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Ecotoxicity responses of the freshwater cnidarian *Hydra attenuata* to 11 rare earth elements.

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Frequently used abbreviations**:**

EC50: effective concentration sub-lethally impacting 50% of the exposed population; LC50: lethal concentration killing 50% of the exposed population; Ln: refers to the 15 rare earth elements also known as lanthanides or a group thereof; NOEC: a no observable effect concentration in an exposed population; REE: rare earth element.

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Abstract

Lanthanides are the major family of rare earth elements (REEs) owing to the essential properties these metallic species provide in diverse fields of today's world economy. They are now being mined and produced as never before. This raises new environmental concerns in terms of their expected future discharges notably to aquatic systems. Interspecies studies of their ecotoxicity are sparse and effects on aquatic life are still poorly understood. Absence of such information for cnidarians, an ecologically relevant freshwater community, thus prompted the present research on REEs toxicity using Hydra attenuata as our animal model. Lethal and sublethal ecotoxicity data generated with the 11 REEs displayed LC50 values ranging from 0.21 to 0.77 mg L−1 and EC50 values ranging from 0.02 to 0.27 mg L−1, thereby confirming the inherent sensitivity of Hydra to REE exposure at environmentally relevant concentrations. Additionally, two properties of REEs were shown to modulate Hydra (sub)lethal toxicity (LC50 and EC50) which decreases with increasing atomic number and with decreasing ionic radius. Compared to studies carried out with different taxonomic groups, Hydra toxicity responses to REEs proved to be among the most sensitive, along with those of other invertebrate species (i.e., Daphnia magna, Ceriodaphnia dubia, Hyalella azteca), suggesting that members of this community are likely more at risk to eventual REE discharges in aquatic environments. Demonstrated Hydra sensitivity to REE exposure strongly justifies their future use in toxicity testing battery approaches to evaluate liquid samples suspected of harbouring REEs.

Introduction

Lanthanides, La (Z = 57) to Lu (Z = 71), form a group of 15 metallic species that, along with yttrium (Z = 39) and scandium (Z = 21), are known as the rare earth elements (REEs). Chemical properties of lanthanides are similar within the group, all closely mirroring that of lanthanum (La), and hence their collective appellation "Ln" referring to any of these metals.

Because of their increasing use in diverse areas linked to the world economy (e.g., agronomy, medicine, industry), mining of REEs and production of REE-containing products have skyrocketed over the past decades thereby raising new environmental concerns for the biosphere (US EPA, 2012). An increased presence of REEs has been shown, for example, in aquatic bodies (Kulaksiz and Bau, 2011a), in sediments (Sneller et al., 2000) and soils (Cao et al., 2000).

More specifically linked to the issue of water quality in ecosystems, REEs have been measured in soil runoff, effluent and in the hydro-sphere itself owing to varied agricultural/industrial practices (Protano and Riccobono, 2002) as well as to medical applications (Bau et al., 2006; Rabiet et al., 2009). In our digital age, the indirect release of REEs to aqueous environments via e-wastes is an additional preoccupation because of

the absence of proper recycling technologies in many countries of the world, thereby posing further potential risks to environmental and human health (Dodson et al., 2012).

With reference to aquatic ecotoxicity, a recent review by a French group flagged 91 references dealing with (freshwater and saltwater) toxicity studies relating exposure of selected lanthanides to biota representative of different biological levels (Gonzalez et al., 2014). As expected, Ln ecotoxicity varied across species tested, influenced in part by experimental conditions imposed by testing procedures. While arthropods, algae and fish received the most attention in the assessment of ecotoxicity on selected REEs in these investigations, cnidarians were virtually left out of any such appraisal. Another recent review focusing solely on Lanthanum demonstrated its bioaccumulation in several types of aquatic biota with varying ecotoxicity responses across levels (microorganisms, protozoans, micro-algae, macrophytes, invertebrate sp., fish), but information on La cnidarian toxicity was lacking (Hermann et al., 2016).

