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Heavy Rare Earth Elements Affect *Sphaerechinus granularis* Sea Urchin Early Life Stages by Multiple Toxicity Endpoints

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Abstract
Heavy rare earth elements (HREEs) were tested for adverse effects to early life stages of the sea urchin *Sphaerechinus granularis*. Embryos were exposed to analytically measured HREE concentrations ranging from 10^{-7} to 10^{-5} M. No significant developmental defect (DD) increases were observed in embryos exposed to 10^{-7} M HREEs, whereas 10^{-5} M HREEs resulted in significant DD increase up to 96% for $HoCl_3$ versus 14% in controls. Embryos exposed to 10^{-6} M HREEs showed the highest DD frequency in embryos exposed to 10^{-6} M $DyCl_3$ and $HoCl_3$. Cytogenetic analysis of HREE-exposed embryos revealed a significant decrease in mitotic activity, with increased mitotic aberrations. When *S. granularis* sperm were exposed to HREEs, the offspring of sperm exposed to 10^{-5} M $GdCl_3$ and $LuCl_3$ showed significant DD increases. The results warrant investigations on HREEs in other test systems, and on REE-containing complex mixtures.

Keywords Heavy rare earth elements · Sea urchins · Embryotoxicity · Cytogenetic damage, offspring damage

The extensive applications of REEs in a number of technologies have made these elements indispensable for daily life (Gambogi and Cordier 2013; EU-OSHA 2013). The potential REE-associated toxicological impacts on environmental health have received considerable attention in recent years (Carpenter et al. 2015; Pagano et al. 2015, 2016; Martino et al. 2017a, b, 2018; Trifuoggi et al. 2017). However, the current literature on REE toxicity is mostly focused on a few light REEs (LREEs such as Y, La, Ce), and on Gd, whereas a very limited body of literature has focused on the adverse effects of heavy REEs (HREEs such as Dy to Lu) (Pagano et al. 2016; Oral et al. 2017). Most studies primarily focused on one or two HREEs (Saitoh et al. 2010; Zhang et al. 2011; Cui et al. 2012; Gao et al. 2015; Vukov et al. 2016), thus preventing a broader comparative evaluation of HREE-associated toxicities.

Heavy rare earth elements are broadly unexplored in environmental toxicology, warranting ad hoc investigations in view of their growing applications in a number of advanced technologies. To date, no published data are available on environmental HREE levels, to the best of our knowledge. Among the extensive applications of HREEs, the production of supermagnetic alloys for either wind turbines or for hybrid vehicle engines raises the attention and demand of two HREEs, namely Dy and Ho. These elements exhibit the highest supermagnetic properties (reviewed by Jensen and Mackintosh 1991) and are most often used in such technological applications. Their environmental occurrence is, however, expected to be minimal or possibly confined to manufacture/use by-products.

Providing comparative toxicity data on HREEs is of priority for monitoring and regulatory programs. This information could help further our understanding of the toxicity mechanisms of these elements which could in turn help in informing mitigation actions aimed at minimizing any adverse health outcomes from HREE exposures.

We recently reported on HREE-associated toxicity in two sea urchin species, *Arbacia lixula* and *Paracentrotus*

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lividus. Endpoints such as embryogenesis, fertilization success, cytogenetic anomalies and redox potential were useful in demonstrating different toxicities across a suite of tested HREEs (Oral et al. 2017). This present follow-up study aims at evaluating the comparative toxicities of six HREEs in the early life stages of another sea urchin species, *Sphaerechinus granularis*, that historically displays higher sensitivity to LREEs (Trifuoggi et al. 2017). Our study will help determine whether or not the differing toxicities from exposure to HREEs and LREEs are species-dependent in echinoids. There are a number of important reasons why the toxicity testing of HREE should be extended to species of echinoids in different families, such as the urchin *S. granularis* versus *P. lividus* and *A. lixula*. Firstly, it has already been shown that different urchin species exhibit a range of sensitivities to toxic materials and oftentimes commonly used test species are actually less sensitive and more robust than other less frequently used species (Trifuoggi et al. 2017; Martino et al. 2017a). Secondly, *S. granularis* lives further offshore and deeper than other species such as *P. lividus* and *A. lixula*. This is extremely relevant as the action of near-shore waves, currents and deposition processes may result in accumulation of toxic material further offshore where species different from those living near-shore may be present. It is therefore extremely pertinent to determine the response to toxicants across a number of species to both establish the level of sensitivity and in terms of environmental relevance to establish if some species may be more at risk based on a combination of their sensitivity and spatial distribution. Altogether, the present report is to complete our comparative toxicity data on HREEs and LREEs across three taxonomically distant echinoid species characterized by different habitats and by different sensitivities to xenobiotics.

