Mixtures of rare earth elements show antagonistic interactions in *Chlamydomonas reinhardtii*

Elise Morel[1], Lei Cui [1], William Zerges[2] and Kevin J. Wilkinson[1*]

[1]Biophysical Environmental Chemistry Group, University of Montreal, P.O. Box 6128, Succ. Centre-Ville, Montreal, QC

[2]Dept. of Biology, Concordia University, 7141 Sherbrooke W., H4B 1R6, Montreal, QC, Canada

*kj.wilkinson@umontreal.ca; ORCID: 0000-0002-7182-3624

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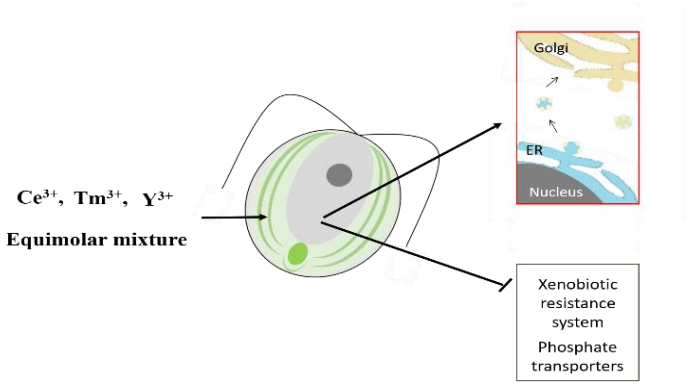
ABSTRACT

In order to better understand the environmental risks of the rare earth elements (REEs), it is necessary to determine their fate and biological effects under environmentally relevant conditions (e.g. at low concentrations, REE mixtures). Here, the unicellular freshwater microalga, *Chlamydomonas reinhardtii*, was exposed for 2 h to one of three soluble REEs (Ce, Tm, Y) salts at 0.5 μM or to an equimolar mixture of these REEs. RNA sequencing revealed common biological effects among the REEs. Known functions of the differentially expressed genes support effects of REEs on protein processing in the endoplasmic reticulum, phosphate transport and the homeostasis of Fe and Ca. The only stress response detected was related to protein misfolding in the endoplasmic reticulum. When the REEs were applied as a mixture, antagonistic effects were overwhelmingly observed with transcriptomic results suggesting that the REEs were initially competing with each other for bio-uptake. Metal biouptake results were consistent with this interpretation. These results suggest that the approach of government agencies to regulate the REEs using biological effects data from single metal exposures may be a largely conservative approach.

KEY WORDS

Rare earth elements, mixture, transcriptomic analysis, microalgae

GRAPHICAL ABSTRACT



INTRODUCTION

Rare earth elements (REEs) are strategic metals used in the development of the low-carbon energy sector.[1, 2] They include the lanthanide metals, in addition to yttrium (Y) and scandium (Sc). In the aquatic environment, REE concentrations vary from ng L^{-1} in non-contaminated waters[3] to mg L^{-1} in the more contaminated ones.[4] Nonetheless, REE contamination levels appear to be increasing in the environment, due largely to increasing e-waste discharges[5] and agricultural applications, such as REE enriched fertilizers.[6, 7] In addition, REE contamination has been observed in streams close to REE mines.[8]

In natural aquatic systems, the light lanthanides (lanthanum (La) to europium (Eu)) are generally found at higher concentrations than the heavy lanthanides (gadolinium (Gd) to lutetium (Lu)), largely due to their greater solubilities.[9] Heavy REEs are generally more strongly complexed than light REEs,[10] potentially decreasing their free ion concentration (and thus their bioavailability). Although there are relatively few exposure data available to define the risk of REEs to environmental and human health, early reports have indicated that the heavy REEs are more toxic than the light ones.[11] For example, La, cerium (Ce) and neodymium (Nd) were less toxic to juvenile rainbow (*Oncorhynchus mykiss*) trout than Y, samarium (Sm), erbium (Er) and Gd with LC50 values in the range of 0.1 to 1 mg L^{-1} for Y (i.e. 1.1 to 11 µM); 1 to 10 mg L^{-1} for Sm, Er and Gd and >40 mg L^{-1} for La, Ce and Nd (i.e. > 286 µM for Ce).[12]

Nonetheless, several factors complicate risk determinations for the REEs including: REE speciation cannot be predicted solely from thermodynamic considerations;[13,14] some REE complexes appear to be bioavailable (e.g. those formed with small hydrophilic ligands);[15,16] and toxicological responses vary across biological species.[10] In addition,

numerous toxicological experiments that have been performed in the presence of phosphate, which is known to precipitate the REEs.[17]

Organisms inhabiting metal-contaminated natural waters are almost always exposed to metal mixtures.[18] This is especially true for metals within the REE series, which are nearly always found together in natural systems.[19-22] In metal mixtures, uptake may be both competitive and non-competitive, resulting in biological uptake and toxicological impacts that can be antagonistic, additive or even synergistic.[18, 23-25] For algae and plants, the few reports on the biouptake of REE mixtures have shown antagonistic effects of one on the uptake of the other.[16, 26] However, biological effects are poorly understood, especially at environmentally relevant REE concentrations and for REE mixtures. The interpretation of bioaccumulation or toxicity results for REE mixtures is complex due to complex interactions at the site(s) of toxicity, interactions among physiological processes and potential chemical interactions with constituents in the media, affecting chemical speciation.[10]

