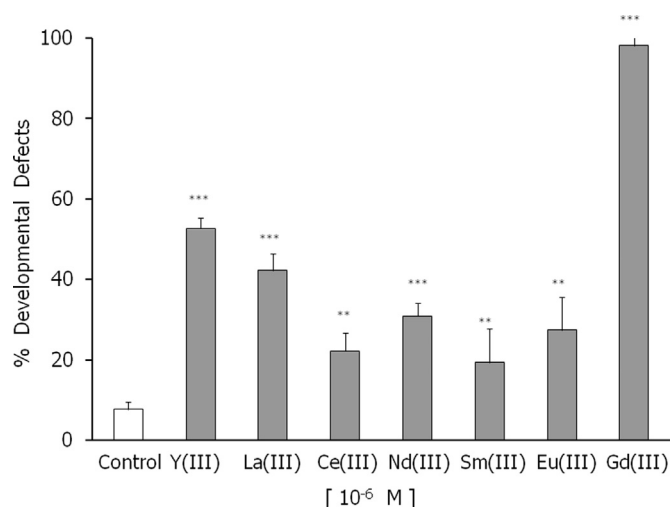
On the other hand, mitotic activity in the offspring of REE-exposed sperm, detected through decreased MPE and increased IE, was significantly inhibited by Ce(III) and, to a lesser extent, by La(III) and Sm(III). No significant mitotoxicity effects were observed in the offspring of Gd(III)-exposed sperm (Fig. 6). Mitotic aberrations showed a significant concentration-related relationship in the offspring of Ce(III)-exposed sperm. No significant aberration increases were detected in the offspring of sperm exposed to La(III), Sm(III), or Gd(III) (Fig. 6).

### 3.4. Anomalies in redox endpoints

As shown in Fig. 7, a significant increase in ROS formation measured by the oxidation of DCFH-DA in *P. lividus* early embryos (5 h post-fertilization) was induced by  $10^{-6}$  M Y(III), Ce(III) and Sm(III) ( $p < 0.05$ ). La(III) and Nd(III) exposure resulted in ROS production similar to control larvae.

Lipid peroxidation, measured as malondialdehyde levels, measured in *P. lividus* pluteus larvae (48 h post-fertilization), was significantly increased following exposures to  $10^{-6}$  M Ce(III) and Gd(III) ( $p < 0.05$ ) when compared to control larvae (Fig. 8). Thus, excess MDA levels were observed for Ce(III) and Gd(III), whereas Y(III), La(III), Sm(III) and Nd(III) failed to induce significantly increased MDA levels.

As shown in Fig. 9, NO levels determined as nitrite levels were significantly increased in *P. lividus* larvae exposed to  $10^{-6}$  M Y(III),



**Fig. 2.** Percent Developmental Defects in larvae reared in  $10^{-6}$  M in REE solutions. Significance noted as: \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

La(III), Ce(III), and Gd(III). Effects ranked as follows:

Y(III) > Gd(III) > La(III) > Ce(III).