Materials and Methods

Trichloride HREE salts were purchased from SIGMA-Aldrich (Italy). Solutions of Dy(III), Ho(III), Er(III), Yb(III), Lu(III), and of Ce(III) were diluted from a 1 M stock solution stored at + 4°C at pH 3 (by HCl addition). These stock solutions were diluted in HCl pH 3 up to the final test concentrations (10^{-4}–10^{-7} M) using filtered seawater (FSW) and 10% scalar dilutions. The FSW was collected offshore Rovinj (North Adriatic Sea, Croatia, 45°04′47″N, 13°38′24″E); salinity 38.1 ± 0.1‰, pH 8.1 ± 0.1.

Nominal versus analytical concentrations were determined by ICP-MS (Aurora Bruker M90, Bremen, Germany) following previously established protocols and QA/QC lab procedures method specific optimizations (including flow parameters, torch alignment, and ion optics) were carried out to maximize the counts per second (cps) of low concentration REE solutions. All samples and standards used were diluted in 2% nitric acid. Analyses were carried out in triplicate.

Sea urchins (*S. granularis*) were collected along the Rovinj coast by the staff of the Center for Marine Research – Ruđer Bošković Institute (Rovinj, Croatia). Gametes were obtained, and embryos were reared as reported previously (Pagano et al. 2001). Controls consisted of embryos reared in natural FSW run as triplicate cultures tagged by random numbers allowing for blind readings. *S. granularis* embryos were reared in trichloride salt solutions of Dy(III), Ho(III), Er(III), Yb(III), Lu(III), and of Ce(III) as a LREE reference at concentrations ranging from 10^{-7} to 10^{-5} M starting 10 min post-fertilization up to the pluteus larval stage, evaluated for developmental defects 72 h post-fertilization. Embryos were incubated in FSW at 18 ± 1°C in Falcon™ Tissue Culture Plates (6 wells, 10 mL/well, code # 353046). Experiments were run with 4–6 replicates.

Plutei were immobilized in 10^{-4} M chromium sulfate 10 min prior to observation (Pagano et al. 1983). The first 100 plutei were scored for the percentages of: (1) normal larvae (N); (2) delayed larvae; (3) malformed larvae (P1); (4) abnormal blastulae or gastrulae (P2), and (5) dead embryos or larvae. Total developmental defects (DD) were scored as (P1 + P2).

Cytogenetic analysis was carried out on 30 cleaving *S. granularis* embryos from four cultures in each treatment schedule following embryo exposure, and triplicate controls. The embryos were fixed in Carnoy's fluid 5 h after fertilization, and stained by acetic carmine. The cytogenetic endpoints both included mitotic activity and mitotic aberrations (Pagano et al. 2001).

A series of experiments were performed on *S. granularis* sperm, by suspending a 50-µL sperm pellet for 10 min in 30 mL FSW containing HREE salts or $CeCl_3$, at concentrations ranging from 10^{-5} to 10^{-4} M; thereafter, 50-µL of sperm suspension were used to inseminate 10 mL of untreated eggs (~ 50 eggs/mL). Fertilization success was measured as % fertilized eggs or fertilization rate (FR) on live cleaving embryos. Thereafter, the embryos/larvae were scored for DD in order to evaluate the effects, if any, of sperm exposure on offspring development.

Results are given as mean ± standard error, or with 95% confidence interval (CI). The half maximal effective concentrations (EC_{50}) were calculated by using a non-linear regression analysis with Cis and the GraphPad Prism software. Statistical assumptions were verified at the onset of each analysis, and a square-root data transformation was applied when underlying statistical assumptions were violated. Differences between groups were determined by two-tailed Student's *t* test or with One-way ANOVAs with Dunnett's multiple comparison test. The variables that were unsuitable for parametric statistics were evaluated with nonparametric

tests including χ^2 test and Mann–Whitney U test. Differences were considered significant when $p < 0.05$.

