Peter Campbell ORCID iD: 0000-0001-7160-4571

Claude Fortin ORCID iD: 0000-0002-2479-1869

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Lanthanum and cerium toxicity to the freshwater green alga *Chlorella fusca*: applicability of the biotic ligand model

Imad Aharchaou, Cédrick Beaubien, Peter G.C. Campbell, and Claude Fortin*

EcotoQ, Institut national de la Recherche scientifique, Centre Eau Terre Environnement (INRS-ETE), Québec, Québec, Canada

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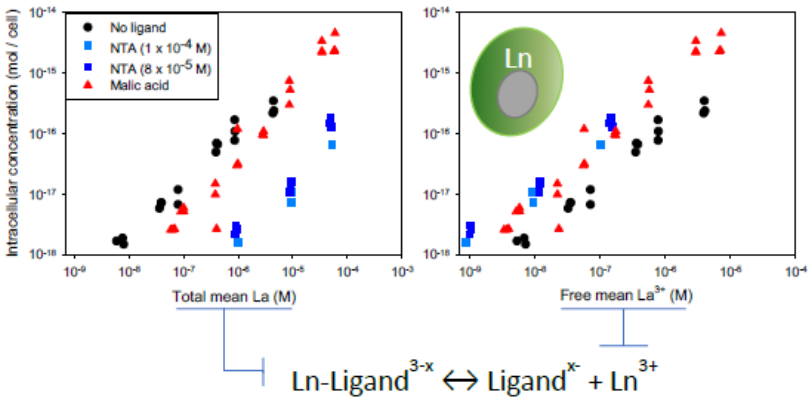
Abstract: The environmental risk assessment of rare earth elements (REE) requires data on their potential toxicity. In this study, the toxicity of lanthanum (La) and cerium (Ce) was studied in relation to metal speciation in solution. For both La and Ce, the use of organic ligands demonstrated that the calculated free ion concentration was a good indicator of toxicity. Whether in the absence or presence of organic ligands, when based

on free ion concentrations, the obtained half maximal effective concentrations were similar. When all generated data were pooled, Ce and La showed identical toxicity thresholds after 120 h of exposure with free ion concentration-based EC_{50} values [95% confidence intervals] of 0.48 [0.38 – 0.60] µM and 0.47 [0.36 – 0.61] µM for La^{3+} and Ce^{3+}, respectively. The inhibition of algal growth was also correlated with the intracellular lanthanide concentrations, regardless of the ligand used. Finally, increasing the ambient calcium concentration protected the test algae by reducing the amount of lanthanide internalized into the cells. These results suggest that, at constant pH (5.5), REE accumulation and toxicity are linked to the free-ion concentration and ambient Ca concentration, as predicted by the biotic ligand model (BLM).

Graphical Abstract

Lanthanum and cerium uptake and toxicity can be predicted by the concentration of its free-ion concentration regardless of the ligand used.



Keywords: Rare earth elements, Lanthanides, Metal speciation, Algae, Growth inhibition, Metal uptake

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*Address correspondence to claude.fortin@ete.inrs.ca

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1 Introduction

The rare earth elements (REE) include 17 elements that comprise Sc, Y, and the lanthanide (Ln) series metals, among which Ce and La are the most abundant (González et al. 2014). The REE are chemically similar elements that are widely used in many high-tech, electronic, and clean energy industries. With the increase of their uses in recent years, an increase in their discharge into aquatic environments is expected (Kulaksiz and Bau 2013). Despite the recent interest in assessing the risks associated with REE metals, little information is available on their bioavailability and toxicity to aquatic organisms. It is generally well accepted that a detailed consideration of metal speciation during exposure is essential to improve our understanding of metal toxicity to aquatic organisms. However, because of the low solubility of REE in the presence of hydroxides (i.e., at circumneutral pH) (El-Akl et al. 2015), phosphates (ubiquitous in algal growth media) and carbonates (i.e., at high pH), assaying toxicity is complex. Indeed, the problem of metal precipitation has been observed in various studies and occurs in most artificial test media (Barry and Meehan 2000; Joonas et al. 2017; Lürling and Tolman 2010). This precipitation is generally due to the presence of phosphate, which is required for algal

growth. In this study we were able to circumvent this problem by using an alga, *Chlorella fusca*, known for its ability to grow for several days in a phosphate-depleted medium, using its internal phosphate reserves. Such an approach resolves the issue of lanthanide precipitation and allows for the quantification of the effects of lanthanides on algal growth in a chemically stable system.

Although the biotic ligand model (BLM) has been successfully used to predict the bioavailability of divalent metals for aquatic organisms (Campbell and Fortin 2013; Erickson 2013; Mebane et al. 2020), its applicability to the trivalent REE metals remains to be demonstrated. As is the case for many other metals, the bioavailability of REE is expected to be highly influenced by the pH and by the presence of other cations in the environment. For example, trivalent Ln^{3+} ions resemble Ca ions in several of their chemical properties (e.g., they bind ionically, have no significant covalent binding and are about the same size) and have been identified as potential competitors for Ca uptake and transport (Barry and Meehan 2000; Herrmann et al. 2016; Tan et al. 2017). Moreover, since REE speciation in natural aquatic environments will be strongly influenced by the presence of inorganic and organic ligands (Weltje et al. 2004), it is important to understand their influence on REE bioavailability and toxicity.

In this study La and Ce were chosen as REE models due to their wide use in the manufacture of super alloys, catalysts, condensers and other materials. They are among the most abundant REEs in the environment (González et al. 2014; Herrmann et al. 2016). Moreover, La and Ce are the REEs for which the highest number of studies is found (González et al. 2014) and more thermodynamic data are available for La and Ce than for many other REEs, allowing us to perform chemical speciation calculations with

confidence. On the basis of all the above considerations, four aspects related to REE risk assessment appear of particular importance for an improved understanding of their toxicity: a) the development of an exposure medium for lanthanides in which no trace of phosphate ions is present and with a slightly acidic pH, in order to minimize the risk of precipitation of the studied metals; b) the assessment of La and Ce toxicity, the most abundant REEs; c) the test of the applicability of the BLM in the prediction of REE toxicity in the presence of different organic ligands; and d) the determination of the effect of Ca concentrations in the exposure media, as this cation has already been identified as a potential competitor with La for internalization in algal cells. We hypothesized that the toxicity of the lanthanides studied in the absence of phosphates would be higher than that previously observed in the literature (Fujiwara et al. 2008; González et al. 2015; McCloskey et al. 1996; Weltje et al. 2004). Also, we anticipated that the toxicity of La and Ce would be similar because of their physicochemical resemblance (Blinova et al. 2018). Based on the BLM paradigm, we hypothesized that the toxicity of REEs could be predicted by the free cation concentrations (Weltje et al. 2004) with a protective effect of Ca (Barry and Meehan 2000; Tan et al. 2017).