In this study, a chemically simple exposure medium was used in order to facilitate the control of REE speciation. Three REEs were examined: Ce (light REE), Tm (heavy REE) and Y (chemically similar REE). Exposure concentrations of 0.5 µM (equivalent to 70.1 $\mu g.L^{-1}$ of Ce, 84.5 $\mu g.L^{-1}$ of Tm and 44.5 $\mu g.L^{-1}$ of Y) were used to simulate moderate contamination. These REE concentrations are slightly higher than the maximum permissive exposure concentrations for freshwater systems (1.8-22.1 $\mu g.L^{-1}$, i.e. ~ 0.01-0.1 µM).[27] Biological effects of the REEs, individually and in a ternary mixture, were revealed by transcriptome profiling (RNA-Seq) analysis performed on *Chlamydomonas reinhardtii*. RNA-Seq has been previously used to gain a more mechanistic understanding of metal

homeostasis in *C. reinhardtii* for both essential nutrients (*e.g.* copper[28], iron[29], zinc[30]) or toxic metals (*e.g.* methyl mercury[31], Cd [32], Pb[33]). In this study, transcriptomic effects of the REEs were inferred from the known functions of the differentially regulated genes in comparison to unexposed microalgae. Expression levels for an equimolar mixture of the REEs were then compared to predictions based upon the single metal exposures.

MATERIALS AND METHODS

MATERIALS

All experiments were performed in polymerware (polypropylene or polycarbonate), which was first soaked in 2% v/v HNO_3 for 24 hours, rinsed 7x with Milli-Q water (total organic carbon < 2 µg L^{-1}; resistivity > 18 MΩ cm) and dried under laminar flow conditions. Chemicals were molecular biology grade or higher, including acetic acid (analytical grade, Fisher Scientific, CA); chloroform (99,8%, Acros organics, CA); nuclease-free water (Qiagen, USA); K_2HPO_4 and KH_2PO_4 (ACS reagent grade, Fisher Chemical, CA); Tris (Tris-(hydroxymethyl)-aminomethane, USP/EPgrade, BDH, CA); EDTA disodium salt (Bioultra grade, Sigma-Aldrich, CA); Isotone (VWR, CA); HNO_3 (67–70%; Aristar Ultra, BDH), NaOH (Acros Organics, CA), NaMES (2-(N-morpholino)ethanesulfonic sodium salt, Acros Organics, CA); NaHEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic sodium salt, Acros Organics, CA). Single element (1.0 g.L^{-1}; $Ce(NO_3)_3$, $Tm(NO_3)_3$, $Y(NO_3)_3$) and multielement (10 mg.L^{-1}; CMS-1) ICP-MS standards were acquired from Inorganic Ventures (USA).

CULTURE AND EXPOSURE CONDITIONS

C. reinhardtii is a green microalga that is ubiquitous to fresh waters and often used for studies examining the toxicology of pollutants in natural waters. Details on its specific culture conditions and preparation for experiments involving trace metals have been described previously.[15] In brief, the wild-type strain CC-125 (aka 137c, *Chlamydomonas* resource center) was cultured in 4×diluted TAP at 20°C under conditions of 12 h light/12 h dark (60 mmol s^{-1} m^{-2}) using orbital shaking (100 rpm), until algae reached their mid-exponential growth phase. Cells were then washed (3×) by pelleting them by centrifugation (2000xg for 3 min) then resuspending them in an exposure medium (see below) that contained no metal. The concentrated cell suspension was then diluted to 6.5-10 x 10^4 cells mL^{-1} (i.e. 0.15 $cm^2.mL^{-1}$) in an exposure solution containing the appropriate metal concentration. Culture densities and cell surface areas were measured using a Multisizer 3 particle counter (50 µm aperture; Beckman Coulter, Mississauga, CA).

Cells were exposed for 2 h to 0.5 µM of **Ce**, **Tm**, **Y** or their equimolar mixture (**Mix**) in 10.0 mM NaHEPES (pH buffered at 7.0 in a solution also containing 10.0 µM $Ca(NO_3)_2$).[34] The exposure duration and concentration were selected to minimize physicochemical modifications to the exposure medium, while being long enough to obtain significant biouptake and resulting genomic response. Indeed, a plateau in the internalization flux was observed at ~0.5 µM for exposures of *C. reinhardtii* to: Ce[35], Nd[36], Sm[16] and Tm[15]. The use of a simplified exposure medium allowed the chemical speciation of the REEs to be precisely controlled, however, it may stress the microalgae at longer time exposure times due to nutrient deficiency (*e.g.* induction of *PSR1* after 4-8 h of phosphate starvation)[37]; therefore, short term exposures (2 h) were favored. Speciation was modeled using Visual MINTEQ v3.1, taking into account equilibration with atmospheric CO_2. In

order to evaluate adsorptive losses and/or contamination, dissolved (< 0.45 μm, nitrocellulose membrane, Millipore, CA) metal concentrations in the experimental media were measured: (i) after preparation of solutions but before the addition of the algae; (ii) following the addition of the algae and (iii) at the end of the 2h algal exposure to the REEs. Four hundred μL of HNO_3 (67–70%) was added to 1 mL of sample followed by 5 h of heating at 80° C (DigiPREP, SCP Science, Baie d'Urfe, CA). Samples were then diluted to 10 mL in Milli-Q water and analyzed by inductively coupled plasma mass spectrometry (ICP-MS, PerkinElmer; NexION 300X, Waltham, US). A calibration curve for each element was run every 20 samples, while blanks and quality control standards were run every 10 samples. Indium was used as the internal standard to correct for instrumental drift.

Cell densities (6.5-10 x 10^4 cells mL^{-1}) were low enough to ensure that REE concentrations did not decrease significantly over the duration of experiment (**Figure S1**). Following the 2 h exposure, cells were pelleted from 200 mL of the exposure medium by centrifugation (2000×g, 2 min, 4°C). Cell pellets were resuspended in 1 mL nuclease-free water before being transferred into 1.5 mL microtubes where cells were again pelleted by centrifugation. Cell pellets were frozen on dry ice and stored at −80 °C.

For metal biouptake experiments, cells were exposed in medium described above for 2 h to 0.5 μM of Ce, Tm, Y or different equimolar binary mixtures (6 biological replicates, each in technical duplicate). Bioaccumulation was stopped at 120 min by adding 5 mL of 0.1 M ethylenediaminetetraacetic acid (EDTA) to 45 mL of the exposure medium at 2 h, in order to stop further bioaccumulation and to wash weakly surface-adsorbed metal from the cell surface. One minute after the addition of EDTA, algae were filtered over two stacked 3 μm nitrocellulose filters (Millipore). The filters were rinsed three times with 5

mL of 0.01 M EDTA. The top filter retained the algae, while the lower one was used to quantify adsorptive losses. Metal biouptake was determined from the difference between two filters. The filters were collected in 15 mL centrifugal tubes and transferred into 0.3 mL of ultrapure HNO_3 (70% v/v) and digested at 85°C overnight. Metal concentrations in the filter digests and in the exposure media were diluted to 1-2% HNO_3 in Milli-Q water prior to analysis by ICP-MS. Flasks were also rinsed with 50 mL of 1% HNO_3 in order to verify mass balances. Mass balances were considered acceptable when recoveries were between 80% and 110%.

TOTAL RNA EXTRACTION

Frozen cell pellets were thawed and immediately resuspended in freshly prepared lysis buffer (0.3 M NaCl, 5.0 mM EDTA, 50 mM Tris-HCl (pH 8.0), 2.0% (w/v) SDS, 3.3 U mL^{-1} proteinase K), where they were incubated at 37°C for 15 min with orbital shaking (300 rpm). Total RNA was isolated by extracting the sample 3x with phenol:chloroform:isoamyl alcohol (25:24:1, pH 6.8), followed by 1x with chloroform:isoamyl alcohol (24:1). At each step, samples were centrifuged (12000xg, 10 min, 4°C) and supernatants were transferred into new tubes. Total RNA was precipitated from the final aqueous phase by isopropanol, then washed with 75% ethanol. After a final centrifugation, the pellet was resuspended in 20-30 µL of nuclease-free water. A 3 µL aliquot was analyzed by automated electrophoresis for RNA quality (RIN number > 7; 1.8 < ratio 260/280 < 2.1; ratio 260/230>1.8; Bioanalyzer, Agilent, Mississauga, CA) and spectroscopy (OD260) in order to determine the RNA concentration (Nanodrop, Wilmington, US). RNA samples were stored at −80°C until RNA-Seq analysis.

RNA-SEQ ANALYSIS

DNase treatment, mRNA selection, library preparation (NEB/KAPA mRNA stranded library preparation) and Illumina sequencing were carried out at the Genome Quebec facilities (www.gqinnovationcenter.com, Montreal, CA). Two lanes on a HiSeq (v.4) were used for the paired-end sequencing (2 × 100 base pairs) of 25 samples (5 replicates for each treatment: Ce, Tm, Y, Mix, controls). All replicates were from independent algal cultures.

For each sample, *ca.* 55 million of reads with their sequences, identification and quality scores were stored in two FastQ files. Read quality was explored with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc), while filtering quality and adapter trimming were carried out with Trim Galore! (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore). Only paired reads obtained after the cleaning step (phred >20, length > 21 bp) were conserved. Reads were aligned to the *C. reinhardtii* genome v5.3 assembly using TopHat2 with standard presets except that intron size was between 30 and 28000 bbp.[38] Approximately 45 million reads were mapped for each sample (around 82% of raw data) with concordant pair alignment accounting for 85% of the total mapped reads in each sample. GeneBody coverage python script (RSeQC) was used to calculate the number of reads for each nucleotide position and to generate a plot illustrating the coverage profile along the gene (**Figure S2**).[39] The number of reads per gene were determined using the Python package HTSeq.[40] Raw RNA sequencing data and the associated count matrix can be found under the data deposit number : GSE176268 (Gene Expression Omnibus platform).

196 Differentially expressed genes (DEGs) were identified using DESeq2[41] for $\log_2$ fold
197 change (Log_2FC) values exceeding |3| and false discovery rates (p_{adj}) < 0.001. Gene
198 annotations were retrieved from MapMan ontology.[42,43] The JGI Comparative Plant
199 Genomics Portal was also used to explore the function of the gene sets of interest, including
200 some of the "not assigned" genes obtained from MapMan.[44] The Fisher exact test was
201 used to identify enriched metabolic pathways. The Algal Functional Annotation Tool was
202 used to convert gene and transcript IDs, when necessary.[45]
203
204 **COMBINED EFFECT MODELING**
205 Due to limitations in the applicability of the Concentration Addition (CA) model
206 for transcriptomic data (e.g. no knowledge of expression ranges for a given gene),[46] an
207 Independent Action (IA) model was used to predict transcriptomic effects associated with
208 the mixture, in spite of expected similar modes of action among the REEs. The conceptual
209 approach depicted by Song *et al.* in the case of binary toxicant mixtures[47] was adapted to a
210 ternary mixture and the combined effects of the mixture treatments were evaluated for each
211 differentially expressed gene (DEG). Additive interactions among the REEs were obtained
212 when the Log_2FC (fold change) observed for a given gene in the mixture treatment was
213 equal to the Log_2FC predicted by the IA model (**Eq. 1**), defined as the sum of the Log_2FC
214 of individual REE treatments (x_i represents the different REEs: Ce, Tm or Y). Gene
215 expression was considered to be synergistic for $|Log_2FC_{observed}\ (Mix)| > |Log_2FC_{predicted}$
216 $(Mix)|$ and antagonistic when $|Log_2FC_{observed}\ (Mix)| < |Log_2FC_{predicted}\ (Mix)|$. Different
217 patterns of combined effects were also discriminated by taking into account the direction
218 of the transcriptional regulation.[47]

219 $$Log_2FC_{observed}\ (Mix) = Log_2FC_{predicted}\ (Mix) = \Sigma\ Log_2FC_{observed}\ (x_i) \qquad (\textbf{Eq. 1})$$

RESULTS AND DISCUSSION

CHEMISTRY OF THE EXPOSURE SOLUTIONS

Speciation calculations indicated that the REEs should exist mainly as free metal ions in the simplified exposure solutions (72% to 85% of the total metal), with small contributions of the carbonate and hydroxide complexes (**Figure S3**). Nonetheless, in preliminary filtration controls of solutions containing the individual metals or the mixtures, important losses of REEs were observed when filtering over 0.45 µm nitrocellulose membranes (91 ± 4% of Ce; 84 ± 4% of Tm and 70 ± 18% of Y). Losses were attributed to the adsorption of the trivalent metals to the flasks or filters, potentially following the formation of metastable (non-equilibrium) particles.[10,35] In spite of a thorough acid wash prior to use, losses were much more important when re-using older polycarbonate flasks, again reinforcing the hypothesis that adsorptive losses were occurring. In order to ensure consistency between added and measured concentrations in the exposure solutions: new flasks were employed; solutions were pre-equilibrated at least 24 hours prior to exposure; and concentrations were measured at the start and the end of the exposure period (**Figure S1**). In this manner, REE concentrations were stabilized, ranging from 0.45-0.49 µM prior to the introduction of the algae (nominal concentration = 0.5 µM).

RNA-SEQ ANALYSES

Transcriptome profiling with RNA-Seq was used to compare effects of Ce, Tm, Y or an equimolar mixture of the three metals (2 h exposure to a nominal REE concentration of 0.5 µM). Of the 19,526 predicted transcripts in *Chlamydomonas reinhardtii*,[48] 16,855 (86%) were detected, indicating sufficient coverage of the genome. Principal component

analysis (PCA), performed on expression levels of these transcripts, revealed that samples exposed to Tm, Y and the ternary mixture could be distinctly grouped from those exposed to Ce (PC2, 33% of variance) (**Figure 1A**). Results of PCA also indicated that the mixture effect was most strongly influenced by Tm (and to a lesser extent by Y).

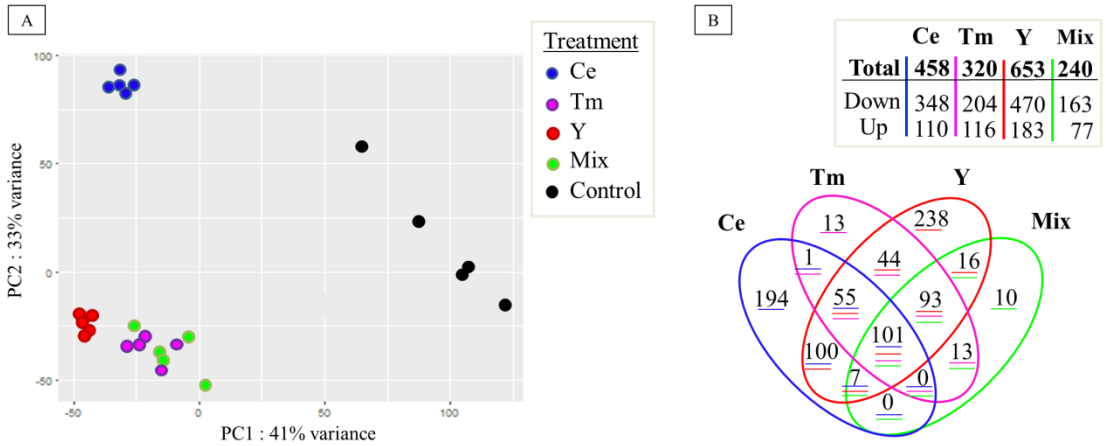


Figure 1. (A) PCA scores from RNASeq analysis resulting from 16,855 detected transcripts following a 2 h exposure of *C. reinhardtii* to 0.5 µM of: Ce (blue), Tm (pink), Y (red), a ternary mixture of the three REE (green) and the control (i.e. no added metal, black). (B) Differentially expressed genes (DEGs) with respect to the control ($Log_2FC > |3|$, $p_{adj} < 0.001$), following a 2 h exposure of *C. reinhardtii* to 0.5 µM of Ce, Y, Tm or their mixture at pH 7.0.

Eight hundred and eighty-four (884) genes showed at least an 8-fold change (FC) with respect to unexposed cells, due to one or more of the treatments ($Log_2FC > |3|$, $p_{adj} < 0.001$) **(Supplemental Data 1)**. Fewer differentially expressed genes (DEGs) were observed in the ternary metal mixture than for any of the individual metal treatments, decreasing in the order Y (653 DEGs) > Ce (458 DEGs) > Tm (320 DEGs) > Mix (240 DEGs) (**Figure 1B**). These differences can only partly be attributed to free ion

concentrations in solution ([Ce^{3+}]>[Y^{3+}]>[Tm^{3+}]) or the biouptake associated with the individual treatments ([Ce]>[Tm]>[Y]) (**Figure 2**) The proportion of up-regulated and down-regulated DEGs were similar in all treatments, with about 70% down-regulated genes (76% for Ce; 72% for Y; 64% for Tm and 68% for the ternary mixture; **Figure 1B**). Below, we explore first the common effects of the REEs, based upon the annotated functions of the differentially expressed genes. Second, we discuss the results that led us to believe that some of the biological effects are distinct for the REEs. Finally, we examined the nature of the interactions in the REE mixture.

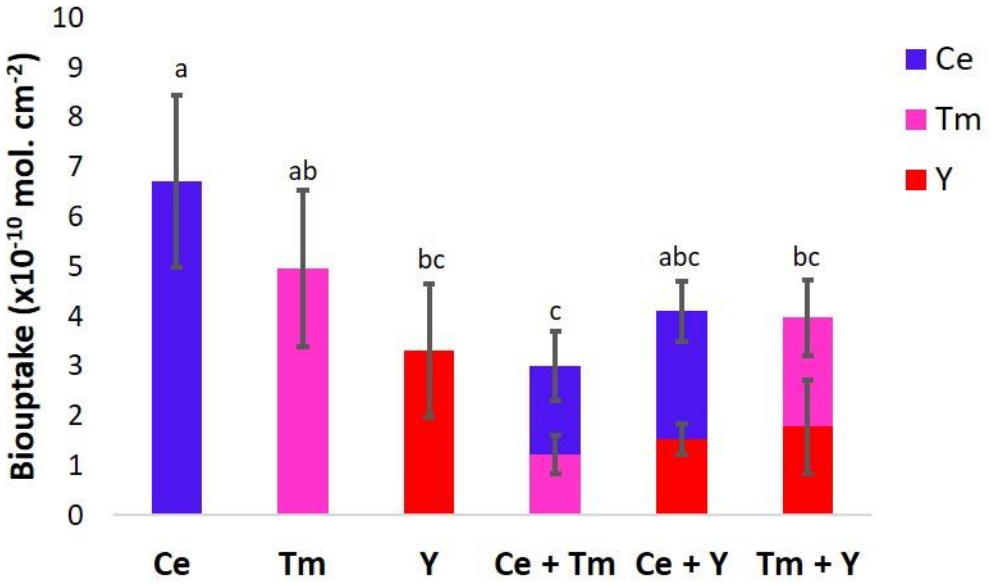


Figure 2. Biouptake of Ce, Tm, Y by *C. reinhardtii* when exposed for 2 h to 0.5 µM of either the individual REEs or equimolar binary mixtures of the REEs. A one-way analysis of variance paired with a Tukey test were used to highlight significant differences between treatments (different letters when $P < 0.05$) (Sigma Plot, v14).

INSIGHT INTO COMMON BIOLOGICAL EFFECTS OF THE REES BASED ON TRANSCRIPTOMIC PROFILING

The REEs tested showed some overlap in their biological effects supporting common REE targets. Of the 884 genes that were differentially expressed with respect to the controls, 156 (18%) were regulated by all three of the REEs (**Figure 1B**, **Supplemental Data 2)**. For each of the REEs and the equimolar ternary mixture, around 70% of the responding genes had no assigned function (**Table 1**). Among the common annotated functions for all three metals, a large number of DEGs were related to protein metabolism, RNA metabolism and the transport of metals and small molecules (**Table 1**). It is not the goal here to discuss, in detail, the mechanism of interaction of the REEs with *Chlamydomonas*. Instead, the common transcriptomic effects are summarized, with a more detailed discussion provided in the Supplementary Information (**S2.2**).

Functional annotation revealed that an important proportion of the commonly induced genes (14 of the 40 up-regulated DEGs) encoded proteins that were involved in protein processing in the endoplasmic reticulum (ER) (**Table S1**). Genes involved in protein targeting to secretory pathways were significantly enriched for all treatments (Fisher exact test, $p < 0.05$) **(Table 1**). These results suggest that REEs impact proteome homeostasis in the endomembrane system.[49] REEs down-regulated genes related to the mechanisms of stress resistance (e.g. xenobiotic resistance system, **Figure 3)**, with the exception of those related to proteins acting in the ER (**Table S1**). Important impacts of the REEs on calcium, phosphate or iron homeostasis in *C. reinhardtii* were also noticed (**Figure 3, Supplemental Data 2**). The nature of the interactions between the REEs and those elements will nonetheless require future investigation (**S2.2)**.

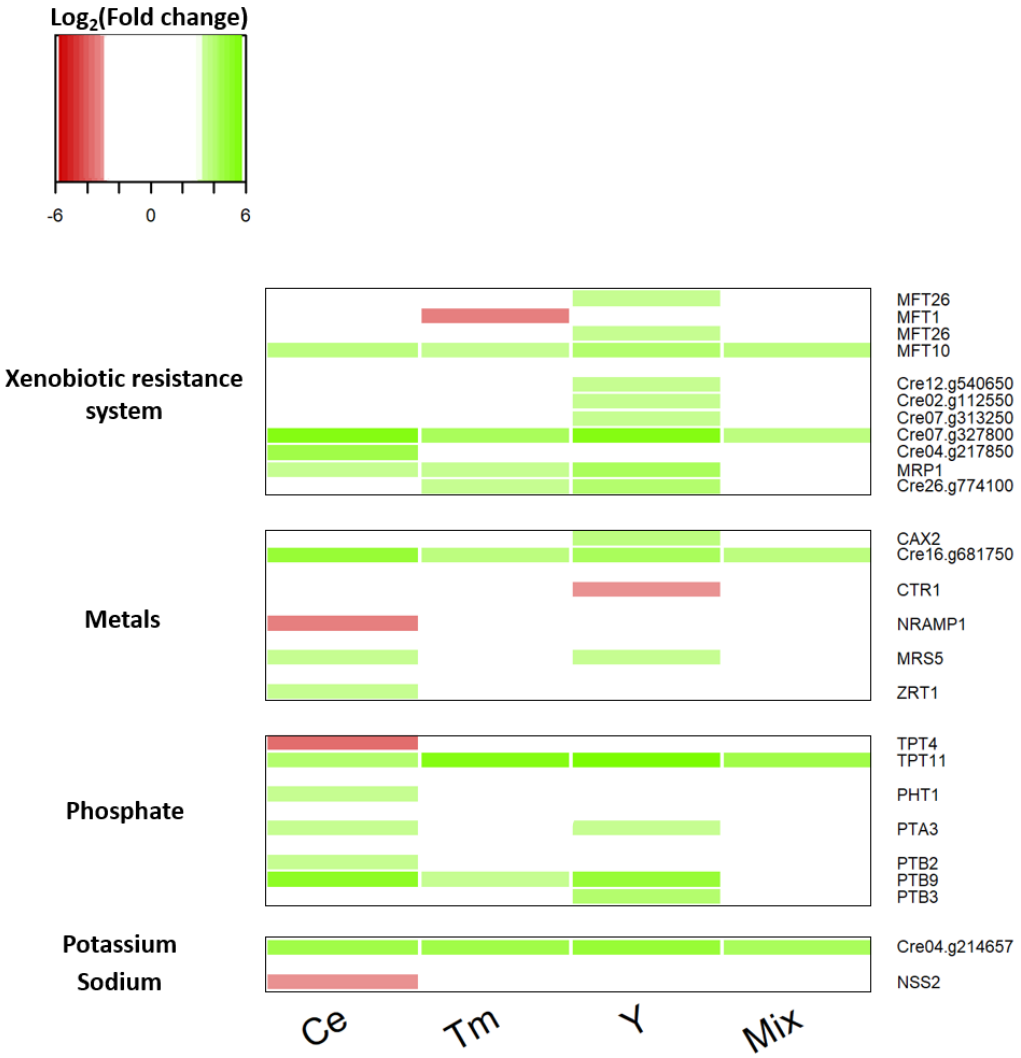
299 **Table 1.** Number of DEGs (with respect to control conditions) for several important
300 metabolic pathways and sub-pathways (MapMan) following a 2 h exposure of *C.*
301 *reinhardtii* to Ce, Tm, Y or their equimolar ternary mixture. Numbers in bold are associated
302 with enriched pathways/sub-pathways (Fisher exact test , $p < 0.05$).

Metabolic pathways	Versus Control			
	Ce	Tm	Y	Mix
Amino acid	**5**	4	6	3
Degradation	0	0	1	0
Synthesis	**5**	3	5	2
*Glutamate synthesis	**4**	1	1	1
Cellular processes	6	6	10	4
Cell development	5	3	7	3
DNA	7	3	12	4
Hormone	1	2	2	1
Lipid	4	5	9	3
Major CHO	1	0	3	0
Minor CHO	4	1	2	1
Miscellaneous enzyme families	6	6	8	4
Not assigned	**317**	224	451	175
Nucleotide	2	1	4	1
Protein	**41**	32	**67**	16
Activation	1	0	3	0
Degradation	9	6	14	2
Folding	6	7	11	5
Posttranslational modification	13	12	23	6
Synthesis	4	0	5	0
Targeting	**8**	**7**	**11**	**3**
Photosynthesis	2	2	7	3
Redox	3	2	2	1
RNA	20	11	28	9
Signaling	8	8	10	5
Stress	**6**	2	4	1
Abiotic	**5**	2	4	1
Biotic	1	0	0	0
Organic acid transformation	1	2	3	2
Tetrapyrrole	1	0	3	1
Transport of metals and small molecules	24	11	25	6

