

# Comparative toxicity of seven rare earth elements in sea urchin early life stages

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**Abstract** The widespread use of rare earth elements (REEs) in a number of technological applications raises unanswered questions related to REE-associated adverse effects. We have previously reported on the multiple impact of some REEs on the early life stages of the sea urchin *Paracentrotus lividus*. The present investigation was to evaluate REE toxicity to early life stages in two unrelated sea urchin species, *Sphaerechinus granularis* and *Arbacia lixula*. The comparative toxicities were tested of seven REEs, namely yttrium, lanthanum, cerium, neodymium, samarium, europium and gadolinium as chloride salts at concentrations ranging from  $10^{-7}$  to  $10^{-4}$  M. The evaluated endpoints included developmental defects and cytogenetic anomalies in REE-exposed embryos/larvae, and decreased fertilization success and offspring damage following sperm exposure. The results showed different toxicity patterns for individual REEs that varied according to test species and to treatment protocol, thus showing

toxicity scaling for the different REEs. Further, the observed effects were compared with those reported for *P. lividus* either following embryo or sperm exposures. *S. granularis* showed a significantly higher sensitivity both compared to *A. lixula* and to *P. lividus*. This study provides clear-cut evidence for distinct toxicity patterns among a series of REEs. The differences in species sensitivity at micromolar REE levels may warrant investigations on species susceptibility to impacts along polluted coasts.

**Keywords** Rare earth elements · Sea urchins · Developmental defects · Cytogenetic anomalies · *Sphaerechinus granularis* · *Arbacia lixula* · *Paracentrotus lividus*

## Introduction

There is growing environmental concern raised by the widespread use of REEs in present-day life due to their presence in a huge number of technological applications as reported previously (US Environmental Protection Agency 2012; Gambogi and Cordier 2013; Pagano et al. 2015a,b). This concern is highlighted by the growing number of publications in recent years on REE-associated toxicity to a number of test organisms and cell systems. It should be noted, however, that the vast majority of publications on REE toxicity is confined to four elements (Gd, Y, Ce, La) out of 17 REEs (reviewed by Pagano et al. 2015a,b).

As a consequence, many questions arise on the associated toxicity of relatively poorly studied REEs. This is especially important considering that some of these elements, e.g., Nd, have established industrial applications and are increasingly used in various industrial processes, indicating the potential for environmental health impacts (Feyerabend et al. 2010; Bleiwas and Gambogi 2013; Gambogi and

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Cordier 2013; Rim 2016). Some reports have investigated comparative toxicities for selected groups of REEs, by suggesting either similar or distinct effects of individual elements (Nakamura et al. 1997; Oral et al. 2010; Cui et al. 2012; González et al. 2015).

Recently, we have reported the effect of some REEs (incl. Y, La, Ce, Nd, Sm, Eu and Gd) on the sea urchin *Paracentrotus lividus* (Pagano et al. 2016). The sea urchin toxicity bioassay model has been utilized for several decades in order to evaluate a number of adverse events related to the marine environment, e.g., ocean acidification (Cipollaro et al. 1986; Lewis et al. 2016), as well as impacting on other, non-marine environments. The observed biological effects on sea urchin early life stages include embryonic and cell differentiation, cell division, fertilization success, transmissible damage from gametes to offspring, and a number of molecular endpoints including, for example, redox metabolism or DNA damage. Indeed, we have found that REEs affect embryogenesis, fertilization, cytogenetic and redox endpoints in *P. lividus* (Pagano et al. 2016).

In this study, we evaluated the effects of these seven REEs on early life stages of two taxonomically unrelated sea urchin species (*Sphaerechinus granularis* and *Arbacia lixula*) using similar experimental protocols under the same conditions. Moreover, the results in terms of embryotoxicity and transmissible damage from sperm to offspring have been compared with those reported for *P. lividus*, in order to rank the susceptibility of the different species to REE-induced harm.

