Cover Letter

Dear Editor,

we would like to submit to Science of the Total Environment Special Issue on Rare Earth Elements the paper: Cerium, gadolinium, lanthanum, and neodymium effects in simplified acid mine discharges to *Raphidocelis subcapitata*, *Lepidium sativum,* and *Vicia faba*

For the first time, we investigated the tole of pH in changing the effects of cerium, gadolinium, lanthanum, and neodymium in a simplified acid mine discharge.

The alteration of rare earth elements (REEs) biogeochemical cycles has increased the potential effects related to their environmental exposure in a one-health perspective. Cerium (Ce), gadolinium (Gd), lanthanum (La), and neodymium (Nd) are frequently related to technological applications and their environmental concentrations are already in the μg/kg – mg/kg (i.e., or L) range depending on the considered matrices (i.e., acid mine discharge (AMD), wastewater, sediment, and soil). The effect of Ce, Gd, La, and Nd was investigated in a simulated AMD (0.01-10.22 mg/L) at pH 4 and 6 considering a battery of photosynthetic organisms (*Raphidocelis subcapitata*, *Lepidium sativum*, and *Vicia faba*) according to a multiple-endpoint approach (growth inhibition, germination index, and mutagenicity). According to modelled chemical speciation, the considered elements were mostly in the trivalent free form (86-88%) at pH 4. Gd, La, and Nd exerted the most relevant toxic effect at pH 4. The pH 6 scenario evidenced a reduction in REEs toxicity level. Mutagenicity was detected only at pH 4 by Gd (up to 3-fold compared to negative controls), La and Nd, while Ce did not show any adverse effect. Toxic effects due to Ce, Gd, La, and Nd can be reduced by controlling the pH, but several gaps into the knowledge still remain about their uptake and trophic transfer, and long-term effects on targeted species.

Prof. Giovanni Libralato on behalf of all co-authors

Cerium, gadolinium, lanthanum, and neodymium effects in simplified acid mine discharges to *Raphidocelis subcapitata*, *Lepidium sativum,* and *Vicia faba*

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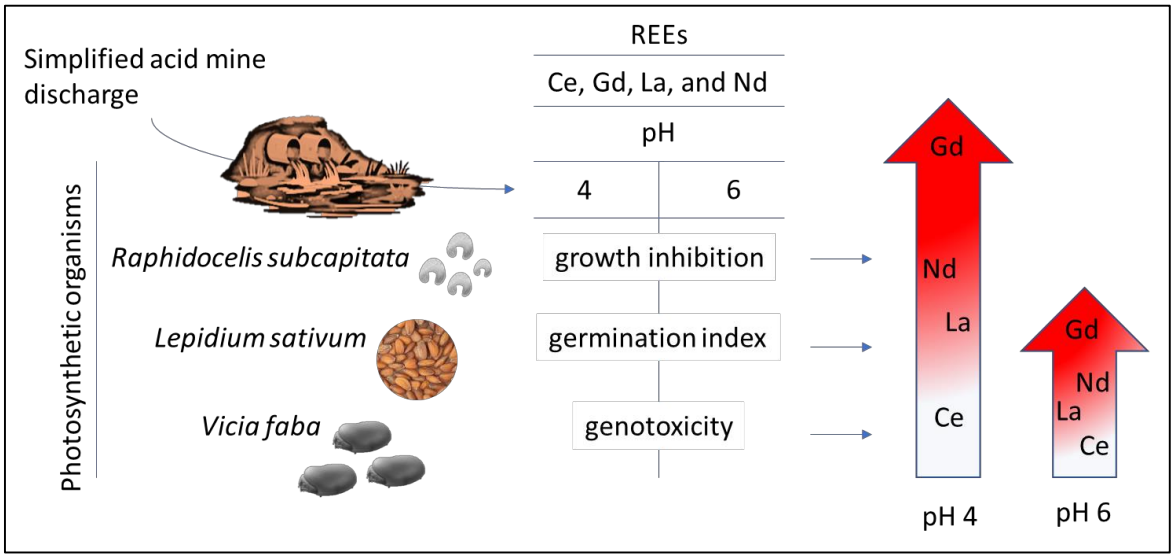
Abstract

The alteration of rare earth elements (REEs) biogeochemical cycles has increased the potential effects related to their environmental exposure in a one-health perspective. Cerium (Ce), gadolinium (Gd), lanthanum (La), and neodymium (Nd) are frequently related to technological applications and their environmental concentrations are already in the μg/kg – mg/kg (i.e., or L) range depending on the considered matrices. The effect of Ce, Gd, La, and Nd was investigated in a simulated AMD (0.01-10.22 mg/L) at pH 4 and 6 considering a battery of photosynthetic organisms (*Raphidocelis subcapitata*, *Lepidium sativum*, and *Vicia faba*) according to a multiple-endpoint approach (growth inhibition, germination index, and mutagenicity). According to modelled chemical speciation, the considered elements were mostly in the trivalent free form (86-88%) at pH 4. Gd, La, and Nd exerted the most relevant toxic effect at pH 4. The pH 6 scenario evidenced a reduction in REEs toxicity level. Mutagenicity was detected only at pH 4 by Gd (up to 3-fold compared to negative controls), La and Nd, while Ce did not show any adverse effect. Toxic effects due to Ce, Gd, La, and Nd can be reduced by controlling the pH, but several gaps of knowledge still remain about their uptake and trophic transfer, and long-term effects on targeted species.