Inexistent ecotoxicity studies related to cnidarian responses toward RREs thus triggered our desire to acquire data in this respect. Since cnidarians (*Hydrozoa*) of the genus *Hydra*, ubiquitous and ecologically relevant in freshwater environments, have been shown to be particularly sensitive to heavy metals (Quinn et al., 2012; Ginou and Holdway, 2013), generating toxicity responses with a *Hydra* species model exposed to Ln metals also appeared entirely justified to evaluate hazard toward this aquatic community.

Herein, we report our findings of Ln ecotoxicity responses generated with *Hydra attenuata*, also known as *Hydra vulgaris* (Pallas, 1766), an animal model used extensively since the 1980s to undertake developmental (Johnson and Gabel, 1982) and diverse toxicity (Fu et al., 1991; Pardos et al., 1999; Pascoe et al., 2002; Quinn et al., 2008) studies. Lastly, it is noteworthy to mention that ever since miniaturization of the testing procedure in microplates was developed in our laboratory, *Hydra* toxicity assays have become simple and cost-effective to perform (Blaise and Kusui, 1997; Trottier et al., 1997).

Materials and Methods

The 11 REEs salts tested were purchased from Sigma-Aldrich. They are listed in Table 1 along with their atomic number and ionic radii characteristics. In preparation for subsequent toxicity testing, each lanthanide salt was processed as follows. First, a 1 g/L stock solution for each was prepared in Hydra medium (made up of 0.15 g CaCl2.2H2O and 0.1 g TES (N-tris [hydroxymethyl]methyl 1-2- aminoethanesulfonic acid) buffer, pH 7.0). The 1 g/L solution was well below the reported solubility limit of the chloride salts in water (Guminski et al., 2016). Then, 400 μL were withdrawn from a 40-mL solution of Hydra medium and 400 μL of each stock solution were re-added to the Hydra medium to make up REEs working solutions of 10 mg L−1for subsequent bioanalysis. Controls consist of 400 μL of distilled water and 400 μL of Hydra medium.

The toxicity testing procedure using Hydra attenuata has been extensively detailed previously (Blaise and Kusui, 1997; Trottier et al., 1997). Hydra were raised and maintained in culture in 20-cm diameter crystallisation bowls at 20–22 °C in the Hydra medium as described above. They were fed daily with Artemia salina brine shrimps. Briefly, exposure tests were conducted in 12-well polystyrene microplates with three animals placed in each of three adjacent wells (n=9) for each of 11 serially-diluted REEs concentrations (10 mg L−1to 0.0098 mg L−1) using Hydra medium as the dilution medium. Experimental animals (n=3 in each well) bathed in 4 mL of Hydra medium (controls) or of REEs dilutions thereof. After a 96-h exposure at room temperature (20–25 °C), changes in Hydra morphology based on the Wilby scale (Wilby, 1989) from controls (normal-sized animals with long and slender tentacles) indicating sublethal (clubbed and/or shortened tentacles) and lethal (tulip and/or disintegrated stages) effects were scored with a stereomicroscope at 6×magnification (the number of hydra with morphological alterations is the endpoint to calculate the EC50). Representative examples for each key morphological changes are included (Fig. 1). Hydra were not fed during the 96-h exposure period.

CETIS software (version 1.8.7.7) was used to determine 96 h-LC50s (Spearman-Karber method; Finney, 1964) and 96-h EC50s (Log-Logit method) values. Pearson-moment correlation analyses were undertaken using Statistica software (version 13.). Significance was set at $p < 0.05$. Correlations with REE ionic radius were performed by using ionic radii values provided in Emsley (1989).

