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A first attempt to evaluate the toxicity to *Phaeodactylum tricornutum* Bohlin exposed to rare earth elements

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The increasing use and demand of rare earth elements in many emerging technologies is leading to a potentially higher input to the marine environment. This study compared for the first time the effect of lanthanum (La), cerium (Ce), neodymium (Nd), samarium (Sm), europium (Eu), gadolinium (Gd), dysprosium (Dy), and erbium (Er) to the microalga *Phaeodactylum tricornutum* Bohlin. The algal growth inhibition was investigated after 72 h of exposure. The median effect concentrations (EC₅₀) ranged from 0.98 mg/L to 13.21 mg/L and elements were ranked as follows: Gd > Ce > Er > La > Eu > Nd > Dy > Sm. The comparison of predicted no effect concentrations (PNEC) for hazard and risk assessment with measured environmental concentrations showed that ecological risks deriving from REEs could be present, but limited to specific environments like estuarine waters. The results support evidence of actions to manage the REE impact in seawater environments, looking to improve the monitoring tailored to the different and dynamic nature of ecosystems.

KEYWORDS

rare earth elements, *phaeodactylum tricornutum*, algal growth, emerging contaminants, risk assessment

Introduction

Rare earth elements (REEs) can have potential harmful effects on environmental and human health, and the related exposure is expected to increase in the near future due to their wide use in technology, medicine, agriculture and industry (Pagano et al., 2019). REEs are considered essential for a large number of applications including the alternative energy sector (Hurst, 2010; Adeel et al., 2019; Cardoso et al., 2019) including permanent catalysis (24%), magnets (23%), polishing (18%), metallurgy (8%), and batteries (8%) (Roskill Information Services Ltd). REEs are strategic resources, but they are not

renewable and are considered to be close to “peaking” (Rustad, 2012; Balaram, 2019) with an application boom that would continue also in the near future (Balaram, 2019). The REEs supply chain is still linear and recycling barely reaches 1% of the world production (Rustad, 2012). In this way, REEs enter the environment via many ways, particularly during disposal of consumer and industrial products (e.g., landfills), discharges from mining and mineral processing, and wastewater from industrial processes that use REEs (Migaszewski and Gałuszka, 2015; Gwenzi et al., 2018; Balaram, 2019). Anthropogenic REEs contamination can be of great concern in hot spots (e.g., ore mine tailings and abandoned mines) (Pagano et al., 2015a). The general alteration of their biogeochemical cycles can transform them into potential emerging contaminants (Pagano et al., 2015a; Pagano et al., 2015b; Gravina et al., 2018; Gwenzi et al., 2018; Balaram, 2019; Galdiero et al., 2019; Pagano et al., 2019; Naccarato et al., 2020). There is still relatively little knowledge of the natural or anthropogenic cycles of REEs in the environment as well as their biological effects compared to cadmium (Cd), mercury (Hg), lead (Pb), chromium (Cr), and nickel (Ni) (Hirano and Suzuki, 1996). However, REEs and heavy metals (HMs) have similar environmental behaviours, can be accumulated by animals and plants and consequently they can pose potential risks in a one-health perspective (Pagano et al., 2015a; Gwenzi et al., 2018). Like HMs, REEs could generate reactive oxygen species (ROS), weaken, or inactivate the antioxidant defence and bind for the high affinity to vital macromolecules (Pagano et al., 2015a; Balali-Mood et al., 2021; Siciliano et al., 2021; Trapasso et al., 2021).

The increased mobilisation and concentration of REEs in water systems could have significant impacts primarily to aquatic organisms (Freitas et al., 2020). The REE-content in seawater can be determined by factors relating to different input sources (e.g., terrestrial, hydrothermal) and scavenging processes related to depth, salinity, and oxygen levels (Greaves et al., 1999; Nozaki, 2001). The distinctive character of the seawater REEs distribution is largely controlled by the uniform trivalent behaviour most REEs (except for Ce and Eu which vary with oxygen levels) and estuarine and oceanic scavenging processes (Nothdurft et al., 2004). In contrast, anthropogenic, strongly chelated, anionic REEs appear to have a conservative behaviour and a long environmental half-life (Kulaksız and Bau, 2007; Lawrence et al., 2009; Kulaksız and Bau, 2013). REEs anomalies due to anthropogenic activities were observed in seawaters in Plymouth Sound, UK (Karadaş et al., 2011), Ibaraki, Japan (Zhu, 2020) and Western Philippine (Goldstein and Jacobsen, 1988).

