Science of the Total Environment

Long-term multi-endpoint exposure of the microalga Raphidocelis subcapitata to lanthanum and cerium

--Manuscript Draft--

Manuscript Number:	STOTEN-D-21-04195R2
Article Type:	VSI:REEs in aquatic systems
Keywords:	Microalgae; Biomarkers; Rare earth elements; Bioconcentration
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Abstract:	Significant release of rare earth elements (REEs) into the environment is mainly due to active or abandoned mining sites, but their presence is globally increasing due to their use in several industrial sectors. The effects on primary producers as Raphidocelis subcapitata are still limited. This research focused on La and Ce as the two most widespread REEs that can be currently found up to hundreds of μg/L in water and wastewater. Microalgae were exposed to La and Ce for 3 days (pH = 7.8) (short-term exposure) to derive the effective concentrations inhibiting the growth on 10% (EC10) of the exposed population. EC10 values (0.5 mg/L of La and 0.4 mg/L of Ce) were used for the 28 days long-term exposure (renewal test) to observe after 7, 14, 21, and 28 days on a multi-endpoint basis microalgae growth inhibition (GI), biomarkers of stress (reactive oxygen species (ROS), superoxide dismutase (SOD), and catalase (CAT)), and bioconcentration. Results evidenced that La and Ce EC10 increased GI (day 28) up to 38% and 28%, respectively. ROS, CAT, and SOD activities showed differential responses from day 7 to day 14, 21, and 28, suggesting, in most of the cases, that La and Ce effects were counteracted (i.e., being the values at day 28 not significantly different, $p > 0.05$, from the relative negative controls), except for La-related ROS activities. La and Ce significantly bioconcentrated in microalgae populations up to 2- and 5-fold (i.e., at day 28 compared to day 7), in that order. Bioconcentrated La and Ce were up to 3157 and 1232 μg/g dry weight (day 28), respectively. These results suggested that low La and Ce concentrations can be slightly toxic to R. subcapitata

Cover Letter

Dear Editor,

we would like to submit to Science of the Total Environment Special Issue on "Rare Earth Elements in aquatic systems" the paper: **Long-term multi-endpoint exposure of *Raphidocelis subcapitata* to lanthanum and cerium.**

For the first time, we investigated the sensitivity of microalgae *R. subcapitata* to La and Ce long-term exposure. (28 days).

Significant release of rare earth elements (REEs) into the environment are mainly due to anthropogenic sources. Hot spots can be related to active or abandoned mining sites, but their presence is globally increasing due to their use in several industrial sectors, especially in electronic devices manufacturing. Currently, the effects on primary producers, like the microalga *Raphidocelis subcapitata*, are still unexplored. This research focused on La and Ce as the two most widespread REEs that can be currently found at environmental concentrations between µg/L and mg/L both in water and sediment on a site-by-site basis. Microalgae were exposed to La and Ce for 3 days (pH = 7.8) (short-term exposure) to derive the effective concentrations inhibiting the growth on 10% (EC10) of the exposed population. EC10 values (0.5 mg/L of La and 0.4 mg/L of Ce) were used for the 28 days long-term exposure (renewal test) to observe after 7, 14, 21, and 28 days on a multi-endpoint basis microalgae growth inhibition (GI), biomarkers of stress (reactive oxygen species (ROS), superoxide dismutase (SOD), and catalase (CAT)), and bioconcentration. Results evidenced than La and Ce EC10 increased GI (day 28) up to 38% and 28%, respectively. ROS, CAT, and SOD activities showed differential responses from day 7 to day 14, 21, and 28, suggesting, in most of the cases, that La and Ce effects were counteracted (i.e., being the values at day 28 not significantly different, $p > 0.05$, from the relative negative controls), except for La-related ROS activities. La and Ce significantly bioconcentrated in microalgae populations up to 2- and 5-fold (i.e., at day 28 compared to day 7), in that order. Bioconcentrated La and Ce were up to 3157 and 1232 µg/g dry weight (day 28), respectively. These results suggested that low La and Ce concentrations can be slightly toxicity

to *R. subcapitata* having the potential to be bioaccumulated and potentially transferred from primary producers to primary consumers (i.e., zooplankton) and further upwards with still unknow effects on the trophic web and, finally, human health.

Prof. Giovanni Libralato on behalf of all co-authors

Long-term multi-endpoint exposure of the microalga *Raphidocelis subcapitata* to lanthanum and cerium

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25 Abstract

26 Significant release of rare earth elements (REEs) into the environment is mainly due to active or
27 abandoned mining sites, but their presence is globally increasing due to their use in several industrial
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33 long-term exposure (renewal test) to observe after 7, 14, 21, and 28 days on a multi-endpoint basis
34 microalgae growth inhibition (GI), biomarkers of stress (reactive oxygen species (ROS), superoxide
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41 that order. Bioconcentrated La and Ce were up to 3157 and 1232 μg/g dry weight (day 28),
42 respectively. These results suggested that low La and Ce concentrations can be slightly toxic to *R.*
43 *subcapitata* having the potential to be bioaccumulated and potentially transferred along the food web.