2 Material and methods

2.1 Cell culture conditions

The unicellular green alga *Chlorella fusca* was used for this study because of its ability to grow on its phosphorus reserves in a phosphate-free medium due to “luxury uptake” of phosphorus. This particular capacity has been used before in several early studies (Helliweli et al. 1983; Parent and Campbell 1994). Axenic cultures of *C. fusca* (CPCC89,

wild strain), originally obtained from the Canadian Phycological Culture Center (University of Waterloo, Canada), were inoculated and grown in a sterile modified high salt medium (MHSM-1, Table S1) at pH 5.5. Algae were kept in an environmental growth chamber (Conviron, CMP4030, Controlled Environments Ltd., Canada) at a constant temperature (20.0 ± 0.1 °C), under continuous light (100 ± µE·m^{-2}·s^{-1}) and with orbital agitation (60 rpm). Once a week, 1-mL aliquots of algal suspension were transferred into 100 mL of freshly prepared and sterile MHSM-1 medium for culture maintenance. All culture plasticware and glassware were soaked for at least 24 h in 10% (v/v) HNO_3, thoroughly rinsed five times with deionized water and three times with ultrapure water and dried under a class 100 laminar flow hood before use. Media were autoclaved at 121 °C for 15 min before use. *2.2 Exposure conditions and speciation modeling*

For the various experiments, exposures were performed in sterile modified culture media containing neither phosphate nor trace metals (MHSM-2, Table S1). Exposure media were spiked to reach different concentrations of La or Ce (0.010 to 100 µM); all tested concentrations were analytically verified and measured concentrations were used for data interpretation; see below. Lanthanum was obtained as $La(NO_3)_3 \cdot 6H_2O$; Acros Organics, 99.99%) and Ce as $Ce(NO_3)_3 \cdot 6H_2O$; Alfa Aesar, 99.99%). The pH was adjusted to 5.5 with 1.0 M NaOH solution (Sigma Aldrich, 97% min) and was buffered with 10 mM of 2-(N-morpholino)-ethanesulfonic acid (MES, Fluka, 99%). Uptake and toxicity of La and Ce were determined in the absence and presence of 1×10^{-4} M of malic acid (Acros Organics, 99%), a natural ligand. In addition, for La, two synthetic ligands were tested: nitrilotriacetic acid (NTA, Sigma Aldrich, 99%; 8×10^{-5} M or 1×10^{-4} M)

and iminodiacetic acid (IDA, Sigma Aldrich; 1×10^{-4} M). All of the experimental media were prepared and left to equilibrate for 24 h before algal inoculation.

In additional experiments, the potential effect of Ca on La and Ce toxicity was investigated in MHSM-Ca medium (Table S1). In order to maintain a constant ionic strength, when the Ca concentration of the medium was increased by the addition of $Ca(NO_3)_2$, the concentration of KNO_3 was reduced and vice versa. The selected concentrations of Ca were of 1 μM, 68 μM and 1 mM with respective KNO_3 concentrations of 4.4 mM, 4.1 mM, and 1.4 mM. The concentrations of La and Ce were selected to obtain a concentration of free ions in solution in the range of concentrations where *Chlorella fusca* growth was inhibited by 50% (EC_{50}) after 120 h of exposure. The total concentration of La in solution was 3.5 μM and that of Ce 6.0 μM (verified analytically) and all the conditions were tested in three to five replicates (n = 3–5) in the presence of malic acid (1×10^{-4} M).

To investigate the temporal stability of Ln concentrations in the exposure solutions, aliquots of La and Ce exposure media were recovered every 24 h, centrifuged for 5 minutes at 5000 rpm and the supernatant was collected, acidified and stored at 4˚C until analysis. A control experiment without algae was also conducted, in which case the centrifugation step was omitted. All the recovered samples were analyzed for total La or Ce.

In all experiments, exponentially growing cells were harvested on a 2-μm polycarbonate filter membrane (Millipore) using a vacuum pressure of 10 cm Hg or less and were rinsed three times with MHSM-2 medium containing the ligand used for

exposure (Table S1), to remove any algal exudates or phosphate present in the original culture medium. Size distribution, average surface area, and cellular density were immediately determined using an electronic particle counter (Multisizer ^{TM} 3 Coulter Counter®; Beckman) after appropriate dilution in Isoton II electrolyte isotonic solution (10 mL as final volume) (Beckman). Cells were then resuspended into the desired exposure solution that had been previously spiked with either La or Ce to give an initial cell density of about 15,000 cells/mL. This initial density was selected to be above the detection limit of the particle counter (4000 cells/mL) and to minimize speciation changes in the media that could be affected by a large algal population (Franklin et al. 2002; Paquet et al. 2015). The speciation of La and Ce in the exposure media was calculated with MINEQL+ (version 5.0); the default MINEQL+ thermodynamic database was updated with the values provided in Leguay et al. (2016). Binding constants for La-IDA (log K_1 = 7.17; log ß$_2$ = 11.69) were taken from the NIST (National Institute of Standards and Technology) Critically Selected Stability Constants of Metal Complexes database (version 8.0). Total average La and Ce concentrations, calculated from the mean of initial and final measured concentrations, as well as nominal concentrations of other ions in solution (Na^+, Ca^{2+}…; Table S1) were used as input data for the thermodynamic speciation calculations; solutions were assumed to be at equilibrium with atmospheric CO_2.

2.3 Toxicity and uptake of La and Ce

Algal growth was monitored over the exposure duration (120 h) by measuring the algal cell density, size distribution and average surface area every 24 h as described above. After 96 h and 120 h incubation periods, the growth yield relative to control was

calculated by normalizing the final cell density of each replicate by that of the control cultures (incubated in the absence of Ln).