Keywords

Phytotoxicity; mutagenicity; rare earth elements; pH; modelled speciation

Graphical abstract



Highlights

- Low pH values can significantly increase the toxicity of Gd, La, and Nd
- Mutagenicity was evidenced for Gd, La, and Nd at pH 4
- Toxicity at pH 6 was significantly lower than at pH 4 on a multi-endpoint basis
- The sensitivity of the considered biological models was: *R. subcapitata* > *V. faba* > *L. sativum*

57 1. Introduction

58 Rare earth elements (REEs) are key components of many emerging technologies in industry,
59 agriculture, and medicine due to their unique physical and chemical properties (Gwenzi et al., 2018;
60 Pagano et al., 2015b; Romero-Freire et al., 2018; Takaya et al., 2018). Concerns are rising about the
61 potential increased alterations of REEs biogeochemical cycles due to Anthropocene (Galdiero et al.,
62 2019a; Moreira et al., 2020). At present, five REEs are labelled as critical for energy production
63 (neodymium, europium, terbium, dysprosium, and yttrium) and two additional ones as nearly critical
64 (cerium and lanthanum) (Romero-Freire et al., 2018; US DOE, 2012). Currently, REEs do not present
65 any threshold limit values for national and international regulations, but they can be considered as
66 new emerging contaminants with still unknown effects (Galdiero et al., 2019a). Two main sources of
67 REEs can be found: 1) direct from active or non-active mining ores activities; 2) indirect from mineral
68 processing and industrial use including the waste cycle (Gwenzi et al., 2018).

69 REEs can be considered as lithophile elements substituting other cations of comparable radius and
70 charge in several mineral structures like silicates, carbonates, oxides, phosphates, and related
71 oxyhydroxysalts (Migaszewski and Gałuszka, 2015). The principal mineral sources of REEs are
72 bastnaesite, monazite, and loparite and the lateritic ion-adsorption clays (Balaram, 2019). Although
73 REEs are abundant in the earth's crust ("not rare"), the ability for mining tends to make them very
74 scarce (de Boer and Lammertsma, 2013; Thomas et al., 2014). REEs are not individual native metals,
75 but they occur together in numerous ore/accessory minerals as either minor or major constituents.
76 Sites in areas impacted by mining activities, not only related to REEs extraction, and industry have
77 been shown to contain REEs with concentrations up to 100 times higher than normal background
78 levels (Gwenzi et al., 2018). Mining activities such as cutting, drilling, blasting, transportation,
79 stockpiling, and processing have been linked to severe environmental and health damages in countries
80 such as China, United States of America (USA), India, Malaysia, and Brazil (Adeel et al., 2019;
81 Balaram, 2019; Galhardi et al., 2020; Liang et al., 2014). Acid mine drainage (AMD) has recently
82 raised a great deal of attention as a potential significant source of REEs directly able to affect

environmental and human health, if not adequately collected and treated. AMD is composed of acidic wastewater (i.e., approximately pH < 5, but it depends on a site-by-site basis) presenting a generally high amount of sulphate and other metals in a dissolved form including rare earth elements (REEs) ranging from ng/L up to thousands of mg/L and more on a site-specific basis (Migaszewski and Gałuszka, 2015). The median concentration of REEs in European Union stream sediment was 198.9 mg/kg (Salminen, 2005), reaching 457.7 µg/L in USA ore mine effluent and 61.3 µg/L in China coal mine effluent, while in surface water their concentration ranged from 75.03 µg/L up to 518.7 µg/L (Migaszewski and Gałuszka, 2015). Zhao et al. (2007) indicated that the REE-sulphate complexes are the main form of dissolved REEs concentration in acid mine wastewater representing more than 60% of the total amount, followed by free metal species form. The presence of REEs in AMD and its acidic pH can increase their mobility and bioavailability in the various environmental compartments with potential negative effects on a one-health approach, especially in mining areas (i.e., both active or abandoned sites) (Cravotta III, 2008; Rim et al., 2013; Stewart et al., 2017; Sun et al., 2017). When the intensity of acidity reaches a baseline ecotoxicity threshold, it can affect organisms through direct acute damage and indirect acidified soil and water (Gonzalez-Gil et al., 2012; Li et al., 2020; Lopes et al., 1999; Xia et al., 2017). Thus, the combined REEs pollution and acid conditions from AMD could adversely affect the structure and function of aquatic ecosystem changing the productivity and the abundance in biomass or even could lead to the elimination of aquatic species (Bott et al., 2012; Kraus and Pomeranz, 2020).