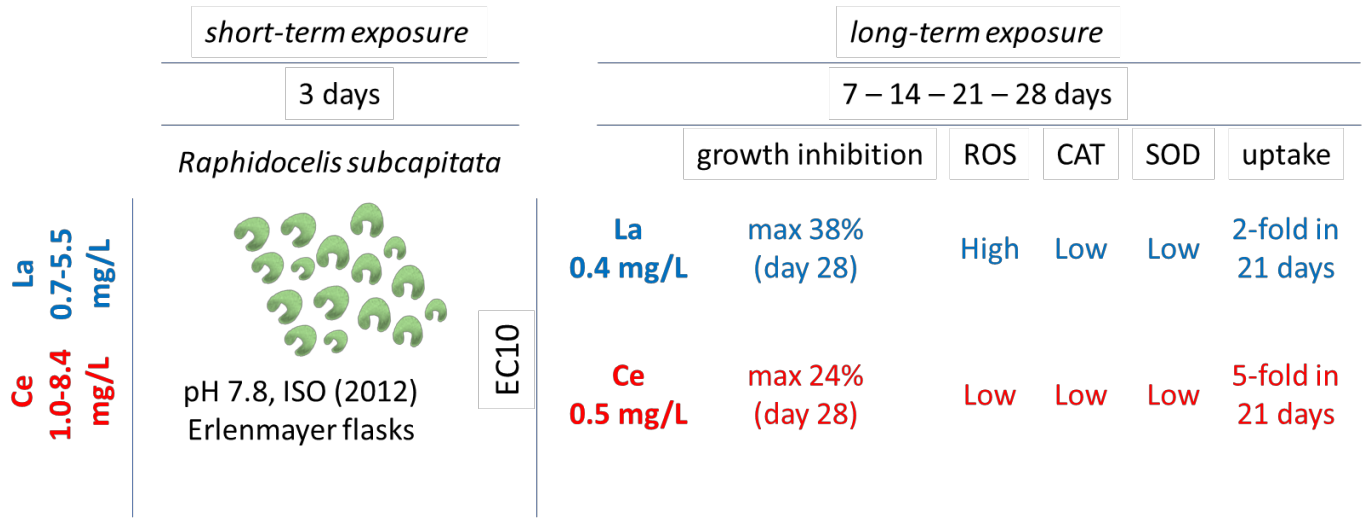
44

45

46 Keywords

47 Microalgae; biomarkers; rare earth elements; bioconcentration

48 Graphical abstract



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53 Highlights

54 • La and Ce cumulatively inhibited microalgae growth up to 38% and 28%, respectively

55 • After 28 days, ROS, CAT, and SOD values were similar to negative controls

56 • In 21 days, La and Ce bioconcentrated up to 2- and 5-fold, respectively

57

1. Introduction

Lanthanides are the major family of rare earth elements (REEs) and due to their essential and unique properties are becoming essential in diverse fields of world economy (Charalampides et al., 2015; Gwenzi et al., 2021; Malhotra et al., 2020). Anthropogenic REEs contamination can be of great concern in hot spots (e.g., ore mine tailings and abandoned mines) (Pagano et al., 2015), but the alteration of their biogeochemical cycles suggests their potential role as widespread emerging contaminants also in agroecosystems (Balaram, 2019; Galdiero et al., 2019; Gravina et al., 2018; Gwenzi et al., 2018; Naccarato et al., 2020; Pagano et al., 2015; Pagano et al., 2019).

About the aquatic environment, their main sources include waste and wastewaters from medical institutions, fertilizers, mining processing, high-technology industries, petroleum refineries, and recycling plants (i.e., e-waste management) (Gwenzi et al., 2018; Gwenzi et al., 2021; Minganti and Drava, 2018; Naccarato et al., 2020; Pagano, 2016; Pagano et al., 2015). According to (Migaszewski and Gałuszka, 2015) review paper, La and Ce ranged between 7.7-80.4 µg/L and 19.4-161 µg/L in wastewater, respectively. In river water, La and Ce concentrations were lower than in wastewater and ranged between 19.7-74 ng/L and 9.67-212 ng/L, in that order (Migaszewski and Gałuszka, 2015), but on a local basis they can be up to 80-200 µg/L (Uchida et al., 2006).

Recent studies demonstrated that REEs exhibit beneficial effects as well as a moderate to high toxicity towards aquatic biota, including bacteria, microalgae, plants, vertebrates, and invertebrates (Adeel et al., 2019; Balaram, 2019; Blinova et al., 2018; Blinova et al., 2020; Herrmann et al., 2016; Oral et al., 2010; Romero-Freire et al., 2019). Their mechanisms of action and behaviour in biological systems are far from being completely understood, but it seems dependent on their concentration and physico-chemical conditions of the exposure media. Similarities in their mode of action were evidenced, but not univocally (Siciliano, 2021), in relation to their ionic radii and coordination numbers with some essential elements, i.e., Ca, Mn, Mg, Fe, and Zn (Valcheva-Traykova et al., 2014).

Several authors evidenced the potential interactions of REEs with biologically active molecules resulting in the excess generation of reactive oxygen species (ROS), inhibition of the antioxidant system, and DNA damages of the exposed aquatic organisms or cultured cells (Blinova et al., 2020; Malhotra et al., 2020), potentially altering the stability, permeability, and functioning of cell membranes (Ramos et al., 2016).

