

# Molecular toxicity of cerium oxide nanoparticles to the freshwater alga *Chlamydomonas reinhardtii* is associated with supra-environmental exposure concentrations

Taylor, Nadine; Merrifield, Ruth; Williams, Timothy; Chipman, James; Lead, Jamie; Viant, Mark

DOI:

[10.3109/17435390.2014.1002868](https://doi.org/10.3109/17435390.2014.1002868)

License:

Creative Commons: Attribution (CC BY)

*Document Version*

Publisher's PDF, also known as Version of record

*Citation for published version (Harvard):*

Taylor, N, Merrifield, R, Williams, T, Chipman, J, Lead, J & Viant, M 2016, 'Molecular toxicity of cerium oxide nanoparticles to the freshwater alga *Chlamydomonas reinhardtii* is associated with supra-environmental exposure concentrations', *Nanotoxicology*, vol. 10, no. 1, pp. 32-41.  
<https://doi.org/10.3109/17435390.2014.1002868>

[Link to publication on Research at Birmingham portal](#)

## **Publisher Rights Statement:**

Checked for repository : 18/03/2015

## **General rights**

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

- Users may freely distribute the URL that is used to identify this publication.
- Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
- User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
- Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

## **Take down policy**

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact [UBIRA@lists.bham.ac.uk](mailto:UBIRA@lists.bham.ac.uk) providing details and we will remove access to the work immediately and investigate.

Download date: 19. Apr. 2024

ORIGINAL ARTICLE

# Molecular toxicity of cerium oxide nanoparticles to the freshwater alga *Chlamydomonas reinhardtii* is associated with supra-environmental exposure concentrations

Nadine S. Taylor<sup>1</sup>, Ruth Merrifield<sup>2,3</sup>, Tim D. Williams<sup>1</sup>, J. Kevin Chipman<sup>1</sup>, Jamie R. Lead<sup>2,3</sup>, and Mark R. Viant<sup>1</sup>

<sup>1</sup>School of Biosciences, University of Birmingham, Edgbaston, Birmingham, UK, <sup>2</sup>SmartState Center for Environmental Nanoscience and Risk, The Arnold School of Public Health, Environmental Health Sciences, University of South Carolina, Columbia, SC, USA, and <sup>3</sup>School of Geography, Earth and Environmental Science, University of Birmingham, Edgbaston, Birmingham, UK

## Abstract

Ceria nanoparticles (NPs) are widely used as fuel catalysts and consequently are likely to enter the environment. Their potential impacts on biota at environmentally relevant concentrations, including uptake and toxicity, remain to be elucidated and quantitative data on which to assess risk are sparse. Therefore, a definitive assessment of the molecular and phenotypic effects of ceria NPs was undertaken, using well-characterised mono-dispersed NPs as their toxicity is likely to be higher, enabling a conservative hazard assessment. Unbiased transcriptomics and metabolomics approaches were used to investigate the potential toxicity of tightly constrained 4–5 nm ceria NPs to the unicellular green alga, *Chlamydomonas reinhardtii*, a sentinel freshwater species. A wide range of exposure concentrations were investigated from predicted environmental levels, to support hazard assessment, to supra-environmental levels to provide insight into molecular toxicity pathways. Ceria NPs were internalised into intracellular vesicles within *C. reinhardtii*, yet caused no significant effect on algal growth at any exposure concentration. Molecular perturbations were only detected at supra-environmental ceria NP-concentrations, primarily down-regulation of photosynthesis and carbon fixation with associated effects on energy metabolism. For acute exposures to small mono-dispersed particles, it can be concluded there should be little concern regarding their dispersal into the environment for this trophic level.

## Keywords

Adverse outcome pathway, gene, mass spectrometry, nanomaterial, nanotoxicology

## History

Received 22 September 2014  
Revised 28 November 2014  
Accepted 22 December 2014  
Published online 5 March 2015

## Introduction

Ceria nanoparticles (NPs) are widely used as catalysts, exploiting their oxygen storing capacity, in particular, as a diesel fuel additive with annual production estimated at up to 700 tonnes (Hendren et al., 2011; Park et al., 2008a; Xia et al., 2008). Other applications include in polishing and coatings and in the biomedical industry for treating inflammation and cancer as well as radiation protection of cells, again exploiting their strong antioxidant properties (Boxall et al., 2007; Celardo et al., 2011; Hoshino et al., 2001; Shah et al., 2012). Consequently these NPs are likely to enter the environment, yet their environmental fate and potential impacts on biota are relatively unknown (Boxall et al., 2007; Van Hoecke et al., 2009). Several uses of ceria NPs lead to non-point source discharge, potentially resulting in low-level and widespread exposure to multiple plant and animal species (Cassee et al., 2011). Recently

the level of ceria NPs in the aquatic environment has been estimated, focusing on diesel as the primary source for environmental discharge (Boxall et al., 2007; Johnson & Park, 2012). Based on actual usage data of the number of vehicles, the consumption of fuel containing ceria NPs, and the fraction that will escape vehicle exhausts, Johnson & Park (2012) predicted that direct deposition into UK waters is as low as 0.02 ng/L, rising to ca. 300 ng/L when considering rainfall data and run-off into water courses that are within 20 m of the roads. This prediction is yet to be confirmed by actual measurements and does little to estimate the levels of ceria NPs that may enter the environment from other industries (Boxall et al., 2007; Hoshino et al., 2001).

While information on the biological effects of ceria NP exposure is available, the findings are somewhat contradictory. Several studies have concluded that there are unlikely to be any adverse health effects of ceria NP exposure, even considering complete release of all ceria NPs ( $4.4 \times 10^6$  particles/cm<sup>3</sup>) when added to diesel fuel at a level of 5000 µg/L (Fall et al., 2007; Park et al., 2008a). Xia et al. (2008) showed that ceria NPs were cytoprotective at 25 µg/L due to their antioxidant properties. Conversely, Park et al. (2008b) observed a cytotoxic effect of ceria NPs on a human lung cell line; however, the concentration range tested (5–40 µg/L) was noticeably higher than expected environmental levels. Thill et al. (2006) and Pelletier et al. (2010) found some evidence of bacterial toxicity at extremely high concentrations (1.2–730 mg/L). Moreover, Zhang et al. (2011)

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Correspondence: Professor Mark R. Viant, School of Biosciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK. Tel: +44 (0)121 414 2219. Fax: +44 (0)121 414 5925. E-mail: M.Viant@bham.ac.uk

discovered that environmentally realistic exposure concentrations (ca. 140–14 000 ng/L) significantly decreased the mean lifespan of nematodes and could induce accumulation of reactive oxygen species (ROS) and oxidative damage in *Caenorhabditis elegans*. In the aquatic environment the situation is similarly conflicting. Van Hoecke et al. (2009) reported no acute toxicity of ceria NPs to two crustaceans (*Daphnia magna* and *Thamnocephalus platyurus*) at concentrations as high as 1 and 5 mg/L, respectively, nor in *Danio rerio* (zebrafish) embryos up to 200 µg/L. At a molecular level, Lee et al. (2009) determined a genotoxic effect of ceria NPs with increased DNA strand breaks in both *D. magna* and *Cophilaxus riparius* at a concentration of 1 mg/L.

Studies investigating the exposure of aquatic plants and algae to ceria NPs are more in agreement. Rogers et al. (2010) determined a 50% effect concentration (EC<sub>50</sub>) for growth inhibition of 10.3 mg/L for *Pseudokirchneriella subcapitata* (a freshwater green alga), while Van Hoecke et al. (2009) determined an EC<sub>10</sub> of 2.6–5.4 mg/L and Rodea-Palomares et al. (2011) an EC<sub>50</sub> of 2.4–29.6 mg/L for the same species. A study using the cyanobacterial strain *Anabaena CPB4337* found an EC<sub>50</sub> of 0.27–6.6 mg/L and several further responses in both *P. subcapitata* and *Anabaena*, including the generation of hydroxyl radicals, decreased chlorophyll concentrations, inhibition of photosynthesis and increased membrane disruption and cell damage, at 10 µg/L (Rodea-Palomares et al., 2012). Interestingly, none of these aquatic toxicity studies utilised predicted environmentally relevant concentrations and no adverse effects on aquatic organisms have been detected below 10 µg/L, which is ca. 30-fold higher than the maximum predicted levels in the aquatic environment (see above). There is a vast array of properties and levels of characterisation that need to be considered when investigating ceria NPs and the majority of these studies are limited in this context, making interpretation difficult (Baalousha et al., 2012a,b). Additionally, nominal exposure concentrations have typically been reported, which do not allow for aggregation, absorptive losses or other dynamic changes during the exposure period.