Relatively scarce information is available to date on REEs-associated biological effects, including bioassays on model organisms, and human health effects (Galdiero et al., 2019b; Gravina et al., 2018; Pagano et al., 2015a; Pagano et al., 2015b). A recognized mechanism of action in REEs-associated health effects relates to modulating oxidative stress including various endpoints such as growth inhibition, cytogenetic effects, and organ-specific damage (Manier et al., 2013; Tai et al., 2010). Even less is known about the REEs toxicity at low pH levels. Only few papers evidenced that low pHs (i.e., mine wastewater) can modify their biological activity increasing aquatic toxicity (Haferburg et al.,

2007; Romero et al., 2010), where a relevant role is also played by organic and inorganic ligands (Thomas et al., 2014). In particular, primary producers can be highly sensitive indicators of toxic effects due to the aquatic exposure to REEs dissolved in AMD. As a parallelism, we must remember that the notorious Itai-Itai disease was caused by the consumption of rice contaminated by cadmium from cultures irrigated with AMD (Inaba et al., 2005). d'Aquino et al. (2009) stated that few data about REEs effects on macrophytes were available from the scientific literature and, to the best of our knowledge, such scarcity persist today. The need to investigate photosynthetic organisms in relation to AMD contamination (i.e., surface water polluted by uncontrolled and untreated AMD, and direct irrigation with contaminated AMD) pushes ahead the present research topic.

This study evaluated the adverse effects of cerium (Ce), lanthanum (La), gadolinium (Gd), and neodymium (Nd) on *Raphidocelis subcapitata* (green microalgae), and *Lepidium sativum* (macrophyte), and *Vicia faba* (macrophyte) considering a background multi-endpoint approach (i.e., growth inhibition, germination index, and genotoxicity) to check the effect of spiked simplified AMD investigating the role of two pH values (4 and 6) in potential toxicity modification.

2. Materials and Methods

2.1 Analytical methods

Trichloride anhydrous salts of Ce (III), La(III), Gd(III), and Nd(III) were purchased from Sigma-Aldrich (Italy). All chemicals were of analytical grade. Testing solutions were prepared from 1 M stock solutions per element in ultra-pure distilled water stored at 4 °C. Stock solutions were diluted to the final test concentrations using freshwater medium (ISO, 2012a) buffered at pH values 4 and 6. The pH was measured with a pH-meter (Mettler Toledo Five Easy, Milan, Italy). The experimental design included 4 exposure concentrations (i.e., 0.01, 0.1, 1, and 10 mg/L – nominal concentrations). Real concentrations were determined by Inductively Coupled plasma mass spectrometry (ICP-MS, Aurora Bruker M90, Bremen, Germany) following previously established protocols and quality assurance and quality control laboratory procedures according to Pagano et al. (2016). The limits of

detection (LOD) and quantification (LOQ) were as follows for Ce, Gd, La, and Nd: 0.0010, 0.0018, 0.0006, and 0.0011 µg/L as LOD; and 0.0035, 0.0058, 0.0021, and 0.0037 µg/L as LOQ. Analyses were carried out in triplicate.

2.2. Algal growth inhibition test

The algal growth inhibition test (72 h) with *R. subcapitata*, formerly known as *Selenastrum capricornutum* and *Pseudokirchneriella subcapitata*, was carried out based on ISO (2012a). The algal density was determined by spectrophotometric analysis (DR5000, Hach Lange GbH, Weinheim, Germany). The percentage inhibition of the cell growth (IG, %) was calculated as the difference between the growth rate of the control and of the sample and expressed as the mean (± standard deviation). Toxicity tests were carried out in triplicate.

2.3 Phytotoxicity test

The *L. sativum* germination and root elongation toxicity tests were performed according to ISO (2012b)). Macrophyte seeds (n = 10) were exposed on filter paper Whatman n. 1 imbibed with 3 mL of testing solution in triplicate in Petri dishes. Samples were incubated at 25 ± 1 °C in darkness and the number of seeds germinated and the length of the developing roots were measured after 3 days. Controls were carried out in distilled water. Germination (%), and root elongation inhibition were combined to calculate the germination index (GI, %) (Libralato et al., 2016).

2.4 Micronucleus test

V. faba was investigated for genotoxicity according to ISO (2013). Macrophyte seeds (n = 5) were exposed on filter paper Whatman n. 1 imbibed with 6 mL of testing solution in triplicate in Petri dishes. .

After incubating in the dark at 22 ± 2 °C for 96 h, the root tips of germinated seeds were fixed for 24 h in 1:3 acetic acid: ethanol solutions, then were cut, stained in Schiff's Reagent using Feulgens

161 method, and squashed on microscope slides (ISO, 2013). The micronucleus frequency MCN (%) was
162 evaluated in 10^3 cells from *V. faba* seeds using ImageJ (Schindelin et al., 2015).
163
164 2.5 Data analyses
165 Median effect concentrations (EC50), EC5 and EC10 were calculated as mean values and relative
166 95% confidence limit values (Galdiero et al., 2019b), for *R. subcapitata* and *L. sativum*. Differences
167 between treatments were assessed via one-way analysis of variance (ANOVA) after the verification
168 of normality (Shapiro-Wilk's test) and homoscedasticity (Levene's test). The *post-hoc* Tukey's test
169 accounted for differences within groups setting the statistical significance at $p < 0.05$. Statistical
170 analysis was carried out via SigmaPlot (Systat Software, San Jose, CA).
171
172