303

304

It should be noted that no clear evidence of acute toxicity was observed for the present exposure conditions. This was expected given the sub-lethal exposure concentrations (0.5 µM) and the short exposure times (2 h) that were used. Indeed, higher REE concentrations and longer exposure times are generally required to observe toxic endpoints such as growth inhibition in microalgae (e.g. EC_{50} of > 2000 µM Ce for *Pseudokirchneriella subcapitata* in a medium containing phosphates where some uncertainties are present with respect to REE speciation).[17] For *C. reinhardtii* exposed to ionic Ce[50], biomarkers such as reactive oxygen species overproduction, changes in the membrane permeability and changes in cell sizes were observed when higher concentrations (1 µM Ce and 5 µM Ce) or longer exposure times were applied (6 h at 0.5 µM). [50] Analysis of transcriptomic effect alone are not sufficient to draw any conclusions on the impact of the REEs on the fitness of the organisms.



317

318 **Figure 3.** Heat maps depicting fold changes in the transcript levels with respect to control
319 ($Log_2FC > |3|$, $p_{adj} < 0.001$) for transport-related genes that were regulated by Ce, Tm, Y
320 or their equimolar ternary mixture, following a 2 h exposure of *C. reinhardtii*. Genes
321 encoding proteins involved in the transport of metabolites, sugars and those forming
322 channels were excluded from this representation (**Supplemental Data 1)**. Red represents
323 the genes that were induced by the treatment ($Log_2FC < -3$), while green represents those
324 that were repressed ($Log_2FC > 3$), white: $-3 \geq Log_2 FC \leq 3$. Acronyms are given for genes with
325 annotated functions.

CE ANOMALY AMONG THE REES

In contrast to the common pathways above, genes involved in amino acid metabolism, stress and numerous non assigned genes were enriched only as a result of the Ce exposures (FET, $p < 0.05$). Further analyses indicated that Ce stimulated glutamate metabolism and regulated abiotic stress related genes, though the genes responded to a lesser extent than the other REEs **(Table 1, Supplemental Data 1).** Indeed, 4 genes encoding enzymes in arginine synthesis (i.e. glutamate metabolism) were up-regulated by Ce ($Log_2FC < (- 3.2)$, $p_{adj} < 0.001$), including one that also responded to Tm and Y. Ce also specifically down-regulated two genes encoding stress-induced heat shock proteins (HSPs): a putative HSP70 (*Cre02.g141186*) and a chloroplast targeted *HSP22C*, in addition to *HSP90B* that was up-regulated by all of the tested REEs (i.e. ER localized).

Finally, although PCA analysis and the Venn's plot strongly indicated a unique response of *Chlamydomonas* to Ce (PC2, 33% of variance) (**Figure 1A**), differences among the REEs represented only 8% of the explained variance when PCA analysis was restricted to the 884 DEGs (PC2: 8% of variance) (**Figure S4**). This indicates that much of the difference between Ce and the two other REEs (Tm and Y) is found in the overall gene expression and not the simply within the subset of differentially expressed genes.

ANTAGONISTIC EFFECTS IN THE REE MIXTURE

For cells exposed to an equimolar mixture of Ce, Tm and Y, only 240 DEGs were significantly up- or down-regulated and among those, 101 were common to DEGs that were seen during the treatments with the individual metals (**Figure 1B**). Given that for any given REE, the mixture contained the same concentration of that metal in addition to the other two complementary metals, the observation that there were far fewer DEGs in the mixture (i.e. 240 DEGs) than for any of the individual REE treatments (i.e. > 320 DEGs) is already strong evidence that competitive interactions were occurring among the metals. Furthermore, cells exposed to the ternary mixture had far more DEGs in common with the Tm exposed cells (207 out of 320 DEGs, 64%) than with those exposed to Y (217 out of 653 DEGs, 33%) or Ce (108 out of 420 DEGs, 25%) (**Figure 1B**).

The expression profiles of the 240 DEGs induced by the ternary mixture were compared to the predictions of an IA model. The results strongly indicated antagonistic interactions among the three REEs with 162 DEGs showing antagonistic downregulation, 75 showing antagonistic upregulation and one showing a synergistic interaction (i.e. enhanced downregulation) (**Supplemental Data 3**). For example, none of the stress and/or damage biomarkers showed additive or synergistic interactions among the REEs in the ternary mixture (**Table 2**). Similar results were obtained for entire dataset of 884 DEGs with >99% of the genes displaying antagonistic interactions among Ce, Tm and Y (**Supplemental Data 3**).

Table 2. Annotated functions and log_2FC of several stress-related genes that were differentially expressed in response to the equimolar REE mixture (Mix) (**Supplemental Data 3**). Metal interactions (synergistic or antagonistic) for a specific gene were deduced from a deviation of the calculated log_2FC value with respect to the expected value obtained for the ternary mixture using an independent action model. Numbers in bold indicate the differentially expressed genes with respect to the controls ($Log_2FC > |3|$, $p_{adj} < 0.001$).

Gene		**Annotated function**	**Observed Log_2 FC**				**Expected Log_2FC**	**Interaction**
ID	**Symbol**	**MapMan**	**Ce**	**Tm**	**Y**	**Mix**	**CA**	
Cre17.g732533		Biodegradation of xenobiotics	-1.7	**-5.2**	**-4.6**	**-4.9**	-11.5	A. up
Cre12.g518200	*PDI1*	Thioredoxin	**-4.2**	**-3.6**	**-4.5**	**-3.1**	-12.2	A. up
Cre02.g090850	*CLPB3*	Stress abiotic	2.2	**3.3**	**4.2**	**3.3**	9.7	A. down
Cre17.g707950	*HEP2*	HSP70s co-chaperones	2.9	**3.5**	**3.6**	**3.4**	10.1	A. down
Cre07.g327450	*DNJ34*		2.7	2.9	**3.2**	**3.1**	8.7	A. down
Cre03.g204577	*DNJ31*		2.6	**3.4**	**3.8**	**3.2**	9.8	A. down
Cre07.g327800		ABC transporter	**5.6**	**4.3**	**5.4**	**3.7**	15.2	A. down
Cre02.g095076	*MFT10*	Major facilitator transporter	**3.5**	**3.3**	**3.9**	**3.4**	10.7	A. down
Cre04.g214657		Osmotic stress potassium transporter	**4.5**	**4.6**	**4.8**	**4.3**	14.0	A. down
Cre08.g367400	*LHCSR 3.2*	Light harvesting for photosynthesis	2.8	**3.4**	**3.8**	**3.2**	10.0	A. down

$Log_2FC < -1$ represent the genes that were induced by the treatment while $Log_2FC > 1$ represents those that were repressed, ABC= ATP Binding Cassette, A.= antagonistic interaction.

Given the high proportion of DEGs that were common between the mixture and the individual REE (25% to 64%, **Figure 1B and above)** and the paucity of DEGs that were specific to the mixture (i.e. 10 DEGs, **Figure 1B**); the near absence of additive or synergistic interactions is surprising. This observation likely results from competitive

interactions that occurred during REE biouptake, as indicated by **Figure 2**. Indeed, for the mixture, similar or lower intracellular concentrations of REEs were observed as compared to exposures for the individual REE. Similar antagonistic binding of La, Ce and Y to the active sites of plant roots have been reported for wheat (*Triticum aestivum),* when exposed to binary mixtures.[26] For microalgae, biouptake experiments in the presence of binary REE mixtures have generally shown that as the concentration and biouptake of one REE is increased, biouptake of the secondary REE is reduced.[16] Furthermore, affinity constants for the binding of REEs to the biological uptake sites are similar, with only a small apparent increase as one goes from left to right in the periodic table (i.e. K_{La} of $10^{6.8}$ M^{-1}; K_{Ce} of $10^{6.9}$ M^{-1}; K_{Sm} of $10^{7.0}$ M^{-1}; K_{Eu} of $10^{7.0}$ M^{-1}).[16] The similarity of the affinity constants is consistent with the observation that the sum of accumulated REEs remained nearly constant for the binary mixtures (**Figure 2**). The observation that fewer genes were significantly differentially expressed and that fold changes were generally lower in the ternary mixture as compared to the individual metal exposures strongly indicates that competitive interactions were occurring at the level of biouptake (common transporter, perhaps related to Ca uptake (**S2.2**), e.g. Cre16.g681750). On the other hand, the presence of DEGs that were specific to a single metal treatment (i.e. 194 of 458 DEGs for Ce and 238 of 653 DEGs for Y; **Figure 1B**) suggests independent targets of the REEs, once they are inside the cells.

IMPLICATIONS FOR THE RISK ASSESSMENT OF RARE EARTH ELEMENTS

The antagonistic effects of REE mixture on both biouptake and the transcriptome of *C. reinhardtii* suggests that estimates of toxicity and effects derived from single metals assays may be largely conservative for the rare earth metals. Given that nearly all contamination of the REE occurs as mixtures, the use of the single metal data will be overprotective. Moreover, our results diminish concern for potential synergistic effects or emergent toxicity of REE mixtures in natural waters. This should be considered in the establishment of governmental regulation. Nonetheless, several caveats need to be noted: data were obtained for a single organism ***(i)***, using short-term exposures ***(ii)*** and at sub-lethal exposure levels. Furthermore ***(iii)***, RNA sequencing alone does not provide sufficient information on the overall fitness of the organism. Finally, the (initial) observation of adsorptive losses to container walls and the untested role of complexes, strongly suggests that great care should be made when extrapolating results obtained in the laboratory to real world conditions.

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ASSOCIATED CONTENT

Supporting Information.pdf Supplemental methods and supplemental results

Supplemental Data.xlsx Differential gene expression analyses, MapMan ontologies, functional complementary information and combined effects modelling.

AUTHOR INFORMATION

Corresponding Author

Kevin J. Wilkinson, Biophysical Environmental Chemistry Group, University of Montreal, P.O. Box 6128, Succ. Centre-Ville, Montreal, QC, kj.wilkinson@umontreal.ca

Author Contributions

The manuscript was written by Elise Morel, critical review and editing were provided by William Zerges and Kevin J. Wilkinson. Lei Cui participated in some aspects of the data acquisition. All authors have given approval to the final version of the manuscript.

ABBREVIATIONS

REEs, rare earth elements; RNA-Seq, RNA sequencing; DEGs, differentially expressed genes; IA, independent action.

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