Results and Discussion

The correspondence between nominal and analytical concentrations of tested salts in FSW, at nominal levels of 10^{-6} and 10^{-5} M, showed that the analytical/nominal concentration ratios ranged from 0.6 to 1.4 [for 10^{-6} M Ce(III) and for 10^{-5} M Dy(III), Er(III) and Lu(III)]; otherwise the analytical/nominal concentration ratios ranged from 0.7 to 1.2, thus with close levels in the order of magnitude (Table 1). Thus, nominal concentration values were considered reliable for concentration-related trends. The time-related concentration decrease of HREEs in FSW solutions, prepared in test culture plates, was measured within 48 h. As shown in Fig. 1, initial concentrations of 10^{-6} and 10^{-5} M HREE solutions in FSW (time 0) were not significantly decreased after 24 h, and only showed a significant decrease ($p < 0.05$) after 48 h. Thus, the test HREE concentrations could be considered stable within 24 h post-fertilization, i.e. from zygote to gastrula stage, both encompassing early embryogenesis and post-hatching differentiation.

The larvae exposed to 10^{-7} M HREEs or Ce(III) failed to show any significant increase in developmental defects (DD) versus controls, as shown in Fig. 2. Exposure to 10^{-5} M HREEs resulted in ≈70% DD [Gd(III) and Lu(III)] to ≈85%–95% DD [Ho(III), Er(III) and Yb(III)] while lesser, though significant, DD increase was observed in larvae reared in 10^{-5} M Ce(III) or Lu(III) (48%–66% DD vs. 14% ± 2% DD in controls; $p < 0.05$). When *S. granularis* embryos were exposed to 10^{-6} M HREEs [including Gd(III), Dy(III), Ho(III), Er(III), and Yb(III)], DD were significantly increased ($p < 0.001$), whereas no significant DD increase was detected in embryos/larvae exposed to Lu(III) or Ce(III) (Figs. 2, 3). Altogether, the results of embryotoxicity experiments were evaluated in terms of EC_{50} for individual HREEs, as shown in Table 2. These EC_{50} values were successfully calculated at micromolar or submicromolar levels in *S. granularis* embryos.

The effects of HREEs and Ce(III) (10^{-5} and 10^{-4} M) were tested on cleaving *S. granularis* embryos (fixed 5 h post-fertilization) by evaluating the inhibition of mitotic activity

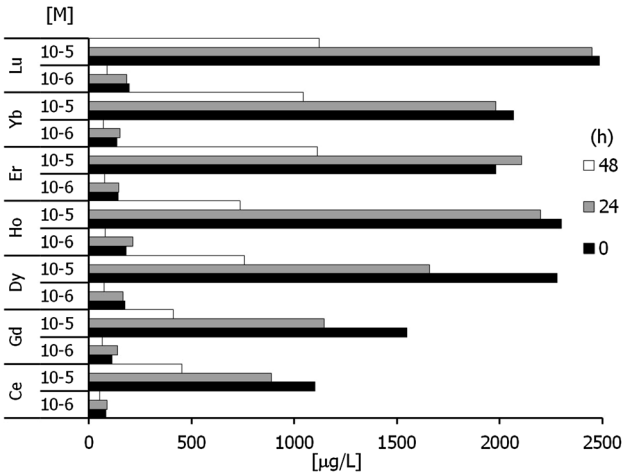

Fig. 1 Time-related decrease of HREE (or Ce) concentrations in culture plates from time 0 and within 24–48 h after dissolution in filtered seawater. Initial nominal concentrations were 10^{-6} and 10^{-5} M; analytical data are expressed as µg/L

Table 1 Correspondence of nominal versus ICP-MS measured analytical HREE concentrations in filtered seawater in the conditions used in the study