Most data on REEs toxicity to aquatic organisms are derived by acute toxicity tests (Blaise et al., 2018; Blinova et al., 2018; González et al., 2015; Trifuoggi et al., 2017). Some data about the chronic toxicity of REEs are present for zooplankton and fish (Blinova et al., 2020), but they are still very limited for unicellular algae (Siciliano, 2021).

Algae are primary producers and key organisms in the food chain allowing the potential biomagnification up to higher trophic levels of REEs with still unknow and unexpected effects on human health (Goecke et al., 2015b; Thomas et al., 2014). Some authors suggested that REEs could be uptaken and concentrated in chloroplasts, where the intracellular lanthanides could cross the internal membrane system until the replacement of magnesium in chlorophyll molecules (Guo et al., 2000; Kang et al., 2000; Ren et al., 2013; Ren et al., 2007; Shen et al., 2002). Only a few microalgae species have been investigated mainly including *Chlorella vulgaris* and *Raphidocelis subcapitata* (Evseeva et al., 2010; Fuma et al., 2005; Goecke et al., 2015b; Hu et al., 2001; Jin et al., 2009; Tai et al., 2010; Yingjun et al., 2012). The median effective concentration (EC50) of lanthanum (La) was > 10.1 mg/L for *Desmodesmus quadricauda* (50% inhibition after 22-23 days at 0.01 mg/L) and *Microcystis aeruginosa* (Jin et al., 2009), and > 5.42 mg/L for *R. subcapitata* (Siciliano, 2021), and 51.72 (47.29-57.93) mg/L (*i.e*, nominal concentrations) (Bergsten-Torralba et al., 2020), and 47.13 (45.30-51-56) mg/L (*i.e*, nominal concentrations) for *C. vulgaris* (Bergsten-Torralba et al., 2020), and 4.38 (4.16-4.62) mg/L (*i.e*, nominal concentrations) for *Nitellopsis obtusa* (Manusadžianas et al., 2020). The toxicity as EC50 of cerium (Ce) as $Ce(NO_3)_3$ was from 3.15 to 6.32 mg/L (*i.e*, nominal concentrations) for *R. subcapitata* (González et al., 2015) (Siciliano, 2021). Effects of Ce to *Desmodesmus quadricauda* at 0.001 mg/L evidenced biostimulation (16%) after 3 days (Goecke et

al., 2015a), while in *Anabaena flosaquae*, after an initial biostimulation (16%, 3 days), showed inhibition (≈ 33%) at 5-10 mg/L after 17 days (Yingjun et al., 2012). Most studies lacked environmentally relevant concentrations and REEs uptake (Blinova et al., 2020; Miazek et al., 2015), like the main physiological mechanisms underlying REEs induced adaptation phenomena (Wang et al., 2014).

This research study investigated for the first time the effect of La and Ce considering a long-term exposure to *R. subcapitata* (*i.e.*, 28 days renewal toxicity test). We investigated environmentally relevant concentrations looking at potential generational adaptations in microalgae supporting bioconcentration of La and Ce and hence their possible transfer up to the food web. The multi-endpoint approach included the assessment of algal growth rate, determination of reactive oxygen species (ROS), enzymatic activity, and uptake from exposure media.

2. Material and methods

2.1 Chemicals, testing solutions, and analytical characterization.

The experiments were carried out using commercially available chemicals: i) lanthanum(III) nitrate hexahydrate ($La(NO_3)_3 \cdot 6H_2O$, purity 97%); and ii) cerium(III) nitrate hexahydrate ($Ce(NO_3)_3 \cdot 6H_2O$, purity 97%) purchased from Sigma-Aldrich (Saint Louis, United States of America). Treatment solutions of La and Ce were prepared by adding REEs' solution (1000 mg/L) to artificial freshwater (ISO, 2012) at least 1 h before the exposure. The pH was measured with a pH-meter (Mettler Toledo Five Easy, Milan, Italy) prior to exposure and samples' collection (day 3, 7, 14, 21, and 28). La and Ce concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS NexION 350X, PerkinElmer, Inc., MA, USA). The limits of detection (LOD) and quantification (LOQ) were for La and Ce as follows: 0.0011 and 0.0010 µg/L as LOD; and 0.0037 and 0.0033 µg/L as LOQ. The calibration referred to the following standards: i) Lanthanum Standard for ICP (*i.e.*, standard reference materials (SRM) from NIST $La(NO_3)_3$ in HNO_3 2-3% 1000 mg/L La