The biological effects and the toxicity of REEs is not fully understood, especially in saltwater. From the scarce literature on REE toxicity to aquatic organisms, only few data are available for marine organisms compared to freshwater ones (González et al., 2015; Freitas et al., 2020). Most

studies demonstrated the ability of REEs to accumulate in marine species belonging to lower trophic levels, and consequently posing potential ecological risks (Pagano et al., 2015b; Ponnurangam et al., 2016; Gwenzi et al., 2018). Currently, no data about REEs exist about *Phaeodactylum tricornutum* Bohlin, that is, a cosmopolitan species found in transitional, marine-coastal and marine waters and considered as a reference model in monitoring seawater pollution and assessment of pure organic and inorganic chemicals (Libralato et al., 2016).

This study investigated the effects of lanthanum (La), cerium (Ce), neodymium (Nd), samarium (Sm), europium (Eu), gadolinium (Gd), dysprosium (Dy), and erbium (Er) on *P. tricornutum* growth explicitly providing the median and 10% effect concentrations (EC₅₀ and EC₁₀). The aims of this study were to: 1) generate ecotoxicological information regarding *P. tricornutum* poorly studied for the REE toxicity, 2) compare, if possible, REEs toxicity with HMs, and 3) weigh up the EC₅₀ with concentrations already found in the seawater to determine the potential risk associated with these elements.

Materials and methods

Chemicals, testing solutions, and analytical characterization

Nitrate standard solutions of 8 REEs (100 mg/L) provided by Sigma-Aldrich (Saint Louis, United States of America) were used for conducting the toxicity trials [La(NO₃)₃ 6H₂O, purity 97%, Ce(NO₃)₃ 6H₂O, purity 97%; Nd(NO₃)₃ 6H₂O, purity 99.9%], Sm (NO₃)₃ 6H₂O, purity 99.9%, Eu(NO₃)₃ 5H₂O, purity 99.9%, Gd(NO₃)₃ 6H₂O, purity 99.9%, Dy(NO₃)₃ xH₂O, purity 99.9% and Er(NO₃)₃ 6H₂O, purity 99.9%. Treatment REE solutions were prepared by diluting REE standard solutions into artificial seawater (ISO, 2016) at least 1 h prior to the exposure followed by the pH measurement (Mettler Toledo Five Easy, Milan, Italy).

Rare earth elements analytical concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS, Aurora M90 Bruker Daltonics Inc.). High-purity water (resistivity of 18.2 MΩ cm) was obtained from a Milli-Q unit (Millipore, United States). Nitric acid (HNO₃, 69% v/v Ultratrace[®] ppb-trace analysis grade) was provided by Scharlau (Barcelona, Spain). All samples analyzed in ICP-MS were prepared in HNO₃ solution (2% v/v). The analysis was performed in Normal Sensitivity mode. Calibration curves for determining REEs ranged from 0.5 to 1,000 µg/L and were constructed daily by analysis of standard solutions prepared immediately before analysis. The internal standard was ¹¹⁵In for both calibration curve and sample analysis.

TABLE 1 Nominal and measured concentrations of rare earth elements and the relative standard deviations; NC = nominal concentrations; MC = measured concentrations (mg/L).