173 3. Results and discussion

174 All endpoints were calculated on real concentrations summarized in Table S1 (Supplementary
175 Materials). The use of Visual MINTEQ 3.1 (Gustafsson, 2012) allowed to model the speciation of
176 Ce, Gd, La, and Nd considering reconstructed freshwater according to ISO (2012a) and the two fixed
177 pH values (4 and 6). According to the database in Supplementary Materials (Table S2), most of the
178 considered REEs were present in the trivalent dissolved free forms (86-88%) at pH 4 (i.e., Ce 88%,
179 Gd 86%, La 87%, and Nd 87%), which are the most bioavailable ones. At pH 6, the free trivalent
180 forms are still present, but with significant reductions compared to pH 4 like for Ce (75%), Gd (58%),
181 La (81%), and Nd (74%). The lower amount of Ce, Gd, La, and Nd in the free form at pH 6 might
182 have influenced their bioavailability and the subsequent effects in the exposed biological models.

183 In Figure 1, the results of the IG (%) of *R. subcapitata* were reported at pH 4 and 6, respectively, for
184 Ce (Figure 1 A and B), Gd (Figure 1 C and D), La (Figure 1 E and F), and Nd (Figure 1 G and H). A
185 linear regression model was considered to fit data concentration-response relationships. All equations
186 and the relative standard errors were included in Figure 1 (A-H). These equations allowed the
187 determination of EC50, EC10, and EC5 that were summarized for both pH values in Table 1.

188 For Ce, La and Nd, biostimulation effects were detected at the first two lowest exposure
189 concentrations for both pH 4 and 6, and also for Gd at pH 6. Microalgae growth impairment occurred
190 for Ce, La, and Nd at 1 and 10 mg/L (nominal concentrations) at pH 4 and 6, and for Gd at pH 6. For
191 microalgae exposed to Gd at pH 4, all exposure concentrations evidenced a concentration-response
192 significant toxic effect up to 73% at 5.72 mg/L. REEs biostimulation effects in unicellular green algae
193 were already reported for nano-CeO_2 considering photosynthesis inhibition and ROS formation as
194 endpoints (Rodea-Palomares et al., 2012) and $Ce(NO_3)_3$ for growth inhibition (Aharchaou et al.,
195 2020).

196 The exposure to Ce at pH 4 showed effects not significantly different from the exposure at pH 6 with
197 a correlation coefficient of $R^2 = 0.93$ (Figure 1 A and B). Only, at 0.137 mg/L the effect of pH 6
198 exposure was still biostimulation and significantly different ($p < 0.001$) from the same concentration

199 at pH 4 treatments being about 25% lower. Ce effects ranged between -10% (0.01 mg/L) and 64%
200 (10.225 mg/L). Indeed, the EC50 of Ce at pH 4 was 3.15 (1.36-7.19) mg/L and Ce EC50 at pH 6 was
201 4.75 (0.04-10.99) mg/L (Table 1). These EC50 values are not significantly different ($p > 0.05$).
202 For Gd, a significant difference in the concentration-response curves can be observed in Figure 1 (C
203 and D), at pH 6 the EC50 value in the investigated concentration range cannot be detected and only
204 EC5 and EC10 values were calculated (i.e., maximum effect of 23% at 5.72 mg/L). At pH 4 (Figure
205 1 C), significant differences ($p <0.001$) between treatments were highlighted. The maximum detected
206 effect was 73% at 5.72 mg/L. Gd EC50 at pH 4 was 0.267 (0.01-5.30 mg/L). For Gd exposure at pH
207 6, only EC5 and EC10 values were calculated (Table 1).
208 For La, effects ranged between -6% (0.01 mg/L) and 21% (5.32 mg/L) (Figure 1 E and F). At 0.01
209 mg/L and 0.12 mg/L of La no significant differences ($p > 0.05$) were observed between treatments at
210 pH 4 and pH 6. At 1.207 mg/L, the inhibitory effect of pH 6 exposure was significantly different (p
211 < 0.01) from the corresponding concentration at pH 4 treatments being about 8% greater. On the
212 contrary, at 5.321 mg/L, the inhibitory effect of pH 6 exposure was significantly different ($p < 0.001$)
213 from the 5.321 mg/L at pH 4 being about 10% lower. The EC50 after 72 h of exposure was not
214 determined in both pH exposure scenarios, while EC5 and EC10 values were summarized in Table
215 1.
216 About *R. subcapitata* exposure to Nd, the effects varied between 7.4% and 56.7% for pH 4, and -
217 0.9% and 59.7% for pH 6 (Figure 1 G and H). Significant differences ($p < 0.05$) were evidenced
218 amongst treatments at the first two lowest exposure concentrations, while no significant differences
219 ($p > 0.05$) were observed within treatments at the remaining concentrations. At 0.005 mg/L, the
220 inhibitory effect of pH 6 exposure was significantly different ($p < 0.01$) from the same concentration
221 at pH 4 treatments being about 8% lower. At 0.075 mg/L, the inhibitory effect of pH 6 exposure was
222 significantly different ($p < 0.001$) from the 0.075 mg/L at pH 4 being about 14% lower. The EC50
223 values of Nd at pH 4 and 6 were not significantly different ($p < 0.01$) being 1.860 mg/L and 1.856
224 mg/L, respectively (Table 1).