In parallel, the total volume of each Erlenmeyer was recovered after being gently filtered through two superimposed 2-µm polycarbonate filters (Millipore). The filtrates were used to determine the exposure concentration at the end of the experiment, and the harvested cells were rinsed three times with 10 mL of a rinse solution (MHSM-R) corresponding to MHSM-2 medium without metals. Afterward, the cells were soaked for 10 min with an extraction-medium (MHSM-E) corresponding to a solution containing only $Ca(NO_3)_2$ (68 µM) and EDTA (1 mM) and then rinsed two more times with 10 mL of rinse solution (MHSM-R). These rinsing steps were designed to remove the metal (La or Ce) bound to the surface of the algal cells. The metal (La or Ce) measured on the upper filter after its acid digestion (see next section) is thus operationally defined as intracellular (Aharchaou et al. 2017; Crémazy et al. 2013). The measured signal of the lower filter was subtracted from the signal of the upper filter to correct for potential metal adsorption on the filter membranes (on average, the La or Ce signal of the lower filter represented 5% of that of the upper filter), and the obtained measured concentrations were normalized by the cell numbers to determine metal accumulation expressed as moles/cell.

2.4 Analytical determination of La and Ce

The filters recovered in the different experiments were acid-digested. They were placed in Teflon beakers with 10 mL of trace metal grade nitric acid, and a watch glass was placed on them before being placed on a hot plate at about 100 °C under a perchloric

acid hood and heated under reflux for 1 h. Afterward, the samples were evaporated to dryness and a volume of 4 mL of trace metal grade perchloric acid (EMD Chemicals) was added to them before being reheated under reflux conditions for 2 h and left under the hood overnight. The following day, the samples were evaporated to dryness and the solid dissolved in 1 mL of trace metal nitric acid by heating on the hot plate. The contents of each beaker were then transferred to 50 mL polypropylene graduated tubes (Sarstedt). Each beaker was rinsed three times with Milli-Q water, its content was added to the 50-mL tube and the volume then completed to 20 mL. Two reference samples (*Lemna minor*, BCR-670; Lake sediment, LKSD-4) were used for quality control and were digested at the same time as the algae and the filters. For BCR-670, the average recoveries were 84 ± 3 % for La and 96 ± 4 % for Ce, while for LKSD-4, the average recoveries were 111 ± 5% for La and 111 ± 7% for Ce.

The Ln concentration in the different samples was determined using inductively coupled plasma mass spectrometry (ICP-MS; XSeries 2, Thermo Scientific). The torch used was made of quartz with an internal diameter injector of 1.8 mm. The nebulizer used was of a micro-flow type and the quartz cyclone nebulization chamber was cooled to 4 °C. For calibration, four multi-elemental solutions of several lanthanides (La, Ce, Nd, and Eu) were prepared with different concentrations between 0.02 and 2 µg/L. The calibration curve was validated with PlasmaCal certified standards C00-061-406 (5% HNO_3, 10 µg/L of La, 9.98 µg/L of Ce, 10.03 µg/L of Nd, 10.05 µg/L of Eu) and C00 061-401 (5% HNO_3, 0.1% HF, 5.04 µg/L of La, 10.04 µg/L of Ce). The isotopes ^{103}Rh and ^{187}Re were used as internal standards to correct for instrumental drift and possible matrix effects. Moreover, two samples of certified standards were passed every 10 to 20

samples to allow for the correction of the variation in the measurements. Indium and uranium were used as an indicator of sensitivity and signal stability and their signal had to be greater than 80 000 counts per second and their coefficient of variation after 10 readings was not to exceed 1%. Also, oxide formation was monitored using the CeO/Ce ratio measurement which was kept below 0.02. The formation of doubly ionized species was followed by the Ba^{2+}/Ba ratio which was kept below 0.05. Optimization of the analysis was performed by adjusting the voltage applied to the lenses, the position of the torch and if necessary, the pressure applied to nebulize the sample. Four measurements were performed from each sample replicate, and the sampler was rinsed with 2% nitric acid for 30 seconds before performing the measurements of the next sample. For the control conditions (e.g., without La or Ce), metal concentrations for both filters and filtrates were below detection limits (0.005 µg/L).

2.5 Data treatment

Two-way ANOVAs with repeated measures followed by Tukey post-hoc comparison were performed to determine the presence of significant differences among the various conditions. P-levels of 0.05 were used as the threshold for statistical significance. Statistical analyses, as well as graphs, were obtained using Sigmaplot (Systat Software, version 12.5). Effective concentrations, EC_{50} and EC_{20} values, were determined using the U.S. EPA's Toxicity Relationship Analysis Program (TRAP, Ver 1.30a) by non-linear regression. The logistic equation with two parameters was used by fixing the relative absence of effect to 1. The EC_{50} and EC_{20} values and confidence intervals are expressed as means [95% lower confidence level (LCL) – 95% upper confidence level (UCL)].

3 Results and discussion

3.1 Chemical speciation and temporal stability of La and Ce concentrations in exposure media

In the absence of any ligand, speciation calculations showed that La and Ce had similar species distribution with the predominance of the free ionic form (90-91%) and sulfato-complexes as the second most abundant species (9-10%; Table 1). In the presence of 1×10^{-4} M of malic acid, La and Ce speciation was dominated by malate complexation (83-95%) and free ionic La and Ce represented only 8 to15% and 4 to 10% of the species distribution, respectively. For La, speciation was also calculated in the presence of 8×10^{-5} M or 1×10^{-4} M of NTA or in the presence of 1×10^{-4} M of IDA. Within the narrow NTA concentration range used, only 0.1 to 0.3% of total La was present as free ionic form and over 99% was bound by NTA. Contrastingly, in the presence of IDA, 88% of total La was present as free ionic form, and only 3% of total La was present as La(IDA), the rest being in the form of sulfato-complexes (Table 1).

At t = 0 h, La and Ce measured concentrations were similar and corresponded to the expected nominal values in both abiotic (absence of algae) and biotic (presence of algae) media. In general, La and Ce total measured concentrations decreased over exposure time, with mean measured final concentrations of 78 ± 24% of initial measured values. For example, in control exposures in the absence of algae, La and Ce concentrations variations remained within 20% of the initial value over the whole exposure duration of 120 h indicating that La or Ce losses to the walls of the test vessel remained negligible over time (Figure S1). In the presence of algae and in the absence of

organic ligands, decreases of La and Ce concentrations were observed. Depending on the exposure concentrations, the losses reached 40% of the initially added quantity in the case of La and 45% in the case of Ce and were more apparent, as expected, when the exposure concentration was low. Such results can be explained by the ability of algae to grow better at low metal concentrations and by the fact that La and Ce losses due to their uptake by algae are more marked when the exposure concentration is low (Paquet et al. 2015). In the presence of both algae and malic acid, La and Ce concentrations remained stable over the experiment duration, indicating effective metal ion buffering (Figure S1). All growth inhibition results were based on the average observed concentrations (calculated from the mean of initial and final measured concentrations), to capture any changes in exposure concentrations.

3.2 Toxicity of La and Ce

In the absence of organic ligands, La and Ce significantly reduced the cellular density over the concentration range tested. For both lanthanides, the longer the exposure duration, the lower the concentration at which the algal density was significantly reduced. In the case of La, after 24 h of exposure, the samples exposed to 5 µM were the only ones to have a cellular density statistically different from the controls. After 48 h of exposure, significant cellular density reduction was observed in samples exposed to a concentration of 500 nM or higher. After 72 and 96 h, the samples exposed to 100 nM of La also became statistically different from the controls. However, after 120 h of exposure, the samples exposed to 100 nM were no longer statistically different from the controls (Figure S2). In the case of Ce, after 24, 48 and 72 h, significant cellular density reduction was observed in samples exposed to a concentration of 500 nM or higher. After 96 h, a

significant effect was observed at 100 nM and not at 500 nM. Moreover, a hormesis effect was observed on algal densities at Ce concentrations of 10, 50 and 100 nM, starting from 48 h of exposure (Figure S3).

The toxicity of La and Ce from the experiments in the absence of organic ligands was expressed in terms of both total dissolved (La and Ce) and calculated free (La^{3+} and Ce^{3+}) concentrations. In the absence of organic ligands, little complexation of La and Ce by nitrate and sulfate occurs (about 1% and 10%, respectively). Consequently, the EC_x values for total dissolved and free ionic metals are very close (Table 2). After 120 h of exposure, EC_{50} values appeared to be similar for both Ln with overlapping confidence intervals. Note however, in the case of Ce, some samples had a relative cell density (cell yields) higher than 1 (Figure S4B). Such results are obtained because algae exposed to nominal concentrations up to 40 nM had a higher cell density than controls after 120 h due to an apparent hormesis effect of Ce (Figure S3). From this dose-response curve distribution, at the total Ce concentrations that result in 50% population reduction relative to the highest population observed (~200 nM), the cell density relative to the control was around 1. On the other hand, the average total Ce concentration corresponding to a relative cell density of 0.5 was about 600 nM (Figure S4B). In order to overcome this issue, we calculated the EC_{50} values considering only the tested concentrations that yielded relative cell density less than 1 (0.5, 1.0 and 5.0 µM Ce). By doing so, Ce appeared to have a similar toxicity as La (Table 2).

In the presence of organic ligands, as expected, the toxicity of La and Ce was affected by the chelation power of each ligand. The growth curves obtained in the presence of NTA and IDA are presented in Figures S5 and S6. After 24 h of exposure to

La (concentration range: 0-70 µM) in the presence of 10^{-4} M malic acid, no significant effect on cell density was observed. After 48 h, the samples exposed to concentrations equal to or higher than 10 µM showed reduced algal densities compared to the controls. Starting from 72 h until the end of exposure, the samples exposed to 3.5 µM became statistically different from the controls (Figure S7). In the case of Ce, after 24 h of exposure, a difference of cell density was observed in the samples exposed to 40 and 70 µM, compared to controls. After 48 and 72 h of exposure, samples with a nominal Ce concentration equal to or greater than 3.5 µM were also different from controls. After 96 h of exposure, the samples exposed to 3.5 µM became non-statistically different from the controls. However, after 120 h these samples became again statistically different from the controls Figure S8). In all cases, La and Ce had no significant effect on algal cell size.

The toxicity of La and Ce from the experiments in the presence of organic ligands (for Ce, only malic acid was used) was expressed in terms of both total dissolved (La and Ce) and calculated free (La^{3+} and Ce^{3+}) concentrations. For both La and Ce, a significant gap was observed between EC_{50} values based on total dissolved or on calculated free ions (Table 2). Indeed, after 120 h of exposure, the EC_{50} values decreased by around 92% and 95% for La and Ce, respectively, when calculated based on free ion concentrations compared to that based on total dissolved La and Ce concentrations. Based on total dissolved concentrations, La appeared more toxic than Ce. As anticipated, in accordance to the BLM, EC_{50} values based on free Ce^{3+} and La^{3+} were much lower than those based on total metal concentrations. Similar findings by Weltje et al. (2004) showed greater total dissolved lutetium EC_{50} values compared to Lu^{3+}-based EC_{50} values for *Vibrio fischeri* in the presence and absence of organic ligands (NTA, malate, oxalate). Similarly,

in this study, for both La and Ce, the total dissolved based EC_{50} values obtained in the presence of malic acid after 120 h were more than 12 times and 20 times higher than those obtained in the absence of the natural organic ligand for La and Ce, respectively. Therefore, malic acid appears to protect against La and Ce toxicity to the algal cells. This ligand effect is further observable in Figure S9 where the cell yield (relative cell density) is plotted against La and Ce total concentration in the presence or absence of malic acid. Thus for a similar exposure concentration (e.g., 1 µM La), the toxic effect was more important in the absence of organic ligands and decreased with increasing chelation power of the tested ligands (IDA < malic acid < NTA). However, the computed EC_{50} values based on free ion concentrations in the presence of malic acid were lower than those in the free-ligand media (Table 2, Figure 1). This could be explained by a significant decrease in Ln concentration over time in the unbuffered medium, demonstrating the importance of the use of metal-buffering ligands in this type of experiment. Since the concentration in the unbuffered medium decreased over time, it follows that if the onset of effects were triggered at the beginning or end of the exposure, the presentation of the results based on the average free concentrations (calculated from the mean of initial and final measured concentrations) might well introduce a bias in the EC_{50} values, thus creating a small gap between the results obtained in the presence and absence of the ligands. Also, the equilibrium constant used for the La-Mal complex (log K = 5.66) is not known with great precision; an examination of published values indicated log K values between 5.25 and 6.04 resulting in a standard deviation of ± 0.3 log units. Modifying the log K value for this complex by 0.3 log units can result in a change in the calculated free La^{3+} concentration by a factor of 2. Considering this

uncertainty, we conclude that the EC_{50} values expressed as free La^{3+} overlap for all conditions tested. Additional EC_{50} values based on the free ion concentrations were determined based on combined data from all the conditions (presence and absence of organic ligands), for La and Ce. The obtained EC_{50} values were of 0.48 [0.38 – 0.60] µM for La^{3+} and of 0.47 [0.36 – 0.61] µM for Ce^{3+} (without hormesis effect data), showing that when the whole generated data set is considered, Ce and La have similar toxicity to the alga after 120 h (La^{3+} EC_{20} = 0.11 [0.08 – 0.15] µM; Ce^{3+} EC_{20} = 0.18 [0.11 – 0.28] µM). Effective concentrations were also computed based on cell yield after 96 h (Table S2) and resulted in similar values as those observed after 120 h (Table 2).

Regardless of the way in which the EC_{50} values are expressed (total dissolved or free cation concentrations), our results are in the low range of the values reported in the literature (Table S3). All the following studies indicate lower REE toxicity than in our study. Stauber and Binet (2000) obtained a 72 h-EC_{50} of 3.2 µM La for the green alga *Raphidocelis subcapitata*. With this same alga, González et al. (2015) obtained a 72 h-EC_{50} of 45 µM Ce. Fujiwara et al. (2008) obtained an EC_{50} after 96 h of exposure of the freshwater green alga *Chlorella kessleri* to La of 313 µM (59 µM when they considered the free La^{3+} concentrations). Similarly, using the bacteria *V. fischeri*, McCloskey et al. (1996) obtained 15 min-EC_{50} values of 1.7 mM and 0.3 mM when considering total La concentrations and its free ion concentrations, respectively. This difference between total and free EC_{50} values was also observed in the case of Ce with algae and rotifers (González et al. 2015). Moreover, similar results have been reported in the case of other lanthanides such as Gd, Lu (González et al. 2015) and Eu (Fujiwara et al. 2008). In another study, using the marine unicellular alga *Skeletonema costatum*, Tai et al. (2010)

obtained similar results for both La and Ce with total dissolved concentrations-based EC_{50} values of 29.2 and 29.7 µM, respectively. Joonas et al. (2017) also tested the toxicity of four REE (La, Ce, Gd, Pr) to the green alga *Raphidocelis subcapitata* and reported comparable growth inhibitory effects for the four elements with EC_{50} values around 9.0 µM. Contrastingly, no adverse effect was found at similar concentration of La, Ce, Gd, and Nd to the green alga *Desmodesmus quadricauda* by Řezanka et al. (2016). Other studies performed with different lanthanides, including La and Ce, on other test organisms can be found in the literature. Herrmann et al. (2016) reviewed the aquatic toxicity of different lanthanides with a particular emphasis on La. Moreover, González et al. (2015) determined the toxicity of three lanthanides (e.g., Ce, Gd and Lu) to different test organisms (e.g., algae, crustacean, and bacteria).

Overall, the EC_{50} values found in the literature are up to 1,000 times higher than those obtained in this study. Several factors may be responsible for this result, including the formation of insoluble La species in exposure media that was observed in the Stauber and Binet (2000) study. Several studies have observed the formation of precipitates in the presence of carbonates and phosphates, which resulted in a significant decrease in REE concentrations in the exposure media (Barry and Meehan 2000; Fujiwara et al. 2008; González et al. 2015; Joonas et al. 2017). More interestingly, some authors have observed that some nutrient concentrations (phosphates and carbonates) also decreased in the media due to the precipitation and they concluded that because algae require these nutrients for their growth, their removal was likely the mechanism behind the adverse effects, rather than any direct effects of the REEs (Joonas et al. 2017; Stauber and Binet 2000). In our case, considering that total dissolved La and Ce concentrations remained

stable over 120 h in the exposure media (Figure S1), no precipitation occurred in our exposure media and the observed effects are directly linked to dissolved La and Ce.

3.3 Applicability of the biotic ligand model

When the toxicity results were expressed in terms of the free La^{3+} and Ce^{3+} concentrations, comparable EC_{50} values were obtained for the different tested conditions, an observation that suggests compliance with the BLM. Thus, in order to evaluate the applicability of the BLM, cell yields were plotted as a function of free cation concentrations, and the results are presented in Figure 1. If La and Ce toxicity follow the BLM, then concentration-response curves, based on free La^{3+} and Ce^{3+} concentrations, will provide a better fit to the data than curves based on total dissolved La and Ce (Figure S9). By comparing the results presented in Figure 1 and Figure S9, we observe that for the different conditions (e.g., absence or presence of organic ligands) and similar exposure concentrations, the plots become closer to each other when expressed as free ion concentrations for both La and Ce. The proximity of the points at concentrations higher than 10^{-7} M Ce^{3+} on the EC_{50} curve in the absence of buffer demonstrates a good estimate of the toxicity of Ce when the exposure conditions are such that the losses of Ce during exposure are small relative to the initial concentration. On the other hand, for the concentrations lower than 10^{-7} M of Ce^{3+}, a divergence in the distribution of the points is observed. The hormesis effect causes this difference between the points in the absence and in the presence of malic acid. Overall, the results confirmed the applicability of the BLM concept for La and Ce: that is, in contrast to total dissolved Ln concentrations, free La^{3+}, and Ce^{3+} concentrations were clearly correlated with the growth response of *C. fusca*.

Studies testing the applicability of models such as the BLM or the free ion activity model (FIAM) for trivalent metals remain scarce compared to those for divalent metals. For example, Weltje et al. (2004) tested the validity of the FIAM for lutetium using the marine bacterium *V. fischeri*. In the presence of synthetic organic ligands, standard concentration-response curves were observed when the concentration was expressed as Lu^{3+} rather than as total Lu, which corroborates our results. In a recent study with the wheat *Triticum aestivum*, Gong et al. (2019) successfully applied the BLM to predict Y and Ce toxicity and account for cation (Ca^{2+} and Mg^{2+}) competition with more than 93% of the variance in toxicity explained. In some recent short-term exposure studies, however, greater accumulation than expected based on the free ion concentration was observed for several REE in the presence of small organic ligands, including malic acid (Tan et al. 2017; Yang et al. 2014; Yang and Wilkinson 2018; Zhao and Wilkinson 2015). Our results do not suggest an increase in toxicity in the presence of malic acid compared to results in its absence, when relative growth is presented as a function of free Ln^{3+}. Moreover, our toxicity results with La^{3+} in the presence of malic acid overlap with those where the non-assimilable NTA buffer was used, suggesting that all the tested ligands are protective against La toxicity (Figure 1). We cannot offer an hypothesis for this discrepancy in results between short- and long-term exposures but the question merits to be explored in the future.

In this work we also measured metal accumulation after 120 h. Cellular yields were plotted against the intracellular concentrations of La and Ce. The obtained results (Figure S10) were close to those obtained when the cellular yields were plotted against free ion concentration (Figure 1) suggesting that the internalization of La and Ce in cells

is the cause of the reduction in cell density in *C. fusca* and that cells are more tolerant to Ce than to La. In the case of La, the EC_{50} in the absence of ligands is 128 [99 – 165] amol/cell and in the presence of malic acid of 118 [89 – 156] amol/cell. It is also interesting to note that, in the case of Ce, the occurrence of a hormesis effect did not alter the onset of toxic effects once a critical internal concentration is reached. The EC_{50} in the absence of malic acid was 441 [368-528] amol/cell (data where an hermetic response was observed were omitted) and in its presence the EC_{50} was 513 [374-705] amol/cell.

Finally, intracellular La and Ce concentrations were plotted against total dissolved and free ion concentrations (Figure 2). No apparent correlation was observed among the different experiments when total dissolved concentrations were used as a measure of exposure (Figures 2A and 2C). However, a good correlation was observed with free ion concentrations (Figures 2B and 2D). For both metals, a small gap remains between uptake data sets as a function of free metal in the presence or absence of ligands. As mentioned earlier, this could be the result of uncertainties in speciation calculation as well as the lack of metal buffering in the absence of ligands.

3.4 Influence of Ca concentrations on La and Ce toxicity

Lanthanides have long been identified as potential inhibitors of Ca transporters (Korotkov et al. 2014; Reed and Bygrave 1974). They exhibit competitive interactions with protons and major cations including Ca^{2+} and Mg^{2+} and they have been shown to interfere with Ca metabolism in cells, presumably because their ionic radii are similar to that of Ca^{2+} (González et al. 2014). The inhibition of Ca metabolism is shared by REEs (Kulaksiz and Bau 2013). Thus, we investigated the effect of Ca concentrations on the

growth of *C. fusca* in exposure media containing La (3.5 µM) or Ce (6.0 µM). Three Ca concentrations (1.0 µM, 68 µM, and 1.0 mM) were tested, and the free ionic La^{3+} and Ce^{3+} proportions remained within a narrow range (i.e., 8-11% for La and 5-6% for Ce) in the three exposure media. The growth curves obtained are shown in Figure S11. Statistical analysis using a two-factor ANOVA with repeated measurements indicates that the cellular yield after 96 h of exposure to the three Ca concentrations tested were statistically different for both La and Ce. After 120 h, in the case of Ce, the media with the three Ca concentrations showed significantly different cellular densities. However, after 120 h of exposure to La, the difference was no longer significant between the algae exposed in the presence of 1.0 mM and 68 µM of Ca due to a greater uncertainty in the measured cell densities for these Ca concentrations. Overall, Ca appears to have a protective effect on *C. fusca* during exposure to La and Ce. In order to test this hypothesis, intracellular concentrations of La and Ce, as well as final cell densities, were determined and compared for the different Ca-containing media. The results of this analysis are presented in Figure 3. The higher the concentration of Ca in the exposure media, the lower was the intracellular concentration of La and Ce. Calcium thus provides a protective effect to the species studied and this protection presumably results from the decrease in internalization of the lanthanides and is reflected in an increase of the cellular density with the Ca concentration. We hypothesize that competition between Ca and the REEs for the same cell membrane carriers is at the origin of this protection. Indeed, the uptake of La and Ce as a function of the Ca concentration follows a typical inhibition pattern. Thus, the Ca affinity constant (K_{Ca}) with La and Ce transporter was determined using a Michaelis-Menten model (equation 1) as developed by François et al. (2007):

$$[M]_{intracell} = \frac{\alpha}{1+K_{Ca}[Ca^{2+}]} \text{ (Equation 1)}$$

where $[M]_{intracell}$ is the measured intracellular concentration of La or Ce in mol/cell, α is a proportionality constant and $[Ca^{2+}]$ is the measured concentration of Ca in the exposure medium in mol/L. It was possible to estimate the K_{Ca} constant using non-linear regression. The obtained K_{Ca} constants for the binding sites of La and Ce were comparable ($4 \pm 2 \times 10^{4.0}$ and $1.8 \pm 0.3 \times 10^{4.0}$, respectively). Interestingly, El-Akl et al. (2015) obtained a K_{Ca} of $2.1 \pm 0.5 \times 10^{4.0}$ for Ce binding sites, a value virtually identical to ours. Similar results were observed for the binding of Ca to the transport sites of other REEs. For example, a stability constant K_{Ca} of $10^{4.0}$ was obtained for Sm binding sites (Tan et al. 2017) whereas the K_{Ca} was lower ($10^{2.6}$) for Nd binding sites (Yang and Wilkinson 2018). Other studies performed on Cd binding sites determined K_{Ca} values of $10^{4.0}$ (Lavoie et al. 2012), $10^{4.5}$ (Kola and Wilkinson 2005) and $10^{4.8}$ (François et al. 2007) which are close to our observed values.

