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Effects of exposure to gadolinium on the development of geographically and phylogenetically distant sea urchins species☆

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ABSTRACT

Gadolinium (Gd), a metal of the lanthanide series used as contrast agent for magnetic resonance imaging, is released into the aquatic environment. We investigated the effects of Gd on the development of four sea urchin species: two from Europe, *Paracentrotus lividus* and *Arbacia lixula*, and two from Australia, *Heliocidaris tuberculata* and *Centrostephanus rodgersii*. Exposure to Gd from fertilization resulted in inhibition or alteration of skeleton growth in the plutei. The similar morphological response to Gd in the four species indicates a similar mechanism underlying abnormal skeletogenesis. Sensitivity to Gd greatly varied, with the EC50 ranging from 56 nM to 132 μM across the four species. These different sensitivities highlight the importance of testing toxicity in several species for risk assessment. The strong negative effects of Gd on calcification in plutei, together with the plethora of marine species that have calcifying larvae, indicates that Gd pollution is urgent issue that needs to be addressed.

1. Introduction

The marine environment is the sink for a range of anthropogenic contaminants, the diversity of which is increasing rapidly as new chemicals are produced and new applications developed. The marine environment receives anthropogenic chemicals originating from terrestrial sources (Islam and Tanaka, 2004). Monitoring chemical pollution in the marine ecosystems and understanding their toxic effects is critical for environmental management (European Marine Board, 2013). Understanding the impacts of chemical pollution in the marine environment requires determination of exposure and chemical concentrations as well as the toxic effects using model organisms (Lyons at al., 2010; Chapman, 2007).

Many pharmaceuticals including therapeutic and prognostic drugs pollute the marine environment because wastewater treatment plants do not adequately remove these compounds. Recently the International Conference on Chemicals Management (ICCM) highlighted the need for global cooperation to build awareness and push for action to address drug pollution (Time To Get Clean, 2015). For example rare elements used in medical applications, such as Gadolinium (Gd), a metal of the lanthanide series of the elements, are released into municipal waste water and then are discharged into aquatic and marine environments. Since the 1980s, chelates of Gd have been used as contrast agents for magnetic resonance imaging (MRI) and were considered safe for humans (Niendorf et al., 1991) until they were linked to nephrogenic systemic fibrosis (NSF) disease (Cowper et al., 2000; Grobner, 2006). Gd^{3+} toxicity appears to be associated with its action as a blocker of Ca^{2+} channels because its ionic radius is nearly equal to that of divalent Ca^{2+} (Sherry et al., 2009). Gd chelates are stable complexes and are not metabolized and so they enter the environment unchanged (Kümmerer and Helmers, 2000). It has been estimated that between 70 and 300 g Gd is released each day into the environment

Abbreviations: hpf, hours post fertilization; Gd, gadolinium; PMCs, primary mesenchyme cells.

☆ In memory of our great colleague and scientist, Dr. Valeria Matranga.

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(Knappe et al., 2005). This poses a potential hazard to the marine biota. Although it is recognized that Gd has negative consequences on human health, the effects of Gd exposure on aquatic organisms are poorly understood. We addressed this gap in knowledge in an investigation of the effects of Gd on the development of sea urchin embryos and larvae.

The sea urchin embryo has long been an important model organism in developmental biology and eco-toxicology to assess the hazard posed by contaminants entering the marine environment (Radenac et al., 2001; Russo et al., 2003; Roccheri et al., 2004; Pinsino et al., 2010; Tellis et al., 2014). A wealth of research with sea urchin embryos and larvae have shown that chemical pollutants impair development and that the larval skeleton is a particularly vulnerable response variable for ecotoxicological tests (Bonaventura et al., 2015; Byrne et al., 2013; Morroni et al., 2016). For instance, exposure to toxic elements (e.g. cadmium and manganese), UVB and X rays inhibits skeletogenesis (Filosto et al., 2008; Pinsino et al., 2011; Bonaventura et al., 2005; Matranga et al., 2010). The sea urchin larval skeleton is produced by the primary mesenchyme cells (PMCs) that migrate into the blastocoel during gastrulation, forming two ventrolateral clusters that secrete the spicule rudiment (Killian and Wilt, 2008; Matranga et al., 2011). Elongation and branching of the spicules produce the three-dimensional pluteus endoskeleton, with specific features depending on the sea urchin species (Zito el al., 2015). We investigated the effect of a range of concentrations of Gd on the development of sea urchin embryos and larvae from four species, two from Europe (*Paracentrotus lividus*, Parechinidae, *Arbacia lixula*, Arbaciidae), and two from eastern Australia (*Heliocidaris tuberculata*, Echinometridae, *Centrostephanus rodgersii*, Diadematidae). This allowed us to compare the responses to Gd and sensitivity across phylogenetically diverse species. As all four species are ecologically important members of rocky reef communities (Boudouresque and Verlaque, 2013; Byrne and Andrew, 2013; Gianguzza and Bonaviri, 2013; Keesing, 2013) and develop through a feeding echinopluteus larva, information of their sensitivity to Gd will aid in the understanding of the hazard posed and of the conserved morphological response across species to provide insights into potential mechanisms of Gd toxicity. The markedly different sensitivities of the four species to Gd is discussed in context of single-species toxicity tests.

2. Materials and methods

2.1. Embryo cultures and Gd exposure conditions

Adult *Paracentrotus lividus* and *Arbacia lixula* were collected along the North-Western coast of Sicily, Italy. *Heliocidaris tuberculata* and *Centrostephanus rodgersii* were collected near Sydney, Australia. Gametes were collected by routine methods (Pinsino et al., 2011; Byrne et al., 2013) and used for fertilization. Four independent experiments were performed for *P. lividus*, *H. tuberculata* and *C. rodgersii* and two for *A. lixula* (see Table 1), with gametes obtained from at least two males and two females. Embryos were reared at 18°–20 °C in Millipore filtered seawater (MFSW) in the presence of antibiotics only for the European species (30 mg/L penicillin and 50 mg/L streptomycin sulfate). Just after fertilization, embryos were exposed to different concentrations of Gadolinium Acetate Tetrahydrate (GAT, Waco). GAT salt was freeze-dried before weighing and dissolved in MFSW. To minimize the volumes to be used for the assessments of Gd toxicity, exposures were carried out in 24-multiwell plates (Cellstar, Greiner Bio-One), with 2000 embryos per well in 2 ml. *P. lividus* was used in preliminary experiments to assess the Gd concentration range causing developmental abnormalities and with respect to results with *Hemicentrotus pulcherrimus, Pseudocentrotus depressus* and *Heliocidaris crassispina* (Saitoh et al., 2010). Specifically, we used doses increasing by a factor of 5 (from 1 to 125 μM) and found about 50% of abnormal embryos at the lowest dose, and 100% lethality at the highest dose tested. A similar approach was used for the other three species under investigation, with final Gd concentrations used ranging from 1 nm to 200 μM. For each experiment 50 embryos from three different wells were sampled at the two developmental stages, gastrula (24 h post fertilization-hpf) and pluteus (48 hpf), as in previous studies (Bonaventura et al., 2005; Pinsino et al., 2011). In some experiments, a total number of 100 or 300 embryos were sampled (see Table 1). Embryos were examined microscopically (Zeiss Axioscop 2 plus or Olympus BX60), photographed using a digital camera and scored for normal/abnormal development (see below).

2.2. Toxicity criteria

The four species were chosen because of their comparable developmental timeline. At 48 hpf, embryos of all species have reached the pluteus stage, where a tri-partite gut and larval arms could be observed in control embryos. Exposure to Gd resulted in major alterations or inhibitions of skeleton growth at the final endpoint (48 hpf). Thus, abnormal embryos were categorized into five morphotypes, as sketched in Fig. 1: CS, complete skeleton: larvae with a regular skeleton; NS, no skeleton; SS, shorter skeleton: skeleton arm rods shorter than controls; AS, asymmetrical skeleton: larvae with a left-right (LR) asymmetry in skeleton rods. For *H. tuberculata* and *C. rodgersii* we identified one additional category, called LP, lost pattern, characterized by an incorrect growth and branching of the skeletal rods. The percentage of each skeleton category in the larvae examined from each of the three wells (per fertilization) was determined and used as the datum for statistical analysis.

2.3. Statistical analysis

The dose-response curves of the four species were calculated plotting the percentages of embryos bearing an abnormal skeleton (Fig. 1: NS, SS, AS and LP embryos) across increasing Gd concentrations. Two ecotoxicological parameters were determined: EC50, the half maximal effective concentration, that represents the concentration where 50% of Gd maximal effect is observed, and the NOEC, no observed effect concentration, the highest concentration of a substance at which no adverse effect is found in exposed organisms. The EC50 values for each Gd-experiment performed were determined using the SigmaPlot 13.0 analysis software (Systat Software, Inc., San Jose, California, USA). The EC50 values were analyzed by the one-way ANOVA on the data for three species (except for *H. tuberculata* due to insufficient replication) with species as the fixed factor. The analyses were performed using the OriginPro 8.1 (OriginLab Corp., Northampton, MA, USA), and the level of significance was set to $P \leq 0.05$. The percentage data determined for each of the morphological categories observed were analysed by the one-way analysis of variance (ANOVA) with Gd concentration as the fixed factor and individual skeletal morphology as the response variable. Tukey's HSD test was used as Post-hoc test for mean comparison. Additionally, two-way ANOVAs were run with Gd concentration and species as the fixed factor and individual skeletal morphology as the response variable. Homogeneity of variance and normality were checked using the Levene's and Shapiro-Wilk tests, respectively.The Shapiro-Wilk normality test showed that all data was significantly drawn from a normally distributed population, except for the concentrations corresponding to percentages all equal to zero or very similar. The data for the skeleton categories of *H. tuberculata* were not analyzed by ANOVA

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Table 1
Summary of experiments performed on the four sea urchin species.

Species	N° Exp	Exp	N° of counts	Gd Concentrations																			
				nanoMolar									microMolar										
P.lividus	4	# 1	1*	0									1	2.5	5	10	20						
		# 2	1*	0									1	2.5	5	10	20						
		# 3	1*	0									1	2.5	5	10	20	40					
		# 4	3	0							250	500	800	1	2.5	5	10	20	40		80	100		
A.lixula	2	# 1	3	0							250	500		1	2.5	5	10	20	40		80	100		
		# 2	3	0							250	500		1	2.5	5	10	20	40		80	100		
H. tuberculata	4	# 1	1+	0										1	2.5	5	10	20						
		# 2	1+	0						125	250	500	800	1	2.5	5	10	20						
		# 3	1+	0	1	10	25	50	100															
		# 4	1+	0	1	10	25	50	100	125	250	500	800											
C. rodgersii	3	# 1	3	0										1	2.5	5	10	20						
		# 2	3	0										1	2.5	5	10	20	40	50	80	100		
		# 3	3	0															40	50	80	100	150	200
		# 4	3	0																			150	200

1*: for *P.lividus*, a total number of 300 embryos were scored for normal/abnormal development.
1+: for *H. tuberculata*, a total number of 100 embryos scored for normal/abnormal development.

tests as there were only two replicates for each Gd concentration (see Table 1).

3. Results

3.1. Gd toxicity dose-response curves in phylogenetically distant species show divergent levels of sensitivity

Our first aim was to determine the general response to Gd in the four species, comparing control embryos raised in normal seawater with embryos raised in Gd-containing seawater at different concentrations. Control embryos developed through the gastrula stage (24 hpf) and subsequently reached the pluteus larvae (48 hpf), characterized by the presence of a fully-developed skeleton (Fig. 1, CS embryos).

In all four species there was a decrease in the percentage of CS embryos, in parallel with the increase of the Gd concentration tested, with species-specific differences in sensitivity. The dose-response curves, obtained by plotting the percentages of embryos with an abnormal skeleton are shown in Fig. 2. The EC50 values were: 56 nM for *H. tuberculata*; 1.18 μM for *P. lividus;* 2.1 μM for *A. lixula* and 132 μM for *C. rodgersii*. Thus, there was a three-order magnitude of difference between the EC50s of *H. tuberculata* and *C. rodgersii* values and a two-order magnitude of difference between the EC50s of *P. lividus*/*A. lixula* and *H. tuberculata*.

The NOEC values, calculated empirically on the basis of the observed effects, were: 1 nM for *H. tuberculata*; 250 nM for *A. lixula;* 250 nM for *P. lividus* and 1 μM for *C. rodgersii*. One-way ANOVA revealed significant differences for the EC50 of *P. lividus*, *A. lixula* and *C. rodgersii* ($F = 482{,}27$; $P = 2{,}32 \cdot 10^{-14}$).

There were significant differences in the percentage of the CS embryos in the control and Gd-exposed embryos (Table 2). The pair-wise comparisons (Tukey HDS Post-hoc Test) showed that the mean percentages of CS embryos for 5, 10 and 20 μM Gd concentrations to 0 μM Gd (control embryos), differed ($P < 0.05$, data not shown). Gd concentrations among sea urchin species had significant effects on the categorized skeletal morphologies (Two-way ANOVA, Table 3).

3.2. Gadolinium exposure perturbs skeleton growth and pattern

At 48 hpf, control embryos of all the four species were plutei

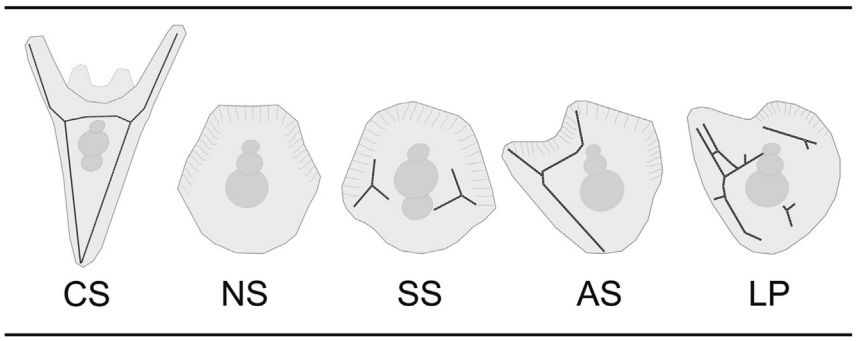

Fig. 1. Sketches of the five morphotypes observed and categorised on the basis of skeleton occurrence, abnormality and asymmetry. CS, Complete Skeleton; NS, No Skeleton; SS, Shorter Skeleton; AS, Asymmetrical Skeleton; LP, Lost Pattern.

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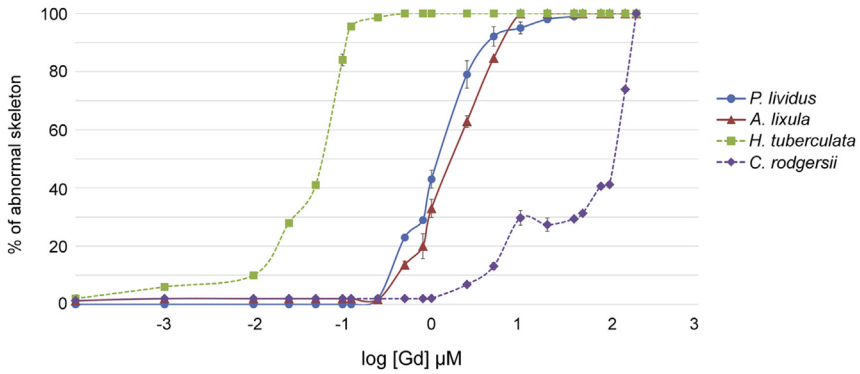

Fig. 2. Concentration-dependent effects of 48 h Gd exposure on abnormal skeleton formation. Percentages of embryos bearing an abnormal skeleton are plotted across increasing Gd concentrations in seawater.

with a tri-partite gut and well-developed skeleton, with species-specific evident differences (Fig. 3 A-D). Specifically, *P. lividus* plutei (Fig. 3A) have an elongated body, with unfenestrated skeletal rods typical of Echinidae (Emlet, 1982; Zito et al., 2015). *A. lixula* plutei (Fig. 3B) have fenestrated postoral rods and a robust apical dentate rostrum with a variable number of 7–9 teeth. The plutei of *H. tuberculata* (Fig. 3C) are more truncated and their skeleton forms a robust and intricate basket-structure, with solid ladder-like fenestrated rods typical of the Echinometridae (Kinjo et al., 2008). The plutei of *C. rodgersii* (Fig. 3D) are generally referred to as two-armed larvae (Soars and Byrne, 2015) because of their very long postoral arms in comparison to the short anterolateral arms, characteristic form of many diadematids (Emlet et al., 2002).

Exposure to Gd had no major effects on early development to gastrulation (24 hpf). Ingression and migration of PMCs occurred with the correct timing, if compared to control embryos, as well as the invagination of the vegetal plate (not shown); at the gastrula stage, we observed a slight delay in biomineral deposition timing (not shown). At 48 hpf, differentiation of ectoderm territories into the columnar epithelium at the animal pole and the squamous epithelium at the vegetal pole also appeared normal (see Fig. 3E, F, K, P). Gd also had no apparent effect on normal tripartite gut development, as shown for *P. lividus* (Fig. 3E, H, L) and *C. rodgersii* embryos (Fig. 3K, O, Q).

After 48 hpf of Gd exposure, impaired skeleton development was evident with a range of skeletal abnormalities (Figs. 1 and 3), including complete skeleton (CS, Fig. 3A-D), no skeleton (NS, Fig. 3E-G), shorter skeleton (SS, Fig. 3H-K) and asymmetrical skeleton (AS, Fig. 3L–O). The lost pattern category was only seen in *C. rodgersii* and *H. tuberculata* (LP, Fig. 3P–Q). A summary of the occurrence of the morphotypes observed in the presence of different concentrations of Gd is shown in Fig. 4. There was a significant effect of Gd concentration on the percentage of the skeletal morphologies observed (Table 2).

For *P. lividus*, the SS category initially increased with the increase of Gd concentration (48.30% ± 0.06 SD at 2.5 µM), as for *A. lixula* (58.82% ± 0.03 SD at 5.0 µM) (Fig. 4A and B). At Gd concentrations higher than 2.5 µM (*P. lividus*) and 5.0 µM (*A. lixula*) the SS category decreased, in parallel with the increase of the NS morphotypes, probably occurring because of the high Gd dose used. The AS morphotype was infrequent in *A. lixula* (Fig. 4B), but common in *P. lividus* with a peak percentage of 43.41% (±0.05 SD) at 20 µM (Fig. 4A). At higher concentrations (100 µM), the incidence of the most severe phenotype (NS) increased (48.85% ± 0.01 SD) in *P. lividus*.

H. tuberculata was the most sensitive species to Gd exposure (Fig. 4C). Beyond the 250 nM threshold there were no CS larvae and the skeleton was absent at concentrations above 5 µM (NS embryos). The peak percentages of the SS, AS and LP morphotypes were reached at 40.80% (±0.03 SD) at 125 nM, 54.38% (±0.03 SD) at 0.5 µM, and 36.00% (±0.0015 SD) at 100 nM, respectively, and decreased at higher Gd doses tested. In contrast, *C. rodgersii* was far less sensitive than the other three species (Fig. 4D). Very low amounts of SS (4.72% ± 0.01 SD) and AS (2.07% ± 0.007 SD) morphotypes were observed at 2.5 µM. The amount of CS embryos

Table 2
Effects of Gd concentrations on the categorized skeletal morphologies (One-way ANOVA).

Specie	Morph.	DF	F value	P value
P. lividus	CS	11	262.60	0
	NS	11	39.49	0
	SS	11	25.95	$1887 \cdot 10^{-15}$
	AS	11	20.27	$1469 \cdot 10^{-13}$
A. lixula	CS	10	763.08	0
	NS	10	171.01	0
	SS	10	91.03	0
	AS	10	37.54	0
C. rodgersii	CS	11	464.65	0
	SS	11	717.87	0
	AS	11	67.99	0
	LP	11	37.40	0

P value < 0.05 indicated that Gd concentrations had significant effects on skeleton morphologies.
DF, degree of freedom: number of Gd concentrations to which embryos were exposed, including zero concentration (control embryos), minus 1.

Table 3
Effects of Gd concentrations among sea urchin species on the skeletal morphologies (Two-way ANOVA).

Morph.	Factors	DF	F value	P value
CS	Gd Conc.	14	96.55	0
	Specie	2	320.70	0
NS	Gd Conc.	14	16.86	0
	Specie	2	103.64	0
SS	Gd Conc.	14	13.00	0
	Specie	2	94.90	0
AS	Gd Conc.	14	73.42	0
	Specie	2	118.14	0

P value < 0.05 indicated that among species, Gd concentration had significant effects on the categorized skeletal morphologies.

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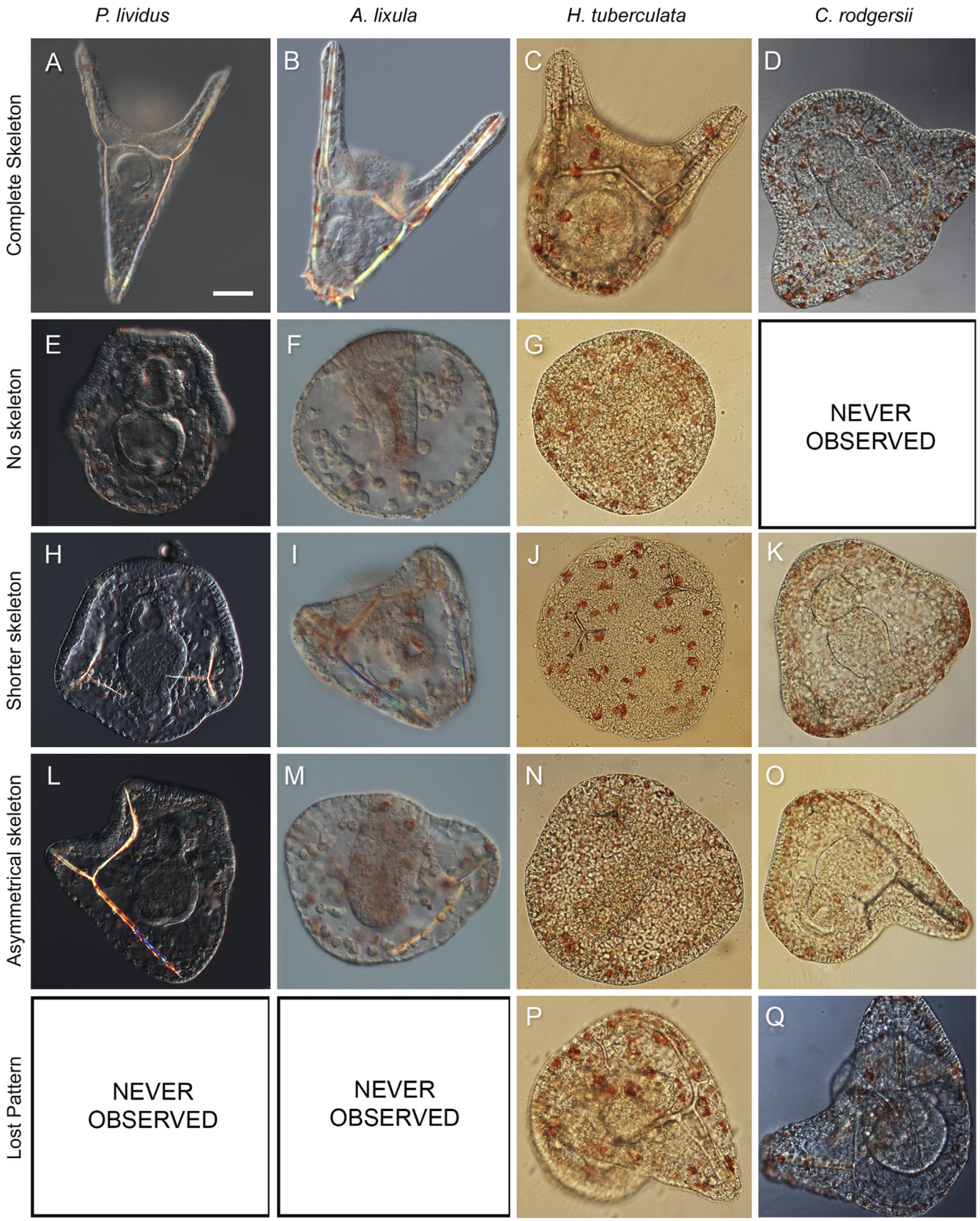

Fig. 3. Morphotypes occurring after Gd exposure show severe impairments of skeleton growth and patterns. Control (A–D) and Gd-exposed (E–Q) embryos after 48 h of development. Bar = 50 μM in A-D, G, J, K, N-Q; 25 μM in E, F, H, I, L, M.

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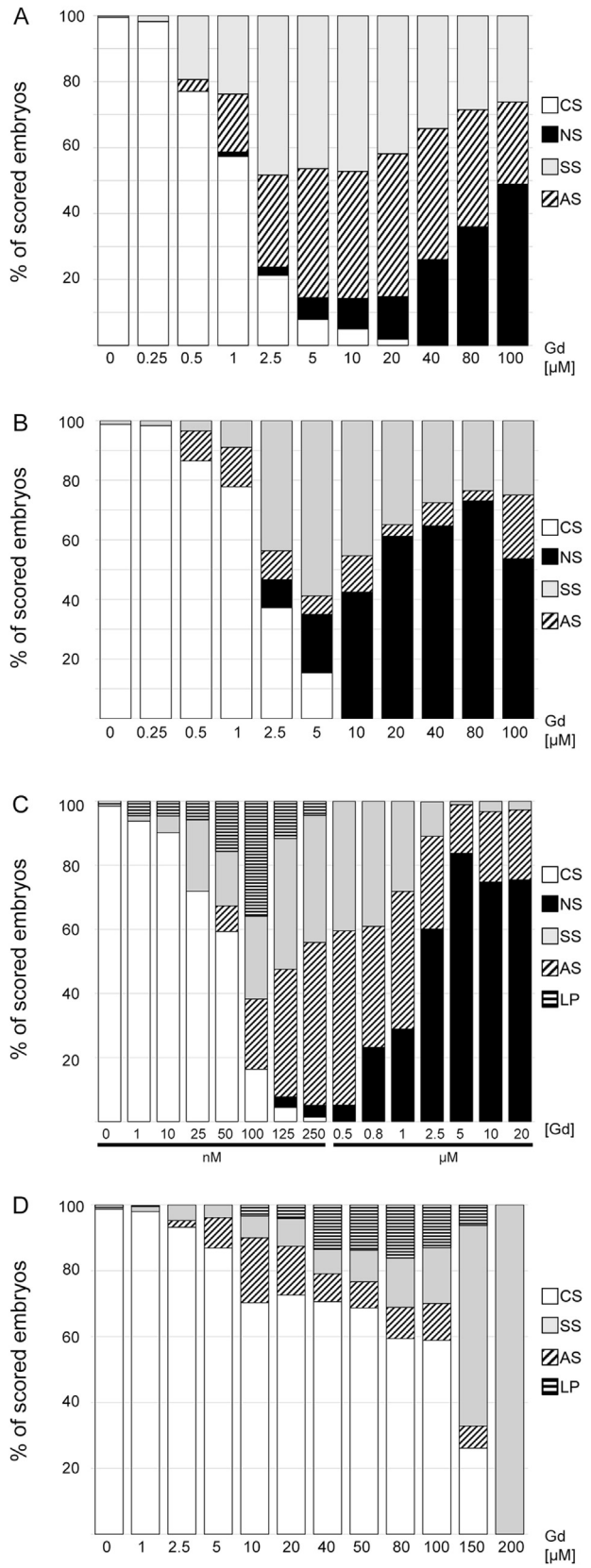

Fig. 4. Impairment of skeleton growth and patters correlates with the exposure to increasing Gd concentrations. *P. lividus* (A), *A. lixula* (B), *H. tuberculata* (C) and *C. rodgersii* (D) embryos after 48 h of development. Bars in the histograms show the percentages of observed phenotyes: white: CS (complete skeleton); black: NS (no skeleton); grey: SS (shorter skeleton); oblique strips: AS (asymmetrical skeleton); horizontal strips: LP (lost pattern). Standard deviation values ranged from 0.0004 to 0.06 for all samples.

decreased in a dose-dependent manner, but as much as 26.14% (±0.02 SD) of the normal morphologies were present even at high Gd concentration (150 μM). Differently from what observed in the other species, the AS category was represented by embryos with a completely full-sized spicule on one side only, never shorter than that of controls, with the highest percentage of 19.74% (±0.01SD) at 10 μM. The LP morphotype reached the amount of 16.10% (±0.02 SD) at 80 μM and then gradually decreased at higher doses. In contrast with what we observed in the other three species, the NS phenotype was never observed in *C. rodgersii* even at the highest concentration tested (200 μM). In this latter treatment, the SS category was 100%.

The effects of Gd concentration with respect to each morphological category for *P. lividus*, *A. lixula* and *C. rodgersii* were significant (Table 2). For *P. lividus,* the data of skeletal morphologies analyzed by ANOVA included values on 0.8 μM Gd concentration (see Table 2) not shown in Fig. 4. The effects of Gd concentrations per each morphological category and across the three species were significant ($P < 0.05$). The pair-wise tests between species (Tukey HSD, Table S1), revealed significant differences. For two species, *P. lividus* and *A. lixula* the responses to Gd of the CS category was not significantly different (Table S1), because the percentage of CS embryos in these species was similar (Figs. 2 and 4, Table S1).

4. Discussion

Gadolinium, along with a plethora of medically used agents released into the marine environment, is an emerging pollutant (Telgmann et al., 2013). Gd pollution is measured as “the Gd anomaly”, calculated as the ratio of the measured Gd concentration in a sample with respect to the background levels of Gd due to geological processes. Positive Gd anomalies were observed in several rivers, lakes and in seawater from different locations (Bau and Dulski, 1996; Nozaki et al., 2000; Elbaz-Poulichet et al., 2002; Zhu et al., 2004; Ogata and Terakado, 2006; Kulaksiz and Bau, 2007). The Gd anomaly is determined using only the total Gd concentration, without information about the speciation of the Gd present in the sample (Bau and Dulski, 1996). Strikingly, the Gd anomalies in seawater around the urban areas with large human populations have increased greatly over time, all over the world, demonstrating that Gd anomalies are caused by the emission release from anthropogenic sources (Zhu et al., 2004). Here we show the toxic effects of Gd on the development of four sea urchin species living in coastal areas around big cities (Palermo, Italy: >1 million inhabitants; Sydney, NSW: >4 million inhabitants), and in particular the perturbation of calcification. As many marine species make a skeleton during their planktonic developmental stage, the strong negative effects of Gd, for some of the sea urchin species at very low concentrations, highlights that Gd pollution is an issue that needs to be addressed.

The four species investigated involved European (*Paracentrotus lividus* and *Arbacia lixula*) and Australian (*Heliocidaris tuberculata* and *Centrostephanus rodgersii*) species that all develop through an echinopluteus larva producing a complex three-dimensional skeleton. As the mechanism of skeletogenesis and digestive tract formation in echinoplutei is conserved across the Echinoidea (Arnone et al., 2015), we expected that the four species would have a similar response to Gd, exhibiting similar phenotypes. On the other hand, due to differences in the extent of skeletogenesis between the species we expected that there might be some differences in absolute sensitivity levels. As expected, the phenotypic response to Gd of impaired skeleton formation was similar across the four species, indicating a similar response mechanism, albeit with different levels of sensitivity with respect to the concentrations used. The response seen for the four species investigated here is

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similar to that reported for three Japanese species, *Hemicentrotus pulcherrimus, Heliocidaris crassispina* and *Pseudocentrotus depressus* (Saitoh et al., 2010), providing further evidence of a conserved mechanism of toxicity of Gd to sea urchin embryos.

Pharmaceuticals are designed to specifically act on biological systems and that's the reason of their high toxicity; Gd ions (Gd^{3+}) toxicity appears to be associated with its action as a blocker of Ca^{2+} channels because its ionic radius is nearly equal to that of divalent Ca^{2+} (Sherry et al., 2009). Some studies suggested that Gd ion concentrations in the micromolar range (between 1 and 200 μM) are able to block Ca^{2+} channels in the membrane of sea urchin eggs (David et al., 1988), *Xenopus* oocytes (Yang and Sachs, 1989) and mammalian cell lines (Broad et al., 1999; Lansman, 1990; Luo et al., 2001). As the skeleton is a carbonate structure, potential blockage of Ca^{2+} channels by Gd may be particularly toxic to the calcification response. Further research in this direction is awaited.

There was several orders of magnitude difference in the EC50 for the most sensitive (*H. tuberculata*) and most tolerant (*C. rodgersii*) species. For all species we observed a strong concentration-dependent impairment of skeletogenesis. The sensitivity to Gd might reflect the differences in the amount of calcite deposited in the spicules with the species that produce more robust complex skeletons (eg. *H. tuberculata* and *A. lixula*) to be more sensitive than species that produce a simpler, lower calcite skeleton (e.g. *P. lividus*). It may be that species that normally produce more calcite are more affected by Gd-impaired uptake of Ca^{2+}. The requirement of Ca^{2+} for the sea urchin biomineralization requires the supply of bicarbonate or carbonate ions, by the formation of an over-saturated micro-environment at the calcification front in the embryo (Stumpp et al., 2012), and a minimal molecular toolkit for mineral deposition in adult tests (Karakostis et al., 2016) which might be the same in the larvae.