Results and discussion

Lethal and sublethal ecotoxicity data generated with the 11 REEs are shown in Table 1. LC50 values are strikingly similar and range from 0.21 to 0.77 mg L−1 (3.7-fold difference). EC50 values display somewhat more variability and range from 0.02 to 0.27 mg L−1, i.e. by slightly over one order of magnitude (13.5-fold difference). The toxicity data plots from which LC50 and EC50 values were derived are shown in Fig. 2. The lighter REEs Y, La, Ce and Pr were generally more toxic than the heavier ones (Nd, Sm, Gd, Tb, Dy, Er) with the exception of the heaviest Lu which was more toxic. With one exception, LC50/EC50 ratios demonstrate that sublethal effects appear at exposure concentrations that are not far from those eliciting lethality. Indeed, 10 of the 11 REEs have ratios ranging from 2.6 to 7.3. In contrast, Pr stands out with an LC50/EC50 ratio of 28. Pr also produced the lowest EC50 value (i.e., 0.020 mg L−1) intimating it would likely be more harmful to Hydra if present in aquatic environments. A further estimate of REEs hazard potential is provided by reporting our data according to the EUDirective 93/67/EEC classification scheme (CEC Commission of the European Communities, 1996). Their span of toxicity responses ranges from "extremely toxic (effects<0.1 mg L−1), "very toxic (effects between 0.1 and 1 mg L−1)", "toxic (effects between 1 and 10 mg L−1)", "harmful (effects between 10 and 100 mg L−1)" and "not toxic (effects> 100 mg L−1)". The REE were thus classified as "extremely toxic" to "very toxic" as all LC50 and EC50 values were found to lie between <0.1 and 1 mg L−1. The lighter lanthanides

(Y, La, Ce, Pr, Nd) appear notably the most hazardous to Hydra based on their sub-lethalitylying in the "extremely toxic" zone.

Based on Table 1 data, it thus appears that REEs demonstrate a rather uniform toxicity response tantamount to their known homogeneous chemical properties, because of their narrow or "generic" toxicity spectrum, which suggests a similar mode of action for REEs. In contrast, an earlier study conducted on 9 metallic nanoparticles with the same Hydra bioassay generated toxicity responses that spanned over three orders of magnitude thereby proposing a more "specific" response for each or some of these particles and hence that likely different modes of action are at play here (Blaise et al., 2008). However, in terms of hazard potential, these nanoparticles proved to be less toxic than REEs, as their responses ranged in the "very toxic" to "harmful" categories based on the (CEC Commission of the European Communities, 1996) classification scheme (0.1–1 and 10–100 mg L−1 respectively).

Comparing REEs Hydra toxicity to that of heavy metals, several studies have shown similar sensitivities as reported in a comprehensive review by Quinn et al. (2012). In one investigation, for example, the mean cadmium and zinc 96 h-LC50 values generated for H. attenuata, were 0.083 and 2.3 mg L−1, respectively (Holdway et al., 2001). In another study, the median 96 h-LC50s of copper, cadmium and zinc for three Hydra species taxonomically related to H. attenuata (i.e., two H. vulgaris strains and H. oligactis) ranged between 0.042 and 14 mg L−1. For the first two of these metals, 96 h-LC50s ranged between 0.042 and 0.52 mg L−1. Zinc, however, an essential element in cellular metabolism, demonstrated less toxicity with 96 h-LC50 of 13–14 mg L−1 (Karntanut and Pascoe, 2002).

Hydra sensitivity to metallic elements is thus indisputable as this study demonstrates their responses to lie in the "extremely toxic" to ''very toxic'' categories for REEs and where the above studies also place them in the same categories for heavy metals (Holdway et al., 2001; Karntanut and Pascoe, 2002) and in the "very toxic" to "harmful" categories for metallic nanoparticles (Blaise et al., 2008) when considering the (CEC Commission of the European Communities, 1996) classification scheme. Hydra appear to lack the metal binding protein metallothionein which does not allow these animals to conjugate and expel metal products, hence promoting their use as sensitive indicators of metal contamination (Andersen et al., 1988; Quinn et al., 2008). Nevertheless, cnidarians possess other capacity to defend against xenobiotics such as cytochrome P450, various conjugating enzymes, ATPdependent efflux transporters and metal stress/oxidative detoxification proteins or transcription factors (Goldstone, 2008). Cnidarians have been shown to express phytochelatins which are another group metalbinding peptides but their relative capacity to bind rare earth is not well understood at present. The presence of heat shock proteins could also provide protection against heavy metals. Given that many metals and other elements often produce oxidative stress, some form of protection could be provided in these organisms.