HREEs	Nominal (M)	Measured analytical concentration (µg/L)	Analytical/nominal (M)
Ce	10^{-6}	83	0.6×10^{-6}
	10^{-5}	1102	0.8×10^{-5}
Gd	10^{-6}	112	0.7×10^{-6}
	10^{-5}	1547	0.9×10^{-5}
Dy	10^{-6}	176	1.1×10^{-6}
	10^{-5}	2279	1.4×10^{-5}
Ho	10^{-6}	179	1.1×10^{-6}
	10^{-5}	2299	1.4×10^{-5}
Er	10^{-6}	141	0.8×10^{-6}
	10^{-5}	1981	1.2×10^{-5}
Yb	10^{-6}	136	0.8×10^{-6}
	10^{-5}	2069	1.2×10^{-5}
Lu	10^{-6}	196	1.1×10^{-6}
	10^{-5}	2485	1.4×10^{-5}

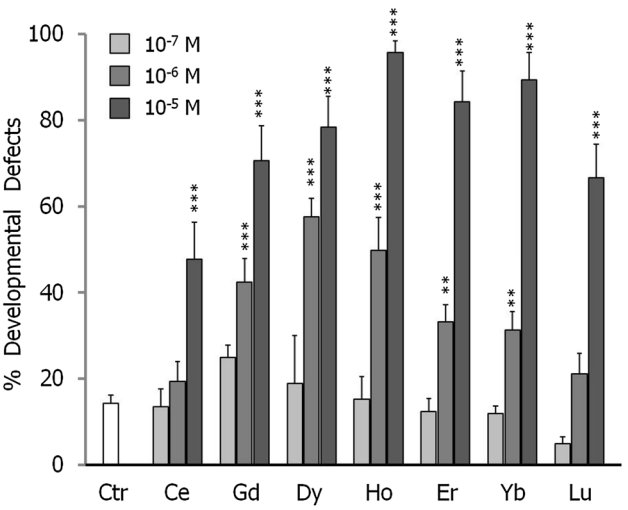

Fig. 2 Concentration-related increase of developmental defects (DD) in *S. granularis* Larvae exposed to HREEs or Ce(III) at levels ranging from 10^{-7} to 10^{-5} M. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

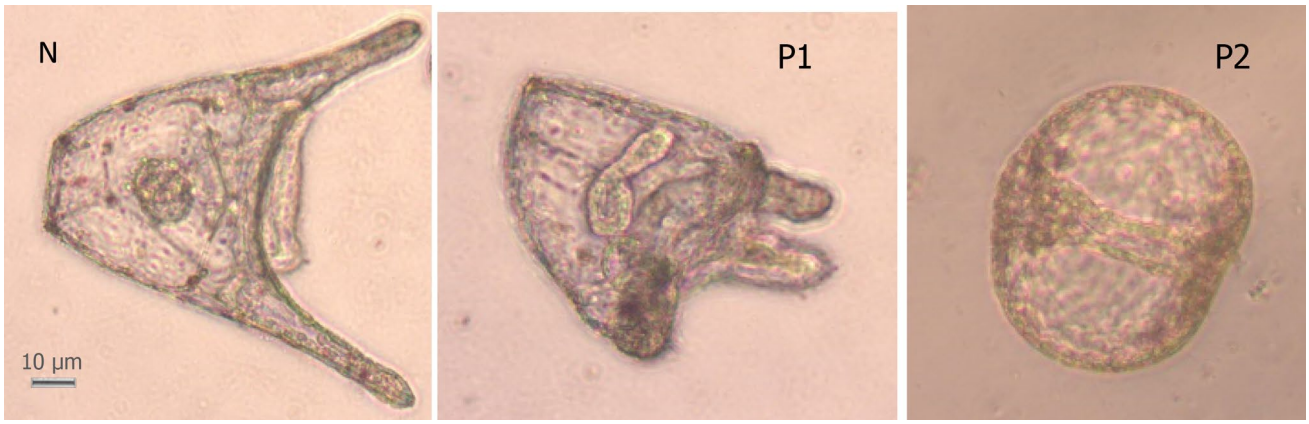

Fig. 3 *Sphaerechinus granularis* larvae were scored for normal (*N*) differentiation, or for larval malformations (*P1*), or developmental arrest at pre-larval stages (*P2*)

Table 2 Embryotoxicity data of individual HREEs showed the half maximal effective concentrations (EC_{50}) and confidence intervals (CIs) that were in micromolar levels