As predicted, *H. tuberculata* embryos were much more sensitive to the Gd exposure then the other three species, suggesting that the need to deposit more calcite and construct a robust larval skeleton may be an indication of sensitivity. The differences in the sensitivity of the species to Gd may also be related to their different habitats and perhaps pollution history of the parental population, a trans-generational effect as seen for the progeny of other invertebrates from polluted sites (Lister et al., 2015). This is a form of developmental plasticity through the influence of environment on gamete quality (Ghalambor et al., 2015; Hamdoun and Epel, 2007). The epigenome may also be involved (Vandegehuchte and Janssen, 2014). The different ability to activate defence strategies, such as the autophagic program (Chiarelli and Roccheri, 2012) and stress proteins induction (Matranga et al., 2011), may contribute to the resilience of the species. Chemical pollutants have been a persistent source of evolutionary challenges throughout the life history of living organisms (Whitehead, 2014). Human-introduced pollutants greatly increased the rate of change of contemporary environment, severely challenging the adaptive potential of many species. Empirical data on the evolutionary potential of a wide range of species are needed to determine their adaptation to the changing world (Hoffmann and Sgrò, 2011). Geographically separated populations within the same species have been shown to differ in their tolerance to climatic conditions, indicating that past selection has resulted in local adaptation to temperature (Kelly et al., 2011) and pH (Langer et al., 2009; Hammond and Hofmann, 2010).

The two European species collected from the same environment, *P. lividus* and *A. lixula,* showed similar sensitivities in response to Gd exposure, in agreement with a previous work where these two species were found equally sensitive to different types of chemicals (Carballeira et al., 2012). In contrast, a recent article on the effects of silver nanoparticles (Ag-NPs) on the development of three sea urchin species demonstrated the species-specific effects of Ag-NPs low concentrations (from 100 down to 1 mg/L) (Burić et al., 2015). These authors found that the three species, *A. lixula*, *P. lividus* and *Sphaerechinus granularis*, differ in their sensitivity to Ag-NPs. The most sensitive species is *A. lixula* whose embryos show an impaired development at the lowest Ag-NP concentrations (1–10 mg/L) tested. It followed *S. granularis*, with an effective Ag-NP concentration range of 10–50 mg/L, and last *P. lividus* (50–100 mg/L). Thus, sympatric species living in the same environment can have different sensitivities to toxicants, as also shown here for the two Australian species and for Japanese species in response to Gd (Saitoh et al., 2010). This indicates that species, despite having a similar environmental history and potential exposure to pollution, might have similar sensitivities to some toxicants and different sensitivities to others.

The differences in sensitivity between the three species may also be influenced by phylogeny. Phylogenetic threes, based on molecular data, show that *C. rodgersii* is a member of the oldest sea urchin lineage among the four examined (Lawrence, 2013), while *H. tuberculata* and *P. lividus* are more recent and more closely related. The hypothesis that *C. rodgersii* is the most robust to the Gd insult may be due to being of a more ancient lineage needs to be assessed. A quantitative genetics study showed the presence of tolerant genotypes in response to concurrent warming and acidification, contributing to the adaptive capacity and resilience of *C. rodgersii* in a changing ocean (Foo et al., 2012). Comparative data on Gd toxicity from the Mediterranean species, *Centrostephanus longispinus* would help discern the potential influence of phylogeny or geographic environment on sensitivity. To understand the relative influence of environmental or phylogenetic history on the different sensitivities of the embryos and larvae of different sea urchins to Gd and other stressors requires data from many other species.

The comparison among effects of Gd on larval skeleton of different species can address important evolutionary questions from a developmental viewpoint, to understand how these differences are generated, and from an ecotoxicological viewpoint, to investigate how such differences influence the response to environmental contaminants.

4.1. Conclusions

In conclusion, we show that four sea urchin species, geographically and phylogenetically distant, had different sensitivity to Gd, but that the effect of this agent on larval phenotype was similar. That the same pollutant can have very different toxicity levels on marine organisms, even within the same taxonomic group, shows that using only one model organism to test the effects of pollutants on the marine environment is not sufficient. Results of pollution assays based on one species within a taxon can be misleading with respect to hazard risk assessment.

Author contributions

Conceived and designed the experiments: CM, MB, VM. Performed the experiments: CM. Performed statistical analysis: RB. Analyzed the data: CM, RB, MCR, MB, VM. Wrote the paper: CM, VM. Revised the manuscript: RB, MCR, MB.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.marenvres.2016.06.001.

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