A few published articles allow us to compare Hydra REEs sensitivity with bioassays undertaken at different biological levels. Exposure (in soft water where toxicity responses

were highest) of the freshwater amphipod Hyalella azteca to the same 11 REEs tested in our study generated 7-d LC50s that ranged from 0.12 to 0.56 mg L−1 (Borgmann et al., 2005). These results are strikingly similar with those obtained herein for Hydra where 96 h-LC50s ranged from 0.21 to 0.77 mg L−1 (see Table 1), thus indicating comparable REEs sensitivities for these two types of aquatic invertebrates.

In another study, exposure of the freshwater chlorophyte Chlorella vulgaris to the same REEs in their chloride salts form produced lowest inhibitory concentrations situated between 2.1 and 10 mg L−1 (Den Dooren De Jong, 1965), thereby assigning their responses as "toxic"according to the CEC classification scheme (CEC Commission of the European Communities, 1996), but indicating that this chlorophyte displayed markedly less sensitivity than our Hydra results. A grey literature publication (i.e., student report) performed on seven REEs (La, Ce, Pr, Nd, Sm, Gd and Dy) reported LC50s for the water flea Daphnia magna and fish Danio rerio ranging between 1.44 and 24 mg L−1and 19–25 mg L−1, respectively (Den Ouden, 1995), hence categorizing them as "toxic" or "harmful" based on CEC gradation. This justifies the need to examine the toxicity of REEs in other species such as microcrustacean, fish and bivalves.

Sneller et al. (2000) examined available toxicity data for eight REEs (Y, La, Ce, Pr, Nd, Sm, Gd, Dy) to determine maximum permissible concentrations for freshwater, saltwater and sediment. For freshwater acute toxicity of these REEs, they reported values ranging from 1.3 to 22 mg L−1 for crustaceans (D. magna 48 h-EC50s), from 14 to 25 mg L−1 for fish (D. rerio 96 h LC50s), and a value of 1.3 mg L−1 for a micro-algal species (Scenedesmus subspicatus 72 h EC50). In terms of freshwater chronic toxicity, they also provided values for four REEs (Y, La, Nd, Dy) ranging from 0.1 to 3.1 for crustaceans (D. magna 21-d NOECs), from 1.2 to 3.8 mg L−1 for fish (D. rerio 30-d NOECs), as well as a value of 0.26 mg L−1 for the carp Cyprinus carpio (21-d NOEC). For these interspecies responses, (CEC Commission of the European Communities, 1996) REE hazard potential thus lies in the "harmful" to "toxic" and in the "toxic" to "very toxic" categories based on acute and chronic toxicity, respectively.

A recent review on the aquatic toxicity of lanthanum (La), offers some insights on multiple endpoint responses (i.e., NOECs, ECx and LCx) of various taxonomic groups for this REE after exposure to various chloride, nitrate, oxide and carbonate salts of La (Hermann et al., 2016). Ciliate, macrophyte and Danio rerio fish toxicity data proved to be the least sensitive to La exposure (endpoint values ranging from 5 to 278 mg L−1). In contrast, for crustaceans (mortality data ranging from 0.55 to 1.59 mg L−1for Daphnia species and from 0.04 to 5 mg L−1 for Ceriodaphnia dubia), micro-algae (growth inhibition data ranging from 0.45 to 16 mg L−1for several chlorophyte species), fishes (96 h-LC50 value of 1.13 mg L−1for the rainbow trout Oncorhynchus mykiss) and decomposers (growth inhibition data for the bacterium Microcystis aeruginosa ranging from 1 to 2.5 mg L−1), toxicity responses were shown to be within, or close to, the 96 h-LC50 Hydra value of 0.21 mg L−1obtained for La in our study.

The LC50s display a decreasing trend in toxicity as REE atomic number increases, except for the last two heaviest REEs (Er and Lu) where an increasing toxicity trend reappears

(Table 1). EC50s also generally exhibit a decreasing trend in toxicity with increasing REE atomic number. In fact, for the 11 REEs, EC50 values are positively correlated with atomic number (Spearman rank r=0.75; p< 0.05) and negatively correlated with ionic radius (Spearman rank r=-0.732; p < 0.05). This suggests that larger ionic radius for trivalent elements contributed to the sublethal toxicity in Hydra.

No similar significant correlation is found for LC50s and atomic number or ionic radius when correlation analysis is conducted on the 11 REEs (Fig. 3A). However, when Er and Lu LC50 values, the two heaviest lanthanides, are excluded, significant correlations are found as for the EC50 data (Fig. 3B). Indeed, LC50 values for the first 9 REEs are now positively correlated with atomic number (r=0.68; p< 0.05) and negatively correlated with ionic radius (r=-0.76; p < 0.05). Overall, it thus appears that atomic number and ionic radius have some influence on REEs Hydra toxicity responses. Similarly, when H. Azteca was exposed to estimated free ions of 14 REEs, toxicity was shown to decrease with increasing atomic number, except for the three heaviest lanthanides (Tm, Yb, Lu) where a reversing trend occurred (Borgmann et al., 2005). To our knowledge, all other (limited) studies performed on groups of lanthanides with freshwater organisms (micro-algae, invertebrates and fish) failed to show discernible trends in toxicity based on REE atomic number (Gonzalez et al., 2014).

In conclusion, this work has shown that H. attenuata is highly sensitive to REE exposure and, based on previous publications, to other metallic compounds in general (i.e., heavy metals and nanometallic particles). When other investigations undertaken at different biological levels were compared with Hydra toxicity testing REEs results, close sensitivities were particularly noted with other invertebrates including D. magna (Sneller et al., 2000), H. Azteca (Borgmann et al., 2005) and C. dubia (Hermann et al., 2016), suggesting that members of this community are likely more at risk to eventual REE discharges in aquatic environments. Additionally, two properties of REEs were shown to modulate Hydra (sub)lethal toxicity which decreases with increasing atomic number and with decreasing ionic radius (Table 1). The influence of 7 REEs (Ce, Er, Sm, La, Y, Nd and Gd) were examined in rainbow trout juveniles also revealed that sub-lethal effects occurred at concentration relatively close to concentrations leading to morbidity (Gagné, 2016). For example, gene expression changes at concentrations between 0.06 mg L−1 for Y (a gene involved growth arrest induced by DNA damage-GADD45) and 40 mg L−1 for Ce (GADD45 and ammonia metabolism glutamate dehydrogenase-GLUD) which suggests that sublethal effects at the gene expression levels involved DNA repair activity and ammonia metabolism at concentrations at one order of magnitude lower than the acute toxic concentration (fish LC20). These sub-lethal concentrations are environmentally realistic since concentrations from 0.001 to 0.14 mg L−1were detected in freshwaters (Weltje et al., 2012) suggesting that these elements could produce effects in the environment. Interestingly, light REEs with atomic number between 57 and 61 where more abundant than the heavier ones. It is noteworthy that low atomic number was associated with higher toxicity (lower LC50 values) and toxicity was higher with higher ionic radius which does not change proportionally with the atomic number (Table 1). Finally, further research is underway to get better insights into REE modes of action as

concerns H. attenuata with biomarker measurements carried out on animals having survived exposure to selected lanthanides."

Acknowledgments

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Table 1. Lanthanides tested, chemical characteristics and corresponding lethal and sublethal ecotoxicity data (expressed in mg/L).

Lanthanide	Atomic number	Ionic radius (for 3$^+$ ions)	96h-LC50 (CI)[a]	96h-EC50 (CI)[b]	LC50/EC50 ratio
Yttrium (III) chloride hexahydrate	39	106	0.22 (0.18-0.28)	0.03 (0.02-0.04)	7.3
Lanthanum (III) chloride heptahydrate	57	122	0.21 (0.19-0.23)	0.07 (0.05-0.09)	3
Cerium (III) chloride heptahydrate	58	107	0.33 (0.24-0.45)	0.05 (0.03-0.07)	6.6
Praseodymium (III) chloride	59	106	0.56 (0.41-0.76)	0.020 (0.016-0.025)	28
Neodynium (III) chloride hexahydrate	60	104	0.31 (0.25-0.39)	0.09 (0.06-0.13)	3.4
Samarium (III) chloride hexahydrate	62	100	0.77 (0.65-0.92)	0.18 (0.13-0.25)	4.3
Gadolinium (III) chloride hexahydrate	64	97	0.52 (0.43-0.63)	0.10 (0.07-0.15)	5.2
Terbium (III) chloride hexahydrate	65	93	0.70 (0.57-0.88)	0.10 (0.07-0.14)	7
Dysprosium (III) chloride hexahydrate	66	91	0.69 (0.56-0.84)	0.27 (0.22-0.32)	2.6
Erbium (III) chloride hexahydrate	68	89	0.40 (0.32-0.57)	0.10 (0.07-0.14)	4
Lutetium (III) chloride hexahydrate	71	85	0.29 (0.22-0.36)	0.10 (0.07-0.15)	2.9

a) Concentration of Ln salts producing a 50% lethality effect (Lethal Concentration or LC50) in exposed *Hydra* after a 96h exposure period, along with their 95% confidence intervals (CI).

b) Concentration of Ln salts producing a 50% sub-lethality effect (Effective Concentration or EC50) in exposed *Hydra* after a 96h exposure period, along with their 95% confidence intervals (CI).
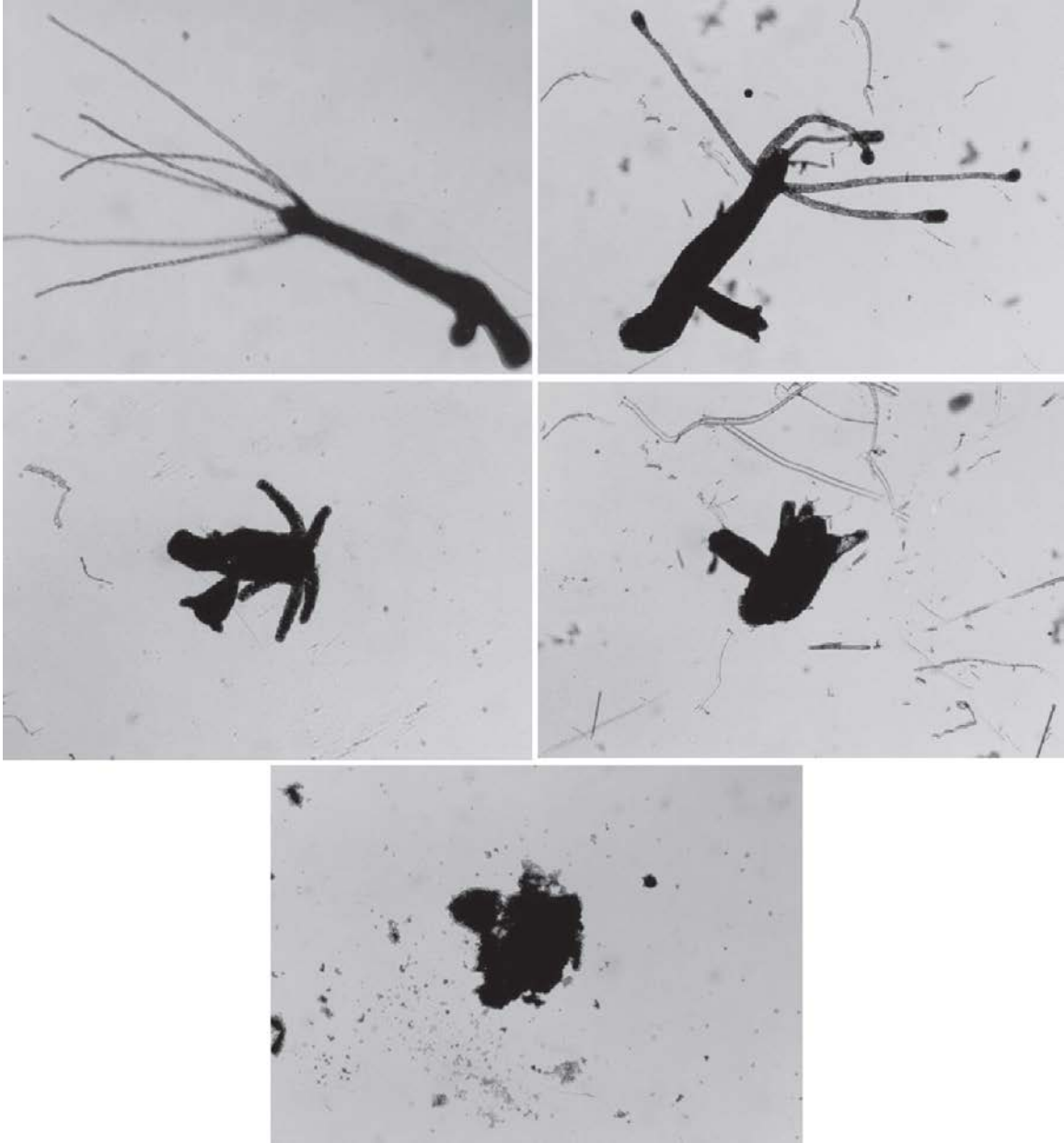
Fig. 1. Morphological stages in Hydra attenuate after exposure to REEs for 96 h (magnification x40): healthy polyp (A); clubbed tentacles (B); shortened tentacles(C); tulip stages (D) and disintegrated stage (E). Scale bar.
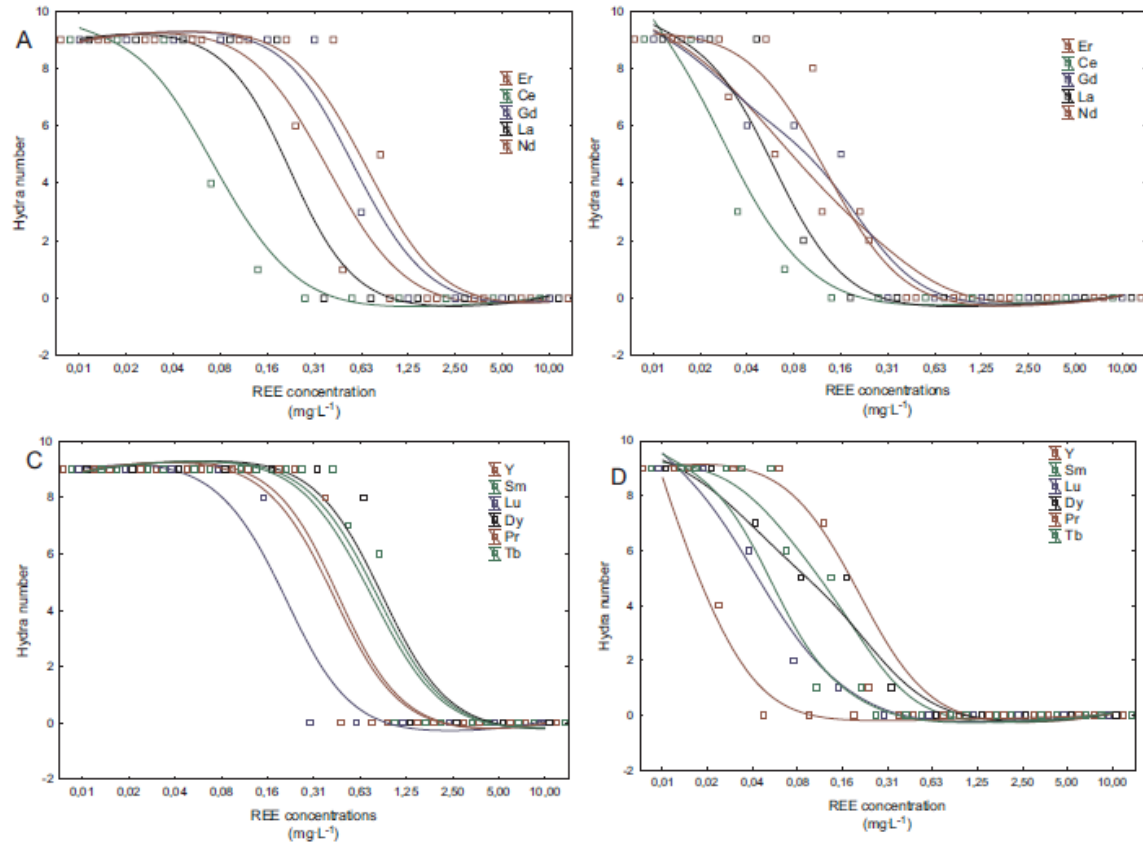
Fig. 2. Hydra attenuata lethality and sublethality ecotoxicity plots for the 11 REE. The data are expressed as the number of Hydra that shows no (sub)lethal morphological changes. The lethality data are shown on the left column (A, C) and consists in the appearance of Hydra in either the tulip and disintegrated stages. The corresponding sublethal data are locater on the right column (B, D) and consists of Hydra with reversible morphological stages (clubbed and shorten tentacles). The derived LC50 and EC50 are reported in Table 1.
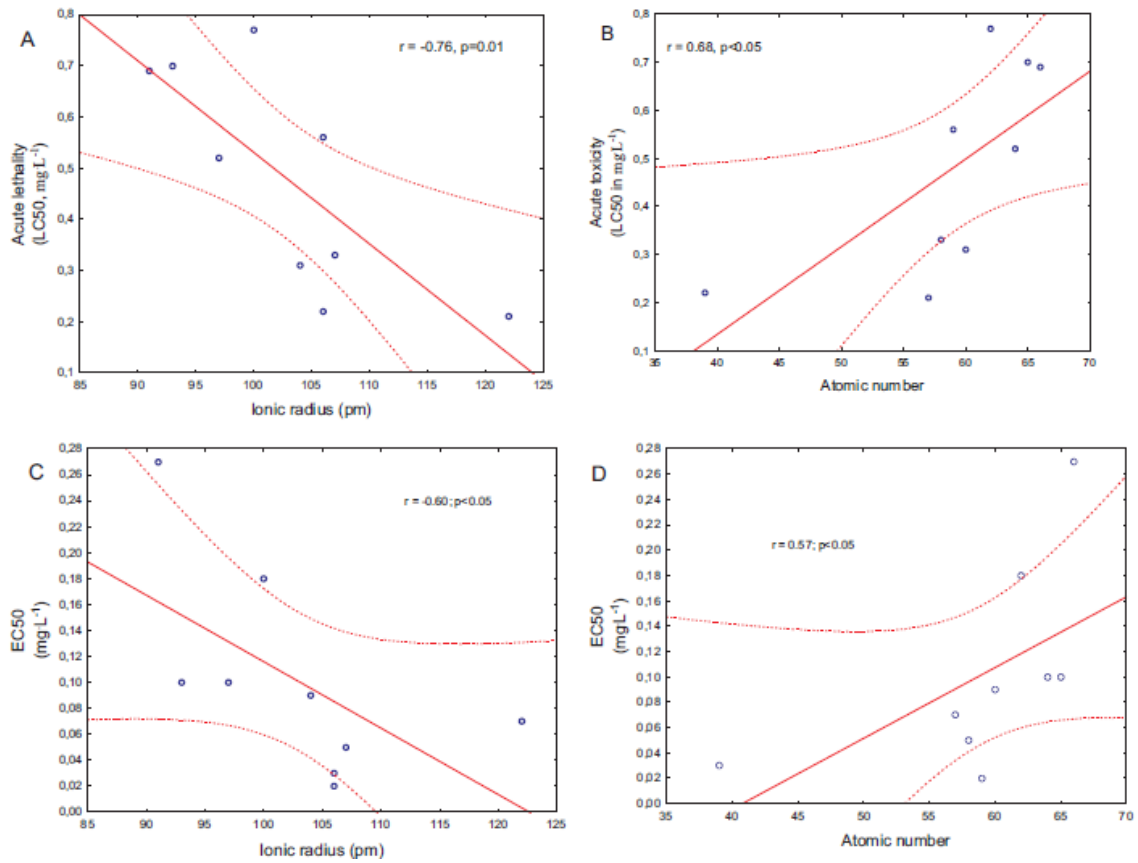
Fig. 3. Correlation of REEs lethality and toxicity with atomic number and ionic radii respectively. Pearson-moment correlations shown between the acute toxicity LC50 value with ionic radius (A) and atomic number (B) and sublethal effects (EC50) with ionic radius (C) and atomic number (D). The dotted lines represent the 95% confidence interval of the regression.