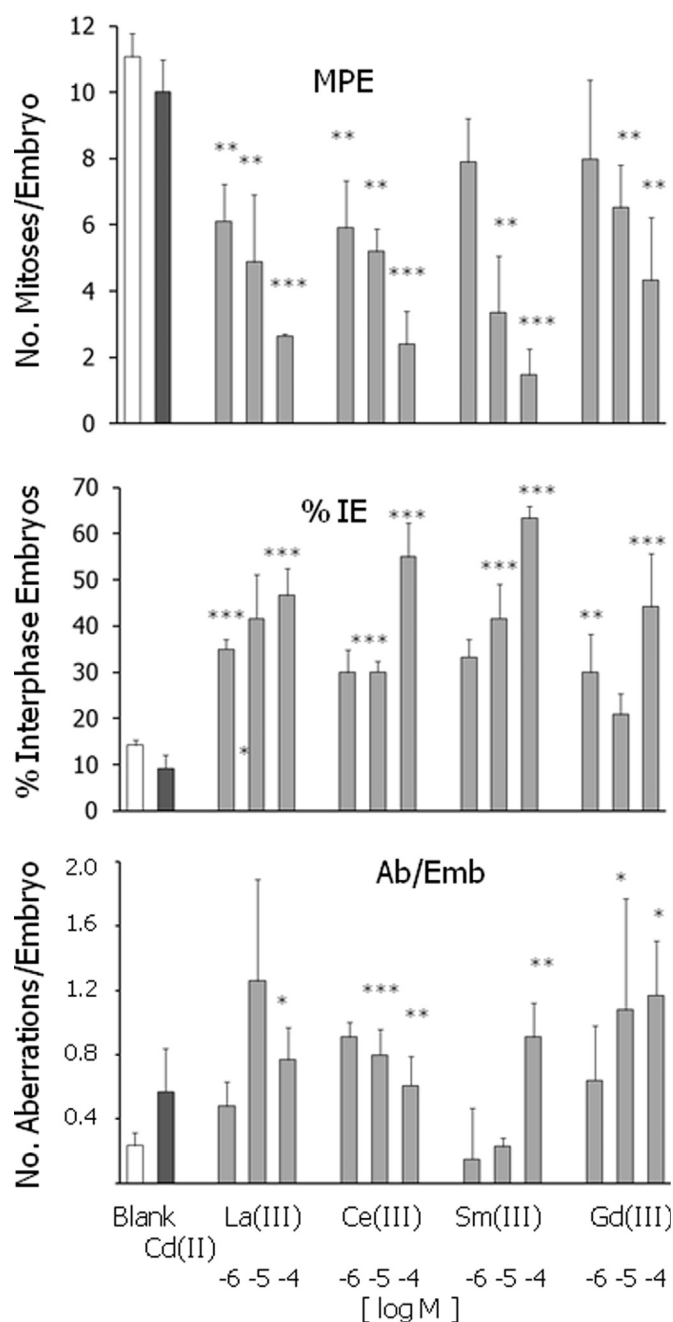
No significant differences between larvae exposed to Nd(III) and Sm(III) and control larvae could be detected. Altogether, increased ROS and/or MDA and/or NO levels were observed in embryos/larvae reared in Y(III), Ce(III) and Gd(III), though with distinct effect patterns. This was the case, e.g., for Gd(III) resulting in excess MDA and NO levels, yet with a non-significant decrease in ROS formation. On the other hand, Ce(III) affected all three redox parameters and Y(III) only ROS and NO levels. On the contrary, Sm(III) and La(III) treatment resulted in the increase of only one parameter, ROS and NO, respectively. Nd(III) consistently failed to induce any significant biological effect when considering various redox endpoints in *P. lividus*.

### 3.5. Results summary

In an attempt to draw a comprehensive outline of the results obtained in the set of treatment procedures and in the different endpoints evaluated, Fig. 10 provides a graphical summary. By exposing embryo/larval cultures to selected REE trichloride salts in the micromolar range, Gd(III), Y(III) and La(III) displayed the most severe effects in inducing developmental defects and Gd(III) and La(III) also cytogenetic anomalies. These REEs also consistently enhanced MDA and NO formation when compared to the other chemical species tested. In turn, these other REEs were effective in exerting developmental and/or cytogenetic defects and/or redox anomalies, though to a lesser extent when compared to Gd(III), Y(III) and La(III).

Following sperm exposure to  $10^{-4}$  M REE, a significant loss of fertilizing capacity was exerted by all tested REEs, except for Ce(III). Eu(III) displayed the most severe spermiotoxicity. The induction of transmissible damage as developmental defects in the offspring was most severe following sperm exposure to Y(III) and La(III). Developmental defects were significantly increased in the offspring of sperm exposed to Ce(III), Nd(III), Sm(III), Eu(III) and Gd(III). This effect was both detected by sperm exposure to  $10^{-4}$  M and was also significant following sperm exposure to  $10^{-5}$  M of La(III), Ce(III) and Sm(III) (Figs. 5 and 10).

Cytogenetic anomalies, though confined to a more limited set of REEs, showed a significant inhibition of mitotic activity and increase of aberrations in the offspring of Ce(III)-exposed sperm.

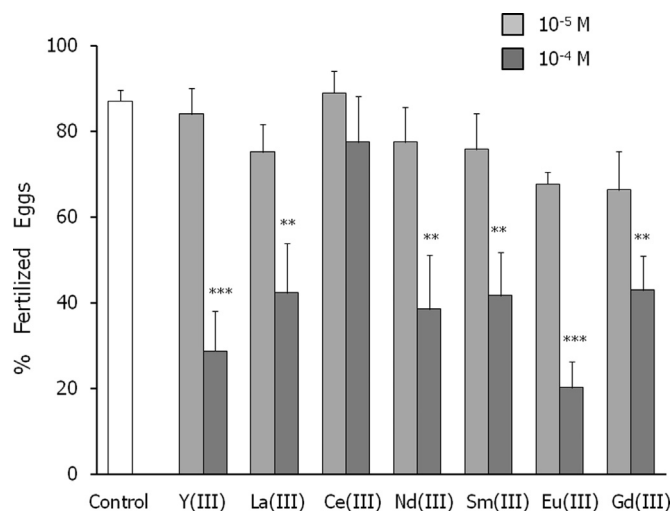


**Fig. 3.** Cytogenetic analysis of *P. lividus* embryos (5-h post-fertilization) exposed to La(III), or Ce(III), or Sm(III), or Gd(III)  $10^{-6}$  to  $10^{-4}$  M. Mean No. Mitoses per Embryo (MPE) and % Interphase Embryos (IE) (lacking active mitoses), showed mitotoxic action. % Embryos displaying  $\geq 1$  mitotic aberrations [E(Ab+)] pointed to induction of cytogenetic anomalies. CdSO<sub>4</sub> was used as reference compound.

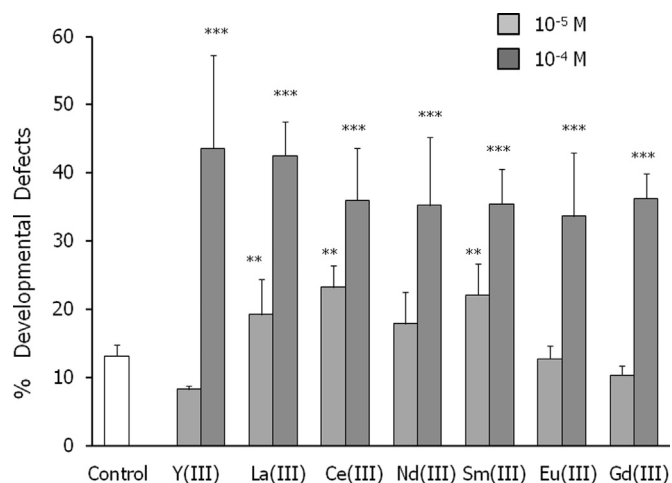
### 4. Discussion

A growing body of literature points to adverse biological effects due to REE environmental exposures (EU-OSHA, 2013; reviewed by Pagano et al. (2015a,b)). Most of the available literature on REE toxicology is confined to a small subset of these elements, thus warranting further investigations on REE-associated health effects.

In marine ecosystems, the offshore background REE levels are known to be minimal, in the picomolar order (Censi et al., 2004); however, REE bioaccumulation in a set of marine biota (plankton, bivalves and turtles) were reported (Strady et al., 2015; Bustamante and Miramand, 2005; Censi et al., 2013). Thus, one may envision that REE-containing effluents may affect coastal sediment



**Fig. 4.** REE-induced inhibition of sperm fertilization success. Except for Ce(III), all tested REEs ( $10^{-4}$  M) resulted in significant decreases of fertilization success.



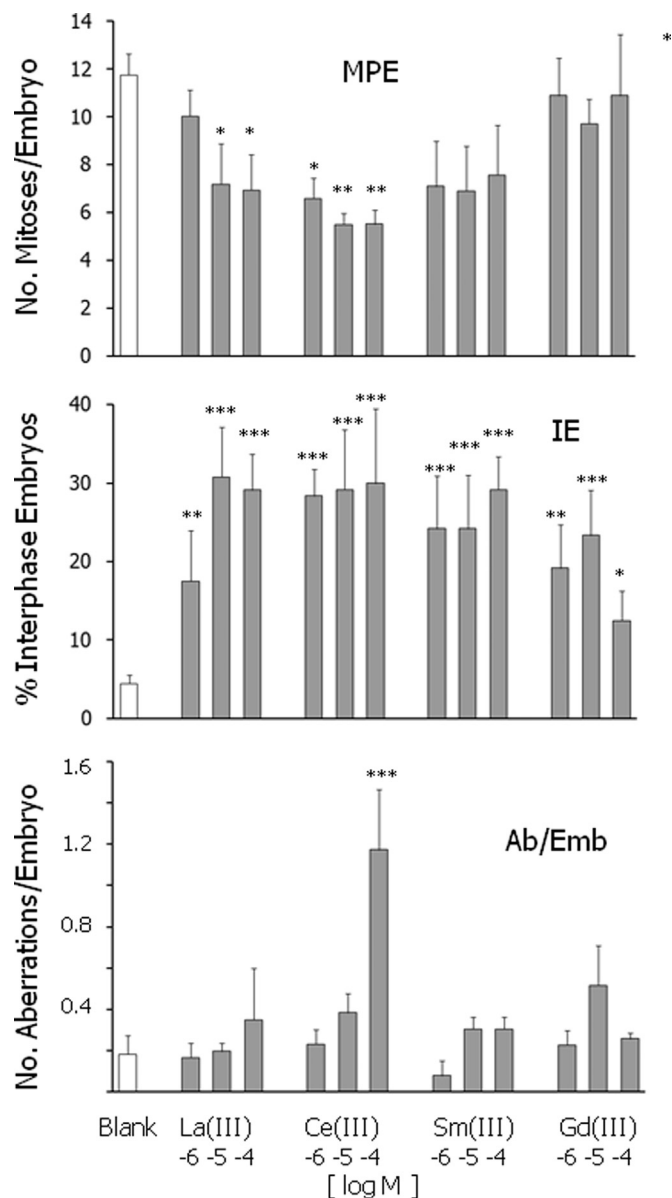
**Fig. 5.** Percent Developmental Defects in the offspring of REE-exposed sperm. Significant transmissible offspring damage was exerted by all tested REEs at the  $10^{-4}$  M level, and also by  $10^{-5}$  M La(III), Ce(III) and Sm(III).

and, hence, littoral benthic biota (Bustamante and Miramand, 2005), including echinoid communities.

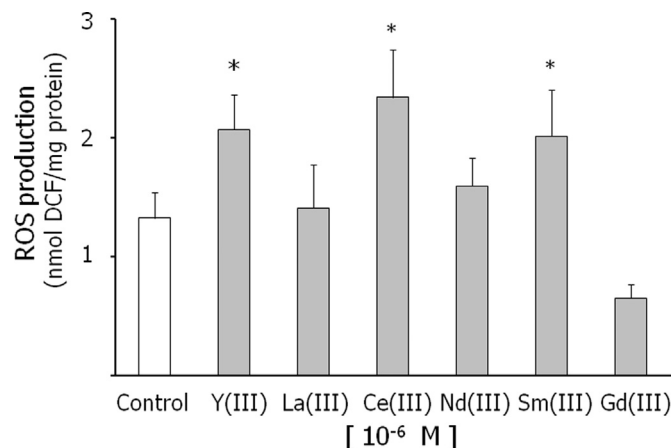
In order to generate a comprehensive toxicity dataset on some selected REEs in a species under controlled laboratory conditions, we tested their effects on *P. lividus* early life stages and measured a series of endpoints. These included fertilization and embryogenesis success, mitotic activity and aberrations, and some selected redox endpoints.

A toxicity ranking list was observed by exposing embryo/larval cultures to REEs. Strongest effects were noted for Gd(III), Y(III) and La(III) (Fig. 10) and included developmental defects, mitotic anomalies and redox endpoints.

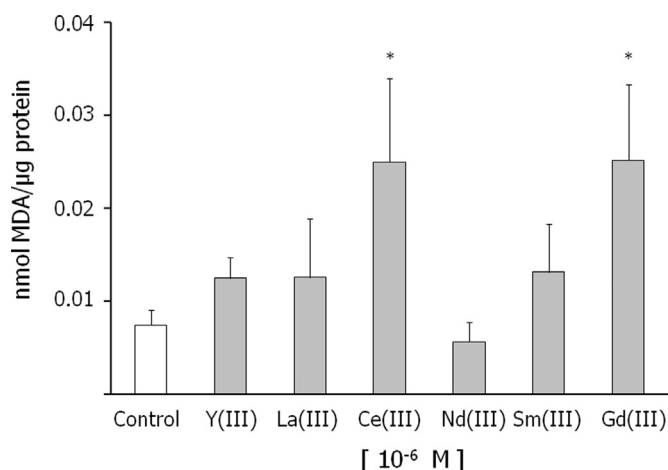
Sperm exposure to REEs resulted in an overall inhibition of fertilization success, especially after exposure to Eu(III). However, Ce(III) did not impact the same endpoints. Most interesting, larval developmental defects following sperm REE exposure led to transmissible offspring damage. This effect was broadly exerted by all tested REEs, with Y(III) and La(III) displaying the strongest effect. This finding may be attributed to induction of dominant lethals, an effect referred to since early genetics studies (Stancati, 1932; Bishop, 1937). In sea urchins, transmissible offspring damage following sperm exposures has been reported by a number of previous studies of several agents, including pharmaceuticals,



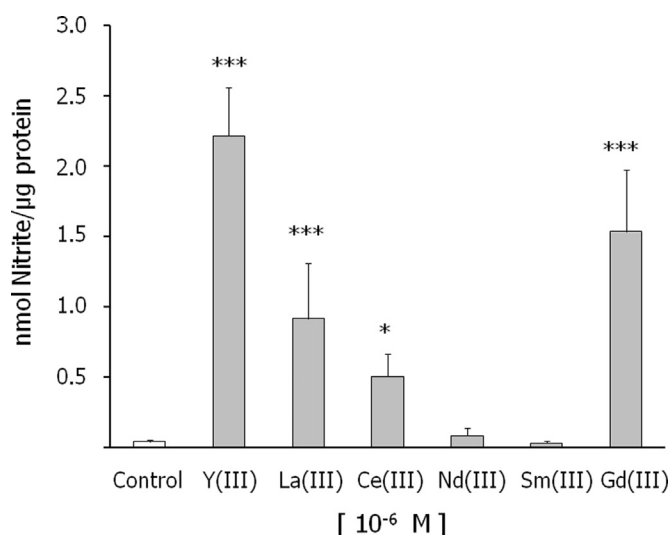
**Fig. 6.** Mitotoxic effects (decreased MPE, increased IE) were exerted in the offspring of La(III)- and Ce(III)-exposed sperm. Excess mitotic aberrations were only found in embryos generated by Ce(III)-exposed sperm.



**Fig. 7.** Formation of reactive oxygen species (ROS) in *P. lividus* embryos (5-h post-fertilization) exposed to  $10^{-6}$  M REEs. Significant excess of ROS formation was found in embryos exposed to Y(III), Ce(III) or Sm(III).



**Fig. 8.** Malondialdehyde (MDA) levels in *P. lividus* larvae (48-h post-fertilization) were significantly increased in  $10^{-6}$  M Ce(III)- and Gd(III)-exposed larvae.



**Fig. 9.** Nitrite (NO) levels in *P. lividus* larvae were significantly increased following exposures to  $10^{-6}$  M Y(III), Gd(III), La(III) and Ce(III).

agrochemicals, several inorganics, and complex mixtures, in an extensive body of literature to be reviewed elsewhere (Pagano et al., in preparation).

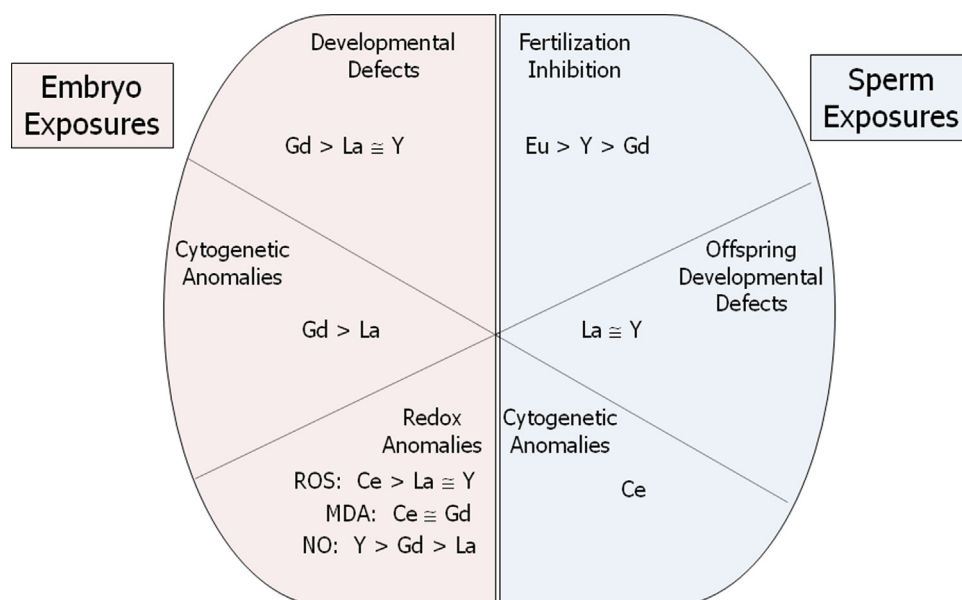
Cytogenetic analysis of REE-exposed embryos provided evidence for both inhibition of mitotic activity and increase of mitotic aberrations induced by La(III), Ce(III), Sm(III) and Gd(III). Unlike REE-exposed embryos, the offspring of REE-exposed sperm displayed mitotoxic effects, yet excess aberrations were only significantly increased in the offspring of Ce(III)-exposed sperm.

Beyond embryological and cytogenetic observations, this study provided evidence for REE-induced increases in oxidative and nitrosative stress, measured as ROS formation and levels of MDA and NO. We have previously reported modulation of redox endpoints in sea urchin embryogenesis following exposures to several xenobiotics (Korkina et al., 2000; Pagano et al., 2001a; Romano et al., 2011; Migliaccio et al., 2015). It is worth noting, however, that oxidative activity is physiologically associated to early embryogenesis, namely to cleavage stage. Thus an antioxidant as methionine resulted in developmental damage following early embryo exposures, while later embryonic (post-hatching) stages were unaffected (Pagano et al., 1997). This background information may suggest a possible explanation for the apparently paradoxical findings of Gd(III)-associated NO formation vs. MDA levels.

Along with the present report, a growing body of literature on REE-associated adverse effects in a number of models (Barry and Meehan, 2000; Huang et al., 2011; Jha and Singh, 1994;1995; Kawagoe et al., 2005; Lurling and Tolman, 2010; Ma et al., 2015; Preaubert et al., 2015; Wang et al., 2012; González et al., 2015) is providing long-awaited information on the comparative toxicity outcomes of exposure to individual REEs. This becomes even more important in a world that is increasingly mining these metals for use in modern technology, and the potential for environmental contamination after these products are discarded to landfills where leachates might start impacting freshwater and terrestrial systems.

## 5. Conclusion

Comparative toxicity testing was performed on a set of REEs in sea urchin early life stages focused on multiple endpoints. Some adverse effects were found across the set of tested REE, such as



**Fig. 10.** Synoptic outline of individual REE exerting highest effects according to the different treatments and endpoints evaluated in this study.

increases in developmental defects both in REE-exposed embryos/larvae and in the offspring of REE-exposed sperm. Moreover, inhibition of sperm fertilization success and of mitotic activity were observed, together with increases in redox endpoints. The results of this study indicate that REEs induce oxidative and nitrosative stress, cytogenetic anomalies and developmental defects in sea urchin embryos. Among tested REEs, Gd(III), Y(III), Ce(III) and La(III) displayed the most severe effects, whereas Nd(III) and Sm(III) resulted in relatively lesser toxicity in the tested endpoints.

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