These data were partly in agreement with previous studies (Liang and Wang, 2013; Thomas et al., 2014; Wang et al., 2014). The comparison of the growth inhibition effects at pH 4 and 6 evidenced only limited differences between the two exposure scenarios, except for Gd where low pH values can increase the effect on the targeted species.

REEs were ranked in increasing order of toxicity at pH 4 according to the estimates obtained. For EC50: La < Ce < Gd < Nd; EC10: La < Ce < Nd < Gd and EC5: La < Ce < Nd < Gd. The toxicity trend evidenced that the most toxic elements at pH 4 were Nd and Gd, while La was the less toxic. At pH 6, the general toxicity decreased and kept similar values compared to pH 4. At both pH, Gd and La EC50 values could not be obtained. REEs were ranked in increasing order of toxicity at pH 6, EC50: La ≈ Gd < Ce < Nd; EC10: Gd < La < Nd < Ce and EC5: Gd < La < Ce < Nd.

In general, the ecotoxicity did not always increase with the increase in the atomic number of the investigated REEs with *R. subcapitata* as reported in previous studies (González et al., 2015; Malhotra et al., 2020; Pagano et al., 2015b), and the general scarcity of experimental data can make it difficult to fully discuss. For example, EC50 value of Ce (4.4 mg/L) according to (González et al., 2015) was very similar to both EC50s at pH 4 and 6, but the pH of testing solutions was not displayed, while the Gd EC50s were significantly different compared to González et al. (2015) (1.257 mg/L).

Several authors (Aharchaou et al., 2020; Joonas et al., 2017; Stauber and Binet, 2000) highlighted that the formation of insoluble REEs species in exposure media or of precipitates in the presence of free ion concentration due to the changes in pH levels might be responsible of the differential responses.

In Figure 2, the results about the GI (%) of *L. sativum* were reported at pH 4 and 6, respectively, for Ce (Figure 2 A and B), Gd (Figure 2 C and D), La (Figure 2 E and F), and Nd (Figure 2 G and H). All detailed data about seed germination and root elongation were provided in Table S3 and Table S4, respectively (Supplementary Materials). GI values between 80% and 120% are considered as acceptable, while, if < 80% or > 120% inhibition or biostimulation effects are identified (Libralato et al., 2016). As a general overview of the obtained results, the GI always evidenced inhibitory effects at the two highest tested concentrations for all the investigated REEs at both pHs. Similarly, all GI values were always > 80% and < 120%, so any biostimulation effect was displayed as well.

Considering the exposure of *L. sativum* to Ce (Table 2S), the number of *L. sativum* germinated seeds was 100% in the control test, but when Ce solutions at 0.137 mg/L, 1.431 mg/L and 10.225 were used the number of seeds was reduced of about 80% both at pH 4 and 6. At 0.01 mg/L, the number of germinated seeds was not significantly different from the negative control (< 10% effect). No significant differences ($p > 0.05$) were observed at 0.137 mg/L and 10.225 mg/L of Ce for both pH values, while at 0.01 mg/L and 1.431 mg/L statistical differences in the effects were evidenced ($p < 0.05$) within pH 4 and pH 6 treatments (Figure 2 A and B). Ce germination index ranged between 81% (0.010 mg/L) and 54% (10.225 mg/L) at pH 4, and between 90% (0.010 mg/L) and 64% (10.225 mg/L) at pH 6.

The number of *L. sativum* germinated seeds after Gd exposure significantly ($p < 0.05$) decreased from 0.154 mg/L, up to 5.721 mg/L. Effects of Gd at pH 4 were not significantly different from those at pH 6 with a correlation coefficient of $R^2 = 0.98$ (Figure 2 C and D). The Gd GI ranged between 83% (0.012 mg/L) and 50% (5.721mg/L) at pH 4, and between 86% (0.012 mg/L) and 60% (5.721mg/L) at pH 6.

About the exposure to La, the number of germinated seeds was reduced up to 90% at pH 4 and 80% at pH 6. No significant differences ($p > 0.05$) were evidenced between treatments at the different pHs in the considered concentration range (Figure E and F). At pH 4, only the lowest exposure concentration (0.012 mg/L) showed a GI significantly different (90%) than all other treatments (63%–

78%) being the only one presenting no effect. At pH 6, the highest concentrations (5.321 mg/L and 1.207 mg/L) showed slight adverse effects ranging between 59% and 66%, while the two lowest treatment presented no effect. In Nd exposure, the number of *L. sativum* germinated seeds was reduced up to 80% at 0.710 and 6.510 mg Nd/L for both pH 4 and 6 (Table S3). Significant differences ($p < 0.05$) were observed only at 6.510 mg/L for both pH values, while at 0.005 mg/L, 0.075 mg/L and 0.710 mg/L no significant difference between treatments was detected ($p > 0.05$) (Table S3). The germination index showed a similar toxicity trend to the previous REEs exposure, but with higher toxicity level (45%) at 6.510 mg/L (pH 4). Indeed, the GI values ranged between 45% and 87% for pH 4, while between 55% and 84% for pH 6.