133 Certipur®□); Cerium Standard for ICP (*i.e.*, SRM from NIST $Ce(NO_3)_3$ in HNO_3 2-3% 1000
134 mg/L Ce Certipur®□). Analyses were carried out in triplicate on samples collected after day 7, 14,
135 21, and 28. About bioaccumulation experiments (i.e., explained in detail in the below sections), filters
136 and organisms were dried at 65 °C for 24 h and digested in aqua regia (HNO_3/HCl = 1:3, v/v) using
137 a microwave oven (START D, Microwave Digestion System, Milestone S.r.l.) and analyzed via ICP-
138 MS including the relative controls.
139
140 *2.2 Cell culture conditions and* R. subcapitata *growth inhibition (GI) test*
141 Axenic cultures of *R. subcapitata* were maintained at the Hygiene Laboratory of the Department of
142 Biology of the University of Naples Federico II in artificial freshwater ISO (2012). Preliminary algal
143 growth inhibition tests (72 h) were performed according to ISO (2012) in order to reflect the
144 physiological status of algal cells (Piovár et al., 2011).
145 Treatment solutions were prepared into each volumetric flask and organized in the following
146 experimental design (i.e., nominal concentrations) to calculate the effective inhibition concentration
147 at 10% (EC10) at pH = 7.8: i) from 0.7 mg/L to 5.5 mg/L for La; ii) from 1.0 mg/L to 8.4 mg/L for
148 Ce.
149 Algae were kept in a climatic growth chamber at constant temperature (24 ± 2 °C) and light conditions
150 (100 ± 10 $\mu Em^{-2} s^{-1}$), and performing continuous shaking during maintenance and testing (50 rpm).
151 After 72 h of exposure, the growth rate relative to the control was calculated by normalizing the final
152 cell density of each replicate to control cultures (incubated in the absence of REEs).
153
154 *2.3 A multi-endpoint experimental approach with* R. subcapitata
155 Modified algal growth inhibition tests (ISO, 2012) were carried out for 28 days exposing microalgae
156 to La and Ce into 250 mL volumetric flasks including four replicates and an inoculum of *R.*
157 *subcapitata* of 10^4 cells/mL. Exposure culturing media were spiked with La or Ce in order to obtain

158 the relative EC10. All tested concentrations were analytically verified. Flasks were incubated for 28
159 days under the same conditions as the growth inhibition test. On day 3, 7, 14, 21, and 28, effects on
160 microalgae were checked considering the optical density (OD) method (i.e., absorbance at 670 nm by
161 Hach Lange DR5000 spectrophotometer). Algae were sampled after 7, 14, 21, and 28 days of
162 exposure to analyze ROS production and the activation of antioxidant defense (superoxide dismutase
163 (SOD) and catalase (CAT)), and to determine La and Ce concentrations bioaccumulated in the algal
164 biomass. Solutions were partially renewed after each sampling period. Algae collection included two
165 main aliquots: i) 100 mL of algae suspension were filtered (0.45 μm polycarbonate Millipore
166 membrane under vacuum pressure) to check bioaccumulation (*i.e.*, filters were rinsed six times with
167 ultra-pure deionized water prior to acid digestion for chemical analysis); ii) 100 mL of algae
168 suspension were centrifuged (1520*g* for 20 min, Beckman TJ-6, rotor 5-92, Milan, Italy) and the
169 pellets were rinsed six times with ultra-pure deionized water prior to ROS, SOD, and CAT analysis.
170 The remaining 50 mL of algae suspension were resuspended in freshly spiked La and Ce culturing
171 media at the respective EC10 values at concentrations > 10^5 cell/mL.
172 A high-pressure homogenization method (French press cell, Thermo Electron Co., Waltham, MA,
173 USA) was applied to the algal biomass at 78 atm to disrupt *R. subcapitata* cell wall. The extracts were
174 suspended in potassium phosphate buffer solution (PBS 1 M at pH 7.4) and centrifuged for 20 min at
175 15000*g* (4 °C). The supernatant was collected, and the protein concentration of each sample was
176 measured using a spectrophotometer (Hach-Lange DR 5000) according to Bradford's method
177 (Bradford, 1976). ROS content was quantified by the ability of free radicals to oxidize the non-
178 fluorescent probe carboxy-H2DFFDA (Sigma Aldrich, Saint Louis, USA) to a fluorescent product
179 that can be measured fluorometrically (Almeida et al., 2017; Almeida et al., 2019). SOD and CAT
180 activities were carried out according to Galdiero et al. (2016).
181
182 *2.4 Statistical analysis*

183 Median effects concentrations (EC50) and effective concentration at 10% inhibition (EC10) were
184 expressed as mean values and the relative 95% confidence limit values for both La and Ce. Growth
185 inhibition data were normalized on negative controls (ISO, 2012). Differences between treatments
186 were assessed via a two-way analysis of variance (ANOVA) after the verification of normality
187 (Shapiro-Wilk (S-W) test) and homoscedasticity (Bartlett's (B) test). If samples are drawn from non-
188 normal populations or do not have equal variances, the non-parametric method Kruskal-Wallis (K-
189 W) ANOVA on ranks was taken into consideration. The *post-hoc* Tukey's test accounted for
190 differences within groups setting the statistical significance at $\alpha = 0.05$. Pearson correlation
191 coefficients ($\alpha = 0.05$) were calculated between the values of biomarkers of stress and La and Ce
192 bioconcentrated in microalgae. Statistical analysis was carried out using SigmaPlot (Systat Software,
193 San Jose, CA) and GraphPad Prism (GraphPad, San Diego, CA, USA).