NC	MC							
	La	Eu	Ce	Gd	Nd	Dy	Sm	Er
0.3	0.33 ± 0.01	0.24 ± 0.01	0.27 ± 0.01	0.38 ± 0.02	0.30 ± 0.013	0.27 ± 0.01	0.31 ± 0.02	0.29 ± 0.14
0.6	0.70 ± 0.03	0.81 ± 0.04	0.63 ± 0.01	0.58 ± 0.03	0.55 ± 0.024	0.56 ± 0.03	0.51 ± 0.03	0.52 ± 0.02
1.2	1.24 ± 0.05	1.59 ± 0.08	1.13 ± 0.03	1.01 ± 0.05	1.42 ± 0.062	1.40 ± 0.08	1.11 ± 0.06	1.28 ± 0.06
2.5	2.03 ± 0.09	2.95 ± 0.12	2.19 ± 0.06	2.06 ± 0.11	2.84 ± 0.124	2.74 ± 0.15	2.28 ± 0.11	2.08 ± 0.09
5	6.00 ± 0.26	5.82 ± 0.28	5.04 ± 0.15	4.08 ± 0.22	6.05 ± 0.26	6.21 ± 0.34	4.26 ± 0.21	4.28 ± 0.20

Phaeodactylum tricornutum bioassay

Axenic cultures of *P. tricornutum* microalgae were cultured at Hygiene Laboratory, Department of Biology at the University of Naples Federico II. Culture of *P. tricornutum* were routinely maintained in axenic artificial seawater medium supplemented with nutrients (ISO, 2016) at $22 \pm 1^\circ\text{C}$ and 4,800 lux on a 16:8 h light-dark cycle. Algal growth inhibition test (72 h) was performed according to ISO 10253:2016 (ISO, 2016) by using multiwell plates. For each concentrations four wells were filled with 2,250 μL of the respective solution ($\text{K}_2\text{Cr}_2\text{O}_7$ positive control, spiked REE solution, synthetic sea water as negative control), 125 μL of the 20-fold culture media and 125 μL of inocula of exponentially growing microalgae. One of the four replicates served as blanks the prepared well plates were then placed on a horizontal shaker with 50 rpm for 72 h at $22 \pm 1^\circ\text{C}$ under continuous light of 6,700 lux. Solutions spiked with REEs ranged between 0.3 mg/L and 5.0 mg/L (nominal concentrations).

After a 72 h of exposure, spectrophotometric measurement of samples at 670 nm (DR 5000 sc, Hach) allowed to determine algal cell density through linear regression equation that described the relationship between optical density and cell density. The growth rate compared to negative control was calculated according to ISO (2016).

Data analysis

After screening assays, testing concentrations were set up in order to allow the calculation of median, 20, 10 and 5% effect concentrations (EC_{50} , EC_{20} , EC_{10} , and EC_5) which were determined using linear and nonlinear regression. For each REE, the best-fitting model was selected based on the mean corrected coefficient of determination (R^2) and by graphical interpretation of the model fit. Differences between treatments were assessed via one-way analysis of variance (ANOVA) after the verification of normality (Shapiro-Wilk's test) and homoscedasticity (Levene's test). Moreover, to further evaluate

the risk of REEs, the estimated concentration causing an effect (EC_{50}) was divided by an assessment factor (AF) to estimate a predicted no-effect concentration (PNEC). The ratio of measured environmental concentration (MEC) listed in Table 3 to the PNEC was calculated to estimate the risk quotient (RQ).

Results

REE nominal vs. analytical concentrations

Analytical concentrations used to calculate concentration-response curves are highlighted in Table 1. The ratio between analytical and nominal concentrations in most samples ranged from 0.8 to 1.2. Results are in accordance with previous studies (Pagano et al., 2016; Oral et al., 2017).

REE toxicity on *P. tricornutum*

In Figure 1, the results about the growth inhibition of *P. tricornutum* are reported for Ce, Dy, Eu, La, Er, Gd, Sm, and Nd. All equations and the relative standard errors are provided in Figure 1 and the determination of EC_{50} , EC_{20} , EC_{10} , and EC_5 are summarized in Table 2.