	EC_{50} (M)	95% CI
Ce(III)	13.0×10^{-6}	$5.5–169\times10^{-6}$
Gd(III)	1.6×10^{-6}	$0.7–3.8\times10^{-6}$
Dy(III)	0.7×10^{-6}	$0.2–1.4\times10^{-6}$
Ho(III)	0.9×10^{-6}	$0.6–1.4\times10^{-6}$
Er(III)	1.9×10^{-6}	$1.2–2.9\times10^{-6}$
Yb(III)	1.8×10^{-6}	$1.3–2.7\times10^{-6}$
Lu (III)	4.5×10^{-6}	$27.5–75.7\times10^{-6}$

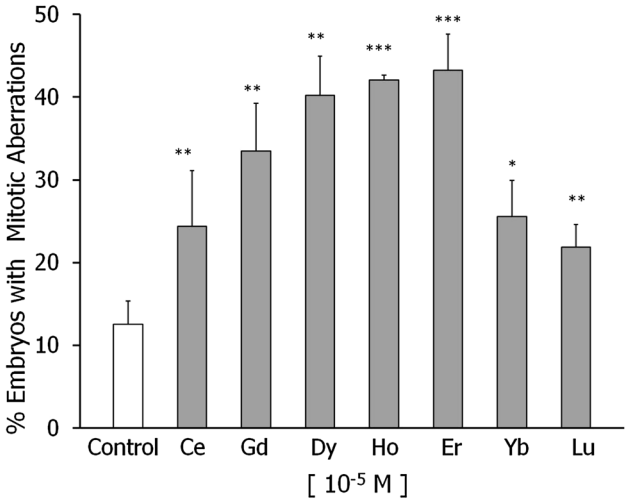

Fig. 4 Induction of mitotic aberrations as % embryos with ≥ 1 mitotic aberrations [E(Ab+)] in *S. granularis* embryos exposed to 10^{-5} M HREEs or Ce(III)

(expressed as percent interphase embryos, IE), and the induction of mitotic abnormalities per embryo (Ab+/Emb). Embryos exposed to Ho(III), Er(III), Yb(III) or Lu(III) at the 10^{-4} M concentration resulted in a significant inhibition of mitotic activity, expressed as IE ($p<0.05$), without significant IE increase following exposures to Ce(III), Gd(III) or Dy(III) (data not shown). The frequencies of mitotic aberrations were significantly increased in embryos exposed to 10^{-5} M HREEs, with most significant effects induced by 10^{-5} M Ho(III) and Er(III) ($p<0.001$) and lesser, though significant effects ($p<0.01–0.05$) in embryos exposed to 10^{-5} M of the other salts (Fig. 4). The same trend in mitotic aberrations was observed in embryos exposed to 10^{-4} M HREEs or Ce(III) (data not shown).

When *S. granularis* sperm were suspended for 10 min in HREEs or Ce(III) at concentrations ranging from 10^{-7} to 10^{-5} M, fertilization success failed to display any significant changes at 10^{-7} and 10^{-6} M concentrations. Sperm exposed to 10^{-5} M HREEs or Ce(III) underwent a dramatic decrease of fertilization success that was most severe for Ce(III), Gd(III) and Dy(III) ($p<0.001$ and $p<0.01$) and slighter, though significant for Yb(III) ($p<0.05$) (data not shown). Following sperm exposures to 10^{-5} M HREEs or Ce(III), significant DD increase was observed in the offspring of sperm exposed to Ce(III), Gd(III) ($p<0.001$), and Lu(III) ($p<0.05$), without significant effects in the offspring of sperm exposed to the other HREEs (Fig. 5).

Considering that HREEs are increasingly used in a wide range of technological applications and industries such as, for example, supermagnetic alloys, there is increasing urgency in generating suitable toxicological information in order to properly understand and model the impacts of exposure to HREEs (Gambogi and Cordier 2013). This could be accomplished through comparative toxicity investigations across several test models to form a baseline from which more comprehensive environmental health assessments may be established.