## Materials and methods

### Chemicals

Trichloride REE salts were from SIGMA-Aldrich (Italy). Solutions of Y(III), La(III), Ce(III), Nd(III), Sm(III), Eu(III) and Gd(III) were diluted from a  $10^{-1}$  M stock solution stored at 4 °C at pH 3 (by HCl addition). In turn, stock solutions were diluted in HCl pH 3 up to the final test concentrations ( $10^{-4}$  to  $10^{-7}$  M) by 10% scalar dilutions. The correspondence of nominal vs. analytical concentrations was determined by ICP-MS analysis using an Aurora M90 Bruker instrument as described in our previous report (Pagano et al. 2016), confirming the correspondence between nominal and analytically determined concentrations. This correspondence was further confirmed in a later study of heavy REEs (Gravina et al. submitted for publication).

### Sea urchins

Sea urchins (*S. granularis*) were collected along the Rovinj coast (north Adriatic Sea, Croatia) by the staff of the Center

for Marine Research - Ruđer Bošković Institute, and *A. lixula* were collected by R. Oral along the Çeşme coast (Aegean Sea, Turkey). Gametes were obtained and embryos were reared as reported previously in Pagano et al. 2001. Embryo exposures to REE trichloride salts at concentrations in the order of  $10^{-7}$  to  $10^{-5}$  M started from zygote (10 min post-fertilization) up to the pluteus larval stage (72 h post-fertilization). Embryos were incubated in REE solutions or FSW at  $18 \pm 1$  °C in Corning™ Falcon™ Polystyrene Microplates (6 wells, 10 ml/well, code # 353046). Embryotoxicity assays were run with at least 3-replicate embryo cultures from different male and female lots. Control cultures were run in triplicate, of which one was “open”, tagged as “1” in order to check the suitability of the whole culture. The other two controls, as well as each treated culture, were tagged by random numbers, in order to achieve blind readings. The same criteria applied to the other treatment schedules, for cytogenetic analysis and for sperm pretreatment.

### Embryological analysis

Embryological analysis was performed on living plutei that were immobilized in  $10^{-4}$  M chromium sulfate for 10 min prior to observation, approx. 72 h after fertilization (Pagano et al. 2001). In each treatment schedule, the first 100 plutei were scored for the percentages of (1) normal larvae (N); (2) retarded larvae (size  $<1/2$  N); (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).

### Cytogenetic analysis

Cytogenetic analysis was carried out on 30 cleaving embryos from triplicate cultures of REE-exposed *S. granularis* embryos, and triplicate controls (each in triplicate cultures) amounting to a total of 9 control cultures. A novel pilot test was also performed on cleaving embryos generated by sperm exposed to three REEs [La(III), Sm(III) and Gd(III)].

*S. granularis* embryos were fixed in Carnoy's fluid (60% ethanol, 30% chloroform and 10% glacial acetic acid) 5 h after fertilization, and stained by 0.5% acetic carmine. Mitotoxicity endpoints included mean number of mitoses per embryo (MPE), and percent interphase embryos (IE). The induction of mitotic aberrations was evaluated by measuring the mean number of mitoses per embryo and percent embryos displaying  $\geq$  mitotic aberrations.

Cytogenetic analysis was not performed in *A. lixula* embryos due to poor transparency of blastomeres preventing proper observation of chromosomes.

### Sperm bioassays

A series of experiments was performed on both *S. granularis* and *A. lixula* sperm. A 50- $\mu$ l sperm pellet was suspended for 10 min (*S. granularis*) or 1 h (*A. lixula*) in 30 ml FSW containing REE salts at concentrations of  $10^{-5}$  to  $10^{-4}$  M. Thereafter, 50  $\mu$ l of REE-containing sperm suspension was used to inseminate 10 ml of untreated eggs (~50 eggs/ml). Thus, by a 200 $\times$  dilution of REE-containing sperm suspension, offspring embryos were reared at REE concentrations of  $5 \times 10^{-8}$  to  $5 \times 10^{-7}$  M.

Fertilization success was measured as percent fertilized eggs and also expressed as fertilization rate (FR) (out of 100 fertilized + unfertilized eggs) on live cleaving embryos within an interval of 1 to 3 h post-fertilization. Thereafter, the embryos generated by REE-treated sperm were cultured up to pluteus stage and scored for developmental defects (72 h post-fertilization) as described above in order to evaluate the effects, if any, of sperm exposure on offspring development.