Currently, few data are available about *L. sativum* exposure to REEs including all endpoints. Wang et al. (2007) reported that Ce^{3+} (14 mg/L), La^{3+} (13.8 mg/L), and Nd^{3+} (14 mg/L) in *Lepidium meyenii* enhanced hyperhydricity and the activities of antioxidative enzymes in adventitious shoots like peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), monodehydroascorbate reductase (MDHAR) and glutathione reductase (GR), but most adventitious shoots grew normally. Thomas et al. (2014) highlighted that La and Ce at "high pH" (5.95 ± 0.02 and 6.74 ± 0.03, in that order) had no impact on seed germination in the tested species at any concentration, whereas Ce supplied at "low pH" (4.08 ± 0.02) induced negative effects (i.e., inhibition concentration on 10% exposed population, IC10, mg/kg dry soil (d.s.)) on seed germination in *Asclepias syriaca* (54.6 mg/kg d.s.), *Desmodium canadense* (165.9 mg/kg d.s.), *Panicum virgatum* (166.8 mg/kg d.s.), *Raphanus sativus* (150.4 mg/kg d.s.), and *Solanum lycopersicum* (195.34 mg/kg d.s.).

The frequency distribution of micronuclei in *V. faba* exposed to Ce (0.010 mg/L), Gd (0.012 mg/L), La (0.012 mg/L), and Nd (0.005 mg/L) was reported in Figure 3 for both pH 4 and 6. No significant differences ($p < 0.05$) were evidenced between the negative controls and the treatments at pH 6. At pH 4, effects were significantly different ($p < 0.05$) from negative controls for La, Nd, and Gd being approximately from three- to four-fold compared to negative controls. The MNF confirmed that Gd

299 at pH 4 is significantly toxic like for *R. subcapitata* and *L. sativum*. For Nd, an increased MNF mitotic
300 and chromosomal aberrations in *V. faba* were also evidenced by Jha and Singh (1994), similarly to
301 our findings. For La, Wang et al. (2011) highlighted some hormetic effects in *V. faba*, but the MNF
302 was not investigated. Only Ce presented no mutagenicity effects either at pH 4 or 6. The pH has a
303 significant role in changing the effects of Gd, La, and Nd to *V. faba* inducing mutagenicity at low
304 values (pH 4).
305
306 **4. Conclusions**
307 The effects of Ce, Gd, La, and Nd were assessed in a simplified acid mine discharge investigating the
308 role of pH 4 and 6 in changing the toxicity profiles of three photosynthetic organisms. A multiple-
309 endpoint approach (i.e., growth inhibition, germination index, and mutagenicity) was used to
310 investigate real exposure scenarios. Results evidenced that pH 4 can increase the toxicity of the
311 selected REEs increasing the amount of free trivalent ions compared to pH 6. In summary, the toxicity
312 trends were as follows: i) for microalgae (i.e., considering the EC50 values): 1) La < Ce < Nd < Gd
313 at pH 4; 2) Nd < Ce < Gd ≈ La at pH 6; ii) for *L. sativum* (i.e., considering the GI(%) at the highest
314 exposure concentration): 1) La < Ce < Gd < Nd at pH 4; 2) Ce < Gd ≈ La ≈ Nd at pH 6; iii) for *V.*
315 *faba* (i.e., MNF): 1) Ce < La ≈ Nd < Gd at pH 4; 2) Ce ≈ La ≈ Nd ≈ Gd at pH 6. The sensitivity of
316 the considered biological models was *R. subcapitata* > *V. faba* > *L. sativum*, suggesting that
317 microalgae can have an important role as well as *V. faba* in the risk assessment of REEs.
318 Gd was the most toxic element at pH 4, followed by La and Nd, and Ce. At pH 6, their effects
319 significantly decreased, and Nd evidenced the highest toxicity. Gd, La and Nd evidenced at pH 4 their
320 potential mutagenicity, that was not present at pH 6. According to the considered exposure scenarios,
321 potential significant negative effects could be exerted especially by Gd, La, and Nd in acidic aquatic
322 environments to microalgae and macrophytes, but they can be reduced by controlling the pH.

Several gaps into the knowledge still remain about REEs toxicity effects and their potential uptake by aquatic species including the transfer through the food web and potential mechanism of adaptation and detoxification.