194 **3. Results and discussion**

195 *3.1. 72 h GI test*

196 Data about 72 h GI were summarized after their normalization on negative controls in Figure 1.
197 Measured concentrations used to calculate concentration-response curves were highlighted in Table
198 1. The pH values of solutions ranged between 7.60-8.00 (mean pH 7.80) all along the monitoring
199 period. For La, the EC50 (± 95% confidence limit values) and EC10 (± 95% confidence limit values)
200 were 1.6 (0.9-2.8) mg/L and 0.4 (0.2-0.8) mg/L, respectively ($Y = 68.72*X + 37.09$; $r^2 = 0.95$; standard
201 error (std.err.) estimate = 6.98). For Ce, the EC50 (±95% confidence limit values) and EC10 (± 95%
202 confidence limit values) were 1.6 (0.9-2.8) mg/L and 0.5 (0.3-0.7) mg/L, respectively ($Y = 75.34*X$
203 $+ 35.38$; $r^2 = 0.9563$; std.err. estimate = 7.2). Lanthanum and Ce showed comparable growth inhibitory
204 effects, with EC50 values of approximately 1.5 mg/L, which is in line with previous findings
205 (González et al., 2015; Joonas et al., 2017; Tai et al., 2010). As a consequence, for the 28 days long-
206 term tests, testing media were spiked with 0.4 mg/L and 0.5 mg/L of La and Ce (i.e., EC10 values),
207 respectively, simulating the exposure deriving from ore mine effluents (Verplanck et al., 2004).

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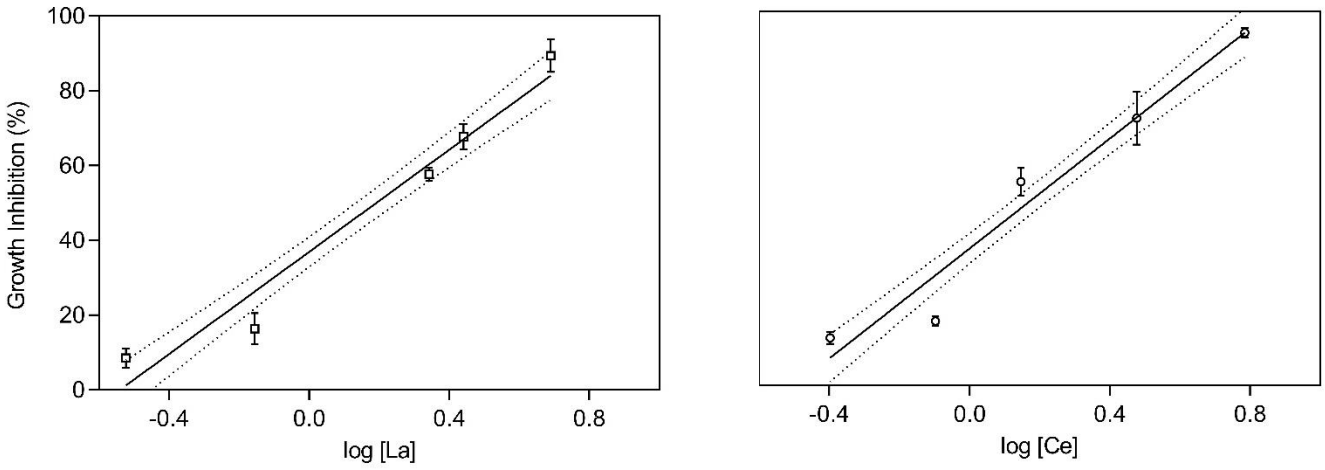


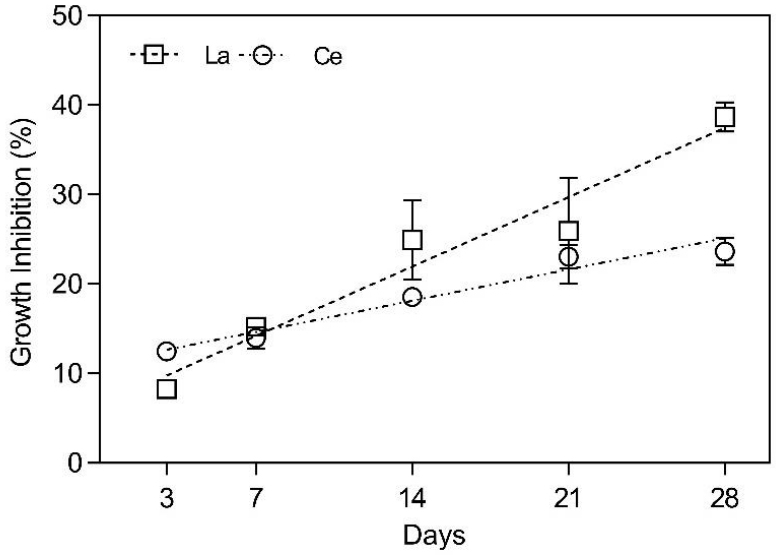
Figure 1 Concentration-response curves for La (Y = 68.72*X + 37.09, R^2=0.9555 std.err. = 6.976) and Ce (Y= 75.34*X+35.38 R^2= 0.9563; std.err. = 8.350) normalized to negative controls after semi-log regression, including 95% limit values (n = 4).