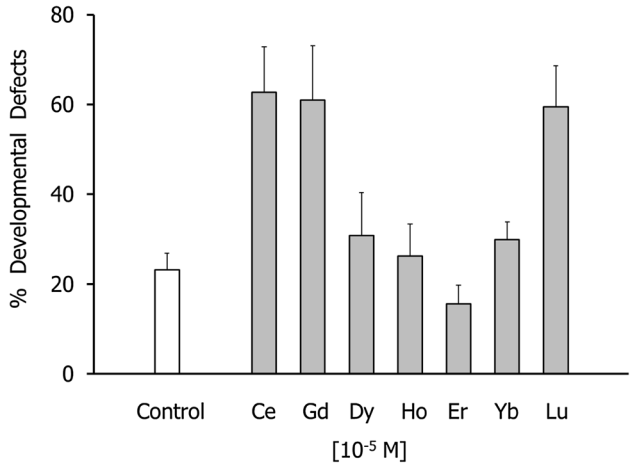

Fig. 5 Increased offspring damage [as % developmental defects (DD)] following *S. granularis* sperm exposure to 10^{-5} M HREEs or Ce(III)

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 key biological events such as cell division and differentiation, genetic damage and redox endpoints (Sea Urchin Genome Sequencing Consortium et al. 2006). Thus, sea urchin bioassays provide useful information on the effects of various xenobiotics including inorganics, organics, pharmaceuticals and complex mixtures (Burić et al. 2015; reviewed by Pagano et al. 2017).

The present study has focused on the effects of six HREEs on *S. granularis* embryos and sperm under controlled laboratory and exposure conditions (Table 1), with time-related concentration decay overlapping with the critical time up to the gastrula stage of development, or approximately 24 h post-fertilization. The tested HREEs resulted in significant concentration-related damage to embryogenesis, and the most severe effects were found for Dy(III) and Ho(III) (Fig. 2). Cytogenetic analysis of HREE-exposed *S. granularis* embryos revealed the induction of mitotic aberrations to different extents by all tested HREEs and by Ce(III). The highest aberration frequencies were induced by 10^{-6} M Dy(III), Ho(III), Gd(III) and Er(III) (Fig. 4), in agreement with previous observations in *P. lividus* and *A. lixula* embryos (Oral et al. 2017).

Sperm exposure to HREEs or to Ce(III) resulted in an overall inhibition of fertilization success, and offspring damage was increased following sperm exposures to Ce(III) and Gd(III) (Fig. 5). Thus, multi-faceted effects have been found for HREEs ranging from genotoxicity to teratogenic impacts.

This study corroborates previous evidence for distinct toxicity patterns of six HREEs in early development of *P. lividus* and *A. lixula*, thus showing close toxicity trends across a range of echinoids species, of different families, by comparing the effects of individual elements. The results reported herein conclude a series of investigations conducted on light and heavy REEs, supporting: (1) the correspondence of nominal and analytical REE levels tested in bioassays and (2) the different toxicities exerted by different REEs (Pagano et al. 2016; Oral et al. 2017; Trifuoggi et al. 2017). The overall results of this series of studies in the sea urchin test system, focused on REE-associated toxicity testify the need for comparative investigations utilizing a set of taxonomically distant species characterized by different ecological features, as in the case of *S. granularis* versus *P. lividus* and *A. lixula*.

In prospect, the present findings should prompt studies in other bioassay models to form a complete picture of REE toxicity aimed at establishing exposure guidelines, and in boosting their applications in broader technologies or industrial processes.

Compliance with Ethical Standards

Conflict of interest The authors have no conflict of interest in the present study.

References

Burić P, Jakšić Ž, Štajner L, Dutour Sikirić M, Jurašin D, Cascio C, Calzolai L, Lyons DM (2015) Effect of silver nanoparticles on Mediterranean sea urchin embryonal development is species specific and depends on moment of first exposure. Mar Environ Res 111:50–59

Carpenter D, Boutin C, Allison JE, Parsons JL, Ellis DM (2015) Uptake and effects of six rare earth elements (REEs) on selected native and crop species growing in contaminated soils. PLoS ONE 10:e0129936

Cui J, Zhang Z, Bai W, Zhang L, He X, Ma Y, Liu Y, Chai Z (2012) Effects of rare earth elements La and Yb on the morphological and functional development of zebrafish embryos. J Environ Sci (China) 24:209–213

EU-OSHA (2013) Priorities for occupational safety and health research in Europe: (2013–2020). European Agency for Safety and Health at Work. ISBN: 978-92-9240-068-2

Gambogi J, Cordier DJ (2013) Rare earths. In: Metals and minerals. U.S. Geological Survey. http://pubs.usgs.gov/of/2013/1072/OFR2013-1072