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Table 1 EC5, EC10, and EC50 values for cerium (Ce), gadolinium (Gd), lanthanum (La), and neodymium (Nd) at pH =4 and 6; values are in mg/L; n.a. = not available; REEs =rare earth elements; EC = effective concentration; average EC values are provided ± 95% confidence limit values in brackets (n = 3).

pH	REEs	EC5	EC10	EC50
4	Ce	0.04 (0.02-0.10)	0.07 (0.03-0.16)	3.15 (1.36-7.19)
	Gd	0.0004 (0.000009-0.012)	0.0008 (0.00002- 0.02)	0.267 (0.009-5.30)
	La	0.256 (0.007-1.15)	0.751 (0.02-3.21)	n.a.
	Nd	0.004 (0.0001-0.096)	0.008 (0.0003-0.187)	1.860 (1.036 to 3.34)
6	Ce	0.09 (0.00- 0.25)	0.014 (0.00- 0.38)	4.75 (0.04- 10.99)
	Gd	0.553 (0.012-0.096)	1.136 (0.023- 0.211)	n.a.
	La	0.196 (0.002-0.37)	0.398 (0.005-0.714)	n.a.
	Nd	0.01 (0.001-2.16)	0.0276 (0.002-3.64)	1.856 (0.973 – 3.54)

Figure 1 Concentration-response relationship at pH 4 and 6 of Ce (A and B), Gd (C and D), La (E and F), and Nd (G and H) exposed to *R. subcapitata*; concentrations in the x-axis are expressed as mg/L: IG = inhibition of growth.