While interactions of nanomaterials with the components of the environment are inevitable, the general consequences of such interactions remain unclear and sparse quantitative data are available on which to assess risk (Lowry et al., 2012; Pelletier et al., 2010; Seaton et al., 2010; Tejamaya et al., 2012). Thus, there still remains a critical need for interdisciplinary approaches to understand the degree of exposures to NPs, their uptake and potential toxic effects on human and environmental health (Cassee et al., 2011; Madl & Pinkerton, 2009; Maynard et al., 2006). Adverse outcome pathways (AOPs) represent a new concept in risk assessment that can bring a wide range of emerging tools and knowledge together to create a link between so-called molecular initiating events and adverse outcomes at higher levels of biological organisation, and have considerable potential to advance regulatory ecotoxicology (Ankley et al., 2010; Volz et al., 2011). Ecotoxicogenomic approaches, including transcriptomics and metabolomics, can provide an unbiased assessment of an organism's response to NPs and hence help to identify these adaptive and toxic molecular response pathways (Ankley et al., 2006; Snape et al., 2004; Sturla et al., 2014). Such 'omics approaches are increasingly being used in aquatic toxicology, including to elucidate the molecular effects of NP exposure (Poma & Di Giorgio, 2008; Poynton et al., 2012; Simon et al., 2013; Tsyusko et al., 2012).

Here we focused on the freshwater environment as an anticipated sink for NP discharges. The behaviour of metal oxide NPs in freshwater is complex, including adsorption onto and intake by biota, dissolution, aggregation and sedimentation. We focused on dispersed NPs as their toxicity is likely to be higher, enabling a conservative assessment of their maximum possible hazard to the

freshwater environment (Klaine et al., 2008). An integrated 'omics approach incorporating both transcriptomics and metabolomics was used to investigate the potential toxicity of tightly constrained and characterised ceria NPs to the unicellular green alga, *Chlamydomonas reinhardtii*, a sentinel species in the freshwater environment. Our aim was to achieve a more comprehensive and definitive assessment of the responses of this sentinel species to ceria NPs than reported previously, using well-defined nanoparticles and unbiased molecular analyses. We investigated a wide range of exposure concentrations from predicted environmental levels, to inform on hazard assessment, to supra-environmental levels to provide insight into the molecular toxicity pathways activated by ceria NP exposure. Consistent with the AOP concept and a weight of evidence approach to environmental risk assessment, these molecular measurements were made alongside standard phenotypic measures of algal growth.

## Experimental section

### Ceria NP synthesis and characterisation in exposure media

The PVP-coated ceria NPs tested in this study were synthesised and characterised as described previously (Merrifield et al., 2013). Larger, PVP-coated "bulk" particles of ca. 500–600 nm were prepared in a similar manner, with identical reagents, but produced by heating an unstirred suspension at 140°C for 4 h. Dissolution rates of Ce from the NPs were determined as described in Supplementary Information (SI).

### Culturing of *Chlamydomonas reinhardtii* and ceria NP uptake and exposure studies

*Chlamydomonas reinhardtii* cultures (CCAP strain 11/32c) were grown and exposed under standard conditions, as described in SI. Specifically, growth inhibition (EC<sub>50</sub>) studies were of 72-h duration, under continuous light as detailed in standard test guidelines (OECD, 2002). Studies were conducted over a wide range of nominal ceria NP concentrations (0.5–80 000 µg/L), also for ionic Ce (CeNO<sub>3</sub>; 0.5–32 mg/L) and free PVP (0.56–56.44 mg/L). 'Omics studies were performed at two dosing regimens: supra-environmental levels (80, 400, 2000 and 10 000 µg/L; *n* = 8) and predicted environmental concentrations (0.029, 0.144 and 0.72 µg/L; *n* = 4), including relevant controls. Measurements of ceria NP uptake by *C. reinhardtii* were conducted using ICP-MS, as described in SI.

### Scanning electron microscopy of *C. reinhardtii*

Following the 72-h exposures, algal cells were collected, fixed, dehydrated, embedded in resin and sectioned on an ultramicrotome, as described in SI. These were imaged on a Jeol 7000 scanning electron microscope using backscatter and STEM modes (see SI). Elemental analysis was performed using an Oxford Instruments INCA EDS system with Si-Li x-ray detector.

### Mass spectrometry-based metabolomics

Following exposure to the ceria NPs, metabolites were extracted from *C. reinhardtii* using a modified version of published methods (see SI). Polar metabolite extracts were dried in a centrifugal concentrator and stored at –80°C. Following resuspension, metabolites were measured using a hybrid 7-T Fourier transform ion cyclotron resonance mass spectrometer (LTQ FT Ultra, Thermo Scientific; negative ion mode using the SIM (selected ion monitoring)-stitching method from *m/z* 70–590), and data were processed as reported previously (see SI). Statistical



analyses using MATLAB (version 7, The Mathworks, Natick, MA), PLS-Toolbox (Eigenvector Research, Wenatchee, WA) and R were used to discover any metabolic perturbations in *C. reinhardtii* in response to treatment as described in SI. MI-Pack software was used to putatively annotate the detected peaks, which were then investigated using MetaboAnalyst 2.0 for biochemical pathway enrichment (See SI).

### Microarray-based transcriptomics

Transcriptomics methods are described in full in SI. Briefly, RNA was prepared from unexposed controls ( $n=4$ ), ionic Ce control ( $\text{CeNO}_3$ ;  $0.008 \mu\text{g/L}$ ;  $n=7$ ) free PVP control ( $1.94 \text{ mg/L}$ ;  $n=4$ ), and two ceria NP concentrations ( $0.144 \mu\text{g/L}$ ;  $n=4$ , and  $10\,000 \mu\text{g/L}$ ;  $n=7$ ). A novel oligonucleotide microarray (Agilent Technologies, Santa Clara, CA) was designed using JGI sequence data and annotation, supplemented by Phytozome and Blast2GO. The  $8 \times 15 \text{ k}$  array design is available from ArrayExpress, accession A-MEXP-2384. Cy3-labeling, hybridisations and scanning were performed (Agilent). Raw microarray data are available at ArrayExpress, accession E-MTAB-2454. Processed signal data were analysed using GeneSpring vGX7.3 (Agilent Technologies), MultiExperimental Viewer (MeV) v4.7 and GE workbench, while SAM analysis was carried out within MeV. Annotation enrichment (Algal Functional Annotation Tool and Blast2GO) compared transcripts statistically significantly altered in expression, as determined by SAM, with the list of all detected transcripts.

### Supporting information

Supporting information is available from the Wiley Online Library and includes energy dispersive spectra of the ceria NPs, additional multivariate analyses of the metabolomics data, full KEGG pathway enrichment analysis of the transcriptomic and metabolomic changes, and lists of significantly changing metabolites and genes in *C. reinhardtii* following exposure to ceria NPs.

## Results

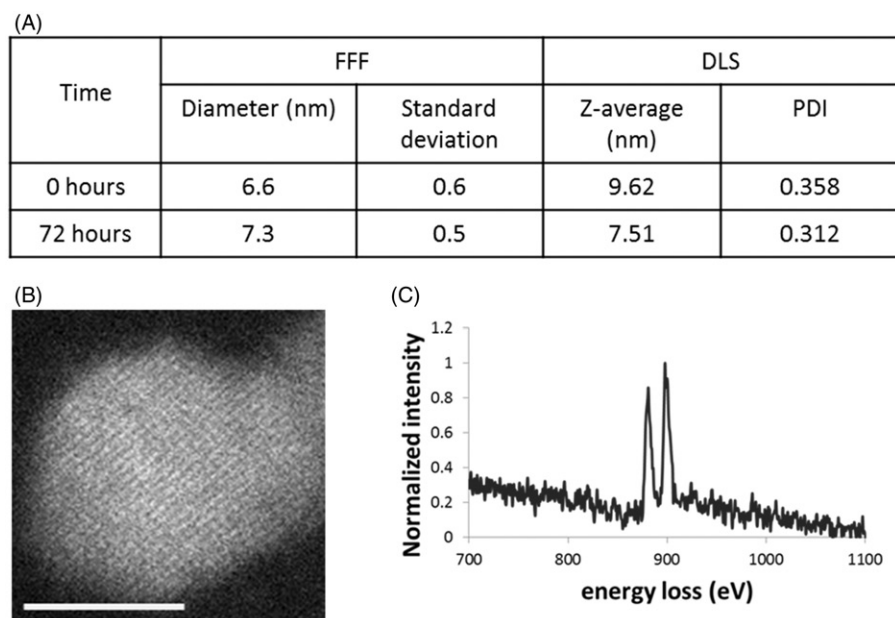
### Ceria NP synthesis and physicochemical characterisation

The thorough characterisation of NPs in complex matrices is challenging but essential for any assessments of exposure and effect (Hassellöv et al., 2008; Tiede et al., 2009). There is a lack of standard methods for assessing such interactions in

ecotoxicological studies (Merrifield et al., 2013; Pelletier et al., 2010). However, information on size, morphology, surface properties, dispersion/aggregation and dissolution and their dynamic changes on exposure must be available to guide the interpretation of any toxicity (Thill et al., 2006; Tiede et al., 2009). The ceria NPs used here were synthesised and fully characterised, as previously described in Merrifield et al. (2013). Briefly, the NPs were polyvinylpyrrolidone (PVP)-coated, made from 99.999% purity grade  $\text{CeNO}_3$  and characterised as 4–5 nm (by transmission electron microscopy; TEM) and 6–7 nm (by field flow fractionation; FFF, and dynamic light scattering; DLS) both as prepared and in the algal exposure media (Figure 1). The difference between the TEM and DLS/FFF is explained by the thin coating of the polymer PVP, consistent with our previous work (Baalousha & Lead, 2012). NP properties were largely, though not entirely, conserved in the *C. reinhardtii* exposure media. In addition to size, aggregation remained minimal, no significant changes in electrophoretic mobility were observed and the NPs remained largely insoluble. However, while the prepared particles were essentially 100% Ce(III), they became ca. 70% Ce(III) and 30% Ce(IV) oxide in the media (measured from the ratio of the white lines in the electron energy loss spectrum; EELS), likely due to sorption of redox active species from the media. The NPs were sterically stabilised by the polymer coating and hence were dispersed under all conditions, despite the near-zero electrophoretic mobility. It was observed that at NP concentrations of 80 mg/L and above the exposure media became opaque and NP aggregates collected at the base of the exposure vessel, indicating their limit of dispersibility. The high stability and minimal dissolution of these NPs made them ideal for ecotoxicological studies (Merrifield et al., 2013). Ceria particles were also made at a larger size (500–600 nm, termed ‘‘bulk’’), identically synthesised and PVP coated, for comparison to the NPs, to evaluate the dependency of any biological responses on nano-structure.

The essentially complete conservation of the tightly constrained particle properties within the exposure media, along with quantitative determination of this conservation, allows for greater understanding of any specific nanoscale effects on toxicity without uncertainties due to particle polydispersity, heterogeneity and dynamic changes caused by the media. Hypothetically, as the coating changes, no doubt the response changes, either due directly to the different coating or indirectly via dynamic changes

Figure 1. (A) Diameter of ceria NPs in exposure media after 0 and 72 h as measured by FFF (mean  $\pm$  SD) and DLS (Z-average and polydispersity index (PDI)); (B) a representative STEM image of ceria NP taken after 72 h under exposure conditions (scale bar 5 nm); and (C) a typical EELS spectrum taken from centre of the nanoparticle.



of the NPs over the exposure period. In addition, the polymer coating and crystalline form suggests these NPs are relevant to ceria NPs used in the polishing, rather than other uses such as diesel combustion, but our primary concern is not representation of specific sectoral uses of NPs but to attempt to understand nanoscale effects in a more quantitative manner.

#### Ceria NPs do not affect *C. reinhardtii* growth rate under conditions tested

Microalgae, including *C. reinhardtii*, are keystone species in aquatic ecosystems and are often used in aquatic toxicity testing (Jamers et al., 2013). In accordance with international standards, we used a measure of growth inhibition over 72 h to indicate toxicity of ceria NPs to *C. reinhardtii* (OECD, 2002). An exceptionally wide range of exposure concentrations (0.5–80 000 µg/L) of ceria NPs were investigated to include both predicted environmental levels and those concentrations at which adverse effects have been reported previously (Figure 2). No inhibition of algal growth was detected at any of the ceria NP concentrations tested (Figure 2A). For ionic cerium (CeNO<sub>3</sub>; positive control), a 50% effect concentration (EC<sub>50</sub>) for growth inhibition of 4.5 mg/L and a “no observed adverse effect level” (NOAEL) on growth of 2 mg/L were determined. These values lie within the range of previously reported concentrations for

CeNO<sub>3</sub> toxicity to freshwater green algae of 0.63–15.5 mg/L (Evseeva et al., 2010; Rogers et al., 2010). Direct toxicity of the free PVP, in the absence of NPs, was also investigated to account for any free PVP introduced from the ceria NP exposures. The lowest concentration of PVP that reduced the algal growth rate was determined to be 56.4 mg/L. Studies were repeated independently, confirming the reproducibility of these growth measures.

#### Ceria NPs are internalised by *C. reinhardtii*

Given the lack of effect of ceria NPs on algal growth, we next sought to determine whether the NPs were taken up by the cells, and if so, to quantify this uptake. *C. reinhardtii* were exposed to 10 mg/L ceria NPs to assess uptake for the standard 72-h growth period used in toxicity assays (OECD, 2002). Ce was separated operationally into internalised/strongly bound NP mass (Figure 3A), loosely bound (Figure 3B), dissolved in media (Figure 3C), and particulate within the media (Figure 3D). Clearly, the majority of the ceria NPs remained within the media as individual nanoparticles, with ca. 1% or less present in the media as dissolved species. This concentration decreased over time, presumably due to absorptive losses rather than uptake, given that the mass of cerium associated with the algal cells was <1% of the dissolved cerium mass. Low masses of cerium

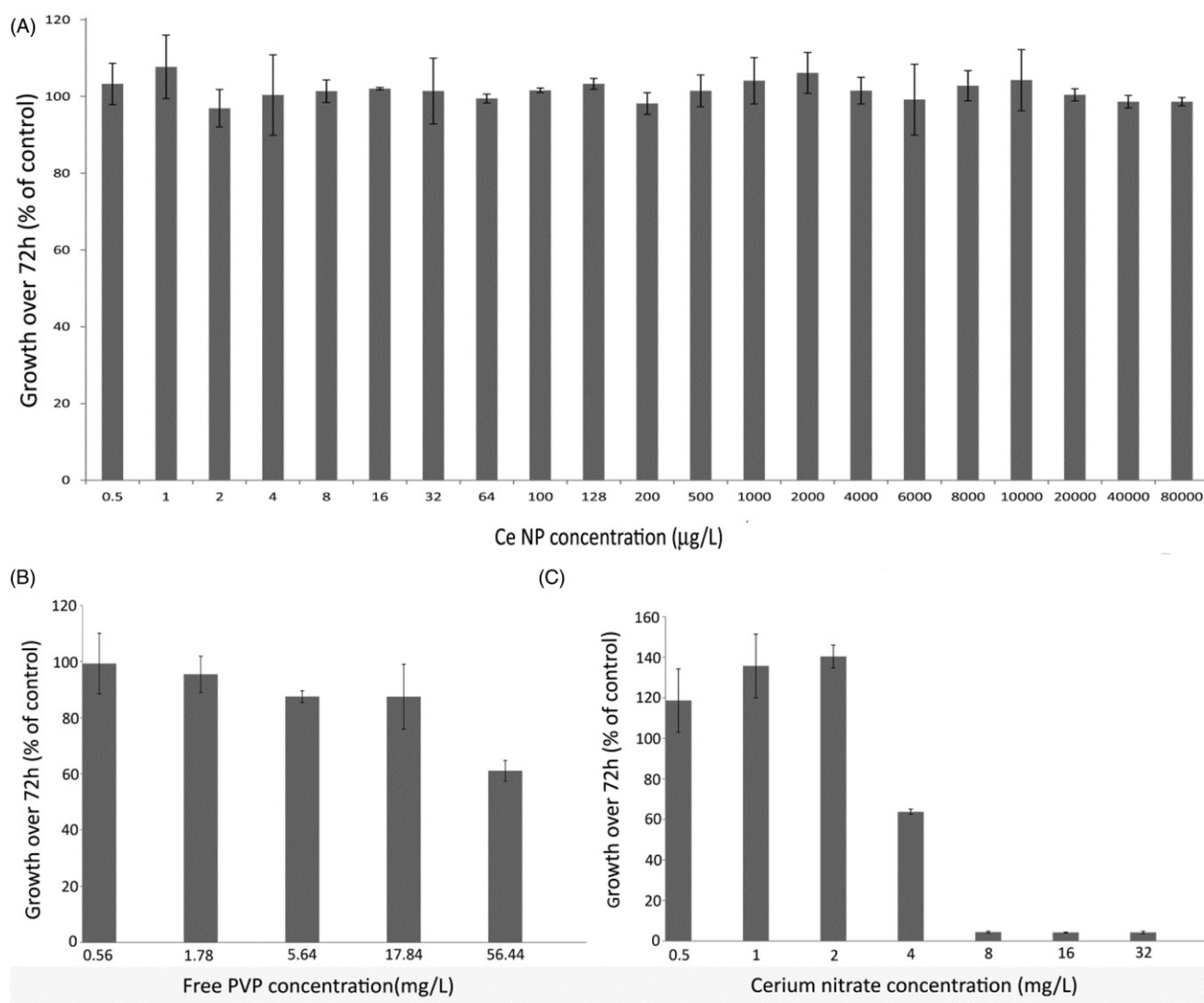


Figure 2. (A) Growth measurements of *C. reinhardtii* exposed to increasing concentrations of ceria NPs, (B) free PVP and (C) cerium nitrate. Data is shown as mean  $\pm$  SD.

were loosely associated with the cells and even lower masses were taken up or strongly associated with the algae. Of the ceria in suspension, <0.1% was internalised within or strongly bound to the algal cells.

To assess whether this uptake of Ce was indeed as a result of cellular internalisation or simply due to adsorption of the NPs to the outside of the *C. reinhardtii*, scanning electron microscopy (SEM) imaging coupled with energy dispersive spectroscopy (EDS) elemental analysis was conducted. Two widely differing ceria NP concentrations were used (80 and 10 000 µg/L), a single bulk ceria concentration (10 000 µg/L), and an unexposed control sample of *C. reinhardtii*; exposure conditions were the same for all the treatments. The measurements showed that the algal cells internalise the ceria NPs at both concentrations, with more Ce detected at the higher exposure concentration (Figure 4A and B). Furthermore, Ce was clearly compartmentalised within intracellular vesicles, an effect not previously reported for ceria in algal cells. Cerium was also detected surrounding the cells in both the highest ceria NP exposed (Figure 4A) and the bulk ceria exposed samples (Figure 4C), although it is not possible to elucidate if the particles were actually bound to the cell surface. No internalised Ce could be detected following exposure to the bulk material and no Ce was detected in the unexposed control samples (Figure 4D). Furthermore, no settling of the particles was observed in either the stock suspension of bulk ceria or at the concentration of bulk particles used in the exposure study.

#### Effects of ceria NPs on gene transcription and metabolism are restricted to supra-environmental exposure concentrations

Several recent studies predict that the environmental levels of ceria NPs are in the ng/L range (ca. 300 ng/L; Boxall et al., 2007;

Johnson & Park, 2012) while the adverse effects of this class of nanomaterial on algal growth occur at supra-environmental levels, at ca. four orders of magnitude higher concentration (2.4–10.3 mg/L; Rodea-Palomares et al., 2011, 2012; Rogers et al., 2010; Van Hoecke et al., 2009). Given this, and our own observations above, we investigated the molecular responses of *C. reinhardtii* at several concentrations of ceria NPs within two widely differing concentration regimens; first, at high supra-environmental levels (80, 2000, 4000 and 10 000 µg/L), reflecting the concentrations that are reported to induce adverse growth effects in algae, and secondly, at lower environmentally realistic values (0.029, 0.144 and 0.72 µg/L) which encompass the estimated environmental levels of ceria NPs in aquatic systems. Additional exposures of *C. reinhardtii* to ionic Ce at 1 µg/L (0.01% of the highest NP concentration to match the dissolution rate of these NPs and 2000 times below the NOAEL; Merrifield et al., 2013), to bulk ceria at 10 000 µg/L (same concentration as the highest ceria NP treatment) and to free PVP at 1.94 mg/L (highest level of free PVP that could result from the highest ceria NP concentration) were included. The algal responses were probed using transcriptomics, to investigate gene regulatory mechanisms, alongside metabolomics to detect any downstream functional molecular responses.

Multivariate analyses using principal components analysis (PCA) of the mass spectrometry-based metabolomics data revealed that there was a significant perturbation of metabolic function within the algal cells when exposed to ceria NPs at supra-environmental concentrations (80, 400, 2000 and 10 000 µg/L; Figures 5A and S2). Consistent with this observation, univariate analyses of individual peak intensities determined that 347 peaks (false discovery rate corrected,  $q < 0.05$ ; of a total of 3379 peaks measured) changed significantly at an exposure concentration of 10 000 µg/L ceria NPs relative to unexposed controls. Of these, 68 were putatively annotated with a known metabolite name

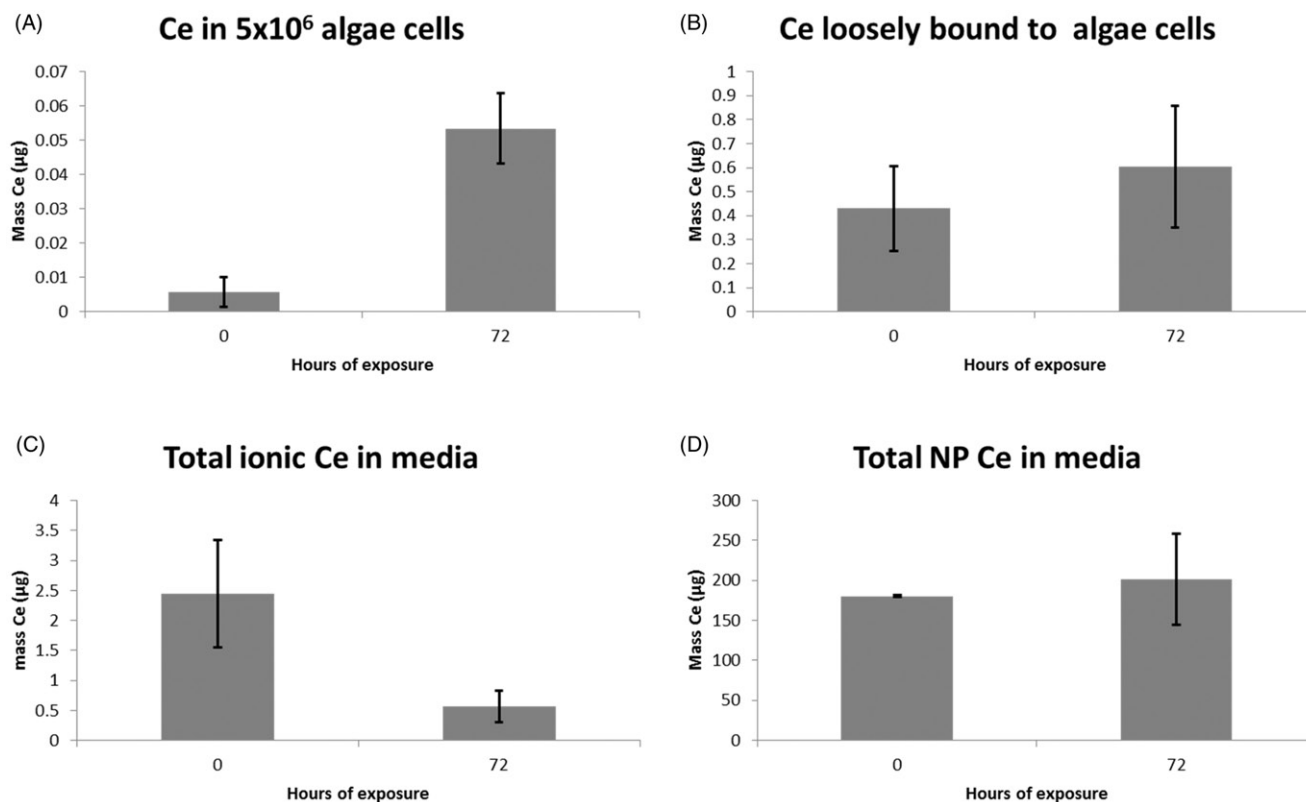


Figure 3. Bar charts showing the increase of cerium (A) within or strongly bound to  $5 \times 10^6$  algae cells, and (B) loosely bound to these cells, together with the masses of (C) dissolved and (D) particulate cerium in the media, all over a standard 72-h exposure period.



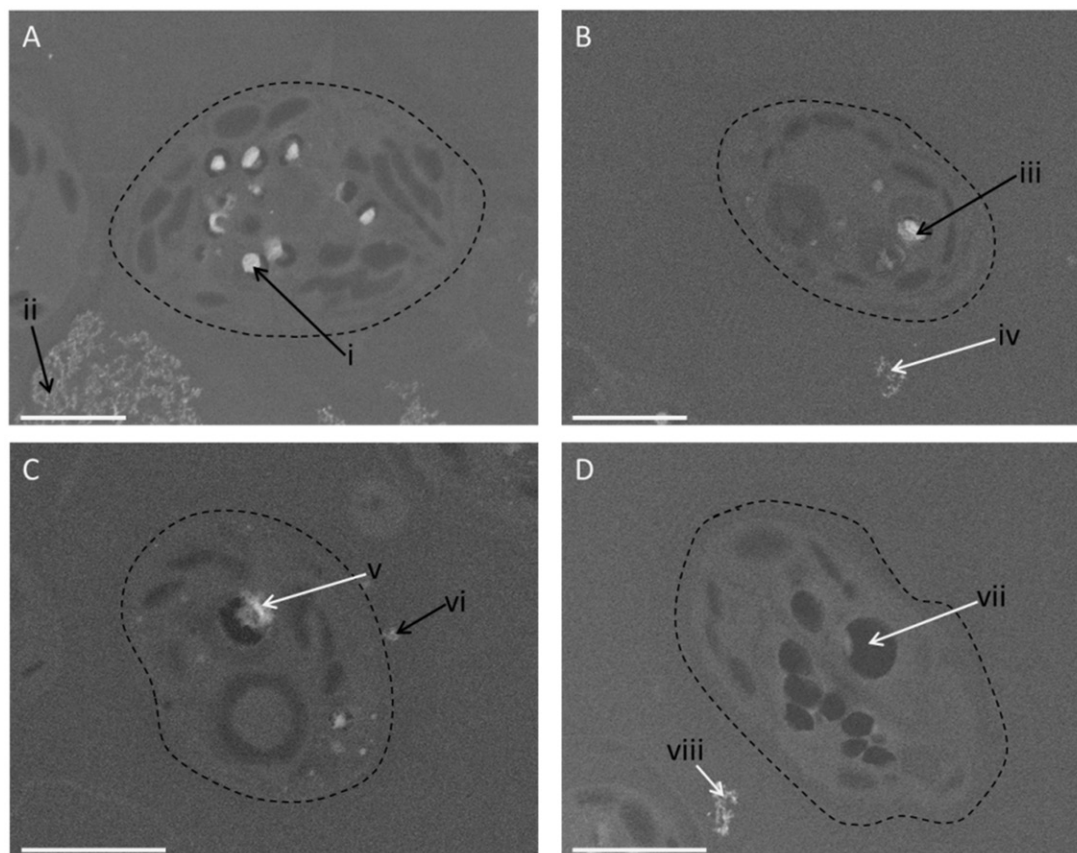


Figure 4. SEM images of algal cells exposed to a high (10 000 µg/L) concentration of ceria NPs (A), a low concentration (80 µg/L) of ceria NPs (B), bulk ceria (10 000 µg/L) (C), and unexposed control cells (D). The black arrows point to the areas where cerium was found using energy dispersive spectroscopy (EDS), while the white arrows are areas where no cerium was found (see Figure S1 for EDS spectra). The dashed line highlights the cell surface. Scale bar in each case is 2 µm.

(Table S1). No metabolic effects were detected at any of the exposure concentrations near the predicted environmental levels of ceria NPs (0.029, 0.144 and 0.72 µg/L; Figures 5C and S3) and no individual peaks changed intensity significantly under this exposure regime. Furthermore, no metabolic effects were detected in the algal cells exposed to ionic Ce, bulk ceria or to free PVP, relative to unexposed controls (Figures S2 and S3), nor did any peaks change intensity significantly relative to controls.

Microarray-based transcriptomics, necessarily conducted on a reduced experimental design but still encompassing environmentally realistic and supra-environmental levels, revealed the same dependency on relatively high concentrations of ceria NPs to induce any effects. Specifically, at a concentration of 10 000 µg/L, ceria NPs exerted significant perturbations on gene transcription, with Statistical Analysis of Microarrays (SAM) revealing that 357 gene transcripts were induced and 707 transcripts were repressed ( $q < 0.05$ ; of a total of 12 211 detected), relative to a combined ionic Ce and untreated control group (Table S2). Similarly, multivariate PCA demonstrated a separation between the transcriptional profiles of 10 000 µg/L ceria NP-treated algal cells compared to control samples (Figure 5B). Consistent with the metabolomics data, in this experiment no significant differences in gene expression ( $q < 0.05$ ) were detected between algal cells exposed to the environmentally realistic 0.144 µg/L ceria NPs and either untreated controls or a combined ionic Ce and untreated control group, and no separation between these treatment groups was apparent in the PCA scores plot (Figure 5D). Potentially, increasing the power of the analysis by examining further replicates could discover gene expression responses to 0.144 µg/L ceria NPs.

Focusing on the exposure of *C. reinhardtii* to the highest concentration of 10 000 µg/L ceria NPs, annotation enrichment analyses were performed for both the transcriptomics and metabolomics datasets. For the transcriptomics, gene ontology (GO) terms significantly enriched among repressed transcripts related predominantly to chloroplast, photosynthesis and response to stress (Figure 6); no GO terms were significantly enriched among induced genes. KEGG pathway enrichment analysis showed that multiple molecular pathways were perturbed after exposure to supra-environmental ceria NP concentrations, the majority of which were represented by both transcripts and metabolites, indicating a consistency between gene expression and subsequent metabolic change (Table 1).

Considering the perturbed pathways, most notable was the statistically significant transcriptional repression of genes encoding chloroplast photosynthetic proteins. For example, transcripts encoding light-harvesting proteins of photosystems I and II *LHCA3,5* and *8*, *LHCB4* and *5*, and *LHCBM2,3,5,6* and *7* were significantly reduced in expression after 10 000 µg/L ceria NP exposure, as were chloroplast-encoded photosystem I transcripts *psa d, e, f, g, h, I, j, k*, and photosystem II transcripts *psb 28, o, p3, s1, w* and *x*. Oxidative stress-responsive transcripts tended to be repressed after NP exposure, with the GO term “response to stress” and the KEGG pathway “glutathione metabolism” significantly enriched amongst down-regulated transcripts. One transcript similar to glutathione peroxidase was induced, but superoxide dismutase *FSD1*, L-ascorbate peroxidase-like, glutathione s-transferases, glutathione reductase *GSHR1* and two peroxiredoxin family transcripts were all repressed. KEGG pathway enrichment analyses identified substantial perturbations to metabolic pathways (Tables 1 and S1),

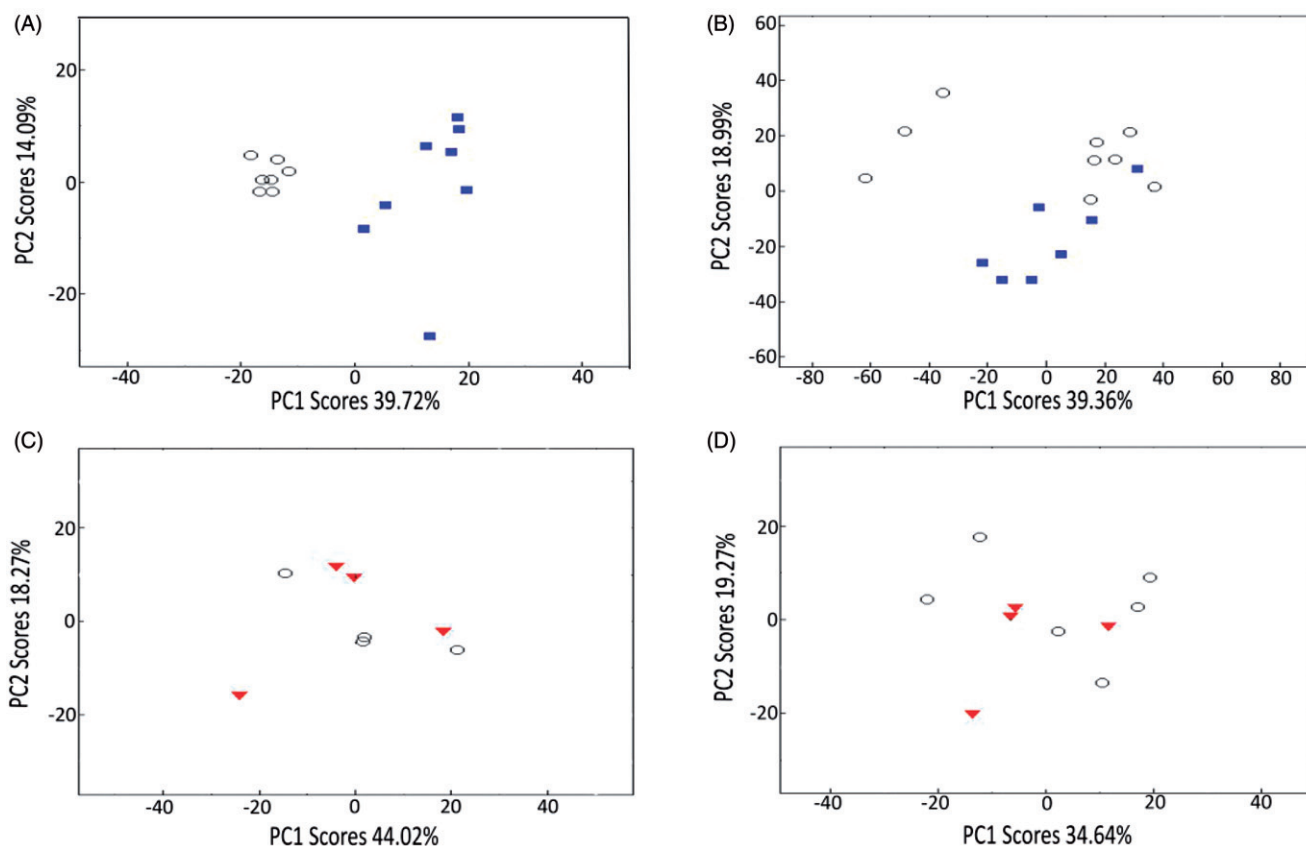


Figure 5. PCA scores plots from analyses of (A) mass spectrometry-based metabolomics dataset and (B) microarray-based transcriptomics dataset measured from *C. reinhardtii* exposed to supra-environmental concentrations of ceria NPs: control (○), 10 000 µg/L ceria NPs (■); and from (C) metabolomics dataset and (D) transcriptomics dataset measured from *C. reinhardtii* exposed to the predicted environmental concentrations of ceria NPs: control (○), 0.144 µg/L ceria NPs (▼). *T*-tests of the PC scores identified that supra-environmental concentrations of ceria NPs significantly perturbed metabolism ( $p$  value:  $6.66 \times 10^{-8}$  along PC1) and gene expression ( $p$  value:  $4.18 \times 10^{-4}$  along PC2) relative to controls, while environmental concentrations of ceria NPs had no effect.

including a consistent repression of transcripts and metabolites in the carbon-fixation pathway (Tables S2 and S3; phosphoribulokinase *PRK1*, sedoheptulose-1,7-bisphosphatase *SEBP1*, ribose-5-phosphate isomerase *RPII* and putatively identified glyceraldehyde-3-phosphate, 3-phosphoglycerate, xylulose-5-phosphate, erythrose-4-phosphate, ribulose-5-phosphate/ribose-5-phosphate) and alterations to CoA and fatty acid biosynthesis, amino acid, nucleotide and carbohydrate metabolism.

## Discussion

### Importance of fully characterising ceria NPs to interpret their uptake and potential toxicity

We report that no inhibition of algal growth was detected at any of the ceria NP concentrations tested (0.5–80 000 µg/L), while an  $EC_{50}$  for growth inhibition of 4.5 mg/L and NOAEL of 2 mg/L were determined for ionic cerium ( $CeNO_3$ ); the lowest concentration of free PVP that reduced growth was 56.4 mg/L (Figure 2). Considering the composition of the highest ceria NP exposure solution, these findings are self-consistent: first, the highest ceria NP exposure was estimated to contain PVP at a maximum concentration of 1.94 mg/L, which is ca. 30 times lower than the concentration of PVP required to reduce growth. Secondly, with a dissolution rate of 0.01% (Merrifield et al., 2013), the highest expected ionic cerium levels in the highest concentration ceria NP exposure is only 8 µg/L, which is over 500 times lower in concentration than our measured  $EC_{50}$  for growth inhibition of *C. reinhardtii* by ionic cerium. Therefore, neither PVP nor

dissolved cerium would be expected to affect growth in the highest ceria NP exposure, and the absence of any effect of these NPs suggests that there is no nano-specific effect on algal growth up to 80 mg/L.

Our finding is perhaps surprising when compared to published  $EC_{50}$  values for algae exposed to ceria NPs, which range from 2.4–10.3 mg/L (Rodea-Palomares et al., 2011, 2012; Rogers et al., 2010; Van Hoecke et al., 2009). However, these earlier studies used commercial ceria NPs that were uncoated, resulting in large aggregates (100–500 nm) with potentially higher dissolution rates. It is conceivable that the adverse effects detected in these studies were caused by direct NP surface (specifically, ceria) interactions with algal cell surfaces, an effect that would not be expected to occur for PVP-coated NPs. In addition, the higher level of toxicity may also have resulted from the much greater rate of dissolution from the uncoated NP core, which was reported as high as 21% by Rogers et al. (2010). The techniques used by Van Hoecke et al. (2009) and Rodea-Palomares et al. (2011, 2012) were unable to detect dissolved ceria and hence dissolution rates could not be determined. However, trace metal analysis of the commercial particles used by Rodea-Palomares et al. (2011, 2012) determined notable levels of other metals in the NP stocks, including dissolved Zn (10–25 µg/L), the toxic effects of which are well documented. This emphasises the necessity to fully characterise NPs in environmentally relevant media to interpret their potential toxicity accurately. The PVP coating used in our study prevents NP aggregation and dissolution and hence allows for a true determination of the adverse effects of ceria NPs on



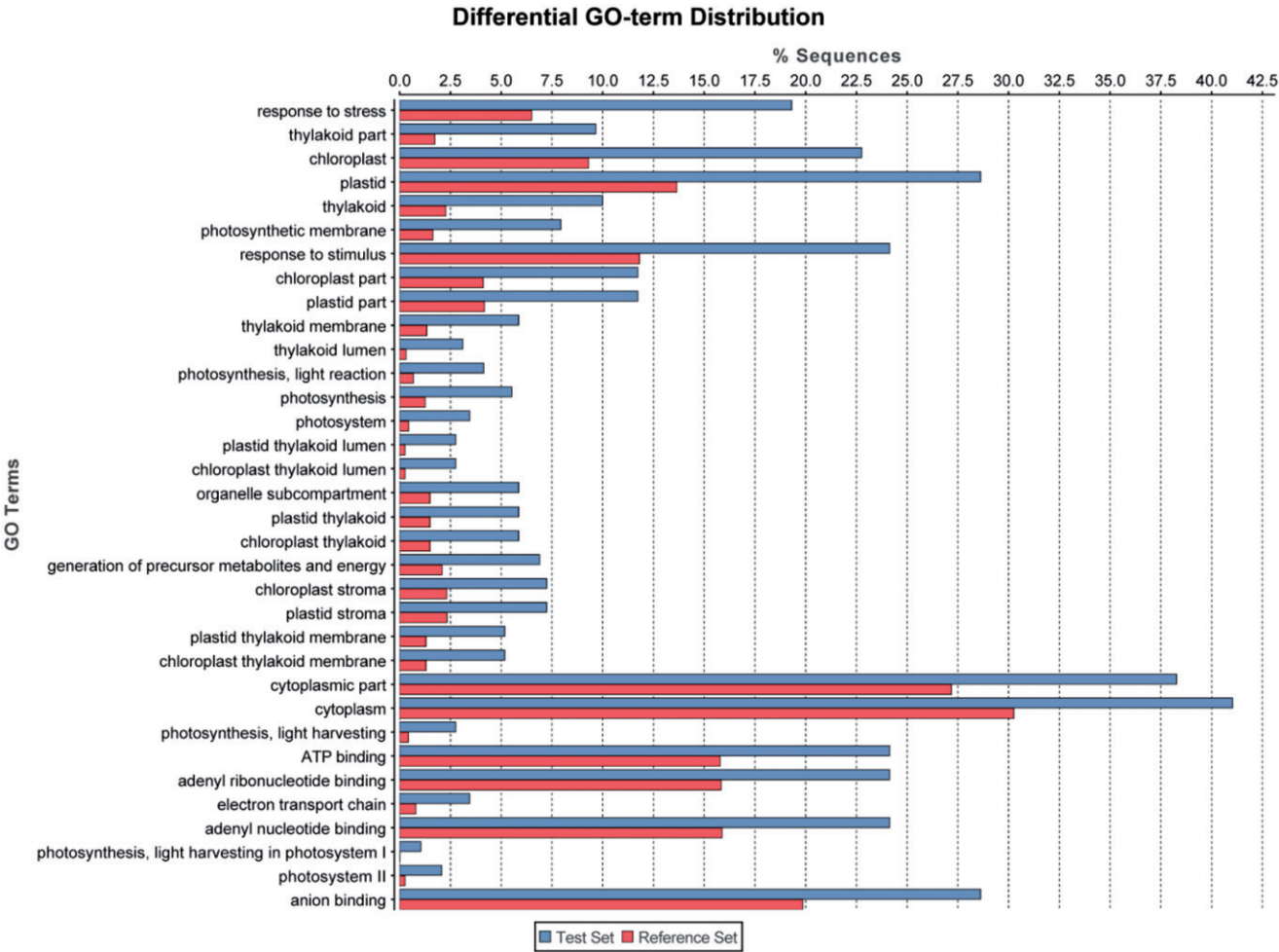


Figure 6. GO terms significantly enriched among gene transcripts repressed in algal cells following a 72-h exposure to 10 000 µg/L ceria NPs (SAM,  $q < 0.05$ ; Test set), as identified by Blast2GO with Fisher's Exact Test at FDR  $< 0.05$  in comparison with all transcripts detected (Reference Set).

Table 1. Top 15 perturbed molecular pathways following KEGG pathway enrichment analysis of the transcriptomic and metabolomic changes elicited in *C. reinhardtii* in response to 10 000 µg/L ceria NPs.

KEGG Pathways	Transcript enrichment			Metabolite enrichment			Combined	
	Hits	<i>q</i> Value	direction	Hits	<i>q</i> Value	direction	Hits	Score
Tyrosine metabolism	4	1.64E-01	↘	2	<b>1.56E-05</b>	↑	6	2.55E-06
Pantothenate and CoA biosynthesis	1	7.59E-01	↓	2	<b>1.56E-05</b>	↗	3	1.18E-05
Valine, leucine and isoleucine biosynthesis	1	7.76E-01	↓	9	<b>1.56E-05</b>	↗	10	1.21E-05
Purine metabolism	9	7.87E-01	↘	3	<b>1.56E-05</b>	↗	12	1.22E-05
Photosynthesis	9	<b>1.37E-05</b>	↓	ND	ND	ND	9	1.37E-05
Pyruvate metabolism	3	6.39E-01	↓	3	<b>3.95E-05</b>	↑	6	2.52E-05
Pyrimidine metabolism	1	9.95E-01	↑	2	<b>3.31E-05</b>	↓	3	3.29E-05
Porphyrin and chlorophyll metabolism	5	2.31E-01	↘	2	<b>1.63E-04</b>	↑	7	3.77E-05
Lysine degradation	4	7.76E-02	↗	2	<b>5.23E-04</b>	—	6	4.06E-05
Fatty acid biosynthesis	4	<b>1.87E-02</b>	↓	2	<b>2.48E-03</b>	↑	6	4.63E-05
Glyoxylate and dicarboxylate metabolism	4	1.24E-01	↓	3	<b>6.87E-04</b>	↓	7	8.50E-05
Isoquinoline alkaloid biosynthesis	2	<b>2.79E-02</b>	—	1	<b>3.43E-03</b>	↑	3	9.59E-05
Glutathione metabolism	6	<b>2.36E-02</b>	↘	1	<b>4.58E-03</b>	↑	7	1.08E-04
Riboflavin metabolism	2	2.11E-01	↓	2	<b>6.87E-04</b>	—	4	1.45E-04
Carbon fixation in photosynthetic organisms	4	2.89E-01	↓	2	<b>6.87E-04</b>	↓	6	1.99E-04

Transcriptomic changes are derived from Algal Functional Annotation Tool and metabolomic changes are derived from Met-PA. Hits show the number of pathway members in the Test Set (gene transcripts differentially expressed in algal cells following a 72-h exposure to 10 000 µg/L ceria NPs, SAM,  $q < 0.05$ ).  $q$  Values denote false discovery rate corrected  $p$  values for enrichment compared with a background of all identified transcripts or metabolites. Arrows indicate the direction of molecular perturbation with a consistent increase in concentration shown as ↑, a consistent decrease as ↓, a majority increase as ↗ and a majority decrease as ↘. Significant pathway enrichments with  $q < 0.05$  are shown in bold; ND denotes no enrichment detected. The combined score was generated by multiplying the  $q$  values for both 'omics approaches and is used for the overall ranking of the pathways. The full list of perturbed pathways is given in Table S1.

*C. reinhardtii*. Furthermore, the extensive characterisation of the ceria NPs used here allows us to rationalise our algal uptake results. Previously it was speculated that NP aggregates that are larger than the pores in algal cell walls (5–20 nm diameter) cannot be taken up (Navarro et al., 2008). This could explain why we have definitively observed the internalisation of the ca. 5 nm mono-dispersed ceria NPs within our algal samples (Figures 3 and 4), unlike in earlier studies in which large ceria aggregates were formed and no internalisation was reported. This again highlights the important role that NP size can have on determining routes of uptake.

### Molecular pathways affected by supra-environmental concentrations of ceria NPs

While neither metabolomics nor transcriptomics detected any significant molecular alterations in response to environmentally relevant concentrations of ceria NPs in this experiment, both approaches detected molecular responses to the supra-environmental level of 10 000 µg/L ceria NPs. These findings add further weight to the argument that the risks to *C. reinhardtii* posed by environmentally realistic concentrations of ceria NPs, for the coated and mono-dispersed particles studied here, are low (Johnson & Park, 2012). At supra-environmental concentrations, however, the PVP-coated nanoparticles induced molecular alterations that were nano-specific, i.e. the changes were not observed as a result of exposure to the isolated PVP coating or dissolved (ionic) Ce. Therefore an understanding of these responses is still valuable within a hazard assessment paradigm.

The observed repression of photosynthesis-related transcription is consistent with the decreased chlorophyll concentration and inhibition of photosynthetic oxygen production found when *P. subcapitata* were exposed to ceria NPs at similar concentrations (Rodea-Palomares et al., 2011). The earlier study also reported an increase in ROS after ceria NP exposure, but there was no evidence of oxidative stress in our study with *C. reinhardtii* implying little effect of ceria NP-induced ROS, rather a decrease in ROS with down-regulation of photosynthesis. Differences between the algal species, the physicochemical properties of the nanoparticles, or their subcellular locations, between this study and the findings reported by Rodea-Palomares et al. (2012) may explain this apparent contradiction. Moreover and importantly, there was a lack of detection of a DNA-damage response despite the inclusion of probes on the microarray representing this biological response. The observed repression of transcripts and metabolites of the carbon fixation pathway and perturbation of CoA and fatty acid biosynthesis, amino acid, nucleotide and carbohydrate metabolism pathways is consistent with repression of photosynthesis. In summary, a 72-h exposure to the supra-environmental concentration of 10 000 µg/L ceria NPs primarily mediated a repression of both photosynthesis and carbon fixation that resulted in wide-ranging changes to cellular metabolism in *C. reinhardtii*.

### Conclusion

We have reported an extensive investigation into the molecular and phenotypic effects of ceria NPs to *C. reinhardtii*, a sentinel algal species in freshwater ecosystems, including all essential components of a nanotoxicology study: synthesis of tightly constrained NPs, physico-chemical characterisation of the nanomaterials in the exposure media; quantification of the uptake of this material into the cells and imaging to determine its location; an assessment of the effects of exposure on the algal phenotype, i.e. its growth rate using standardised OECD protocols; and extensive molecular measurements spanning both regulatory (gene expression) and functional (metabolic) responses. We report clear evidence for the

internalisation of the mono-dispersed, PVP-coated, ceria NPs into intracellular vesicles within the *C. reinhardtii*. Despite this, no significant effect on algal growth rate was measured at any of the wide-range of ceria NP exposure concentrations which included predicted environmental levels. Furthermore, molecular perturbations were only detected when *C. reinhardtii* was exposed to very high concentrations of the ceria NPs, ca. 3000 times above the predicted environmental levels of this nanomaterial. The principal molecular changes associated with ceria NP exposure include a down-regulation of photosynthesis and carbon fixation with associated effects on energy metabolism. We recommend that these molecular changes can direct the construction of adverse outcome pathways in regard to on-going hazard assessment of ceria NPs. Overall we conclude that for acute exposures to small (4–5 nm) mono-dispersed particles, there should be little concern regarding their dispersal into the environment for this specific trophic level. However, our discovery of the internalisation of the ceria by the algal cells raises further questions. First, what are the molecular and phenotypic effects of the ceria NPs in a longer term exposure scenario, and second, what are the repercussions of this internalisation within the aquatic food chain with regards to the bioavailability and potential toxicity of ceria to primary consumers? Such questions must be addressed as part of a rigorous hazard assessment of this important and widely used class of nanomaterial.

### Acknowledgements

We acknowledge Paul Stanley and Theresa Morris for their assistance with the electron microscopy and the Birmingham Functional Genomics laboratory staff for helping with the transcriptomics.

### Declaration of interest

We thank the UK Natural Environment Research Council (NE/H008764/1 and NE/H013148/1) and the Center for Environmental Nanoscience and Risk for financial support. The FT-ICR mass spectrometer used in this research was obtained through the Birmingham Science City Translational Medicine: Experimental Medicine Network of Excellence project, with support from Advantage West Midlands (AWM).

### References

- Ankley GT, Daston GP, Degitz SJ, Denslow ND, Hoke RA, Kennedy SW, et al. 2006. Toxicogenomics in regulatory ecotoxicology. *Environ Sci Technol* 40:4055–64.
- Ankley GT, Bennett RS, Erickson RJ, Hoff DJ, Hornung MW, Johnson RD, et al. 2010. Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. *Environ Toxicol Chem* 29:730–41.
- Baalousha M, Lead JL. 2012. Rationalizing nanomaterial sizes measured by atomic force microscopy, flow field-flow fractionation and dynamic light scattering: sample preparation, polydispersity and particle structure. *Environ Sci Technol* 46:6134–42.
- Baalousha M, Ju-Nam Y, Cole PA, Gaiser B, Fernandes TF, Hriljac JA, et al. 2012a. Characterization of cerium oxide nanoparticles – part 1: size measurements. *Environ Toxicol Chem* 31:983–93.
- Baalousha M, Ju-Nam Y, Cole PA, Gaiser B, Fernandes TF, Hriljac JA. 2012b. Characterization of cerium oxide nanoparticles – part 2: nonsize measurements. *Environ Toxicol Chem* 31:994–1003.
- Boxall ABA, Chaudhry Q, Sinclair C, Jones A, Aitken R, Jefferson B, et al. 2007. Current and Future predicted environmental exposure to engineered nanoparticles. Central Science Laboratory Report for Department of Environment Food and Rural Affairs, Central Science Laboratory, York, UK.
- Cassee FR, Van Balen EC, Singh C, Green D, Muijsers H, Weinstein J, et al. 2011. Exposure, health and ecological effects review of engineered nanoscale cerium and cerium oxide associated with its use as a fuel additive. *Crit Rev Toxicol* 41:213–29.

- Celardo I, Pedersen JZ, Traversa E, Ghibelli L. 2011. Pharmacological potential of cerium oxide nanoparticles. *Nanoscale* 3:1411–20.
- Evseeva T, Geras'kin S, Majstrenko T, Brown J, Belykh E. 2010. Comparative estimation of  $^{232}\text{Th}$  and stable Ce(III) toxicity and detoxification pathways in freshwater alga *Chlorella vulgaris*. *Chemosphere* 81:1320–7.
- Fall M, Guerbet M, Park B, Gouriou F, Dionnet F, Morin J. 2007. Evaluation of cerium oxide and cerium oxide based fuel additive safety on organotypic cultures of lung slices. *Nanotoxicology* 1:227–34.
- Hassellöv M, Readman JW, Ranville JF, Tiede K. 2008. Nanoparticle analysis and characterization in environmental risk assessment of engineered nanoparticles. *Ecotoxicology* 17:344–61.
- Hendren CO, Mesnard X, Dröge J, Wiesner MR. 2011. Estimating production data for five engineered nanomaterials as a basis for exposure assessment. *Environ Sci Technol* 45:2562–9.
- Hoshino T, Kurata Y, Terasaki Y, Susa K. 2001. Mechanism of polishing of  $\text{SiO}_2$  films by  $\text{CeO}_2$  particles. *J Non-Cryst Solids* 283:129–36.
- Jamers A, Blust R, De Coen W, Griffin JL, Jones OAH. 2013. Copper toxicity in the microalga *Chlamydomonas reinhardtii*: an integrated approach. *Biomaterials* 26:731–40.
- Johnson AC, Park B. 2012. Predicting contamination by the fuel additive cerium oxide engineered nanoparticles within the United Kingdom and the associated risks. *Environ Toxicol Chem* 31:2582–7.
- Klaine SJ, Alvarez PJJ, Batley GE, Fernandes TF, Handy RH, Lyon DY, et al. 2008. Nanomaterials in the environment: behaviour, fate bioavailability and effects. *Environ Toxicol Chem* 27:1825–51.
- Lee S, Kim S, Choi J. 2009. Genotoxicity and ecotoxicity assays using the freshwater crustacean *Daphnia magna* and the larva of the aquatic midge *Chironomus riparius* to screen the ecological risks of nanoparticle exposure. *Environ Toxicol Pharmacol* 28:86–91.
- Lowry GV, Espinasse BP, Badireddy AR, Richardson CJ, Reinsch BC, Bryant LD, et al. 2012. Long-term transformation and fate of manufactured Ag nanoparticles in a simulated large scale freshwater emergent wetland. *Environ Sci Technol* 46:7027–36.
- Madl AK, Pinkerton KE. 2009. Health effects of inhaled engineered and incidental nanoparticles. *Crit Rev Toxicol* 39:629–58.
- Maynard AD, Aitken RJ, Butz T, Colvin V, Donaldson K, Oberdörster G, et al. 2006. Safe handling of nanotechnology. *Nature* 444:267–9.
- Merrifield RC, Wang ZW, Palmer RE, Lead JR. 2013. Synthesis and characterisation of polyvinylpyrrolidone-coated cerium oxide nanoparticles. *Environ Sci Technol* 47:12426–33.
- Navarro E, Baun A, Behra R, Hartmann NB, Filser J, Miao A-J, et al. 2008. Environmental behaviour and ecotoxicity of engineered nanoparticles to algae, plants and fungi. *Ecotoxicology* 17:372–86.
- OECD. 2002. Guidelines for Testing of Chemicals, No 201, Freshwater Alga and Cyanobacteria, Growth Inhibition Test. Organisation for Economic Cooperation and Development, Paris, France.
- Park B, Donaldson K, Duffin R, Tran L, Kelly F, Mudway I, et al. 2008a. Hazard and risk assessment of a nanoparticulate cerium-oxide based diesel fuel additive – A case study. *Inhal Toxicol* 20:547–66.
- Park E, Choi J, Park Y, Park K. 2008b. Oxidative stress induced by cerium oxide nanoparticles in cultured BEAS-2B cells. *Toxicology* 245:90–100.
- Pelletier DA, Suresh AK, Holton GA, McKeown CK, Wang W, Gu B, et al. 2010. Effects of engineered cerium oxide nanoparticles on bacterial growth and viability. *Appl Environ Microbiol* 76:7981–9.
- Poma A, Di Giorgio ML. 2008. Toxicogenomics to improve comprehension of the mechanisms underlying responses of in vitro and in vivo systems to nanomaterials: a review. *Curr Genomics* 9:571–85.
- Poynton HC, Lazorchak JM, Impellitteri CA, Blalock BJ, Rogers K, Allen HJ, et al. 2012. Toxicogenomic responses of nanotoxicity in *Daphnia magna* exposed to silver nitrate and coated silver nanoparticles. *Environ Sci Technol* 46:6288–96.
- Rogers NJ, Franklin NM, Apte SA, Batley GE, Angel BM, Lead JR, et al. 2010. Physico-chemical behaviour and algal toxicity of nanoparticulate  $\text{CeO}_2$  in freshwater. *Environ Chem* 7:50–60.
- Rodea-Palmares I, Boltes K, Fernández-Pinas F, Leganés F, García-Calvo E, Santiago J, et al. 2011. Physicochemical characterization and ecotoxicological assessment of  $\text{CeO}_2$  nanoparticles using two aquatic microorganisms. *Toxicol Sci* 119:135–45.
- Rodea-Palmares I, Gonzalo S, Santiago-Morales J, Leganés F, García-Calvo E, Rosal R, et al. 2012. An insight into the mechanisms of nanoceria toxicity in aquatic photosynthetic organisms. *Aquat Toxicol* 122:133–43.
- Seaton A, Tran L, Aitken R, Donaldson K. 2010. Nanoparticles, human health hazard and regulation. *J R Soc Interface* 7:S119–29.
- Shah V, Shas S, Shah H, Rispoli FJ, McDonnell KT, Workeneh S, et al. 2012. Antibacterial activity of polymer coated cerium oxide nanoparticles. *Plos One* 7:1–13.
- Simon DF, Domingos RF, Hauser C, Hutchins CM, Zerges W, Wilkinson KJ. 2013. Transcriptome sequencing of the effects of metal nanoparticle exposure on the transcriptome of *Chlamydomonas reinhardtii*. *Appl Environ Microbiol* 79:4744–85.
- Snape JR, Maund SJ, Pickford DB, Hutchinson TH. 2004. Ecotoxicogenomics: the challenge of integrating genomics into aquatic and terrestrial ecotoxicology. *Aquat Toxicol* 67:143–54.
- Sturla SJ, Boobis AR, FitzGerald RE, Hoeng J, Kavlock RJ, Schirmer K, et al. 2014. Systems toxicology: from basic research to risk assessment. *Chem Res Toxicol* 27:314–29.
- Tejamaya M, Römer I, Merrifield RC, Lead JL. 2012. Stability of citrate, PVP and PEG coated silver nanoparticles in ecotoxicology media. *Environ Sci Technol* 46:7011–17.
- Thill A, Zeyons O, Spalla O, Chauvat F, Rose J, Auffan M, et al. 2006. Cytotoxicity of  $\text{CeO}_2$  nanoparticles for *Escherichia coli*. Physico-chemical insight of the cytotoxicity mechanism. *Environ Sci Technol* 40:6151–6.
- Tiede K, Hassellöv M, Breitbarth E, Chaudry Q, Boxall ABA. 2009. Considerations for environmental fate and ecotoxicity testing to support environmental risk assessments for engineered nanoparticles. *J Chromatogr A* 1216:503–9.
- Tsyusko OV, Unrine JM, Spurgeon D, Blalock BJ, Starnes D, Tseng M. 2012. Toxicogenomic responses of the model organism *Caenorhabditis elegans* to gold nanoparticles. *Environ Sci Technol* 46:4115–24.
- Van Hoecke K, Quik JTK, Mankiewicz-Boczek J, De Schampelaere KAC, Elsaesser A, Ven Der Meeren P, et al. 2009. Fate and effects of  $\text{CeO}_2$  nanoparticles in aquatic ecotoxicity tests. *Environ. Sci Technol* 43:4537–46.
- Volz DC, Belanger S, Embry M, Padilla S, Sanderson H, Schimer K, et al. 2011. Adverse outcome pathways during early fish development: a conceptual framework for identification of chemical screening and prioritization strategies. *Toxicol Sci* 123:349–58.
- Xia T, Kovochich M, Liong M, Mädler L, Gilbert B, Shi H, et al. 2008. Comparison of the mechanism of toxicity of zinc oxide and cerium oxide nanoparticles based on dissolution and oxidative stress properties. *ACS Nano* 10:2121–34.
- Zhang H, He X, Zhang Z, Zhang P, Li Y, Ma Y, et al. 2011. Nano- $\text{CeO}_2$  exhibits adverse effects at environmental relevant concentrations. *Environ Sci Technol* 45:3725–30.

Supplementary material available online  
Supplementary Information