La		Ce	
Nominal	Measured	Nominal	Measured
0.7	0.30 ± 0.04	1.0	0.40 ± 0.04
1.4	0.70 ± 0.03	1.4	0.80 ± 0.02
2.2	2.20 ± 0.09	1.7	1.40 ± 0.08
2.8	2.80 ± 0.09	5.6	3.00 ± 0.05
5.5	4.90 ± 0.06	8.4	6.10 ± 0.07

Table 1 Nominal and measured (ICP-MS) La and Ce concentrations (mg/L) and the relative std.err. (n = 3).

218 *3.3. Long-term exposure effects*

219 Data about La and Ce cumulative growth inhibition after 3, 7, 14, 21, and 28 days were summarized
220 in Figure 2 after data normalization on negative controls. Data were normally distributed (S-W) and
221 presented equal variances (B test). For La, GI increased three times from day 3 (8%) to day 14 (27%),
222 being constant approximately for one week, then increased again at the end of the exposure period
223 (38%). At the end of each week, the GI of Ce tended to slightly increase doubling from 12% (day 3)
224 to 24% (day 28), thus suggesting an increased susceptibility along time. No significant differences
225 (ANOVA, $p > 0.05$) were observed after day 3, 7, 14, and 21 between La and Ce. A statistically
226 significant difference ($p < 0.05$) was found on day 28, where La was more toxic than Ce.
227 The slow increase of toxic effects during the 28 days exposure period can suggest the presence of
228 nutrient depletion phenomena rather than toxicity *per se*. It was reported that REEs could sequester
229 essential nutrients such as phosphates producing death by starvation (Lürling and van Oosterhout,
230 2013; Yuan et al., 2009). This hypothesis needs further investigations to be confirmed since this effect
231 could influence the EC50 of REEs, thus, potentially, environmental decision-making procedures.



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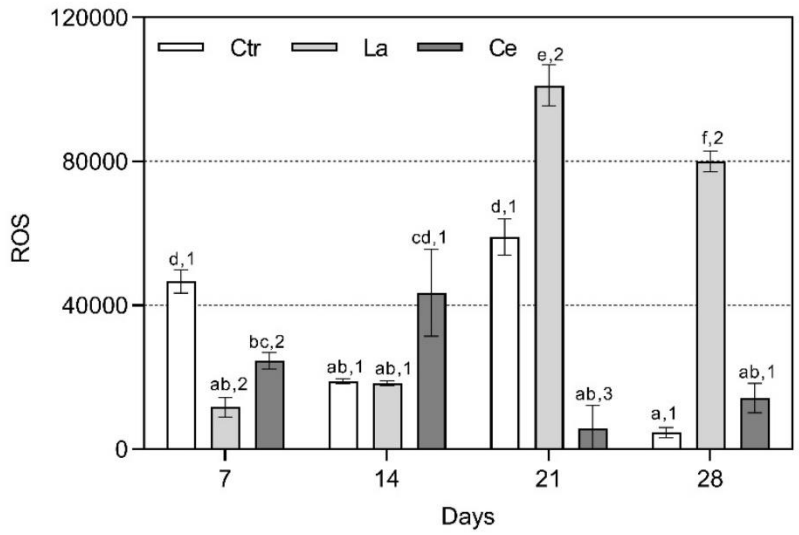
233 Figure 2 La (0.4 mg/L) and Ce (0.5 mg/L) cumulative growth inhibition of *R. subcapitata* after 3, 7,
234 14, 21, and 28 days of exposure normalized to negative controls (± std.err.; n = 4); La: Y =

235 0.4991*X + 11.15, R^2 = 0.7890, std.err. = 2.307; Ce: Y = 1.108*X + 6.446, R^2 = 0.7714, std.err. =
236 5.773.

237

238 Results about ROS were summarized for La and Ce after data normalization on protein activity in
239 Figure 3. Data were normally distributed (S-W), and presented equal variances (B test). Statistical
240 comparisons between effects due to contact time duration (7, 14, 21 and 28 days) were included in
241 the same figure (*post-hoc* Tukey's test).
242 For La, ROS production tended to increase during the 28 days exposure period and after day 14 was
243 greater (days 21 and 28) than the negative control, evidencing the absence of
244 adaptation/detoxification mechanisms. At day 7, La induced less ROS production, while at day 14 no
245 statistical difference was found comparing the ROS value from the control group. At days 21 and 28,
246 ROS production drastically increased.
247 Cerium exposure did not evidence any specific ROS trend with values substantially comparable
248 between contact times. At 7, 21, and 28 days, Ce induced less, or comparable ROS levels compared
249 to negative controls. Only at day 14, the microalgae exposed to Ce produced more ROS than the
250 negative controls. Compared to La, *R. subcapitata* might follow a different detoxification strategy to
251 contrast and/or clear the oxidative damage caused by ROS.



252

253 Figure 3 ROS production in *R. subcapitata* exposed to La and Ce lasting 28 days after normalization
254 on protein content. Results are presented as mean ± std.err. (n = 4) in U/mg protein. Letters (a-f)
255 indicate significant differences between exposure times (7, 14, 21, and 28) within treatments (La and
256 Ce), while numbers (1-3) highlighted significant differences within exposure times (7, 14, 21, and
257 28) between treatments (La and Ce) ($p < 0.05$, Tukey's test).