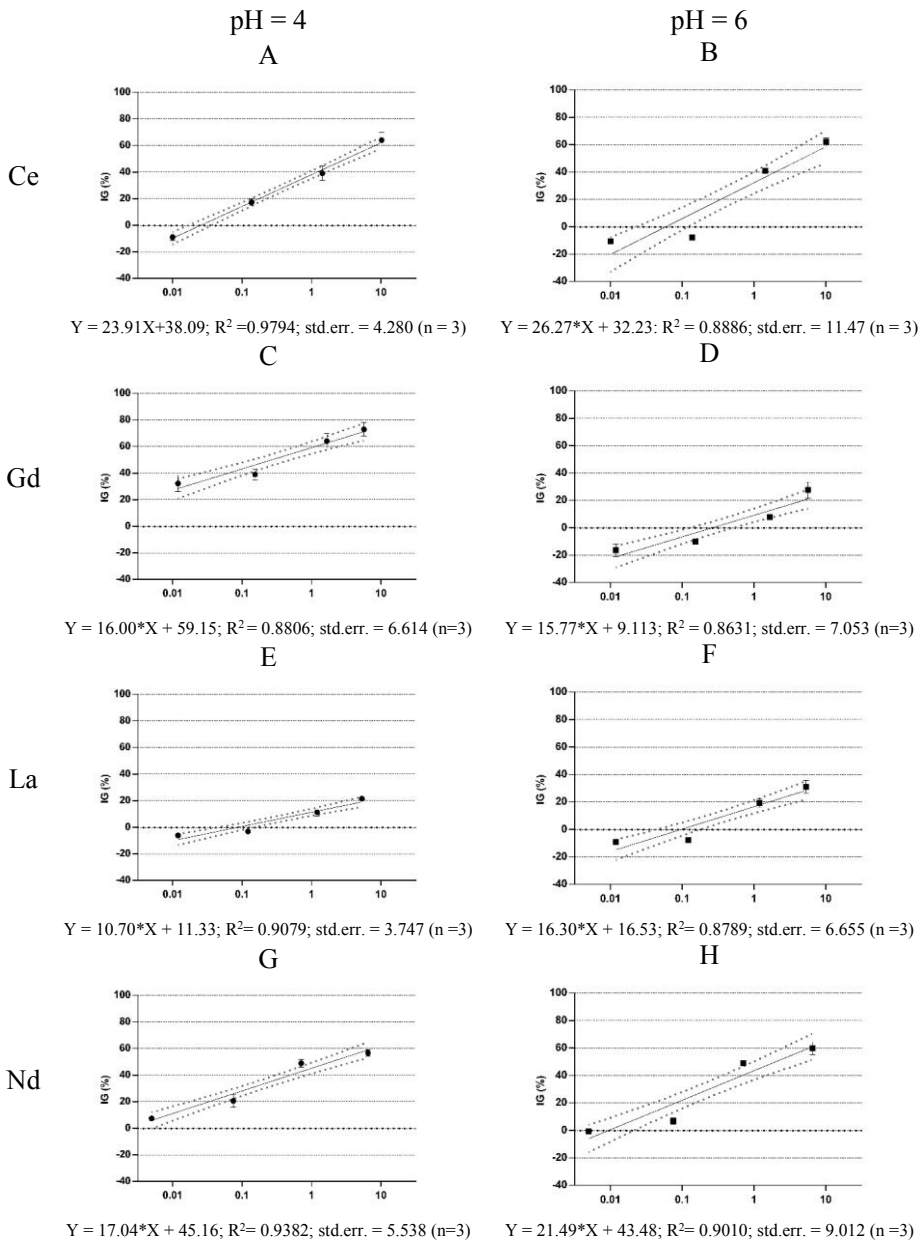
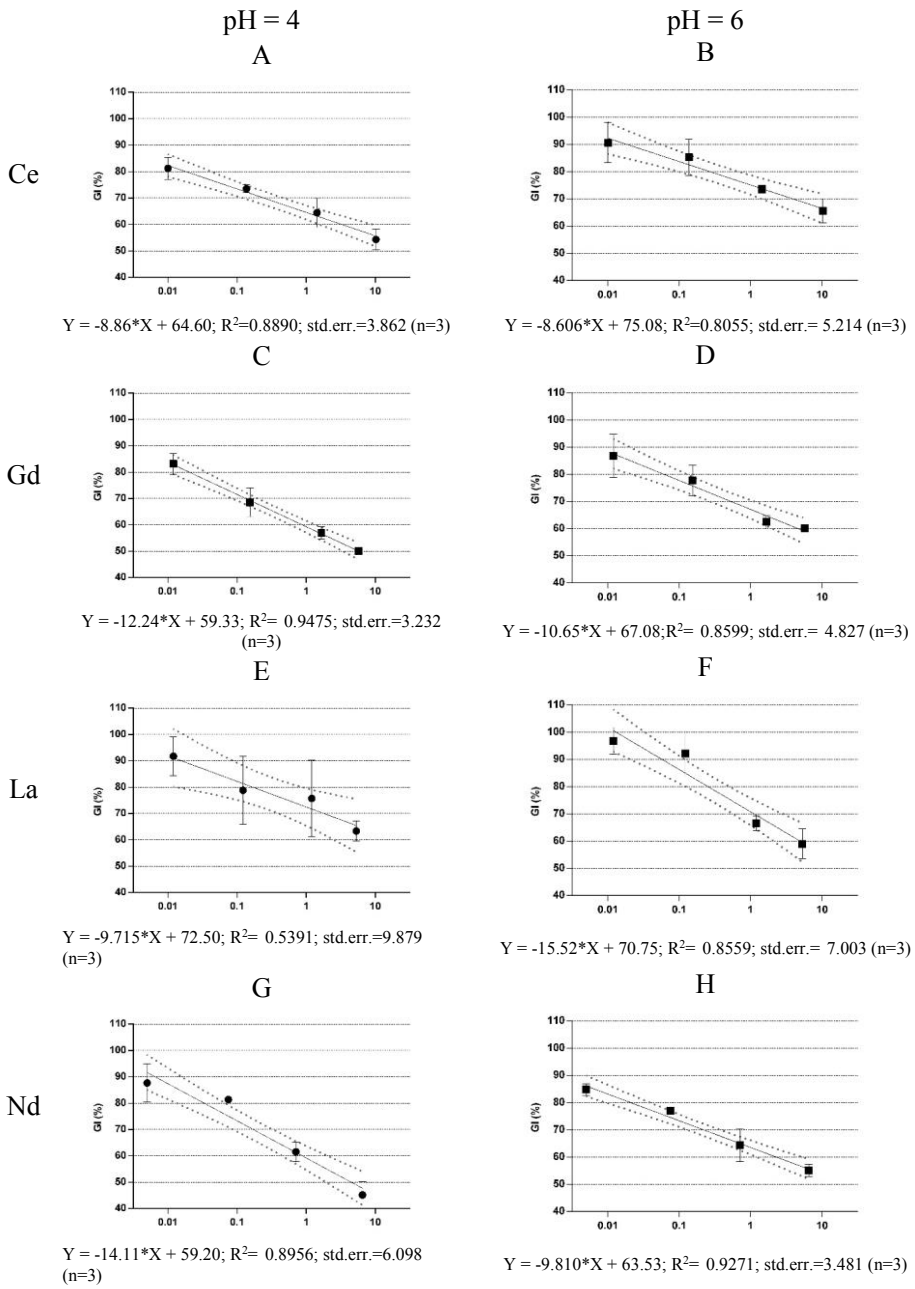
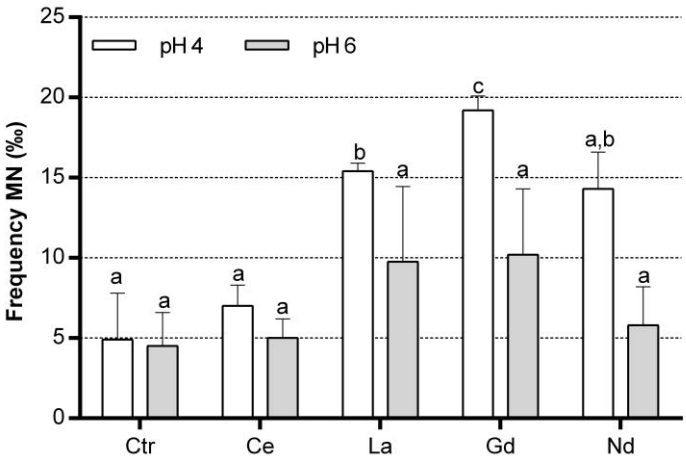


Figure 2 Concentration-response relationship at pH 4 and 6 of Ce (A and B), Gd (C and D), La (E and F), and Nd (G and H) exposed to *L. sativum*; concentrations in the x-axis are expressed as mg/L; GI = germination index.



Formatted Table

Figure 3 Frequency of micronuclei (MC) in *V. faba* root exposed to Ce (0.010 mg/L), Gd (0.012 mg/L), La (0.012 mg/L), and Nd (0.005 mg/L) at pH = 4 and 6; letters (a-c) correspond to significantly different data (Tukey's test, $p < 0.05$); ctr = negative control; error bars indicate standard errors (n=3).



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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: