Distinguishing the effects of Ce nanoparticles from their dissolution products - Identification of transcriptomic biomarkers that are specific for ionic Ce in *Chlamydomonas reinhardtii*

Elise Morel[1], Jessica Dozois[1], Vera I. Slaveykova[2] and Kevin J. Wilkinson[1,Ψ]

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

[2] Environmental Biogeochemistry and Ecotoxicology, Department F.-A. Forel for Environmental and Aquatic Sciences, School of Earth and Environmental Sciences, Faculty of Science, University of Geneva, Uni Carl Vogt, 66, boulevard Carl-Vogt, CH-1211 Genève 4, Switzerland

[Ψ] kj.wilkinson@umontreal.ca; ORCID: 0000-0002-7182-3624

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Abstract

Cerium (Ce) is a rare earth element that is incorporated in numerous consumer products, either in its cationic form or as engineered nanoparticles (ENPs). Given the propensity of small oxide particles to dissolve, it is unclear if biological responses induced by ENPs will be due to the nanoparticles themselves or rather to their dissolution. This study provides the foundation for the development of transcriptomic biomarkers that are specific for ionic Ce in the freshwater alga, *Chlamydomonas reinhardtii.* exposed either to ionic Ce or to two different types of small Ce ENPs (uncoated, ~10 nm or citrate coated, ~ 4 nm). Quantitative reverse transcription PCR was used to analyse mRNA levels of four ionic Ce specific genes (*Cre17g.737300, MMP6, GTR12* and *HSP22E*) that were previously identified by whole transcriptome analysis in addition to two oxidative stress biomarkers (*APX1* and *GPX5*). Expression was characterized for exposures to 0.03 to 3 µM Ce, for 60 to 360 minutes and for pH 5.0-8.0. Near linear concentration-response curves were obtained for the ionic Ce and as a function of exposure time. Some variability in the transcriptomic response was observed as a function of pH, which was attributed to the formation of metastable Ce species in solution. Oxidative stress biomarkers analysed at transcriptomic and cellular levels confirmed that different effects were induced for dissolved Ce in comparison to Ce ENPs. The measured expression levels confirmed that changes in Ce speciation and the dissolution of Ce ENPs greatly influence Ce bioavailability.

Introduction

Approximately one consumer product containing engineered nanoparticles (ENPs) is created every day.[1] Their increasing use and diversity of applications (biology, medicine, electronics, optics, cosmetics, textiles, painting, etc.) is largely explained by their unique properties, including a high specific surface area.[2] Nonetheless, once emitted into environmental matrices, their properties change. In order to decrease their excess surface energy, they will undergo modifications such as agglomeration, adsorption, dissolution or even changes to their crystal structure.[3] These modifications will dictate the intrinsic properties of the ENPs and their interfacial reactivities.[4]

One of the major difficulties in evaluating the environmental effects of the metal-based ENPs is distinguishing between the effects of dissolution products and the nanoparticles themselves.[5,6,7] Indeed, many of the metal-based nanoparticles dissolve significantly, especially at environmentally relevant (i.e. low) concentrations.[8,9] Several authors have postulated that the effects of dissolved Ce to phytoplankton could be neglected with respect to the ENPs.[10,11,12] Others have shown Ce ENPs to have a lower acute toxicity than ionic Ce.[13,14] One of the difficulties is that it is technically difficult to quantify ENP dissolution at low particle concentrations.[9] An alternate strategy would be to distinguish dissolved metal and ENPs at the level of the cell by using biomarkers that are specific to one form or the other of the metal. The use of biomarkers has the added advantage of contributing to our mechanistic understanding of the bioavailability of the toxic species, which can be helpful for assessing environmental risk via an adverse outcome pathways approach.

Ideally, a biomarker should give a sensitive and specific molecular signal in response to an environmentally relevant exposure condition.[15] In reality, biomarkers typically respond over a limited concentration range and in some cases, non-linearly with respect to environmental stressors. In addition, environmental media are generally complex and variable, with physicochemical factors such as temperature, pH, water hardness, organic matter content having the potential to influence the activation and intensity of the biomarkers. Therefore, in ecotoxicology, multiple biomarkers are generally used in order to identify effects concentrations.[16] As much as possible, targets should be related to a relevant biological pathway and induction should be related to the dose of the stressor. Nonetheless, it is necessary to keep the number of molecular targets included in the bioassay low enough so that analysis is both affordable and practical.

Whole transcriptomic analysis (RNA-Seq) has been used previously to distinguish the effects of different metal oxide nanoparticles,[17] the effects of particle size,[18] the effects of particle coatings[19] and nanoparticles at different stages of their life cycle.[20] The present study contributes to the development of a transcriptomic bioassay for ionic Ce, by quantifying mRNA levels of genes that were identified as potential ionic Ce biomarkers in the freshwater eukaryotic green alga *Chlamydomonas reinhardtii*.[19] Quantitative reverse transcription PCR (RT-*q*PCR) was performed on a number of promising biomarkers as a function of exposure time, dose and pH.[21] The specificity of the response to ionic Ce was validated by analyzing mRNA levels of selected targets in response to uncoated and citrate stabilized Ce ENPs.

Materials and Methods

Ce forms of interest

$Ce(NO_3)_3$ (ionic Ce) was purchased from Inorganic Ventures (1.0 g L^{-1}; ICP-MS standard). Uncoated Ce ENPs (nominally 15 - 30 nm) were purchased from Nanostructured & Amorphous Materials as a powder (1406RE). Triammonium citrate stabilized Ce ENPs (nominally 10 nm) were obtained from Byk (Nanobyk®-3810). The measured Ce concentration of the stock suspension of the citrate coated Ce ENPs was 188 ± 3 g L^{-1} Ce ENPs. Detailed characterization and preparation of the Ce ENPs can be found in Morel *et al.* (2020).[19]

Culture and preparation of the microalgae

C. reinhardtii is a green microalga that is ubiquitous to fresh waters and is often used for water quality monitoring and studies examining the toxicology of pollutants in natural waters. Details on its specific culture and preparation for experiments involving trace metals can be found in Zhao and Wilkinson (2015).[22] In brief, wild type CC-125 (aka 137c) from the *Chlamydomonas* resource center was grown 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 in 4×diluted TAP. The cells were then washed 3x with a simplified exposure medium (see below) that contained no Ce. Cell concentrate was added to the exposure solutions in order to obtain 6.5-10 x 10^4 cells mL^{-1} (i.e. 0.15 $cm^2.mL^{-1}$). Cell concentrations and cell surface areas were measured using a Multisizer 3 particle counter (50 mm aperture; Beckman Coulter).

Algal exposures

Simplified experimental media were used during the exposure so that the chemical speciation of Ce could be precisely controlled. For example, phosphates can precipitate Ce and thus were removed from the exposure media. The absence of phosphates for short time experiments has been shown to not induce any deleterious effects to the microalgae.[23] Furthermore, 10^{-5} M $Ca(NO_3)_2$ was added to the exposure media in order to help preserve cell wall integrity.[23] Exposures were conducted in triplicate (at least) with independent batches of microalgae and fresh exposure media that were prepared daily. Control treatments were conducted in similar exposure medium as the treated cells but without the addition of any Ce forms.

For the time series exposures, cells were exposed to 0.5 µM of Ce (70.1 µg Ce L^{-1} for the ionic Ce; 86.1 µg CeO_2 L^{-1} for the Ce ENPs (composed of ~90% CeO_2)[19] in a medium containing 10.0 mM NaHEPES (pH 7.0) and 10^{-5} M $Ca(NO_3)_2$. Cells were sampled at 3, 10, 20, 40, 60, 120 and 360 minutes for the determination of Ce biouptake and at 60, 120, 240 and 360 minutes for the analysis of mRNA expression. Cells were pelleted by centrifugation (2000×g, 2 min., 4°C) from 200 mL of the exposure solution. Cell pellets were then resuspended in 1 mL of nuclease-free water before being transferred into 1.5 mL microtubes, where they were again separated (2000×g, 1 min., 4°C), prior to being frozen on dry ice for 10 min. and stored at −80°C until RNA extraction.

For the concentration-response exposures, *C. reinhardtii* was exposed for 2 hours to 0.03, 0.05, 0.3, 0.5 or 3 µM Ce (nominal Ce concentrations for both ionic Ce and Ce ENPs) in 10.0 mM NaHEPES (pH 7.0) and 10^{-5} M $Ca(NO_3)_2$. For exposures examining the effect of pH, Ce was held constant (0.5 µM Ce) while pH was varied by changing the

pH buffer: 10.0 mM NaHEPES (pH 7.0, pH 8.0); 10.0 mM NaMES (pH 5.0, pH 6.0). Cell pellets were isolated as described above, for the analysis of mRNA expression.

Ce determinations

Cerium concentrations in the exposure media were quantified by inductively coupled plasma mass spectrometry (ICP-MS, Perkin Elmer Nexion 300X).[19] Dissolved Ce was distinguished from colloidal (ENP) forms using centrifugal ultrafiltration (Amicon ultra-4, 3 kDa molar mass cutoff) by centrifuging 4 mL samples for 20 min. at 3700×g. In order to minimize adsorptive losses to the ultrafiltration membrane, the filtrate was collected and analyzed only after the third centrifugation cycle. Mass balances were determined from Ce concentrations that were measured: (i) in the filtrate; (ii) in the solution remaining above the filter; and (iii) in an acid (69% v/v HNO_3) extraction of the filter. Ce speciation in the exposure media was calculated with Visual Minteq (v3.1) using measured Ce concentrations and a partial pressure of $4.0x10^{-4}$ atm for CO_2 (to take into account atmospheric contributions of carbonate/bicarbonate).

Ce biouptake

For the analysis of Ce biouptake, 5 mL of 0.1 M EDTA was added to 45 mL of the exposure medium in order to stop biouptake and to simultaneously wash the adsorbed Ce from the cell wall, presumably leaving only internalized Ce.[24] After 1 min. in EDTA, the solution was filtered through a nitrocellulose filter (pore size 3.0 μm, Millipore), which was then rinsed three more times with 5 mL of 0.01 M EDTA. A similar filtration protocol was carried out on exposure solutions without added algae, in order to quantify adsorptive losses to the filter. Furthermore, 1 mL of the exposure medium was sampled immediately

before and during algal exposition. Algal cells and filters were digested by adding 400 µL of ultrapure HNO_3 (67–70%) and 300 µL of ultrapure H_2O_2 (30%), prior to heating the mixture at 80° C for 5 h (DigiPREP, SCP science).

Digests were diluted in MilliQ water and analyzed by ICP-MS. During ICP-MS analysis, a Ce calibration curve was run every 20 samples while blanks and quality control standards were run every 10 samples. Indium was used as an internal standard to correct instrumental drift.

RNA extraction

Frozen cell pellets were thawed and then immediately resuspended in freshly prepared lysis buffer (0.3 M NaCl, 5.0 mM EDTA, 50 mM Tris-HCl (pH 8.0), 2.0% SDS (wt/v), 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 (12,000xg, 10 min., 4°C) and supernatants were transferred into new tubes. Total RNA was precipitated from the final aqueous phase by isopropanol addition, then washed with 75% ethanol. After a final centrifugation, the pellet was resuspended in 20-30 µL of nuclease-free water. An aliquot (3 µL) 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) and spectroscopy (OD260) in order to determine the concentration of RNA (Nanodrop). RNA samples were stored at −80°C until RNA RT-*q*PCR analysis.

Reverse transcriptase quantitative real-time PCR (RT-qPCR)

Real-time PCR procedures and analysis followed MIQE guidelines.[25] Primers and fluorescent probe sets for the Taqman assay were designed using the Roche Universal Probe Library website (www.universalprobelibrary.com). Specificity of the probes was verified using the grep function of the Linux exploitation system with primer sequences searched in two annotation files containing either the transcript or the gene sequences of *C. reinhardtii* (genome v5.3 assembly), allowing for exon overlaps. Exon positions and transcript isoforms were determined for each gene using the JGI Comparative Plant Genomics Portal.[26] Total extracted RNA was converted into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Controls without reverse transcriptase were prepared in order to verify the absence of contaminating DNA. *q*PCR reactions were performed using the Taqman Fast *q*PCR MasterMix (Applied Biosystems) with a 1/5 dilution of reverse transcription products and primer and probe concentrations of 250 nM and 100 nM, respectively, in a final volume of 10 µL. 'No template' controls were also prepared in order to control for contamination. Enzyme activation was conducted for 20 s at 95°C and followed by 40 dual temperature amplification cycles (1 s at 95°C and 20 s at 60°C) using the QuantStudio 7 Flex Real-Time PCR Systems (IRIC Genomics Platform). For each primer pair and probe set, amplification efficiency was assessed using a standard curve and validated when >85%.

To generate the standard curve, *q*PCR reactions were performed using cDNA from a mix of RNA samples using a serial dilution of 1/5, 1/25, 1/125 and 1/625. Data were analyzed using the QuantStudio Real-Time PCR software (v1.3). Relative mRNA levels

were analyzed using the $2^{-\Delta\Delta CT}$ method with the threshold cycle (CT). Non-induced control genes: *RACK1*[27] (CT around 20) and *APG6* (CT around 27 and no observable changes in intensity[19]) were used for normalization of the *q*PCR data (**Table S1**). Fold changes between 0.5 and 2.0 were attributed to technical and biological variability rather than a treatment effect.

Reactive oxygen species (ROS) production and oxidative stress

Flow cytometry was employed to follow the oxidative stress and membrane damage in microalgae exposed under similar conditions as described above, except that nominal concentrations were 0, 0.05, 0.1, 0,5, 1 and 5 µM for the ionic Ce and 0, 0,5, 1 and 10 µM for the Ce ENPs. Three 1 mL or 0.5 mL aliquots were taken from each of algal suspensions following 30, 90, 210 or 330 min. of exposure. The 1 mL aliquot was directly analyzed using flow cytometry (BD Accuri C6 Plus, BD) equipped with a 488 nm argon excitation laser and a CSampler (Accuri cytometers Inc., Michigan). A flow rate of 35 µL min^{-1} was used to measure cell density and biological parameters including: (i) size and granularity of *C. reinhardtii* using either forward laser scattering (0 ± 15°, forward scatter, FSC) or side scattering (90 ± 15°, side scatter, SSC) and (ii) autofluorescence of chlorophyll (> 670 nm, FL3). Oxidative stress and membrane damage of the exposed algae were determined following staining with CellROX green and propidium iodide (PI). Two 0.5 mL aliquots were stained with 5 µM of CellROX green (Life Technologies Europe BV, Zug, Switzerland) or 7 µM propidium iodide (PI; Sigma – Aldrich, Buchs, Switzerland) in the dark for 30 min. Negative controls ([Ce] = 0 M) were performed for each exposure time and for each type of measurement. Positive controls were prepared from 0.5 mL of an algal aliquot that was: (i) not exposed to Ce; (ii) incubated for 30 min. in the dark with cumene

hydroperoxide (1.2 mM) or (iii) placed in warm water (T°> 50°C) for 30 min., prior to incubation with the fluorescent markers (CellROX, PI). CellROX green stained cells were followed using green fluorescence channel (530 ± 15 nm, FL1) and PI stained cells via the yellow / orange fluorescence channel (585 ± 20 nm, FL2). Analyses were conducted in triplicate with independent batches of algae and exposure media that were prepared on different days.

For each acquisition, a threshold of 10,000 events in the gate corresponding to algal cells was selected. Details on the flow cytometry gating strategy can be found in the Supplemental information (**Figure S1** and **Figure S2**), however, it mainly followed procedures described in Cheloni et *al.* (2013, 2014).[28,29] The proportions of cells that were present in the different gates of interest were retrieved with the CFlow Plus program, in addition to the average values of the different parameters of interest.

Statistical analyses

Statistical analyses were performed in SigmaPlot (v12.0). Data points presented are determined from biological replicates (i.e. independent cultures), while error bars show the standard deviations. A one way analysis of variance (ANOVA) paired with the Holm-Sidak test were used to compare measured fold change to the threshold values (i.e. RT-qPCR) or to compare treated and untreated microalgae (i.e. cytometry data) with significance defined by * for $p<0.05$, ** for $p<0.01$ and *** for $p<0.001$. This method was also applied for all pairwise multiple comparisons with significant differences highlighted by the different letters when $p<0.05$.

Results and Discussion

Identification of exposure biomarkers for ionic Ce

Based upon RNA-Seq analysis,[19] a number of candidate transcripts were identified as being specific to ionic Ce. Among the 57 transcripts that were shown to be clearly regulated by Ce,[19] 8 were selected based on the magnitude of their regulation by ionic Ce in regards to controls (i.e. the 4 most up-regulated and the 4 most down-regulated). Further filtering based upon the analysis of their raw expression profiles (**Figure S3**) and their functional annotations led to the selection of four transcripts: three that were induced following exposure to ionic Ce (*Cre17.g737300, MMP6* and *GTR12*) and a single transcript (*HSP22E*) that was repressed in its presence (**Table 1**). Transcript levels of ROS-induced genes *APX1*[30] and *GPX5*[31] were added in order to test for occurrence of oxidative stress.

RNA-Seq results were first validated using RT-*q*PCR assays (**Table SI**), that were conducted on independent samples of cells, which were exposed to similar exposure conditions (i.e. 0.5 µM Ce in HEPES, pH 7.0, 120 min. exposure). While the direction of regulation (induction or repression) was similar using RNA-Seq and RT-*q*PCR, the magnitude of the regulation (i.e. fold change values) generally differed (**Table 1**). Differences were attributed to both technical and biological variabilities in the experiments.

Ce biouptake and the induced transcriptomic signals as a function of time

Ce biouptake increased over the first two hours of exposure but appeared to stabilize at longer times (**Figure 1**), consistent with prior results with this microalga.[32] Given that Ce concentrations in the medium were constant over the entire exposure period (**Figure S4)**, the decreased uptake flux (decrease in slope in Fig. 1) was likely due to

biological regulation of the uptake (resulting from an increased efflux or decreased influx) as opposed to chemical effects (precipitation of Ce, depletion of Ce in the medium, etc.).

The mRNA levels of the four most sensitive biomarkers (**Table 1**) were quantified during an exposure to 0.5 µM Ce over 360 min. **(Figure 2)**. Three patterns of mRNA expression were observed: *Cre17.g737300* increased over time to reach a plateau at 240 min. (**Figure 2a**); the expression level of *HSP22E* was repressed over time (**Figure 2d**); whereas the expression levels remained fairly stable for *GTR12* and *MMP6*, over the entire exposure period (**Figure 2b, 2c**). For the oxidative stress marker genes, a significant repression of *APX1* was observed at very short exposure times (Holm-Sidak test, $p<0.01$) but then values returned to control levels by 120 min. (**Figure S5a**). Expression levels of *GPX5* were stable and showed no sign of being regulated by ionic Ce (**Figure S5b**).

Concentration-response relationship

For a 120 min. exposure to ionic Ce, biomarker expression was examined as a function of the measured concentrations of Ce (nominally 0.03 to 3 µM). Increased expression was observed as a function of concentration for all potential biomarker genes, up to 2-3 µM Ce (**Figure 3**). *APX1* appeared to be repressed only for the highest exposure concentration (ionic Ce ~ 3 µM) (**Figure S6a**), whereas expression levels of *GPX5* were stable and showed no sign of being regulated by ionic Ce over the entire tested concentration range (**Figure S6b**).

The no effects (mRNA) concentration[16] for a 120 min. exposure was between 0.2 µM and 0.5 µM Ce for *MMP6* (Holm-Sidak test, $p<0.05$) and *HSP22E* (Holm-Sidak test, $p<0.05$) and between 0.5 and 1 µM for *Cre17.g737300* (Holm-Sidak test, $p<0.001$) and

279 *GTR12* (Holm-Sidak test, $p<0.05$) (**Figure 3**). As expected, these values are lower than the
280 values required to observe significant biological effects at the cellular level. For example,
281 an EC_{10} of 9.4 µM was observed for a 72 hours fluorescence inhibition assay for
282 *Pseudokirchneriella subcapitata*.[33] The predicted "no-effects" concentration (PNEC)
283 estimated for a Ce salt was around 0.4 µM for an ecosystem level study by the same
284 authors, which is in good agreement with the transcriptomic results obtained in this study.[33]
285 Finally, Collin *et al.* (2014) estimated a PNEC value of 5.8 nM for Ce ENPs (cerium
286 dioxide).[13]

287 ***Role of pH on the transcriptomic signal***

288 Transcript levels of the biomarkers were evaluated for pH variations from 5.0 to
289 8.0 for 0.5 µM ionic Ce and a 120 min. exposure time. Based upon thermodynamic
290 calculations (Visual Minteq), free Ce in solution will vary from 99.9% to 5.9% of the total
291 Ce in solution, over the pH range of 5.0 to 8.0. Measured Ce concentrations and calculated
292 ionic Ce were fairly constant between pH 5.0 and 7.0 (**Figure 4**), however, metastable
293 colloidal species, not predicted by equilibrium calculations, are thought to form, especially
294 at the higher pH.[19,32] For example, significant decreases of 20% of the total Ce (Holm-
295 Sidak test, $p<0.01$) and nearly all of the dissolved Ce (Holm-Sidak test, $p<0.001$) were
296 observed when increasing the pH from 7.0 to 8.0 (**Figure 4**), suggesting losses by
297 adsorption on the flask walls and/or the formation of sedimenting precipitates at pH 8.0.
298 Indeed, the near absence of dissolved Ce at pH 8.0 broadly agreed with the transcriptomic
299 responses observed for all of the biomarkers, which were not different from control values
300 at pH 8.0 (**Figure 5, Figure S7**).

301

Biomarker responses for the Ce ENPs

Very little or no dissolved Ce could be detected in the ENP suspensions at pH 7.0 (**Table 2)**, although this fraction did appear to increase at the lower pH for the citrate coated ENPs. In spite of these very low concentrations (and proportions) of dissolved Ce, biouptake was ~10x higher for the ENPs than it was for dissolved Ce (**Figure S8**), for a similar total Ce concentration in the exposure medium. Y intercepts (i.e. t=0) from the biouptake measurements were greater for ENP biouptake experiments than for the ionic cerium, which probably reflected an important adsorption of the Ce ENPs onto the cell surface.

In spite of a much greater sorption/biouptake in the presence of ENPs, induction did not differ from control responses for any of the selected biomarkers, either as a function of time (**Figure 6**) or concentration (**Figure 7**), strongly indicating a specificity for dissolved Ce. This result is important as it shows that these biomarkers could be used to distinguish between the effects of the ENPs and the effects of dissolved Ce. It also demonstrates that there is a clear mechanistic difference between the effect of dissolved Ce and that of Ce ENPs. Note that in spite of our efforts, no specific biomarkers for the Ce ENPs were identified to date. *GTR12* did respond to the citrate coated Ce ENPs, but only at the most acidic pH (pH 5.0, Holm-Sidak test, $p<0.01$) (**Figure S9**). As postulated earlier,[19] some of the transcriptomic effects observed for the citrate coated Ce ENPs may have come from increased citrate in the medium, rather than from a specific ENP effect, however, this result implied that the effects of these Ce ENPs were due to their dissolution products rather than to the ENP themselves. Admittedly, this point would require a more thorough examination of all potential biomarkers (*Chlamydomonas* has 19,526 predicted

transcripts).[34,35] As expected, based upon the weak response for the ENPs, little variation in the transcriptomic response was observed for the oxidative stress biomarkers (**Figure S10**).

Validation of the transcriptomic signatures

mRNA levels generally changed in a single direction as a function of both exposure time and Ce concentration, which is an important point when identifying useful biomarkers. Furthermore, the conditions that were employed in this study did not induce important metabolic changes in *C. reinhardtii* (i.e. no changes in the metabolic pathways that responded to Ce). On the other hand, exposures to ionic Ce led to an overexpression of the reactive oxygen species (ROS) and a modification in the membrane permeability of *C. reinhardtii*, when determined by flow cytometry (**Figure 8 a, d**). For example, after a 120 min. exposure to 1 µM ionic Ce, 10% (Holm-Sidak test, $p \leq 0.001$) of the microalgae presented an excess of ROS and while 7% (Holm-Sidak test, $p \leq 0.05$) showed signs of membrane damage. These physiological effects may result from a direct effect of the ionic Ce on oxidative stress related targets such as *APX1*, which was rapidly repressed by ionic Ce (i.e. 60 min.), especially with increasing concentration. Ce can also indirectly produce ROS through enzymatic inhibition via substitution of the metal cofactors. In this perspective, the highly expressed *MMP6* which translate to a protease make sense as the non-functional enzymes must be removed as fast as possible by the cytoplasm. For the short exposure times used here, similar effects were not observed when the microalgae were exposed to Ce ENPs, even at higher concentrations (> 1 µM Ce) (**Figure 8 b, c, e, f**). Indeed, ROS generation and membrane damage have really only been reported in *C. reinhardtii* for much longer exposure times (>12h) and relatively high concentrations of

Ce ENPs.[36] In contrast to the ionic Ce, the absence of changes in ROS or biomarker induction in the presence of ENP suggests that the two Ce forms act differently, in agreement with our previous results using *C. reinhardtii*.[19] To further confirm the hypothesis that substantial Ce ENPs remain sorbed to the outer cell wall, further experiments using variable extracting agents should be performed.

Nonetheless, not all of the Ce biomarkers were affected in the same manner by exposure time, which may have been related to the specific biological pathways within which the investigated genes were involved. For example, in the case of *HSP22E* and *APX1*, both were induced at early exposure times (i.e. maximum of down-regulation was observed at 60 min.) and both encode chloroplast-targeted proteins that are induced during oxidative stress.[37] Given that significant modifications to cell size were observed within 60 min. following the exposure of *C. reinhardtii* to 1 μM ($p \leq 0.05$) and 5 μM ($p \leq 0.001$) of Ce (**Figure S11a**), the induction of *Cre17g.737300* that was observed as a function of time and concentration may have reflected perturbations in cell turgor at the higher concentrations of ionic Ce. Indeed, while no precise biological function for the protein that is encoded by *Cre17g.737300* has been yet identified in *C. reinhardtii*, high sulfur keratin-associated transmembrane proteins have been reported to be up-regulated in maize during hydric stress.[38] The absence of such an effect (at both the transcriptomic and cellular levels) when the microalgae were exposed to environmentally relevant concentrations of Ce ENPs (**Figure S11b,c**) suggests that the regulation of genes related to cellular processes[19] may allow *C. reinhardtii* to adapt well to short-term and low concentration exposures of the ENPs.

Summary and environmental implications

Due to their high specificity and relative linearity with the respect the concentration of ionic Ce (over an environmentally relevant concentration range), four biomarkers (*Cre17.g737300*, *GTR12*, *MMP6* and *HSP22E*) were identified as being specific to dissolved Ce (likely Ce^{3+}) in *C. reinhardtii*. With their different sensitivities, their simultaneous use could be an appropriate strategy for identifying bioavailable Ce to *C. reinhardtii* and perhaps other biological species. Indeed, the low concentrations of dissolved Ce that co-occurred in the suspensions of citrate coated Ce ENPs at pH 5.0 were identified based upon the induction of *GTR12*.

One caveat should be noted. A much greater variability in mRNA levels was observed when the pH of media varied. This result likely reflected the complexity of Ce speciation, even in simple aqueous media, resulting from the formation of metastable species, which are likely highly charged and likely to sorb to multiple surfaces including cell walls and exposure container walls.

Finally, the specificity of this transcriptomic signature will need to be validated in the presence of other rare earth metals since in nature, they are almost always found as metal mixtures.[39] The presence of ligands such as phosphate or natural organic matter should also be validated as they are both likely to attenuate the bioavailability of Ce[40] and/or the metabolism of the microalgae.[41]

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Table 1. Functional information (MapMan ontology[42,43] and JGI Comparative Plant Genomics Portal (Phytozome)[26]) and fold change in mRNA levels for selected differentially expressed genes (Fold-change > |2.0|) after a 120 min. exposure of *C. reinhardtii* to 0.5 µM of ionic Ce (pH 7.0), as determined using RNA-Seq (n=3) and RT-qPCR (n=5 to 7). Four potential ionic Ce exposure biomarkers (*Cre17.g737300, MMP6, GTR12, HSP22E*), 2 oxidative stress biomarkers (*APX1, GPX5*) and 2 endogen controls (*APG6, RACK1*) were examined.

Identification		Functional information		Fold change	
ID	**Symbol**	**Mapman**	**Phytozome**	**RNA-Seq**	**RT-*q*PCR**
Cre17.g737300	-	Not assigned	Ultrahigh sulfur keratin-associated protein	10.7 ± 0.8	5.7 ± 1.0
Cre16.g692200	*MMP6*	Protein degradation	Metalloproteinase of VMP family	7.3 ± 0.8	13.9 ± 7.0
Cre06.g302050	*GTR12*	Minor CHO metabolism	1,3-beta-D-glucan synthase	4.9 ± 0.8	17.6 ± 8.2
Cre14.g617450	*HSP22E*	Protein folding	Heat shock protein 22E	0.3 ± 0.9	0.3 ± 0.1
Cre02.g087700	*APX1*	Redox	Ascorbate peroxidase	-	0.4 ± 0.2
Cre10.g458450	*GPX5*	Not assigned	Glutathione peroxidase	-	-
Cre01.g020250	*APG6*	Protein degradation/Cell organization	Beclin 1	-	-
Cre06.g278222	*RACK1*	Development	Receptor of activated protein kinase C	-	-

Table 2. Total Ce concentrations and percentages of dissolved Ce as a function of pH after 120 min. exposure of *C. reinhardtii* to 0.5 µM Ce in the form of either citrate coated Ce ENPs or uncoated Ce ENPs (n= 2 to 6).

pH	Total Ce concentration (µM)		Dissolved Ce (%)	
	Citrate coated	Uncoated	Citrate coated	Uncoated
5.0	0.28 ± 0.09	0.35 ± 0.09	10.0 ± 8.9	ND
6.0	0.42 ± 0.07	0.42 ± 0.04	1.4 ± 1.7	1.1 ± 0.4
7.0	0.38 ± 0.02	0.42 ± 0.07	0.2 ± 0.2	ND
8.0	0.28 ± 0.09	0.39 ± 0.04	ND	ND

ND= Not detected.

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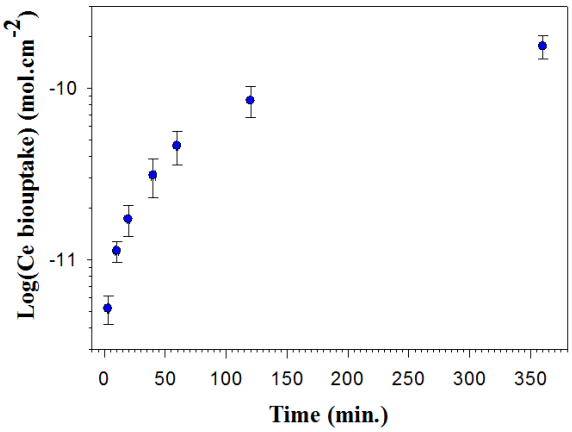

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565 Figure 1. Ce biouptake by *C. reinhardtii* as a function of exposure time for 0.5 µM of ionic
566 Ce (n=2 to 3).

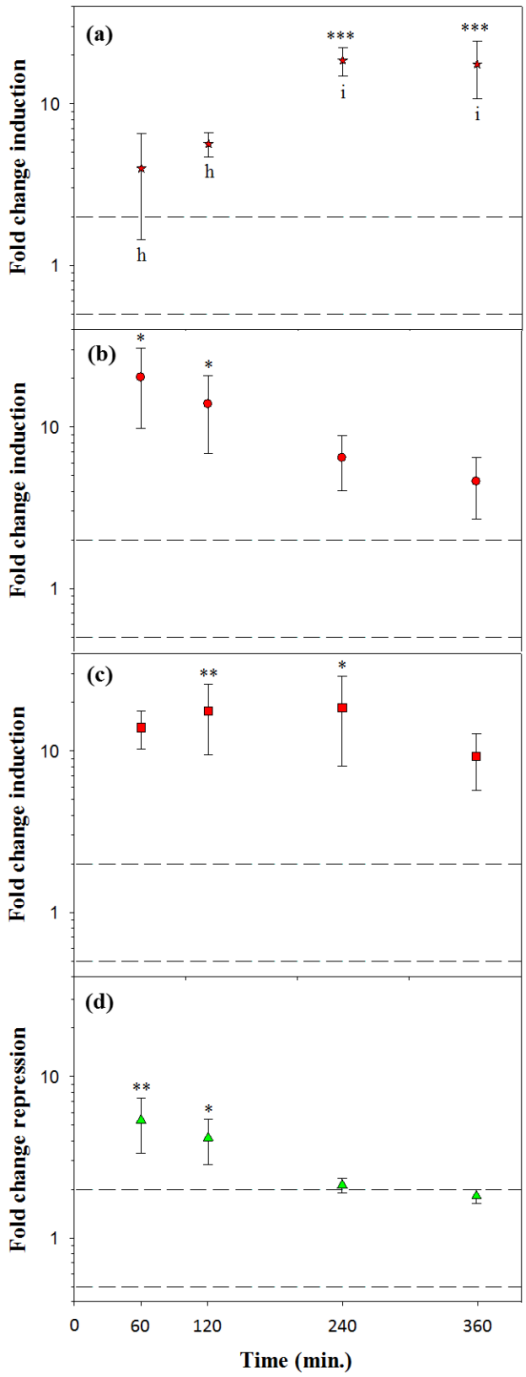


Figure 2. Fold change induction of (a) *Cre17.g737300*, (b) *MMP6*, (c) *GTR12*, and fold change repression of (d) *HSP22E* as a function of exposure time for *C. reinhardtii* exposed to 0.5 µM of ionic Ce. Induced biomarkers are indicated by red points, while repressed biomarker (=1/fold change induction) is represented by the green points (n=2 to 7). The dotted lines define the area in which the fold changes of mRNA levels are considered to occur randomly due to technical and biological variability (0.5>fold change<2.0).

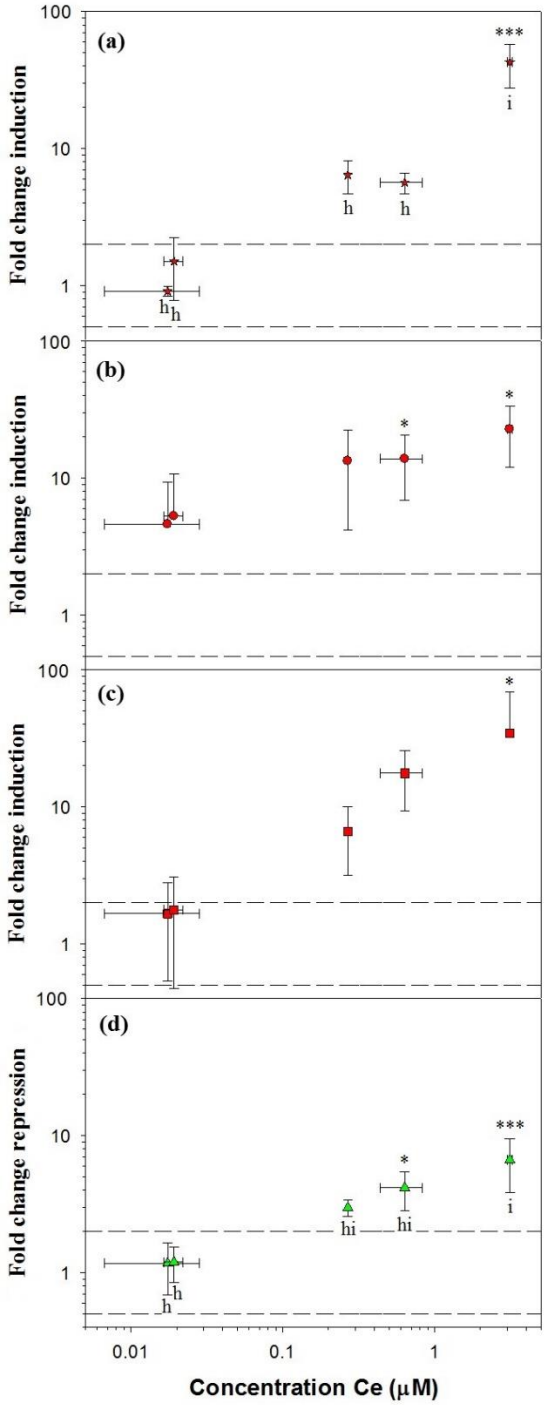
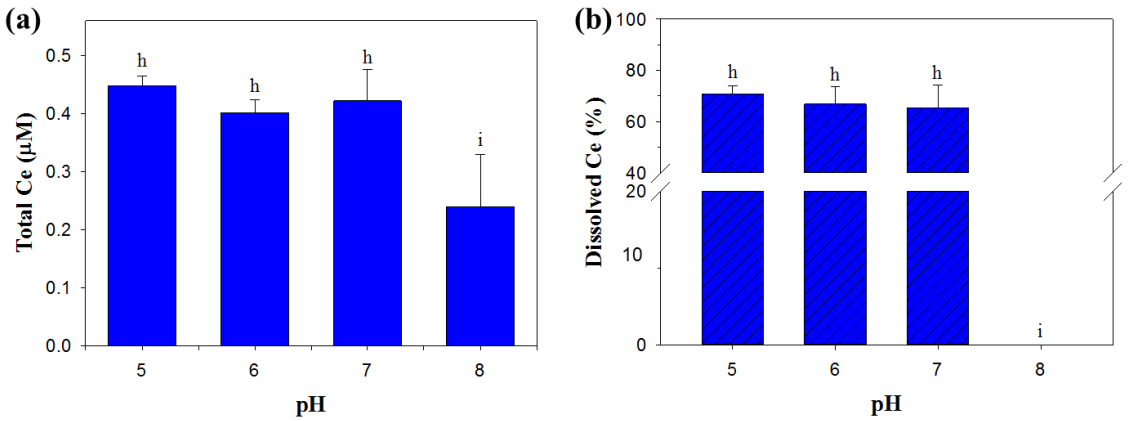


Figure 3. Fold change induction of (a) *Cre17.g737300*, (b) *MMP6*, (c) *GTR12*, and fold change repression of (d) *HSP22E* as a function of concentration for a 120 min. exposure of *C. reinhardtii* to ionic Ce (n= 2 to 7). Induced biomarkers are indicated by red points, while the repressed biomarker (=1/fold change induction) is represented by green points. The dotted lines define the area in which fold changes of mRNA levels are considered to occur randomly due to technical and biological variability (0.5>fold change<2.0).



582

583 Figure 4 (a) Total Ce concentration in the exposure media and (b) proportion of dissolved
584 Ce as a function of pH for a 120 min. exposure of *C. reinhardtii* to 0.5 µM ionic Ce (n= 2
585 to 6).

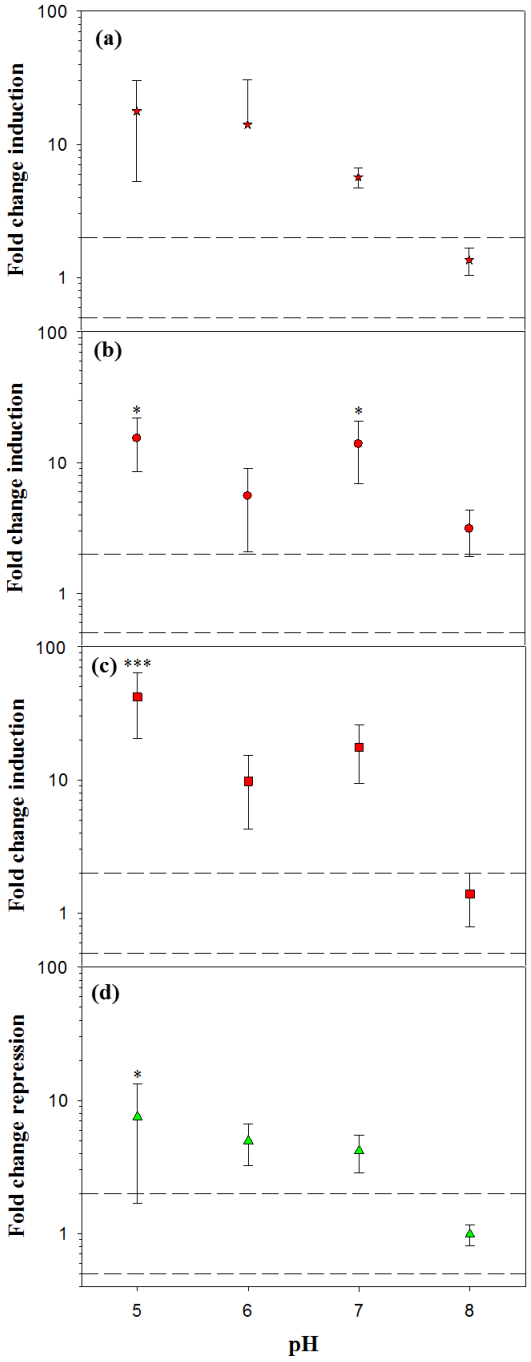


Figure 5. Fold change induction of (a) *Cre17.g737300*, (b) *MMP6*, (c) *GTR12*, and fold change repression of (d) *HSP22E* as function of pH for a 120 min. exposure of *C. reinhardtii* to 0.5 µM of ionic Ce. Induced biomarkers are indicated by red points, while repressed biomarker (=1/fold change induction) is represented by the green points (n= 2 to 7). The dotted lines define the area in which fold changes of mRNA levels are considered to occur randomly due to technical and biological variability (0.5>fold change<2.0).

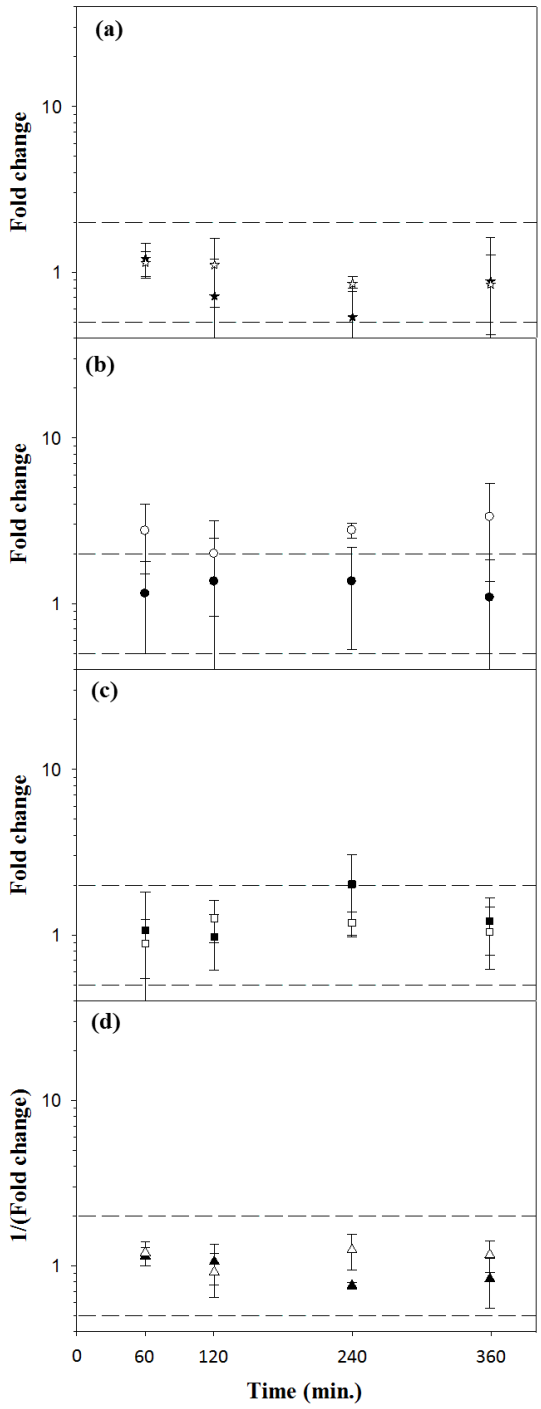
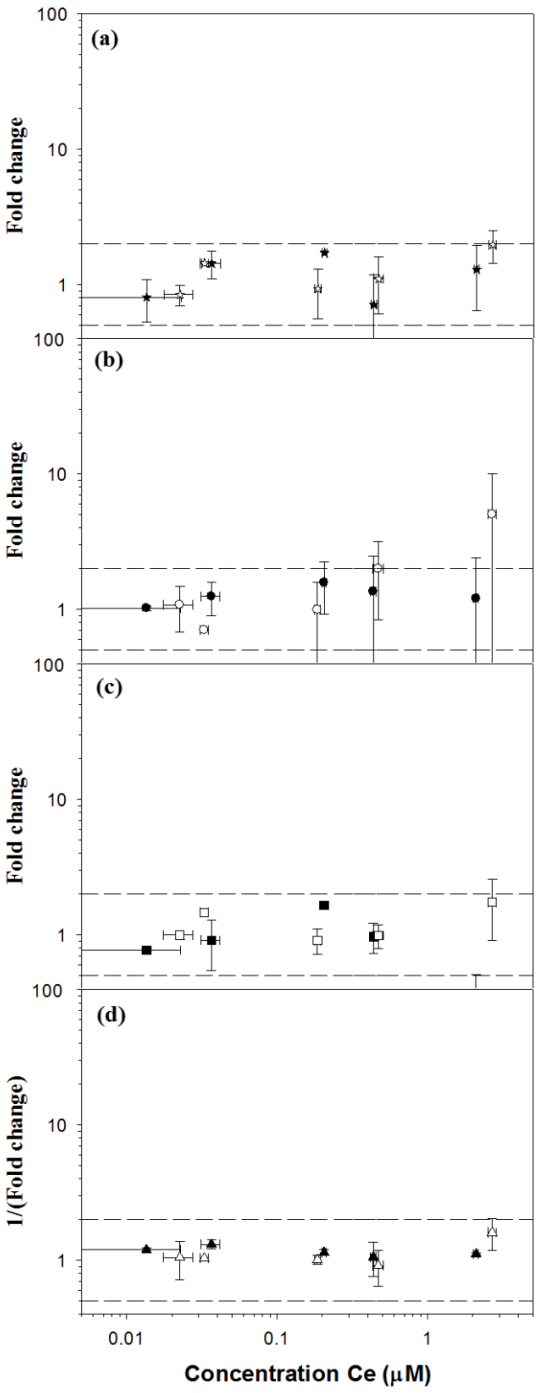
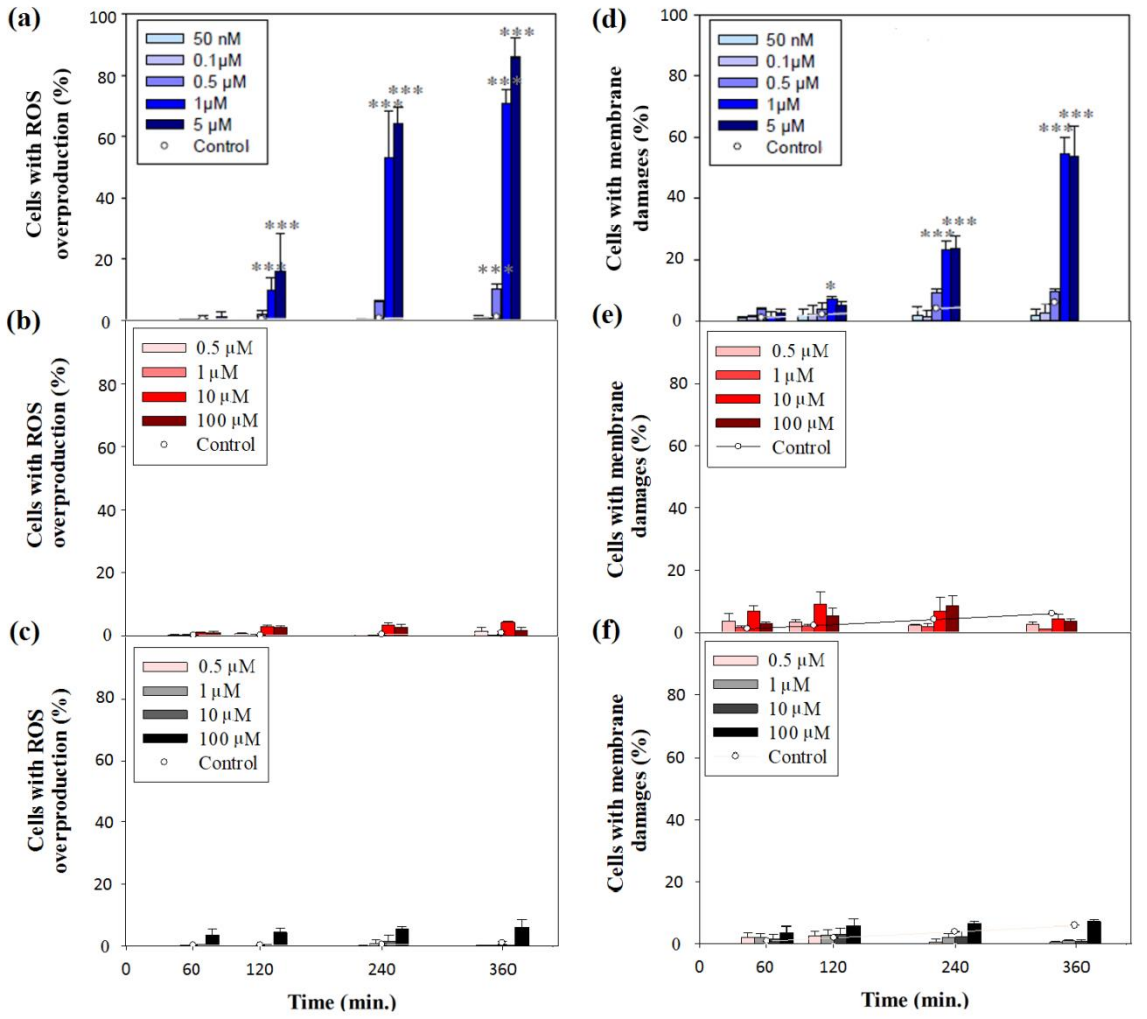


Figure 6. Fold change in mRNA levels of (a) *Cre17.g737300*, (b) *MMP6*, (c) *GTR12*, and reciprocal of fold change in mRNA levels of (d) *HSP22E* as a function of exposure time for *C. reinhardtii* exposed to 0.5 µM of total nominal Ce (dissolved and ENP) for citrate coated Ce ENPs (empty symbols) and uncoated Ce ENPs (full symbols) (n=2 to 7). The dotted lines define an area in which the fold change mRNA levels are considered to occur randomly due to technical and biological variability (0.5>fold change<2.0).



602

603 Figure 7. Fold change in mRNA levels of of (a) *Cre17.g737300*, (b) *MMP6*, (c) *GTR12*,
604 and reciprocal of fold change in mRNA levels of (d) *HSP22E* as a function of the Ce
605 concentration for a 120 min. exposure of *C. reinhardtii* to citrate coated Ce ENPs (empty
606 symbols) and uncoated Ce ENPs (full symbols) (n=2 to 7). The dotted lines define the area
607 in which fold changes of mRNA levels are considered to occur randomly due to technical
608 and biological variability (0.5>fold change<2.0).



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610 Figure 8. (a, b, c) ROS overproduction and (d, e, f) membrane damage for *C. reinhardtii*
611 as a function of time and concentration for exposures to (a, d) ionic Ce, (b, e) citrate
612 coated Ce ENPs and (c, f) uncoated Ce ENPs at pH 7.0 (n=2 to 3).

613