4 Conclusions

Our results showed similar toxicity and accumulation levels for La and Ce for a given free-metal ion concentration when all other parameters are constant (e.g. pH, hardness). Increasing the concentration of Ca in the medium reduced the internalization of the Ln and vice versa. The only point that differentiates these two lanthanides in the present study is that Ce, in the absence of an organic ligand, induced an hormesis effect on the green alga *Chlorella fusca*, whereas this phenomenon was not observed for La. The reason why an hormesis effect was only observed in the absence of organic ligands

remains unknown and the exact cause of metal-induced hormesis remains unknown (see review by Calabrese (2008)).

The observed toxicity thresholds of La and Ce were 10 to 1000 times lower than in the literature (cf. Table S3). This difference was likely caused by several factors including 1) the non-consideration of the speciation of the metal in solution in various early studies, 2) the absence of formation of insoluble phosphate species in our exposure media, 3) the presence of "protective" chemical species such as Ca at various levels in the different studies and/or, 4) a marked variability in species sensitivity. The results obtained in this study suggest that the BLM framework can be used for the environmental risk assessment of REEs. We demonstrated that a rigorous control of REE speciation in the algal exposure media was needed if one wanted to understand the links between the speciation of the REEs and their uptake and toxicity. This opens a door of opportunity to develop a BLM-based toxicity prediction model for REEs.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

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Data availability statement—Data, associated metadata, and calculation tools are available from the corresponding author (claude.fortin@ete.inrs.ca).

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Figures

Figure 1. Cell yield (measured cell density in exposed samples divided by control cell density) after 120 h of exposure to La (A) or Ce (B) as a function of calculated La and Ce free ion concentrations in solution. Exposures performed in the absence of organic ligands (black), and in the presence of NTA (blue), IDA (green) or malic acid (red).

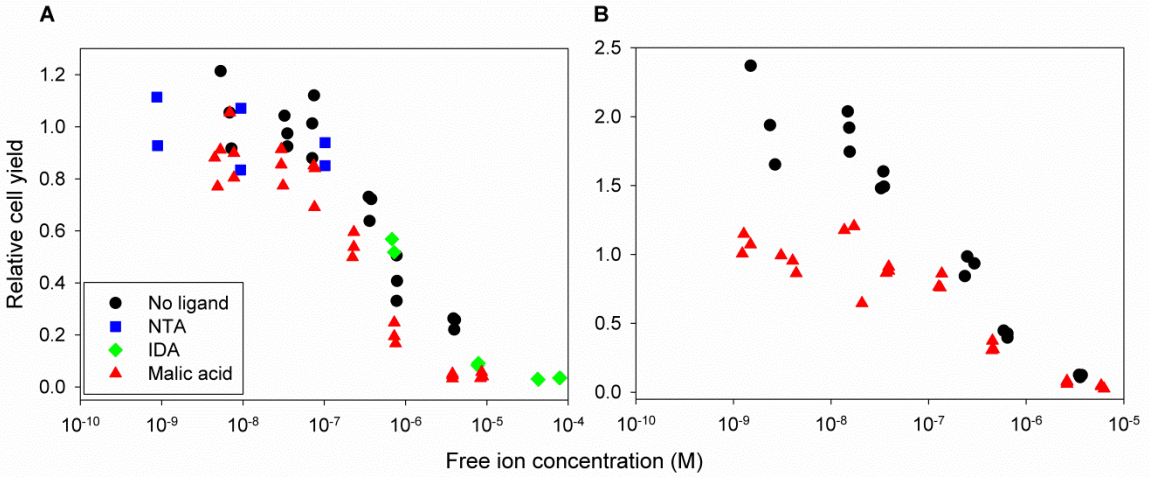

Figure 2. Intracellular La or Ce concentration after 120 h of exposure as a function of total mean La (A) or Ce (C) measured concentrations or calculated free La^{3+} (B) or Ce^{3+} (D) concentrations. Exposures performed in the absence of organic ligands (black), and in the presence of NTA (blue), or malic acid (red).

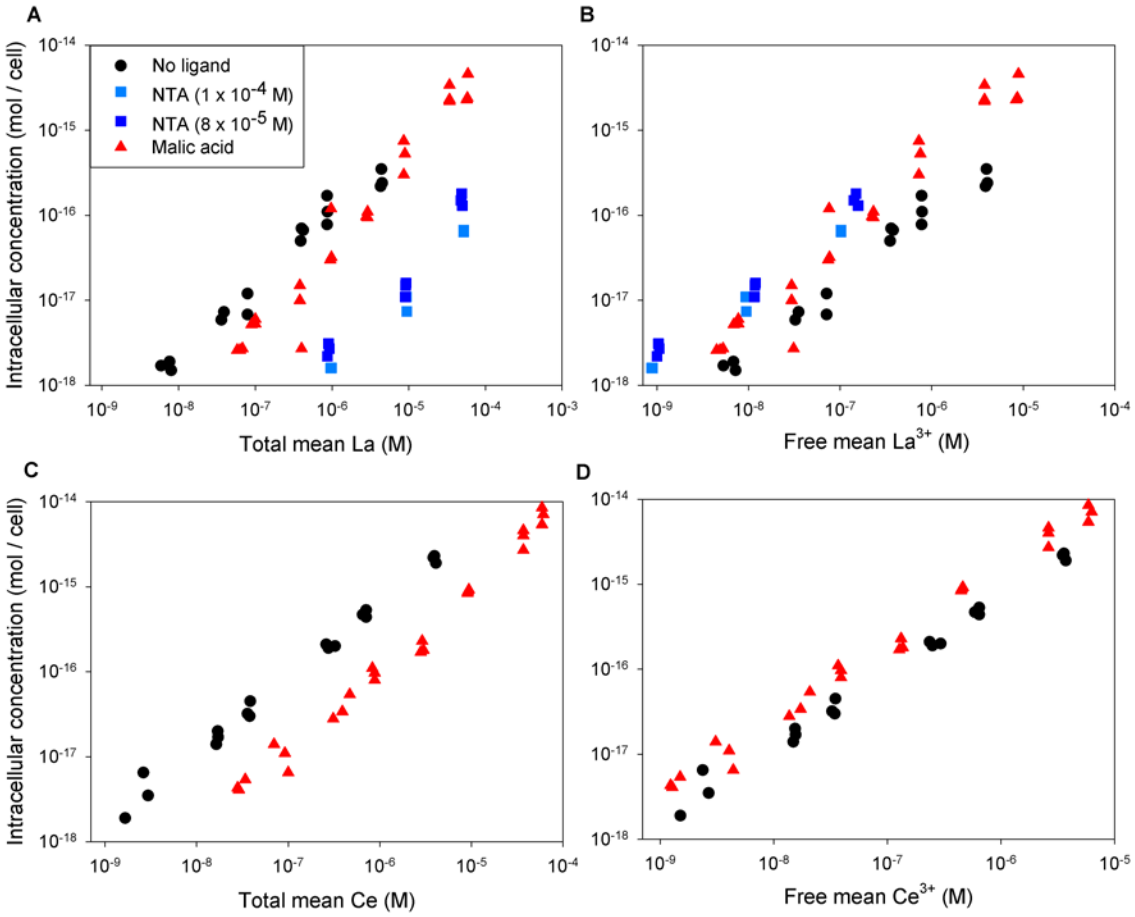

Figure 3. Cellular density and intracellular La or Ce concentrations after 120 h of exposure to La (3.5 µM) or Ce (6.0 µM) in the presence of different Ca concentrations. All conditions were tested in five replicates in the presence of 10^{-4} M of malic acid and error bars indicate one standard deviation.

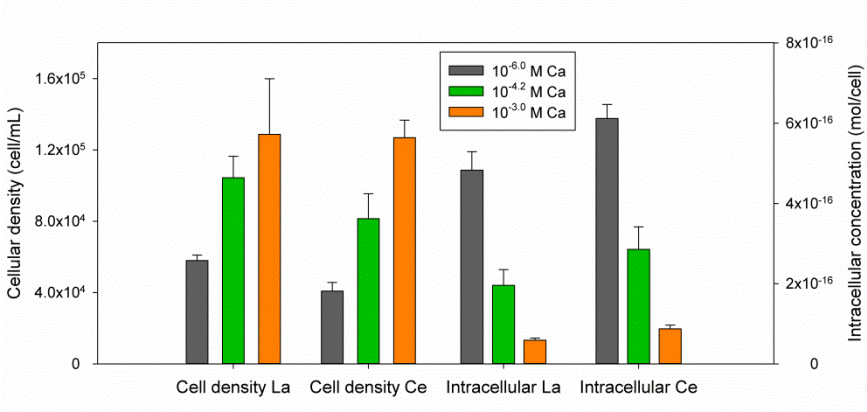

Table 1. Main species of La and Ce and their calculated abundance percentages in solution in the absence or presence of different ligands. Speciation calculated with MINEQL+ 5.0 software for pH 5.5 using 1.0 µM as La or Ce input concentrations and other species total concentrations reported in Table S1 as input concentrations. The default MINEQL+ thermodynamic database was updated with the values provided in Leguay et al. (2016). Binding constants for La-IDA (log K_1 = 7.17; log β_2 = 11.69) were taken from the NIST (National Institute of Standards and Technology) Critically Selected Stability Constants of Metal Complexes database (version 8.0).

	No ligand	**Malic acid (1×10^{-4} M)**	**NTA (8×10^{-5} M)**	**NTA (1×10^{-4} M)**	**IDA (1×10^{-4} M)**
La	La^{3+} (90%)	La^{3+} (7.8-15%)	La^{3+} (0.1-0.3%)	La^{3+} (0.1%)	La^{3+} (88-89%)
	$LaSO_4^+$ (10%)	$LaMal^+$ (80-84%)	La(NTA) (96-98%)	La(NTA) (95-97%)	$LaSO_4^+$ (8.5-9.4%)
		$La\ Mal_2^-$ (3.4-	$La(NTA)_2^{3-}$ (1.7-	$La(NTA)_2^{3-}$ (2.5-	$La(IDA)^+$

		7.2%)	4.1%)	5.1%)	(2.8%)
Ce	Ce^{3+} (91%) $CeSO_4^+$ (9.3%)	Ce^{3+} (4.4-10%) $CeMal^+$ (83-85%) $CeMal_2^-$ (5.2-12%)			

Table 2. Toxicity of La and Ce to *Chlorella fusca* after exposure over 120 h, in the absence or presence of ligands in MHSM-2 medium. Results are expressed as EC_{50} or EC_{20} with upper and lower 95% confidence intervals in brackets and all values are given in µM. Data for Ce in the absence of ligands showed a hormesis effect at total concentrations up to 40 nM and were not considered in the dose-response regression analysis. When a data set did not follow a dose-response pattern, the EC_x could not be determined (N.D.).

			120 h	
Condition	**Effective concentration**	**REE**	**Total (µM)**	**Free ion (µM)**
No ligand	**EC_{20}**	**La**	0.23 [0.13 - 0.40]	0.20 [0.12 - 0.36]
	EC_{50}		0.86 [0.60 - 1.23]	0.78 [0.54 - 1.11]
	EC_{20}	**Ce**	0.40 [0.29 - 0.55]	0.36 [0.26 - 0.50]
	EC_{50}		0.63 [0.54 - 0.74]	0.57 [0.49 - 0.67]
Malic acid	**EC_{20}**	**La**	0.74 [0.50 - 1.10]	0.05 [0.04 - 0.08]
	EC_{50}		2.89 [2.24 - 3.72]	0.23 [0.18 - 0.31]
	EC_{20}	**Ce**	2.40 [1.52 - 3.79]	0.11 [0.06 - 0.17]
	EC_{50}		6.13 [4.57 - 8.23]	0.30 [0.21 - 0.41]

NTA or IDA	**EC_{20}**	**La**	N. D.	0.12 [0.08 – 0.18]
	EC_{50}		N. D.	0.70 [0.49 – 0.99]
All conditions	**EC_{20}**	**La**	N. D.	0.11 [0.08 – 0.15]
	EC_{50}		N. D.	0.48 [0.38 – 0.60]
	EC_{20}	**Ce**	0.61 [0.28 – 1.36]	0.18 [0.11 – 0.28]
	EC_{50}		2.79 [1.64 – 4.75]	0.47 [0.36 – 0.61]