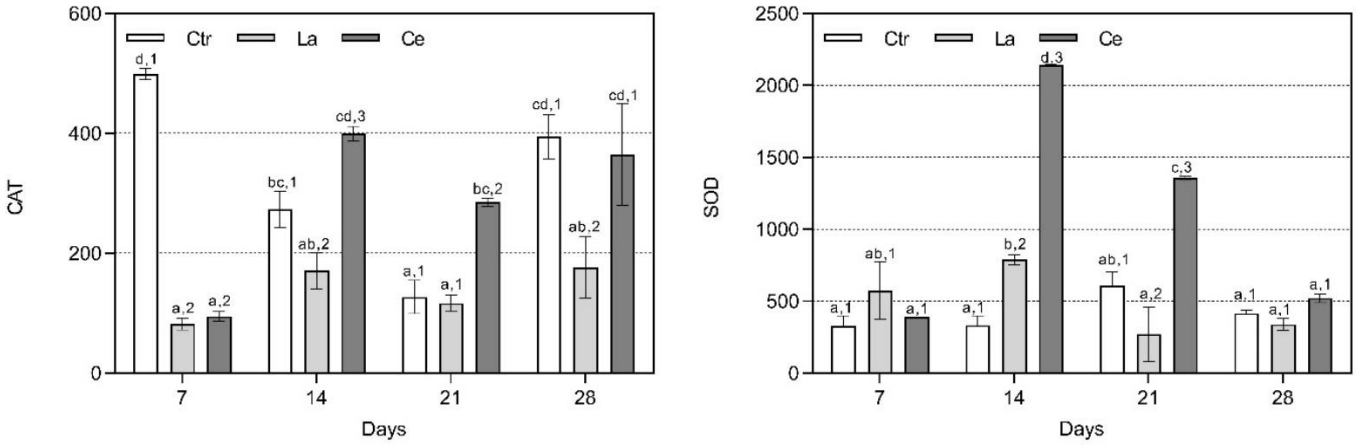
258

259 Data about CAT and SOD were summarized in Figure 4 (A and B, in that order) for both La and Ce
260 after data normalization on protein content. Data were normally distributed (S-W), but they did not
261 present equal variances (B test), so the K-W test was carried out. Generally, La and Ce had different
262 effects on the activities of antioxidant enzymes at most of the considered scenarios compared to
263 negative controls.
264 The levels of CAT (Figure 4A) and SOD (Figure 4B) activities after La exposure were slightly
265 enhanced after day 7 and remained constant approximately for the entire period of exposure from day
266 14 to day 28. CAT and SOD contents reached a maximum at day 14 with a value of 170 U/mg protein
267 and 780 U/mg protein, respectively.
268 About Ce exposure, the contents of CAT (Figure 3A) and SOD (Figure 3B) were at their minimum
269 level. The highest CAT (400 U/mg protein) and SOD (2000 U/mg protein) activities appeared as a
270 consequence of algal exposure to Ce after 14 days. At day 21 and 28, CAT activities were not
271 significantly different ($p > 0.05$). About SOD, at day 21 the activity significantly ($p < 0.05$) decreased
272 compared to day 14, reaching values in day 28 comparable to day 7 (i.e., being similar to negative
273 control value too). Thus after 28 days of exposure, the levels of both CAT and SOD, being not
274 significantly different from the respective negative controls, could suggest the reduction of oxidative
275 stress via other detoxification mechanisms like for example phytochelatins (PCs) production (He et
276 al., 2005) or bioconcentration. He et al. (2005) observed that both calcium and lanthanum can
277 influence the expression of PC synthase gene and cadmium absorption in *Lactuca sativa*. In

particular, La(III) was able to enhance the mRNA level of *LsPCS1* (i.e., phytochelatin synthase gene) and PCs accumulation. Other causes could be related to inactivation of enzymes by ROS, decrease in synthesis of enzyme, or change in the assembly of its subunits (Cheng et al., 2016). Comparatively, the activities of CAT and SOD in Ce exposure were higher than in La, suggesting that a lower antioxidative capacity was required to eliminate ROS generated by La-based treatments.

Currently, little information about the mechanism of response to environmental stress in microalgae exposed to REEs is known. La and Ce treatments increased the oxidative stress and the activities of antioxidant enzymes (i.e., CAT and SOD) contributing to the elimination of ROS with peak activities at the intermediate monitoring periods (day 14 and 21). At the end of the exposure period (day 28), both La and Ce presented activity values similar or lower than the respective negative controls suggesting the potential development of tolerance to La and Ce due to the generational succession of *R. subcapitata* in 28 days (i.e., the culture was always kept in log-phase). This is something new compared to the existing knowledge about microalgae stress response also to other metals like cadmium, copper, chromium, and lead especially due to the extension of the exposure period from 15 days up to 28 days (Danouche et al., 2020). This is a challenging aspect for microalgae assemblages that could be further investigated considering suitable tolerance genes (i.e., rate of creation of tolerance genes by mutation, fitness cost of tolerance, and size of the population) on which the selection could act as already observed for other metals (e.g., copper from mining sites), but in macrophytes (Macnair, 1993).

297 +



298

299 Figure 4 Antioxidant enzyme activities after normalization on protein content; CAT (A) and SOD (B)
300 were expressed as U/mg protein. Results are presented as mean ± std.err. (n = 4). Letters (a-d) indicate
301 significant differences between exposure times (7, 14, 21, and 28) within treatments (La and Ce),
302 while numbers (1-3) highlighted significant differences within exposure times (7, 14, 21, and 28)
303 between treatments (La and Ce) ($p < 0.05$, Tukey's test).