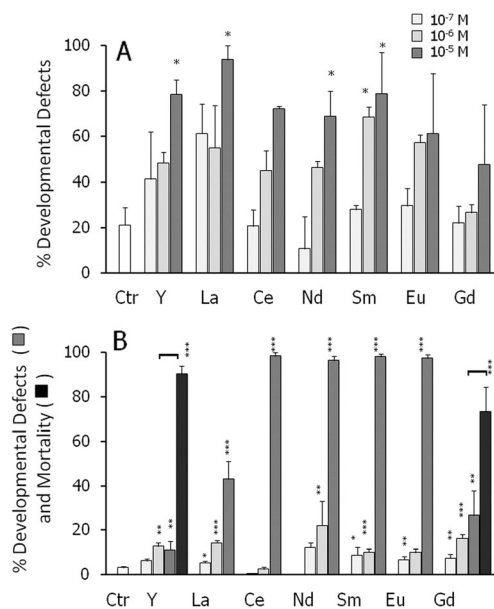
### Statistical analysis

Statistical analysis was carried out by using a GraphPad Prism software (GraphPad, San Diego, CA, USA). 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. Experimental data were fitted to a log-logistic model with variable slope. 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 and treatment groups were determined through an unpaired two-tailed Student's *t* test or with one-way Anova with Dunnett's multiple comparison test as a post hoc analysis. The variables that were unsuitable for a parametric approach (cytogenetic analysis) were evaluated with nonparametric tests:  $\chi^2$  test and Mann–Whitney *U* test. Differences were considered significant when  $p < 0.05$ . To carry out several simultaneous comparisons, Tukey's and Bonferroni's methods were used.

## Results

### Embryotoxicity tests

By rearing *S. granularis* embryos in REE chloride salts at levels ranging from  $10^{-7}$  to  $10^{-5}$  M, concentration-related increases in developmental defects (% DD) were observed, as shown in Fig. 1a. The highest DD values were significantly increased in *S. granularis* exposed to La(III), Y(III), Nd(III) and Sm(III), whereas Gd(III) resulted in the lowest DD values

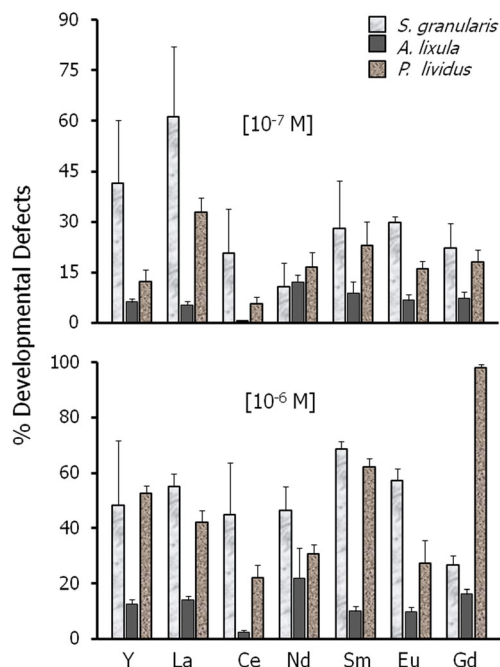


**Fig. 1** a Percent developmental defects (DD) in *S. granularis* embryos/larvae reared in REEs at concentrations ranging  $10^{-7}$  to  $10^{-5}$  M. \* $p < 0.05$ . b DD and % mortality in *A. lixula* embryos/larvae reared in REEs at concentrations ranging  $10^{-7}$  to  $10^{-5}$  M. This highest concentration showed, for Y(III) and Gd(III), 100% DD + mortality. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$

that were close to controls at Gd(III) levels up to  $10^{-6}$  M, with a non-statistically significant increase at the  $10^{-5}$  M Gd(III) level. No relevant mortality was detected in *S. granularis* larvae up to  $10^{-5}$  M REEs (mostly <1%), except for Y(III), La(III) and Ce(III) showing sporadic excess mortality in individual cultures (data not shown).

When *A. lixula* embryos were exposed to REEs in the same concentration range ( $10^{-7}$  to  $10^{-5}$  M), the DD values were significantly increased ( $p < 0.05$  to  $p < 0.01$ ) even at  $10^{-6}$  M REE concentrations, and resulted in 100% acute toxicity [DD (■) + mortality (■)] in larvae exposed to  $10^{-5}$  M Y(III) and Gd(III) (Fig. 1b).

The inter-species findings of sub-acute REE exposures ( $10^{-7}$  and  $10^{-6}$  M) of embryos from *S. granularis* and *A. lixula* were compared, together with the previously published results of *P. lividus* embryotoxicity assays at the same REE concentrations (Pagano et al. 2016). As shown in Fig. 2, *S. granularis* larvae almost invariably resulted in the highest DD rates, with the exception of  $10^{-6}$  M Gd(III), which showed the highest developmental toxicity in *P. lividus* larvae. A significantly higher toxicity in embryos of the three species was exerted by  $10^{-7}$  M La(III) and Y(III) and, in particular, *S. granularis* embryos displayed highest sensitivity vs. *P. lividus* and *A. lixula* embryos. *P. lividus* embryos exposed to  $10^{-6}$  M Gd(III) displayed highest sensitivity in contrasts with the relatively low sensitivity to the other tested REEs when compared to *S. granularis* embryos (Fig. 2 and Supplement # 1).



**Fig. 2** Comparisons among DD in embryos/larvae of *S. granularis*, *A. lixula* and *P. lividus* reared in  $10^{-7}$  and  $10^{-6}$  M REEs. Data from *P. lividus* are from Pagano et al. 2016, showing significantly higher toxicity for Gd(III)

As shown in Table 1,  $EC_{50}$  data showed the highest sensitivity of *S. granularis* embryos/larvae for most of tested REEs, with the exception of Gd(III) resulting in most severe effects in *P. lividus* embryos/larvae.

### Cytogenetic analysis

*S. granularis* embryos were exposed to REEs at concentrations ranging from  $10^{-6}$  to  $10^{-4}$  M and were evaluated for changes in mitotic activity and for frequencies of mitotic aberrations. Embryo exposure to REEs failed to increase mitotic aberrations (data not shown). However, a mitotoxic effect was found, in terms of % interphase embryos (i.e., failing to show any mitotic figures), as shown in Fig. 3, with concentration-related trends for 5 out of 7 tested REEs [La(III), Ce(III), Nd(III), Sm(III) and Gd(III)].

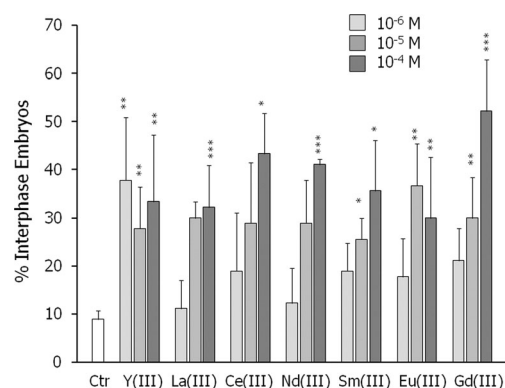
### Sperm pretreatment

Both exposures of *S. granularis* and of *A. lixula* sperm to REEs failed to result in any significant decrease of sperm fertilization success (data not shown). The offspring of REE-exposed sperm resulted in increased malformations in both species, as shown in Fig. 4. The offspring of REE-exposed ( $10^{-5}$  and  $10^{-4}$  M) *S. granularis* sperm was most severely affected by  $10^{-4}$  M La(III), Nd(III), Gd(III) and Eu(III) ( $t$  values = 5.9, 8.3 and 6.4 respectively with  $df = 22$ )

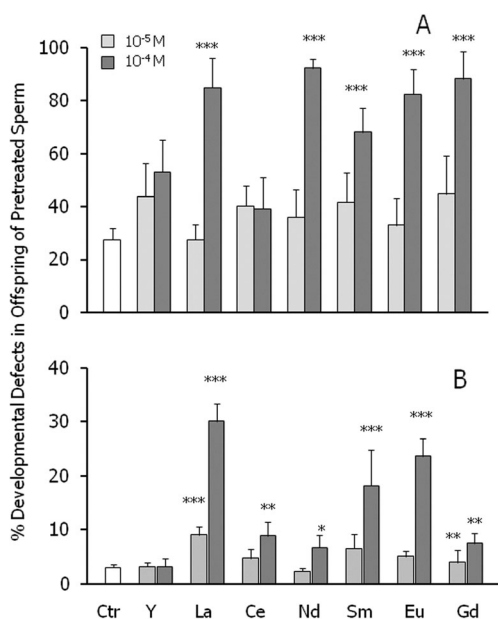
**Table 1** Comparative embryotoxicity data expressed as  $EC_{50}$  and related parameters in *S. granularis*, *A. lixula* and *P. lividus*

	$EC_{50}$	95% CI
Y (III)		
<i>S. granularis</i>	$4.7 \times 10^{-7}$	1.3–11.7 $10^{-7}$
<i>A. lixula</i>	$40 \times 10^{-7}$	28–100 $10^{-7}$
<i>P. lividus</i>	$7.9 \times 10^{-7}$	2.9–22.2 $10^{-7}$
La (III)		
<i>S. granularis</i>	$0.6 \times 10^{-7}$	0.01–2.9 $10^{-7}$
<i>A. lixula</i>	$155 \times 10^{-7}$	120–225 $10^{-7}$
<i>P. lividus</i>	$6.6 \times 10^{-7}$	0.97–45.44 $10^{-7}$
Ce (III)		
<i>S. granularis</i>	$13.3 \times 10^{-7}$	8.6–20.1 $10^{-7}$
<i>A. lixula</i>	$19 \times 10^{-7}$	17–50 $10^{-7}$
<i>P. lividus</i>	$53 \times 10^{-7}$	19–144 $10^{-7}$
Nd (III)		
<i>S. granularis</i>	$17 \times 10^{-7}$	8.9–32.8 $10^{-7}$
<i>A. lixula</i>	$15 \times 10^{-7}$	12–17 $10^{-7}$
<i>P. lividus</i>	$15.1 \times 10^{-7}$	4.5–52 $10^{-7}$
Sm (III)		
<i>S. granularis</i>	$4.1 \times 10^{-7}$	1.0–11 $10^{-7}$
<i>A. lixula</i>	$19.1 \times 10^{-7}$	17–21 $10^{-7}$
<i>P. lividus</i>	48.4	10 $10^{-7}$
Eu (III)		
<i>S. granularis</i>	$9.6 \times 10^{-7}$	3.8–21 $10^{-7}$
<i>A. lixula</i>	$14.7 \times 10^{-7}$	0–17 $10^{-7}$
<i>P. lividus</i>	$19.6 \times 10^{-7}$	6.7–56.8 $10^{-7}$
Gd (III)		
<i>S. granularis</i>	$55.6 \times 10^{-7}$	23–76 $10^{-7}$
<i>A. lixula</i>	$15.5 \times 10^{-7}$	13.4–17.6 $10^{-7}$
<i>P. lividus</i>	$1.97 \times 10^{-7}$	0.28–13.76 $10^{-7}$

( $p < 0.001$ ), whereas a non-significant increase in developmental defects (DD) was observed in the offspring of Ce(III)-exposed sperm (Fig. 4a). The offspring of REE-exposed ( $10^{-5}$



**Fig. 3** Mitotoxic effects in *S. granularis* embryos as % interphase embryos, lacking active mitoses



**Fig. 4** a A and B (capital) Offspring damage as DD following *S. granularis* sperm exposure to REEs, 10<sup>-5</sup> to 10<sup>-4</sup> M. b Offspring damage as DD following *A. lixula* sperm exposure to REEs, 10<sup>-5</sup> to 10<sup>-4</sup> M

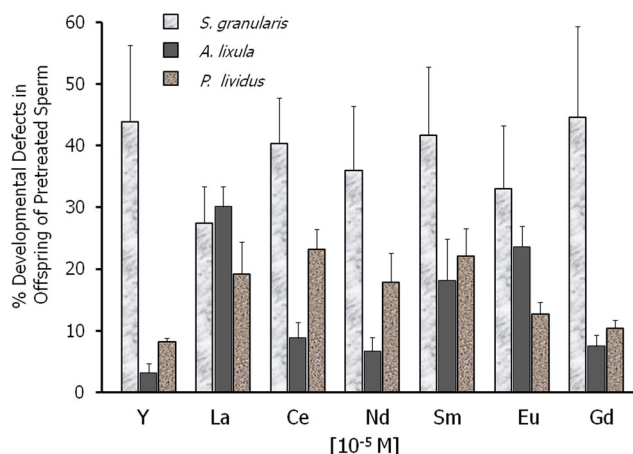
and 10<sup>-4</sup> M) *A. lixula* sperm resulted in significantly increased DD following sperm exposures to La(III), Sm(III) and Eu(III), whereas lesser or non-significant offspring damage was observed following sperm exposures to Y(III), Ce(III), Nd(III) and Gd(III) (Fig. 4b).

A pilot test was carried out by measuring cytogenetic abnormalities in embryos generated by sperm exposed to three REEs [La(III), Sm(III) and Gd(III)] at concentrations ranging from 10<sup>-6</sup> to 10<sup>-4</sup> M. Again, no increase in mitotic aberrations was detected, while a significant mitotoxic effect was observed as percent Interphase Embryos (lacking active mitoses), as shown in Supplement # 2.

By comparing the offspring damage observed in the three sea urchin species, again *S. granularis* showed significantly higher sensitivity both compared to *A. lixula* and to *P. lividus* (Pagano et al. 2016). The offspring of *A. lixula* sperm showed the lowest sensitivity, with the exception for the offspring of La(III)-exposed sperm that showed overlapping—and higher—sensitivity for *A. lixula* and *S. granularis* compared to *P. lividus* (Fig. 5).

By comparing the different offspring damage effects ranking of the seven tested REEs across the three sea urchin species, highest sensitivity of *S. granularis* to almost all of tested REEs was observed at the 10<sup>-5</sup> M concentration, except for La(III) which resulted in comparable effects in *S. granularis* and *A. lixula* (see Supplement # 3).

By summarizing the findings of embryotoxicity and offspring damage following REE embryo or sperm exposures, Table 2 shows the highest sensitivity of *S. granularis* embryos and sperm compared to the other two sea urchin species.



**Fig. 5** Comparisons among DD in offspring embryos/larvae of the three sea urchin species, following sperm exposure to REEs, 10<sup>-5</sup> M. Data from *P. lividus* are from Pagano et al. 2016

**Discussion**

The available literature is focused on the toxicity of four REEs (Gd, Y, La and Ce), while the relative scarcity of reports on the other REEs represents a challenge to the investigations on REE-associated toxicity. By considering that several REEs are not actually “rare” in terms of geological occurrence, mining and subsequent technological applications, the task of acquiring suitable toxicological information becomes a mandatory challenge. This must be accomplished by appropriate comparative toxicity investigations across several test models, environmental health assessments and broadly unexplored epidemiological investigations (Snow et al. 2014; Liu et al. 2015; Rim 2016; Li et al. 2016; Wang et al. 2016).

In the framework of toxicity testing systems, sea urchin bioassays have a unique role in characterizing a number of endpoints that are relevant across various taxa, involving a number of key biological events such as cell division and differentiation, genetic damage and redox endpoints. Thus, sea urchin bioassays may provide useful hints and contributions in the overall evaluations of potential modes of action of exposures to various xenobiotics across more than just marine species. This statement relies on an extensive database of

**Table 2** Highest effects scores as developmental defects induced by individual REEs in the three sea urchin species either following embryo exposures or in the offspring of REE-exposed sperm [from Figs. 2, 4, and 5 (*A. lixula* and *S. granularis*), and from Pagano et al., 2016 (*P. lividus*)]

	Embryotoxicity	Offspring damage
<i>A. lixula</i>	Y ≅ Gd	La > Eu > Sm
<i>S. granularis</i>	La > Y > Sm	La ≅ Nd ≅ Gd
<i>P. lividus</i>	Gd > La ≅ Y	La ≅ Y

literature on the use of sea urchin bioassays gathered over several decades and focused on inorganics, organics, pharmaceuticals and complex mixtures (reviewed by Pagano et al. 2017a,b). Thus, the present study provides a dataset which should not be regarded as limited to sea urchins and marine organisms, as far as it provides information on a set of key events such as cell division and differentiation, and transmissible (genetic) effects.