As a general overview, the algal growth always evidenced inhibitory effects at all the tested concentrations, except for La and Dy, which displayed biostimulation effect at the two lowest tested concentrations. Cells of *P. tricornutum* exposed to La and Dy concentrations between 0.3 and 0.7 mg/L after 72 h showed greater cell density than the control group. The elements that caused the highest growth inhibition at the lowest concentrations were Gd and Ce (27 and 24%, respectively).

Cell density of *P. tricornutum* at the highest concentrations exposed of Ce, La, Gd and Er showed the highest inhibition of growth with inhibitions of 88, 91, 98, and 96% respectively (Figure 1). In contrast, the highest concentrations of Eu, Nd, Dy, and Sm showed the lowest growth inhibition 72, 62, 71, and 53%, respectively (Figure 1).

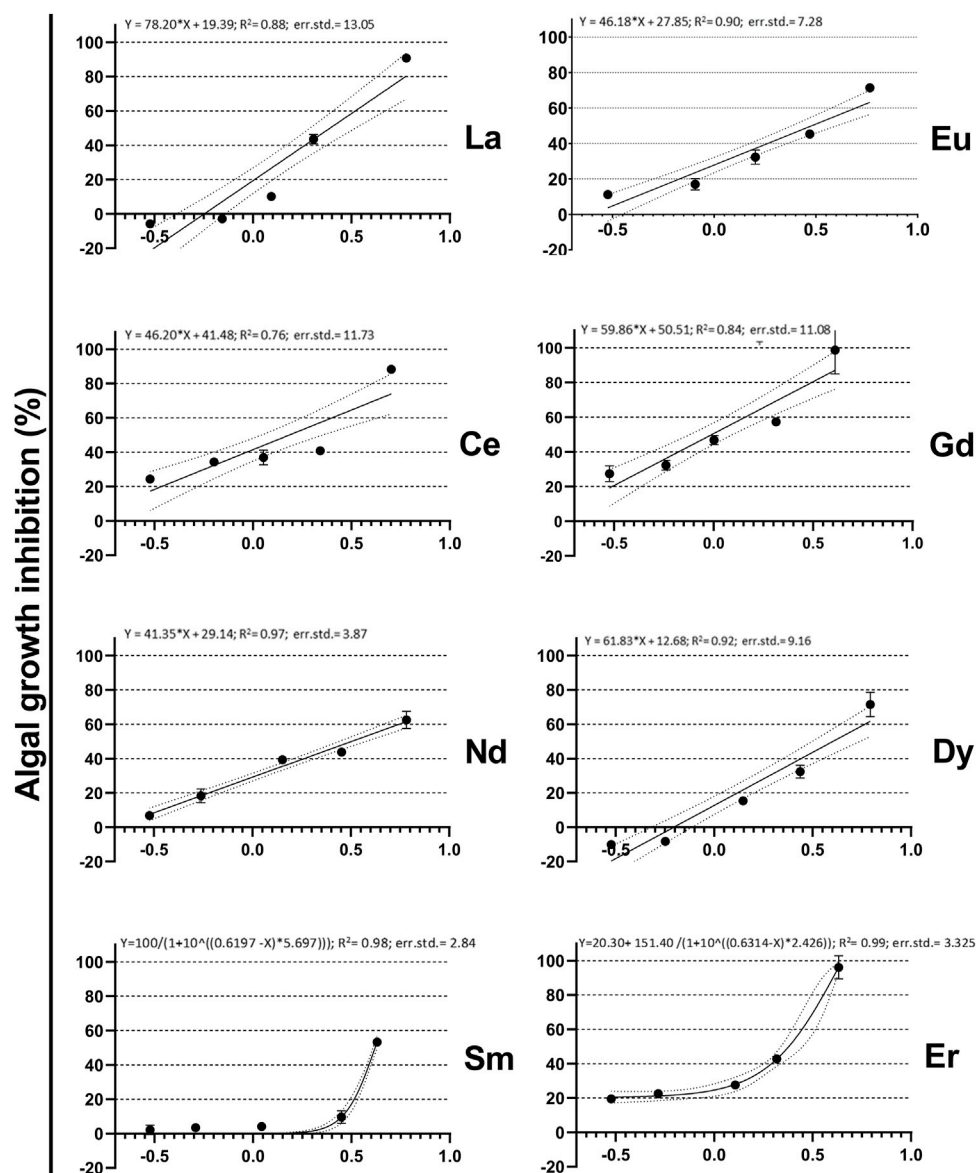


FIGURE 1

Concentration-response relationship of La, Eu, Ce, Gd, Nd, Dy, Sm, and Er exposed to *P. tricornutum*; x-axis log scaled concentrations are in mg/L. Solid line indicates the line of best fit, the dashed lines represent the 95% confidence intervals for linear regression (La, Eu, Ce, Gd, Nd, and Dy) and for nonlinear regression (Sm, and Er).

The toxicity always showed a dose-response relationship. Both Dy and La displayed biostimulation at low concentrations, while the highest relative inhibition was observed at the highest concentration.

The EC_{50} values ($\pm 95\%$ confidence limit values) obtained for all the tested elements were summarised in Table 2 including EC_5 , EC_{10} and E_{20} . Based on the calculated EC_{50} values, the following toxicity relationship was established: $Gd > Ce > Er > La > Eu > Nd > Dy > Sm$.

Comparative risk assessment

About risk assessment, RQs for each REEs were estimated using values of MEC considering three different scenarios from the literature (coastal seawater, open seawater and estuarine seawater) (Wysocka and Vassileva, 2017; Zhu, 2020) and PNEC (Table 3). Wennmalm and Gunnarsson (2009) and Verlicchi et al. (2012) classified the environmental risk into different levels according to the RQs obtained. The

TABLE 2 EC₅, EC₁₀, EC₂₀, and EC₅₀ values for Ce, Dy, Eu, La and Nd on *P. tricornutum*; values are in mg/L; n.a. = not available; REEs = rare earth elements; EC = effective concentration; average EC values are provided \pm 95% confidence limit values in brackets ($n = 3$).

REEs	EC ₅	EC ₁₀	EC ₂₀	EC ₅₀
La	0.65 (0.24–1.89)	0.76 (0.28–2.19)	1.02 (0.37–2.97)	2.46 (0.87–7.34)
Eu	0.32 (0.11–0.84)	0.41 (0.15–1.07)	0.68 (0.25–1.74)	3.02 (1.16–7.41)
Ce	0.16 (0.04–0.78)	0.21 (0.05–1.01)	0.34 (0.08–1.68)	1.53 (0.32–7.75)
Gd	0.17 (0.07–0.46)	0.21 (0.08–0.56)	0.31 (0.12–0.83)	0.98 (0.37–2.67)
Nd	0.26 (0.17–0.40)	0.34 (0.23–0.53)	0.60 (0.39–0.92)	3.19 (2.09–4.88)
Dy	0.75 (0.31–1.81)	0.90 (0.38–2.18)	1.31 (0.55–3.16)	4.01 (1.67–9.70)
Sm	0.60 (0.06–8.18)	0.85 (0.08–11.20)	1.68 (0.16–20.99)	13.21 (1.03–138.28)
Er	0.27 (0.05–1.44)	0.33 (0.06–1.75)	0.48 (0.09–2.57)	1.55 (0.28–8.20)

TABLE 3 Calculated risk quotients for REEs in seawater.

REE	PNEC [ng/L]	MEC [ng/L]	RQ				
			Open seawater (Zhu, 2020)	Coastal seawater (Zhu, 2020)	Estuarine water (Wysocka and Vassileva, 2017)	Open sea water	Coastal seawater
La	2,460	99.30	166.00	794.83	0.0404	0.0675	0.3231
Eu	3,020	2.50	2.07	59.06	0.0008	0.0007	0.0196
Ce	1,530	33.50	276.00	704.75	0.0219	0.1804	0.4606
Gd	980	13.90	10.10	311.32	0.0142	0.0103	0.3177
Nd	3,190	58.80	31.00	855.91	0.0184	0.0097	0.2683
Dy	4,010	14.60	10.80	356.38	0.0036	0.0027	0.0889
Sm	13210	10.80	7.50	778.84	0.0008	0.0006	0.0590
Er	1,550	12.50	8.20	288.31	0.0081	0.0053	0.1860

classification follows: if $RQ < 0.1$ the environmental risk level is considered insignificant, an RQ from 0.1–1.0 is considered low, an RQ from 1.0–10 is considered moderate, and an $RQ > 10$ is considered high risk. For groups of relatively unknown sensitivity such as saltwater species and for acute laboratory studies, the assessment factor is between the range of 10–1,000 (Fairbrother, 2008). Because of the scarce data available about the toxicity of REEs to marine species, the factor of this study was set at 1,000.

In all scenarios, RQ s were below 1 and none of the investigated REEs were classified as high risk. The estimated RQ s varied from 0.0006 to 0.1803 with a mean value of 0.0346 in the coastal seawaters, from 0.0008 to 0.0403 in the open seawaters with a mean value of 0.0135 and from 0.0196 to 0.4606 in the estuarine waters with a mean value of 0.2154, indicating, in the latter case, a potential low risk posed by the REEs on the marine organisms. Only Ce posed low risks to aquatic organisms in the coastal seawaters, while all the other elements resulted in insignificant risk. Moreover, for open seawaters, all the REEs resulted in insignificant risks, including Ce, La and Nd, which are considered the most abundant REEs in the environment (Freitas et al., 2020; Adeel et al., 2019). Overall, of the 8 REEs, more than 60% exhibited potential ecotoxicological risks in the estuarine

waters due to their relative high concentrations and/or toxicities. The results agreed with the previous study of (González et al., 2015) that REEs in the different exposure environmental conditions had ecological risks limited to some hotspots.

Discussion

To the best of our knowledge, this is the first study reporting a large set of REEs toxicity data towards *P. tricornutum*. Previous data are available only from Sun et al. (2019) on La but considering only nominal concentrations. The EC₅₀ of La from this study (2.46 mg/L) is more than 4 times lower than that obtained by (Sun et al., 2019) (10.08 mg/L), being such difference attributable to the use in our paper of measured concentrations. However, the values obtained in both studies do not differ greatly and can therefore still be comparable in the effects on the growth. Moreover, to further evaluate the data obtained and interpret the toxicological relations for tested REEs, the results obtained were compared to those obtained by (Tai et al., 2010) on diatom *Skeletonema costatum*. From this study, the EC₅₀ of Ce (1.53 mg/L) was lower than those obtained by

TABLE 4 Toxicity response of *P. tricornutum* to metals in 72-h growth inhibition bioassays. *Measured, #Nominal Concentrations.

Element	72-h EC ₅₀	References
Cadmium (Cd(NO ₃) ₂ · 4H ₂ O)	5.37 [#]	Horvatić and Peršić, (2007)
Cobalt (Co(NO ₃) ₂)	19.57 [#]	
Cobalt (CoCl ₂)	1.19 [#]	
Zinc (ZnSO ₄ ·7H ₂ O)	41.85 [#]	
Mercury (HgSO ₄)	0.03 [#]	
Mercury (HgCl ₂)	1.16 × 10 ^{-5#}	
Nickel (NiSO ₄ ·6H ₂ O)	7.28 [#]	
Copper (CuSO ₄ ·5H ₂ O)	0.035	Moreno-Garrido et al., (2000)
	0.008*	Levy et al., (2007)
	0.010 [#]	Franklin et al., (2001)
	0.565*	Liping and Zheng, (2008)

Tai et al. (2010) (4.2 mg/L) and the Dy values obtained were very similar in value (4.01 and 4.6 mg/L).

Moreover, to understand better the sensitivity of *P. tricornutum* to metals, the EC₅₀ values obtained were also compared to data available on the toxicity of heavy metals (Table 4). The ecotoxicity of HMs (e.g., Cd, Pb) is higher than that of REEs, when compared under similar experimental conditions and species. From the results presented in this study compared to those obtained by (Horvatić and Peršić, 2007), the element with the highest toxicity was Gd (0.98 mg/L), which differed around 4 orders of magnitude with the element with the highest toxicity mercury chloride (1.16·10⁻⁵ mg/L), had a similar toxicity to cobalt chloride (1.19 mg/L) and had a higher toxicity than cadmium nitrate (5.37 mg/L). Moreover, (Horvatić and Peršić, 2007), also found that low concentrations of Cd and Co had a stimulatory effect on the growth rate from concentrations between 0.02 and 1.25 mg/L for Co and 0.16–0.31 mg/L for Cd, which are in a similar range to those obtained for Dy and La, which presented a biostimulation effect from concentrations between 0.3 and 0.7 mg/L. Results displayed, indicate that HMs such as copper and mercury are more toxic than the tested REEs, nevertheless, the results obtained are in a similar range to the remaining HMs presented. Elements such as Hg, Pb, Co and Cr have shown to be the most toxic elements affecting the growth of *P. tricornutum* (Cabrita et al., 2016), this might be explained by the fact that the cells incorporate these elements and the internalization causes damages in terms of cell division and growth (Deng et al., 2013; Cabrita et al., 2014; Cabrita et al., 2016).

The outcome of the environmental risk assessment of investigated REEs was different depending on the MECs of coastal, oceanic, and estuarine waters used to calculate the RQs. The evaluation results showed that RQ values were gradually increasing from ocean to estuary. The RQs were less than 0.1 in coastal and oceanic waters, except for Ce, which meant ecological environmental risk of REEs to marine species was not of concern. While for coastal and oceanic scenarios,

hydrodynamics and significant dilution could explain the lower REE concentrations, it was notable that REEs concentrations were higher at estuarine waters, which could be attributed to the local sewage discharge and anthropic activities.

Either way, the RQs in the estuarine waters for REEs were always higher than 0.1. In this scenario, based on the RQ approach, the REEs assessed herein appear to be of raising marine concern, especially with regard to La and Ce, which could be explained based on the more frequent use of Ce and La that can promote their occurrence in environment as well as seawater. This work reinforces the urgent need for more data embracing other REEs and other species, in order to avoid under- or over- estimation when assessing the environmental risks of these metals.

Conclusion

Rare earth elements (Ce, La, Eu, Sm, Gd, Dy, Er, and Lu) effects to *P. tricornutum* growth inhibition were investigated. Results showed similar toxicity levels between light, medium and heavy REEs.

Toxicity can be compared to the effect of heavy metals on *P. tricornutum*. Although the differences in orders of magnitude between some elements, it can be observed that both groups of elements can have a bio-stimulatory effect but an inhibitory effect at higher concentrations. Further studies to evaluate the effects of low and constant exposure is suggested.

Further, this study presents a first insight on the effect of several REEs on the unicellular species *P. tricornutum*, which can be considered as a potential candidate to further study the effect of REEs on more complex matrices such as artificial sea water.

Based on *P. tricornutum* toxicity results, the presence of REEs in the marine environment does not seem to represent a widespread environmental risk except at some hotspots which may become more severe with the increase of REE application.

This approach could be used to further investigate more relevant endpoints, which could lead to indirect effects on the food chain and to study predictable patterns in bioavailability, bioaccumulation and ecotoxicity, as well as studies addressing the combined effect of several stressors such as REEs mixtures.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

AS: Conceptualization, Formal analysis, Data curation, Writing- original draft- review and editing. MS: Formal analysis. AP: Formal analysis. EP: Formal analysis,

Data curation, Writing- original draft- review and editing. MTR: Formal analysis AB: Formal analysis, Data curation, Writing- original draft. LS: Formal analysis, Data curation. EG: Formal analysis. MTO: Funding acquisition. MG: Supervision, Funding acquisition- review and editing. GL: Writing—original draft, Supervision, Funding acquisition- review and editing.

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