Gao JL, Wu M, Liu W, Feng ZJ, Zhang YZ, Jiang FL, Liu Y, Dai J (2015) Dysfunction of rice mitochondrial membrane induced by Yb^{3+}. J Membr Biol 248:1159–1165

Jensen J, Mackintosh AR (1991) Rare earth magnetism. Clarendon Press, Oxford

Martino C, Bonaventura R, Byrne M, Roccheri M, Matranga V (2017a) Effects of exposure to gadolinium on the development of geographically and phylogenetically distant sea urchin species. Mar Environ Res 128:98–106

Martino C, Chiarelli R, Bosco L, Roccheri MC (2017b) Induction of skeletal abnormalities and autophagy in *Paracentrotus lividus* sea urchin embryos exposed to gadolinium. Mar Environ Res 130:12–20

Martino C, Costa C, Roccheri MC Koop D, Scudiero R, Byrne M (2018) Gadolinium perturbs expression of skeletogenic genes,

calcium uptake and larval development in phylogenetically distant sea urchin species. Aquat Toxicol 194(3):57–66

Oral R, Pagano G, Siciliano A, Gravina M, Palumbo A, Castellano I, Migliaccio O, Thomas PJ, Guida M, Tommasi F, Trifuoggi M (2017) Heavy rare earth elements affect early life stages in *Paracentrotus lividus* and *Arbacia lixula* sea urchins. Environ Res 154:240–246

Pagano G, Esposito A, Bove P, de Angelis M, Rota A, Giordano GG (1983) The effects of hexavalent and trivalent chromium on fertilization and development in sea urchins. Environ Res 30:442–452

Pagano G, Korkina LG, Iaccarino M, De Biase A, Deeva IB, Doronin YK, Guida M, Melluso G, Meriç S, Oral R, Trieff NM, Warnau M (2001) Developmental, cytogenetic and biochemical effects of spiked or environmentally polluted sediments in sea urchin bioassays. In: Garrigues P, Walker CH, Barth H (eds) Biomarkers in marine ecosystems: a practical approach. Elsevier, Amsterdam, pp 85–129

Pagano G, Guida M, Tommasi F, Oral R (2015) Health effects and toxicity mechanisms of rare earth elements – knowledge gaps and research prospects. Ecotoxicol Environ Saf 115C:40–48

Pagano G, Guida M, Siciliano A, Oral R, Koçbaş F, Palumbo A, Castellano I, Migliaccio O, Thomas PJ, Trifuoggi M (2016) Comparative toxicities of selected rare earth elements: sea urchin embryogenesis and fertilization damage with redox and cytogenetic effects. Environ Res 147:453–460

Pagano G, Guida M, Trifuoggi M, Thomas PJ, Palumbo A, Romano G, Oral R (2017) Sea urchin bioassays in toxicity testing: I. Inorganics, organics, complex mixtures and natural products. Expert Opin Environ Biol 6:1

Saitoh M, Kuroda R, Muranaka Y, Uto N, Murai J, Kuroda H (2010) Asymmetric inhibition of spicule formation in sea urchin embryos with low concentrations of gadolinium ion. Dev Growth Differ 52:735–746

Sea Urchin Genome Sequencing Consortium, Sodergren E et al. (2006) The genome of the sea urchin *Strongylocentrotus purpuratus*. Science 314:941–952

Trifuoggi M, Pagano G, Guida M, Palumbo A, Siciliano A, Gravina M, Lyons DM, Burić P, Levak M, Thomas PJ, Giarra A, Oral R (2017) Comparative toxicity of seven rare earth elements in sea urchin early life stages. Environ Sci Poll Res 24:20803–20810

Vukov O, Smith DS, McGeer JC (2016) Acute dysprosium toxicity to *Daphnia pulex* and *Hyalella azteca* and development of the biotic ligand approach. Aquat Toxicol 170:142–151

Zhang P, Ma Y, Zhang Z, He X, Guo Z, Tai R, Ding Y, Zhao Y, Chai Z (2011) Comparative toxicity of nanoparticulate/bulk Yb_2O_3 and $YbCl_3$ to cucumber (*Cucumis sativus*). Environ Sci Technol 46:1834–1841