This study attempted to evaluate the effects on sea urchin early life stages of seven REEs in terms of developmental defects in REE-exposed pluteus larvae or in the offspring of REE-exposed sperm. Previous studies reported on the effects of several REEs in plants or in cell systems, in terms of bioaccumulation (Carpenter et al. 2015; Huang et al. 2011), yet without clear-cut distinctions in-between the behaviors of different REEs and their modes of action. In this context, suitable procedures to obtain reliable and comparable data for individual REEs were recommended by González et al. (2014,2015), and ad hoc studies are warranted both in toxicity bioassays and in mammalian studies.

We found that some of the tested REEs affected embryogenesis in embryos exposed to  $10^{-6}$  M (Fig. 2). Micromolar REE concentrations may be regarded as realistic in REE-polluted marine sediment (Bustamante and Miramand 2005), or in other REE-containing complex mixtures as, e.g., bauxite residues (Karadağ et al. 2009; Wang et al. 2010) or fly ash from coal combustion (Franus et al. 2015; Taggart et al. 2016). Thus, ad hoc investigations on REE toxicity in other test models may be timely. Our findings showed that individual REEs give rise to different toxicity patterns displaying a clear toxicity gradient with, e.g., La(III) displaying the highest toxicity to sea urchin developing embryos among the tested REEs.

Sperm exposure to REEs, though compatible with fertilization success, resulted in offspring damage expressed through increased developmental defects and embryonic/larval mortality. Again, distinct effects were noted among the tested REEs, and distinct effects in the three species (Fig. 5). Altogether, these results support the notion that a generalization of REE-induced adverse effects would not be appropriate, and should prompt ad hoc investigations on distinct REE toxicities in other bioassay models.

A noteworthy finding of the present study relates to the different REE sensitivities of the three sea urchin species. *S. granularis* displayed significantly higher sensitivity compared to *A. lixula* and *P. lividus*, both following embryo and sperm exposures. Burić et al. (2015) tested the effects of silver nanoparticles on fertilization and early development of the same sea urchin species, and found *A. lixula* to be the most sensitive species to silver nanoparticles. Other studies reported on different echinoid species sensitivities, by testing salinity as a confounding factor, or antibiotics and disinfectants associated with fish farming (Carballeira et al. 2011,2012). A recent report by Martino et al. (2016) found different species

sensitivities to Gd(III) in four unrelated sea urchin species. Our results corroborate their study as we have shown variable sensitivities of exposure to  $10^{-6}$  M Gd(III) across our three tested species. The greatest Gd(III)-induced effect was noted for *P. lividus* embryo exposure (Fig. 2), while displaying minor, non-significant effects on *S. granularis* and *A. lixula* embryos. This result was in contrast with the findings of the other REEs, where *S. granularis* and *A. lixula* appeared more sensitive than *P. lividus*. This paradoxical finding deserves further investigation.

Beyond the present REE-focused study, it may be noted that the observed enhanced sensitivity of *S. granularis* to REEs might be an indication of a more general sensitivity of this species to environmental pollution. Indeed, since 2015 and to date, a scanty occurrence of *S. granularis* has been unexpectedly observed in the Bay of Naples as this species had been present—and utilized in a number of our studies—for several decades (from Pagano et al. 1983 to De Nicola et al. 2007). This observation might warrant studies at the community level.

## Conclusion

The tested REEs induced multiple damage to sea urchin early life stages, including developmental defects in REE-exposed embryos and in the offspring of REE-exposed sperm, moreover by inhibiting cell division in cleaving embryos. The effects pointed to different toxicities of individual tested REEs, and to different sensitivities of three sea urchin species.

This study corroborates previous evidence for distinct toxicity patterns of seven light REEs in sea urchin early development, by prompting studies in other bioassay models.

Differing species sensitivity to REE-induced effects was found confirming previous reports, and pointing to the need to extrapolate these findings to ecosystem health and integrity while considering species fitness in areas with apparently minor, yet effective environmental pollution, warranting ad hoc investigations.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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