304

305 Results about La and Ce uptake in *R. subcapitata* were summarized in Figure 5 after their
306 normalization to negative controls (i.e., 0.210 ± 0.010 µgLa/g dry weight (d.w.); 0.368 ± 0.030
307 µgCe/g d.w.). Bioconcentration data were best fitted via an exponential growth (3 parameters) curve
308 ($f=y_0+a*\exp(b*x)$) (see Supplementary Materials for details). The average La content per unit mass
309 was of 2058 µg/g d.w. (i.e., mean of day 7, 14, 21, and 28 values) ranging from 1442 ± 459, 1684 ±
310 347, 1947 ± 296, 3157 ± 265 µg/g d.w. at day 7, 14, 21, and 28. The amount of La in microalgae
311 constantly increased from day 7 to day 28, substantially doubling its value in 21 days (day 28).
312 The average Ce content per unit mass in *R. subcapitata* was of 353 µg/g d.w. ranging from 237 ± 59,
313 284 ± 35, 442 ± 131, 1232 ± 120 d.w. µg/g at day 7, 14, 21, and 28. Its content in microalgae slightly

increased between day 7 and day 21 reaching the highest level in day 28. Cerium was able to bioconcentrate increasing its initial concentration (day 7) in microalgae by 5-fold in 21 days.

No significant Pearson correlations ($p > 0.05$) between the biomarkers of stress and bioconcentrated La and Ce were found. The association between the induction of tolerance and bioconcentration could represent for La and Ce, and potentially for other REEs, the key for bioaccumulation and biomagnification through the food chain. MacMillan et al. (2019) evidenced that freshwater zooplankton can bioconcentrate REEs from several environmental drivers including water column and bottom sediment, especially at higher dissolved organic carbon ratios and lower pH values, as also confirmed in Siciliano (2021). No information is currently available about trophic transfer of REEs from primary producers to primary consumers (i.e., zooplankton), but we can suspect that the potential convergence of tolerance acquisition and bioconcentration from water of La and Ce in *R. subcapitatata* could strongly increase the potential biomagnification through the food chain, also with possible repercussion on food safety and human health.

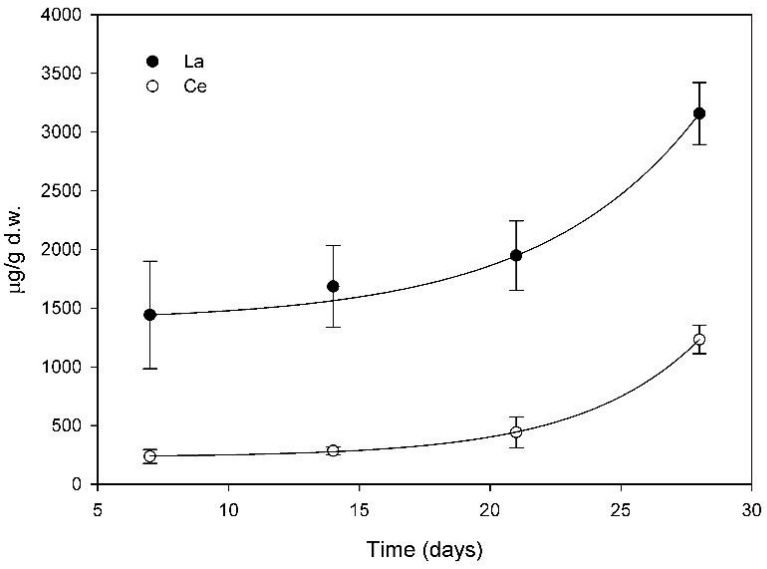


Figure 5 La and Ce uptake (µg/g) in algal biomass at day 7, 14, 21, and 28 after normalization on negative controls; data are in µg/g (± std.err.; n = 3).

Conclusions

This research focused on the effects of La and Ce as potential new emerging contaminants as a consequence of the alteration of their natural biogeochemical cycles. Populations of *R. subcapitata* were exposed to La and Ce serial concentrations (3 days) to define their concentration-response curves and the relative EC10. La and Ce EC10 values were used to spike the microalgae growth media for the 28 days long-term exposure to monitor their effects on growth inhibition, biomarkers of stress (ROS, SOD, and CAT), and the potential to bioconcentrate. La and Ce are able to slightly increase microalgae growth inhibition in 28 days (i.e., 38% and 28%, in that order), allowing them, at the same time, to bioconcentrate up 3157 and 1232 μg/g dry weight, respectively. CAT and SOD presented relatively low activity levels, like for ROS in the case of Ce. ROS showed higher activities, but without any clear and specific correlations with toxicity data. Microalgae as primary producers showed to bioconcentrate La and Ce from spiked water, suggesting that further investigations on primary consumers (i.e., zooplankton) are necessary in order to verify their potential biomagnification through the food chain up to human beings. Further studies are also required to investigate the association between the induction of tolerance and bioconcentration in *R. subcapitata*.

Acknowledgments

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 857989.

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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: