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Physico-chemical behaviour and algal toxicity of nanoparticulate CeO$_2$ in freshwater

Nicola J. Rogers,$^{A,B,D}$ Natasha M. Franklin,$^{A}$ Simon C. Apte,$^B$ Graeme E. Batley,$^{A,B}$ Brad M. Angel,$^{A,B}$ Jamie R. Lead$^C$ and Mohammed Baalousha$^C$

$^A$Nanosafety Theme, CSIRO Future Manufacturing Flagship, Locked Bag 2007, Kirrawee, NSW 2232, Australia.
$^B$Centre for Environmental Contaminants Research, CSIRO Land and Water, Locked Bag 2007, Kirrawee, NSW 2232, Australia.
$^C$School of Geography, Earth and Environmental Sciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK.
$^D$Corresponding author. Email: nicola.rogers@csiro.au

Environmental context. It cannot be assumed that nanomaterials entering aquatic environments will have the same impacts on aquatic biota as their macroscopic particle equivalents. If their toxicities are different, this will have implications for the way in which nanomaterial usage is regulated. Algae, at the bottom of the food chain, are likely to be a sensitive indicator of toxic effects. Understanding the physical and chemical factors controlling nanoparticle toxicity to algae will assist in evaluating their ecological risk.

Abstract. In assessing the risks posed by nanomaterials in the environment, the overriding research challenges are to determine if nanomaterials are more toxic than the bulk forms of the same material, and the extent to which toxicity is governed by particle size and reactivity. In this study, the toxicity of nanoparticulate CeO$_2$ (nominally 10–20 nm) to the freshwater alga Pseudokirchneriella subcapitata was compared to the same material at the micron size (nominally <5 μm). Growth inhibition experiments revealed inhibitory concentration values, giving 50% reduction in algal growth rate after 72 h (IC$_{50}$), of 10.3 ± 1.7 and 66 ± 22 mg L$^{-1}$ for the nanoparticles and bulk materials respectively. Cells exposed to CeO$_2$ particles were permeable to the DNA-binding dye SYTOX Green in a concentration-dependent manner indicating damage to the cell membrane. Screening assays to assess the oxidative activity of the particles showed that the light illumination conditions used during standard assays are sufficient to stimulate photocatalytic activity of CeO$_2$ particles, causing the generation of hydroxyl radicals and peroxidation of a model plant fatty acid. No oxidative activity or lipid peroxidation was observed in the dark. These findings indicate that inhibitory mode of action of CeO$_2$ to P subcapitata is mediated by a cell-particle interaction causing membrane damage. The effect is most likely photochemically induced and is enhanced for the nanoparticulate form of the CeO$_2$.

Additional keywords: cerium oxide, microalgae, nanoparticle, photocatalytic activity.

Introduction

The growing use of manufactured nanomaterials in consumer products is raising questions as to whether nanosized materials should be regulated differently to macroscopic forms of the same compounds in terms of the risks they pose both to human and ecosystem health. To address this issue requires an understanding of the fate and toxicity of nanomaterials, together with appropriate measurement protocols to reliably evaluate nanoparticle behaviour in environmental matrices. The findings in this area to date have been summarised in several recent reviews$^{[1–3]}$; however, such is the progress that even recent reviews are quickly dating. At the same time, the Organisation for Economic Cooperation and Development (OECD), through its Working Party on Manufactured Nanomaterials (see http://www.oecd.org/document/8/0,3343, en_21571361_41212117_41226376_1_1_1_1,00.html, accessed 29 September 2009), is incorporating research findings into testing programs to underpin future risk assessments.

In aquatic systems, the most important control on nanomaterial impacts is the extent to which they retain nano size or lose it through aggregation. This has confounded the assessment of their toxicity to aquatic biota. So too has a failure to consider their solubility. Investigation of the toxicity of nanoparticulate zinc oxide has demonstrated that all of the observed toxicity could be explained by dissolved zinc resulting from nanoparticle dissolution, despite the fact that zinc oxide is considered to be ‘insoluble’ in water$^{[4–5]}$. To demonstrate nanoparticle toxicity required a material for which solubility was not likely to be an issue, but which showed measurable toxicity. Options included titanium dioxide, cerium oxide, bismuth oxide and silver. A screening of the solubility and toxicity of these compounds led to the choice of cerium oxide (CeO$_2$). The toxicity of cerium has been poorly studied. It can exist both as cerium(III) and cerium(IV) in solution; however, at the pH of natural waters (6–8), cerium(III) is hydrolysed relatively rapidly, which in the presence of oxygen will oxidise to...
cerium(IV), most likely as Ce(OH)$_2^+$.[6] The only algal toxicity data showed cerium(III) to have very low toxicity to Chlorella vulgaris,[7] whereas cerium(IV) showed moderate toxicity to the amphipod Hyalella azteca.[8] Nanoparticulate cerium oxide was shown to be not acutely toxic to the cladoceran Daphnia magna,[9,10] however, our initial screening showed toxicity to the freshwater alga Pseudokirchneriella subcapitata at mg L$^{-1}$ concentrations.

The choice of CeO$_2$ was reinforced by its wide potential usage, particularly as an additive to diesel fuels where it improves the combustion efficiency of engine carbon deposits, reducing particulate emissions and improving fuel efficiency.[9] It has also been shown to be an effective photocatalyst for water decomposition.[11]

This paper describes investigations of the toxic effects of commercially available nanoparticulate and micron-sized CeO$_2$ to the freshwater alga Pseudokirchneriella subcapitata. Extensive physico-chemical characterisation including particle size, shape, aggregation state, surface chemistry and chemical reactivity was used to elucidate possible mechanisms of particle toxicity and the extent to which nanosize represented an increased environmental risk.

**Experimental**

**Particle characterisation studies**

Uncoated cerium(IV) oxide nanopowder, having a nominal particle diameter specified as 10–20 nm and surface area of 80 m$^2$ g$^{-1}$, and macro-particulate cerium(IV) oxide powder (99.9%) with a diameter specified as <5 µm, were obtained from Sigma-Aldrich (St Louis, MO, USA). Concentrated suspensions of CeO$_2$ (20–100 mg L$^{-1}$) were prepared and characterised using dynamic light scattering (DLS), measurements of zeta-potential and point of zero charge, transmission electron microscopy (TEM), and equilibrium dialysis. Dynamic light scattering (DLS) and zeta-potential analyses of nanoparticle suspensions were obtained using a Malvern Instruments Zetasizer Nano ZS (4 mW He-Ne laser at 633 nm, with MPT-2 autotitratior, Malvern Instruments Ltd, Worcestershire, UK) at 25°C. Appropriate amounts of CeO$_2$ particles were added to a synthetic freshwater algid medium, identical to that used for all algal bioassays.[14] The low hardness and near-neutral pH (pH 6.5) of the medium was typical of natural freshwaters. All suspensions were initially sonicated for 30 min and vigorously shaken before analysis to break up visible clumps and resuspend any sedimented particles. Samples were immediately placed in clean disposable cuvettes and at least three consecutive measurements were performed at 25°C, with each consisting of six runs of 20-s duration. Initially unfiltered suspensions were used to identify the entire particle size distribution. Samples were subsequently filtered through a 0.45-µm membrane filter to remove large particles that were interfering with the analysis. Samples that did not pass the instrument's internal quality criteria were disregarded.

Brunauer–Emmett–Teller (BET) surface area measurements were determined by multi-point gas adsorption using a Micromeritics ASAP 2400 surface area analyser (Norcross, GA, USA). Nitrogen was used as the adsorbate at 77 K. Prior to analysis, samples were vacuum degassed, at 200°C, to an ultimate vacuum of <10 Pa.

**Particle chemistry**

High resolution TEM using an FEI Tecnai F20 Field Emission gun (FEG) provided direct visual information about particle size, shape and structure. The microscope operating conditions were as follows: an accelerating voltage of 200 keV, extraction voltage of 3800 eV, gun lens 2–3 (apparent size) and spot size 2–3. The aperture of the second condenser lens was nominally 50 µm and the objective aperture was nominally 40 µm. The spatial resolution was better than 0.24 nm point to point with 0.12-nm line resolution. Contrasted brightfield (CBF) was used for morphological and structural analysis.[12,13] TEM micrographs were collected on a Gatan TV camera. Digital Micrograph software was used to measure particle diameter and more than 100 particles or aggregates were measured for each sample. TEM samples were prepared by ultracentrifugation at 150 000 g at 25°C (30 000 rpm) using a Beckman ultracentrifuge (L7–65 Ultracentrifuge, Beckman Coulter (UK) Ltd, High Wycombe, UK) with a swing-out rotor SW40Ti.

Coupling the TEM with an X-ray energy dispersive spectrometer (X-EDS) (Oxford Instruments, Oxfordshire, UK) allowed determination of the chemical or elemental composition and purity of individual particles. Coupling to a parallel electron energy loss spectrometer (PEELS 666, Gatan, Inc., Pleasanton, CA, USA) enabled analysis of the local atomic structure of the specimen, regardless of the sample structure (crystalline or amorphous), and the electronic structure, bonding and nearest neighbour distribution of the atom in a given sample. This was used to provide information on the cerium oxidation state.

Ratios of cerium(III) to cerium(IV) were obtained from PEELS spectra[14] collected with a Gatan PEELS 666 and EL/P data acquisition system (Gatan Inc.). The energy spread of the incident electron beam (as measured by the full width at half maximum (FWHM) of the zero-loss peak) was ~1 eV. All spectra were collected in diffraction mode (i.e. image coupling to the spectrometer) and a selected area aperture of 10 µm was used to block non-collimated electrons to minimise sample degradation. The PEELS spectrum Ce M-edge was collected with an illumination angle 2α = 8.2 mrad, a collection angle 2β = 8.9, a 1.0-mm PEELS aperture and 0.2 eV per channel energy dispersion. Ceria particles are very beam sensitive, with reduction of Ce$^{IV}$ to Ce$^{III}$ with time through irradiation-induced reduction. A time series of PEELS spectra was collected every 3 s for 3 min. No irradiation damage was observed within the first 9 s, and therefore the collection time for these spectra was confined to 3 s to prevent any irradiation damage. The Ce M4–5 core-loss ionisation edges and the corresponding low-loss PEELS spectrum, including the zero loss peak were acquired consecutively from the same specimen region.

X-ray diffraction and TOPAS Reitveld refinement was used to perform profile analysis on the data to estimate crystallite dimensions from peak width. XRDP patterns were recorded with a PANalytical X’Pert Pro microprocessor-controlled diffractometer using Fe filtered Co Kα radiation, 1/4° divergence slit, 1/2° anti-scatter slit and X’Celerator fast Si strip detector. The diffraction patterns were recorded in steps of 0.016° 2θ with a 0.5-s counting time per step, and logged to data files on a PC for analysis using HighScore Plus and XPLOT.

**Chemical analyses**

Nanoparticle dissolution was assessed by dialysis[4] using membranes of 1000 Dalton molecular weight cut-off (~1-nm nominal pore size) filled with 10 mL of Milli-Q water and placed in an external test suspension containing either nanoparticulate or bulk CeO$_2$ particles (100 mg L$^{-1}$) in 0.01 M Ca(NO$_3$)$_2$ solution buffered to pH 6.5 with 2 mM PIPES (piperazine-N,N’-bis(ethanesulfonic acid); Sigma-Aldrich, St Louis, MO).
Dialysis cells were added to the test suspension and left to continually stir in a temperature- and light-controlled incubator under the same conditions used for the algal bioassays. To identify the influence of possible cerium trace impurities (e.g. cerium nitrate) from the CeO2 powders on the dialysed cerium fraction, additional samples of nano-CeO2 and bulk CeO2 were repeatedly washed with Milli-Q water, oven-dried, and new suspensions (100 mg L\(^{-1}\)) prepared and measured for dissolved cerium, in this case using 0.1-µm filterable cerium as a surrogate for dissolved (dialysed) cerium over the same sampling period.

Samples for dissolved cerium analysis were acidified (0.2%) with Tracepur HNO3 (Merck), and measured by inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7500 CE). The instrument was calibrated using a five-point cerium standard (blank, 12.5, 25.0, 50.0, 100 µg L\(^{-1}\)). The detection limit for dissolved cerium was 0.007 µg L\(^{-1}\).

The redox potentials of nanoparticle suspensions (100 mg L\(^{-1}\)) were measured using a WTW pH 320 m and SenTix® ORP platinum electrode with standard solutions.

**Algal bioassays**

The comparative toxicity of nanoparticulate and bulk CeO2 was assessed using *Pseudokirchneriella subcapitata* ATCC 22662 cultured axenically in US Environmental Protection Agency (EPA) medium on a 24-h light cycle (Philips TL 40W cool white fluorescent lighting, Danvers, MA, 70 µmol photons m\(^{-2}\) s\(^{-1}\) at 24°C.

Full details of the bioassay procedure have been described previously.[4] The test medium was the standard US EPA medium without ethylenediaminetetraacetic acid (EDTA), prefiltered through a 0.22-µm membrane filter. The medium had an alkalinity of 9 mg L\(^{-1}\) as CaCO\(_3\) and a water hardness of 15 mg L\(^{-1}\) as CaCO\(_3\). To minimise pH increases as a result of algal growth over 72 h, the medium was buffered with 2 mM PIPES to maintain the pH at 6.5 ± 0.1. Temperature and light conditions for the toxicity tests were identical to those used for culture maintenance. Tests solutions were continually shaken throughout the bioassay using a rotary shaker at 100 rpm.

Stock suspensions (0.1, 1, 5, 10 g L\(^{-1}\)) of nanoparticulate and bulk CeO2 (Sigma-Aldrich) were prepared in the buffered algal test medium at pH 6.5. All stock suspensions were sonicated for 30 min before spiking into the test vessels. Controls, together with at least five contaminant concentrations (each in triplicate) were prepared with total CeO2 concentrations ranging from 1 to 200 mg L\(^{-1}\).

Tests were undertaken in 30 mL borosilicate glass mini-vials using 6 mL test medium. Additional mini-vials were prepared at each concentration for pH measurement at the beginning and end of the test and for determination of dissolved (0.1-µm filterable) cerium. Turbidity controls were also included to account for possible effects of light attenuation on algal growth due to the presence of the particles. To physically separate the algae from the particles during toxicity testing, mini-vials containing cells in growth media but no particles were placed inside a larger vial containing either growth medium (control) or a suspension of the particles in growth medium.

Exponentially-growing cells were harvested by centrifuging (700g at 25°C, 7 min) and washed three times with the test medium. Each minivial was inoculated with a known concentration of pre-washed *P. subcapitata* cells to give the initial cell density of 2–4 \(\times 10^4\) cells mL\(^{-1}\). Cell counts were obtained using a four-colour BD-FACSCalibur™ (Becton Dickinson Biosciences, San Jose, CA) flow cytometer as previously described (OECD Working Party on Manufactured Nanomaterials website). IC\(_{50}\) values (i.e. the inhibitory concentration giving 50% reduction in algal growth rate after 72 h compared to the controls) were calculated using Linear Interpolation Method (ICP) analysis (ToxCalc Version 5.0.23C, Tidepool Software, McKinleyville, CA).

To assess membrane permeability, the DNA-binding dye SYTOX® Green (S7-020, Molecular Probes, USA) was used. A 25-µl aliquot of SYTOX® Green (1 µM) was added to 0.5 mL of algal cells to give a final dye concentration of 0.05 µM. The sample was well mixed and left to equilibrate in the dark for 5 min before flow cytometric analysis. Changes in fluorescence emission at 530 nm were measured using the BD-FACSCalibur™ flow cytometer (FL1 channel) and were compared to control cells to determine membrane damage by the toxicant. Two membrane integrity states were defined, with M1 indicating healthy control cells and M2 membrane permeabilisation. Shifts in the population from M1 into M2 were expressed as a percentage and used to identify membrane damage. Heat-killed cells, which had been boiled for 30 min, were used as a positive control showing a 100% shift from M1 to M2.

**Screening assays for reactive oxygen species**

A series of screening assays were developed to determine the potential for the CeO2 particles to produce reactive oxygen species (ROS). The general oxidative activity of the particles was evaluated using potassium iodide, where 5 mL of 0.25 M KI was added to 30 mL of CeO2 particles (1 g L\(^{-1}\)) in the algal growth medium. The solutions were placed in the algal incubator for 72 h under the same light regime used in the growth bioassays. A positive result for oxidative activity was recorded by the appearance of a yellow colour in solution, but the solution remained colourless in the absence of oxidative activity. The results for CeO2 particles were qualitatively evaluated against the activity of a suspension of titanium dioxide nanoparticles (1 g L\(^{-1}\)) due to its well documented photocatalytic oxidative activity.[15] The production of superoxide radicals by the particles was evaluated in the same way using nitroblue tetrazolium (NBT) (Sigma-Aldrich) as the indicator.[16] Final concentration ~60 µM. An endpoint colour change from yellow to purple indicated a positive result. Negative (no particles) and positive controls (1 mM H\(_2\)O\(_2\) for oxidative activity (KI assay) or 1 g L\(^{-1}\) TiO\(_2\) nanoparticles or both) were included with each screening assay. The production of hydroxyl radicals by CeO2 particles was measured using a coumarin assay.[17] A stock solution 0.5 mM coumarin (1,2-benzopyrone, Sigma-Aldrich) was prepared in algal growth medium ensuring that the reagent was fully dissolved. Cerium dioxide nano or bulk particles were added to achieve a final concentration of 1 g L\(^{-1}\). The resulting suspensions were thoroughly shaken and placed in the algal incubator for 72 h under the same light regime used in the growth bioassays. The particles were removed by centrifugation or 0.1-µm filtration. The fluorescence of the supernatant solutions was measured at an excitation wavelength of 328 nm, and an emission wavelength of 450 nm (5-nm bandwidth) on a Perkin-Elmer L S 55 Fluorescence Spectrometer. Negative (no particles) and positive (1 g L\(^{-1}\) TiO\(_2\) nanoparticles) controls were included in each assay.

Lipid peroxidation was measured with a thiobarbituric acid reactive substances (TBARS) assay using linoleic acid as a model plant fatty acid.[18] A stock solution of linoleic acid (0.1 M) was prepared in ethanol and kept refrigerated. Nanoparticle stock suspensions (5 g L\(^{-1}\)) were prepared and sonicated as described.
for the bioassay. Thiobarbituric acid (TBA) reagent was freshly prepared daily by adding 0.09 g TBA to 30 mL trichloroacetic acid (4%) and heating in a water bath at 45°C for 30 min. Test solutions (10 mL) contained 3 mM linoleic acid and 100 mg L⁻¹ particles in deionised water and were incubated either in the dark or in the algal growth cabinet using the same light conditions described for the algal bioassays. Following incubation the test suspensions were filtered (0.1 µm) to remove particles. The filtrate (1 mL) was added to TBA reagent (2 mL) and placed in a boiling water bath for 10 min for colour development. Absorbance values were measured at 515 nm on a Perkin Elmer Lambda 45 UV/VIS spectrometer. The method was calibrated using a series of 1,1,3,3-tetramethoxypropane standards (0–50 mmol L⁻¹).

Results and discussion

Particle characterisation studies

Characterisation of the CeO₂ particles (nanoparticulate and bulk) was performed using a combination of techniques in order to provide information on the particles’ chemistry, surface area, morphological shape and size distribution. TEM analysis (Fig. 1) illustrates the significant difference in particle size of the two cerium oxide powders studied. Despite both suspensions showing considerable aggregation in the algal test medium at pH 6.5, the primary particle size and resulting aggregates were substantially smaller for the nanopowder (Table 1) resulting in flocs ranging from ∼20 to 500 nm, compared to micron-sized for the bulk suspensions. DLS data confirmed that the nanoparticles (100 mg L⁻¹ suspensions) did not exist as single particles in the algal test medium at pH 6.5 and estimates of particle size distributions in unfiltered suspensions were not obtainable due to the poor correlation of the data. The mean nanoparticle size distribution generated by DLS after filtering the sample was ∼190 ± 30 nm. The CeO₂ particles (20 mg L⁻¹) carried a net negative charge in the algal test media with zeta potential measurements of approximately −13 to −18 mV and −18 to −25 at pH 6.5 for nanoparticulate and bulk CeO₂ respectively. Typically, a zeta potential between −30 mV and +30 mV indicates that the particles are

![Fig. 1. Transmission electron microscopy (TEM) images of nanoparticulate (a) and bulk CeO₂ (b) in PIPES-buffered algal growth medium (pH 6.5).](image)

Table 1. Physical and chemical properties of nanoparticulate and bulk CeO₂

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Nano CeO₂</th>
<th>Bulk CeO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size (nm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary particles (TEM)²</td>
<td>7–25</td>
<td>200–600</td>
</tr>
<tr>
<td>Aggregates (TEM)²</td>
<td>20–500</td>
<td>Micron-sized</td>
</tr>
<tr>
<td>Aggregates (DLS) unfiltered</td>
<td>Poor correlation data</td>
<td>n/d</td>
</tr>
<tr>
<td>Aggregates (DLS) 0.45-µm filtered⁵</td>
<td>190 ± 30</td>
<td>n/d</td>
</tr>
<tr>
<td>Morphology</td>
<td>Octahedral, truncated octahedral</td>
<td>Octahedral, truncated octahedral</td>
</tr>
<tr>
<td>Crystalline size (nm)</td>
<td>16 nm (~80%); 80 nm (~20%)</td>
<td>260 nm</td>
</tr>
<tr>
<td>Crystalline structure</td>
<td>Cerianite</td>
<td>Cerianite</td>
</tr>
<tr>
<td>BET surface area (m² g⁻¹)</td>
<td>75.8</td>
<td>3.1</td>
</tr>
<tr>
<td>Zeta potential (pH 6.5) (mV)</td>
<td>−13 to −18</td>
<td>−18 to −25</td>
</tr>
<tr>
<td>Solubility (µg Ce L⁻¹)</td>
<td>&lt;3</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Percent Ce³⁺ (NaNO₃)³</td>
<td>62 ± 29</td>
<td>0 ± 7</td>
</tr>
<tr>
<td>Percent Ce³⁺ (algal growth medium)³⁷</td>
<td>48 ± 32</td>
<td>n/d</td>
</tr>
<tr>
<td>Redox potential (Eh) (mV)</td>
<td>649</td>
<td>649</td>
</tr>
</tbody>
</table>

²Range of primary (individual) particle size of at least 100 particles.
³Range of aggregate size of at least 100 aggregates.
⁴Mean and standard deviation of 6 replicates.
⁵Mean and standard deviation of at least 10 particles.
unstable and will tend to aggregate. No point of zero charge (PZC) was observed in the algal test medium (pH 3.0–11.0) with the particles displaying a negative charge across the entire pH range whilst in 10 mM NaNO₃, the PZC was at approximately pH 9.0 and the nanoparticles displayed a positive charge at pH 6.5.  

The results of XRD analysis showed that both CeO₂ samples were composed of pure (>99%) cerianite (cubic CeO₂) with the bulk particles displaying sharp peaks indicating very crystalline, coarse crystallites, while the nanoparticles displayed broad peaks indicating a very fine crystallite size. Analysis of the peak profiles using TOPAS (Bruker) software indicated that the bulk sample contained crystallites with mean dimensions of ∼260 nm. In contrast, two distinct crystallite sizes were observed for the nanoparticle sample with the majority of the sample (∼80%) having a mean crystallite dimension of ∼16 nm, with the remainder (∼20%) having larger dimensions of ∼80 nm. Crystallite size does not necessarily equate with particle size since it is the mean size of the smallest diffraction domain within the crystal; however, as particle sizes get smaller, the particle size approaches the crystallite domain size.  

The BET surface areas of nanoparticulate and bulk CeO₂ were 75.8 and 3.1 m² g⁻¹ respectively in agreement with the particle sizes of the dispersed nanomaterial and bulk CeO₂.  

Dissolution of the nanoparticulate and bulk CeO₂ powders in algal growth medium at pH 6.5 was determined using equilibrium dialysis. Negligible solubility was observed for nano CeO₂ over 72 h with a dialysed cerium concentration of <3 µg L⁻¹. For bulk CeO₂, an initial spike in the dialysed cerium concentration of ∼90 µg L⁻¹ was observed at the first sampling (after 6 h), which gradually decreased in subsequent samplings to <10 µg L⁻¹ by 192 h. A further experiment using washed bulk CeO₂ powder again indicated negligible dialysable cerium (<3 µg L⁻¹).  

Parallel Electron energy loss spectrometry (PEELS) measures the energy loss as due to electrons interacting with the sample as they transmit through the specimen and therefore measures through the thickness of the particles to ∼0.5 µm. A comparison of the PEELS data for each of the CeO₂ samples showed that there was 62 ± 29% cerium(III) present in the nanoparticulates, but negligible amounts on the bulk particles (Table 1), which is generally within the previously reported range. Previous studies using X-ray photoelectron spectroscopy (XPS) and X-ray adsorption near edge spectroscopy (XANES), have shown that the Ce³⁺ : Ce⁴⁺ ratio increases as particle size decreases with cerium(III) content estimated at 1% (10 nm) and 6% (6 nm).¹¹⁹ In biological medium, the surface cerium(III) content of CeO₂ (7 nm) was estimated to be 21 ± 4% using XANES²²⁰ but other studies found no measurable cerium(III) on 14–30 nm particles in abiotic biological medium.¹⁰ So reduction to cerium(III) is clearly variable, depending on factors such as particle source and synthesis route, particle size, the environment in which the measurements are made, and also the measurement technique.¹¹⁹ The measurements give a good indication of the surface chemistry of the nanomaterial but the surface chemistry of the bulk material may be masked by the greater contribution of the core chemistry to the PEELS measurements. At least some of the ‘core material’ will not be involved with interactions with biological surfaces.

**Toxicity of nanoparticulate and bulk CeO₂ to P. subcapitata**

The results of toxicity testing of nanoparticulate and bulk CeO₂ using P. subcapitata are shown in Fig. 2 and Table 2. For nanoparticulate CeO₂, inhibition of algal growth rate occurred at a concentration of ∼1 mg L⁻¹, with a mean 72 h IC₅₀ value observed at ca. 10 mg L⁻¹. The bulk CeO₂ was ∼6.5 times less toxic than nano CeO₂ showing a shift in the concentration response curves to the right and an increase in the 72 h IC₅₀ to 66 mg L⁻¹. The IC₅₀ for dissolved cerium(III) was 0.63 mg L⁻¹. Given the very low solubility of CeO₂ reported above (<0.01 mg L⁻¹ dialysable cerium), it is clear that dissolved cerium is not responsible for the observed toxicity.
Chemistry and toxicity of particulate CeO₂ in freshwater

Table 2. Toxicity of nanoparticulate and bulk CeO₂ to P. subcapitata
IC₅₀, the inhibitory concentration values giving 50% reduction in algal growth rate after 72 h. n/a, not applicable

<table>
<thead>
<tr>
<th>Material</th>
<th>72-h IC₅₀ Mass (mg L⁻¹)</th>
<th>Surface area (m² L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CeO₂ (nano)A</td>
<td>10.3 ± 1.7</td>
<td>0.78 ± 0.13</td>
</tr>
<tr>
<td>CeO₂ (bulk)b</td>
<td>66 ± 22</td>
<td>0.20 ± 0.07</td>
</tr>
<tr>
<td>Ce(NO₃)₃</td>
<td>0.63 (0.60–0.65)</td>
<td>n/a</td>
</tr>
<tr>
<td>Pre-exposed CeO₂ (nano)c</td>
<td>&gt;50</td>
<td>–</td>
</tr>
<tr>
<td>Pre-exposed CeO₂ (bulk)c</td>
<td>&gt;100</td>
<td>–</td>
</tr>
</tbody>
</table>

AValues represent the mean ± s.d. of 3 separate bioassays. B95% confidence interval. CMedia exposed to particles for 48 h then filtered to remove CeO₂ before bioassay.

When toxicity is normalised to BET surface area, bulk CeO₂ is ∼4 times more toxic than the nanoparticles (Table 2). This contrasts with the findings of Van Hoecke et al. [10] who found that the different toxicities of CeO₂ nanoparticles within a narrow size range (14, 20, 29 nm) could be explained by the difference in surface areas. The appropriateness of this normalisation to our data is, however, questionable since the particles represent a much wider size range and we know that there is extensive aggregation of nanoparticles in solution. The surface area available for interaction with aggregated nanoparticles will likely be much lower than that estimated on the basis of gas adsorption to dry particles.

Potential mechanisms for enhanced toxicity of CeO₂ nanoparticles

Our results clearly show enhanced toxicity of nanoparticulate CeO₂ compared to micron-sized material on a mass basis (t-test, P > 0.05), despite the extensive particle aggregation observed in suspension. To understand these effects further, and in order to make future predictions about the potential toxicity of new nanomaterials, it is important to understand the mechanism(s) underlying these observed differences in toxicity. There are several different mechanisms by which particulate toxicants might inhibit algal growth (cell division) rate. Indirect, or artefactual, inhibition occurs when the toxicant interacts with the environment of the cell, for example by sequestering nutrients or changing pH or redox potential in the external milieu. Direct inhibition involves interaction of the toxicant with the cell itself and can be either chemical, such as inhibition of metabolism, membrane transport processes or mitosis, or physical, for example mechanical damage to the cell membrane. A combination of these pathways may even be present. The production of reactive oxygen species by nanoparticles would cause chemically induced toxicity but the propensity to generate ROS is a consequence of the physical structure and reactive surface chemistry of very small particles. It was also important to determine if the same mechanism(s) was responsible for the toxicity of both forms of the material and just potentiated by the nano form, or if two different mechanisms were involved.

Indirect inhibition (artefacts)

Redox conditions can affect the toxicity of nanomaterials [21]. Suspensions containing bulk and nanoparticulate CeO₂ in algal growth medium had similar redox potentials; however, this does not necessarily reflect differences in surface redox activity.

Table 3. Removal of micronutrients from test solutions by CeO₂ particles after 48 h
P medium was exposed to particles for 72 h before phosphorus analysis

<table>
<thead>
<tr>
<th>Particle</th>
<th>Concentration (mg L⁻¹)</th>
<th>Micronutrient concentration (percentage of control medium)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P Mn Fe Co Zn Mo</td>
<td></td>
</tr>
<tr>
<td>Nano</td>
<td>5 87 95 16 97 97 11</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>63 93 7 68 103 &lt;10</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>3 89 19 93 128 &lt;10</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>6 – – – – –</td>
<td></td>
</tr>
<tr>
<td>Bulk</td>
<td>10 96 95 &lt;1 87 99 44</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>67 96 &lt;1 90 101 34</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>41 85 &lt;1 86 100 7</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>8 – – – – –</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. Complexation of phosphorus by in the algal growth medium by cerium oxide particles (72-h exposure) (a), and 72-h growth of P. subcapitata with decreasing growth medium phosphate concentration (b). A, no added nitrate.

Potential toxicity artefacts due to solution turbidity causing shading of the algal cells were eliminated based on results from turbidity controls included with each toxicity experiment. These showed no effect on algal growth rate, indicating that particles in suspension did not influence light intensity received by the cells (data not shown).

Toxicity differences could be caused by depletion of phosphate reaching the algal cell surface due to adsorption to the particle surfaces. Suspensions of CeO₂ were prepared in algal growth medium and duplicate sub-samples were filtered through a 0.1-μm filter at 2 h and 72 h. The filtrate was acidified (0.2% HNO₃) and the phosphorus concentration measured by
Algal growth medium was exposed to increasing concentrations of nanoparticles. Algal growth bioassays were then performed using media which had been exposed to nanoparticles for 48 h prior to filtration. None of the nanoparticles used in this study showed a significant effect on algal growth rate, while a 95% reduction in phosphate concentration in the growth medium did not significantly affect algal growth rate, while a 50% reduction in phosphate concentration in the growth medium did not significantly affect algal growth rate, while a 95% reduction in medium phosphate concentration resulted in a 50% reduction in algal growth rate. In the total absence of phosphate (with and without added nitrate), the cells still maintained over 50% of control growth.

Chemical inhibition

The chemical factors most likely to play a role in the enhanced toxicity of nanoparticulate CeO$_2$ include the redox chemistry of cerium in the particles, and the potential to generate reactive oxygen species (ROS) both of which may induce oxidative stress and cell toxicity. Reduction of cerium(IV) on the surface of CeO$_2$ nanoparticles, linked to cytotoxic effects mediated by oxidative stress, has been observed during contact between CeO$_2$ nanoparticles and bacteria$^{[27]}$ and mammalian cells$^{[20,28]}$ but these studies did not consider cellular interactions with bulk CeO$_2$. In this study there clearly was some interaction between bulk CeO$_2$ and the algal cells in order for there to be an inhibitory response. As discussed earlier, measurements using PEELS in the absence of the algae showed significantly higher (t-test, $P < 0.05$) percentages of trivalent cerium present in the nanoparticles compared to the bulk material used in this study. The surface chemistry alone is therefore unable to explain the enhanced toxicity of the nanoparticles as a greater proportion of nanomaterial was already reduced at the start of the toxicity experiments.

Toxicity may, however, be related to the surface chemistry and the fraction of the particle surface that is available to interact with the algal cell. Pseudokirchneriella subcapitata has a cell surface area of $\sim 70 \mu$m$^2$ or $7.0 \times 10^3$ nm$^2$ $^{[29]}$. The primary particles are $\sim 15$-nm diameter and $\sim 3 \times 10^5$ particles could potentially fit onto the surface of the cell. For the bulk particles ($\sim 400$ nm) only $\sim 4 \times 10^5$ particles would fit onto the surface of an alga, $\sim 700$ times fewer particles than the nanomaterial. The particles are, however, aggregated in algal growth medium, with

### Table 4. Membrane damage to *P. subcapitata* exposed to CeO$_2$ measured by SYTOX® Green binding

<table>
<thead>
<tr>
<th>Test solution</th>
<th>Concentration (mg L$^{-1}$)</th>
<th>Growth (percentage of control)</th>
<th>Percentage of cells in M2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>100</td>
<td>4 ± 1.2</td>
</tr>
<tr>
<td>Heat-killed cells</td>
<td>0</td>
<td>NA</td>
<td>100 ± n/d</td>
</tr>
<tr>
<td>Nano CeO$_2$</td>
<td>1</td>
<td>70</td>
<td>8 ± 3.6</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>64</td>
<td>13 ± 5.8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>51</td>
<td>14 ± 4.4</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>23</td>
<td>47 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>15</td>
<td>50 ± 1.8</td>
</tr>
<tr>
<td>Bulk CeO$_2$</td>
<td>5</td>
<td>100</td>
<td>9 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>100</td>
<td>9 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>78</td>
<td>20 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>65</td>
<td>42 ± 10</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>46</td>
<td>37 ± 13</td>
</tr>
</tbody>
</table>

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aggregate sizes of ~200 nm and micron sized, for the nano and bulk materials respectively, allowing 1.8 \times 10^3 nano and 70 bulk particles (25 times fewer) to fit around the cell. These calculations are approximations and only indicative of relative toxicity, as TEM and light microscopy images did not indicate that the cells were uniformly covered by the particles. Furthermore, only a fraction of the surface of the particles would be in contact, and available to interact with the algal cell. The nanoparticles are still not as toxic as expected on the basis of particle packing around the cell but nevertheless this is a better predictor of the relative toxicities of the nano and bulk forms of the material. If oxidative stress, via reduction of Ce^{IV} at or near the cell membrane, is the dominant mechanism, then the nanoparticles with a much greater surface interaction with the cell membrane would be most toxic on a mass basis, and the bulk particles with a high Ce^{IV} content may be relatively more toxic than predicted from calculations of the biologically available surface area. If so, a combination of specific surface area and Ce^{IV}:Ce^{III} ratio (i.e. total Ce^{IV} on the available surface) could be the relevant parameter for toxicity.

There is some inconsistency in the literature on the biological effects of nanoparticulate CeO$_2$ as to whether it acts as a free radical scavenger and antioxidant\cite{30} or causes oxidative stress via the production of reactive oxygen species (ROS)\cite{20,28}. A series of simple chemical tests were therefore developed for

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
\textbf{Assay} & \textbf{Assay} & \textbf{Assay} & \textbf{FU} \\
\hline
\textbf{Potassium iodide (KI): general oxidative activity (130 h)} & \textbf{Nitroblue tetrazolium (NBT): superoxide production (130 h)} & \textbf{Coumarin: hydroxyl radical production (96 h)} & \\
\hline
\textbf{Qualitative} & \textbf{Qualitative} & \textbf{Qualitative} & \\
\textbf{Algal incubator} & \textbf{Algal incubator} & \textbf{Algal incubator} & \\
\textbf{Dark} & \textbf{Dark} & \textbf{Dark} & \\
\hline
Control & – & – & – & n/d & 13 & 13 \\
1 mM H$_2$O$_2$ & +++ & n/d & n/d & n/d & 13 & 13 \\
Bulk TiO$_2$ & – & – & – & n/d & 13 & 13 \\
Nano TiO$_2$ (anatase) & +++ & – & – & n/d & 13 & 13 \\
Nano TiO$_2$ (rutile) & n/d & n/d & n/d & n/d & 13 & 13 \\
Bulk CeO$_2$ & – & – & – & n/d & 13 & 13 \\
Nano CeO$_2$ & +++ & – & – & n/d & 13 & 13 \\
\hline
\end{tabular}
\caption{Summary of oxidative activity of CeO$_2$ particles compared with TiO$_2$ controls}
\end{table}
Fig. 5. Peroxidation of linoleic acid by CeO$_2$ particles measured by thiobarbituric acid reactive substances (TBARS) assay. Photoactivation of lipid peroxidation by CeO$_2$ nanoparticles (24 h) under light conditions used in the algal bioassay (a), and TBARS produced at 24 and 48 h compared with a TiO$_2$ positive control under light conditions used in the algal bioassay (b).

Use as a screening procedure for assessing particle oxidative activity. These comprised the qualitative assessment of oxidative activity, and superoxide radical production and measurements of hydroxyl radical production. Titanium dioxide in the presence of light was used as a positive control due to its well documented oxidative activity.\textsuperscript{15,16} The results of these screening assays are summarised in Table 5. Under the conditions used, oxidative activity of the CeO$_2$ particles was observed in the potassium iodide assay with the activity of the nanoparticles apparently greater than the bulk although both appeared less active than the TiO$_2$ particles or hydrogen peroxide positive controls. No detectable superoxide anion production was observed for CeO$_2$ particles compared to TiO$_2$ particles. However, hydroxyl radicals were also produced by both nano and bulk CeO$_2$ particles, but again both were less active than TiO$_2$ particles. Oxidative activity and hydroxyl radical production was not observed in the dark, indicating the photocatalytic nature of these reactions. The photocatalytic activity of cerium dioxide in bulk and nanoparticulate form is well established with several studies showing that the materials are able to absorb UV light and generate a range of reaction products including hydroxyl radicals.\textsuperscript{11,31,32} Our study demonstrates that the light illumination conditions used during standard algal bioassays are sufficient to stimulate photocatalytic activity and cause the generation of hydroxyl radicals.

If ROS production, or redox activity, is a mechanism of CeO$_2$ toxicity then, due to the extremely short half-lives these
species, there must also be close proximity between the algae and the CeO$_2$ particles. Cerium dioxide nanoparticles have previously been observed to adsorb to bacterial cells$^{[20]}$ and to cluster around the surface of algal cells$^{[10]}$. TiO$_2$ is more photoactive under the bioassay illumination conditions but less inhibitory to the growth of P. subcapitata (IC$_{50} > 1000$ mg L$^{-1}$) than CeO$_2$. TiO$_2$ nanoparticles have previously been shown to closely associate with algal cells$^{[5]}$, so either a different particle-cell interaction is present in our system or oxidative stress is not an important mechanism of action. Alternatively, the algal interaction or catalytic mechanism of the CeO$_2$ particles is very different from that of TiO$_2$; or the concentrated particle suspension (1000 mg L$^{-1}$) used in the screening assays is not representative of the activity of the particles in the less concentrated suspensions (<100 mg L$^{-1}$) used for the algal bioassays.

The flow cytometric data indicate that nano CeO$_2$ increases membrane permeability more than bulk CeO$_2$, an observation which could be explained by an oxidative stress mechanism. Although the aggregated particles are not likely to be getting into the algal cell, damage to membrane lipids (peroxidation) could initiate a surface disruption. Linoleic acid, a common component of plant lipids, was used as a model compound to investigate the potential for lipid peroxidation without the complication of cell-particle interactions. Fig. 5 shows the peroxidation of linoleic acid by CeO$_2$ particles (100 mg L$^{-1}$) over 24 and 48 h measured using a thiobarbituric acid reactive substances (TBARS) assay. Again nano TiO$_2$ was used as a positive control for the assay. All the particles produced a positive TBARS response compared to the control under the light conditions used for the algal bioassay with significant effects (t-test, P < 0.05) for the TiO$_2$ and nanoparticulate CeO$_2$ (Fig. 5a). The difference in magnitude of the responses between TiO$_2$ and CeO$_2$ was much less in the TBARS assay (~1.5 times) than seen in the coumarin assay for hydroxyl radicals (~6 times), which suggests that not all of the hydroxyl radicals generated by the TiO$_2$ are available for peroxidation reactions in this model system. No significant TBARS were produced by CeO$_2$ nanoparticles in the dark compared to the control in 24 h (Fig. 5b). This photocatalytic effect appeared to be much less for the bulk particles where no significant difference in TBARS production was observed between the light and dark exposures.

Thus the inhibitory mode of action of CeO$_2$ on the growth of P. subcapitata appears to be mediated by a cell-particle interaction causing membrane damage. The effect is enhanced for the nanoparticle form of the material and is most likely photochymically induced.

Conclusions
The data presented here indicate that the toxicity of cerium oxide particles may be mediated by several factors including particle size, surface area, phosphate complexation, surface chemistry and chemical reactivity. Nanoparticulate CeO$_2$ was found to be more toxic than the bulk form of the material on a mass basis. The IC$_{50}$ value for both materials was in the mg L$^{-1}$ concentration range. This is several orders of magnitude above CeO$_2$ concentrations currently predicted in the environment from its use as a diesel additive$^{[33]}$ but such concentrations might occur during a localised pollution event such as a fuel spillage. Nevertheless, in analysing our findings, the overriding question was to address the question of whether size is an issue in nanoparticle toxicity. If the observed toxicity of CeO$_2$ is a peculiarity of cerium, its surface chemistry and reactivity, then the question of whether size matters remains unresolved. The published data on the in vitro (eco)toxicological effects are diverse and at times conflicting, suggesting that, for a reactive material like CeO$_2$, toxicity may be dependant on several factors including particle size, specific surface area and bioavailable surface area, source and synthesis route leading to differences in surface reactivity and chemistry and the exposure medium. A generalised predictor of size-related toxicity is therefore not likely, and compound specific studies will be required.

Oxidative stress has previously been proposed as a mechanism for nanoparticulate CeO$_2$ toxicity.$^{[20,27,28]}$ What is clear from our data, however, is that production of hydroxyl radicals and initiation of lipid peroxidation are photocatalytic. It is also possible that all the oxidative activity of CeO$_2$ particles (assessed by the KI assay) is mediated by hydroxyl radical production as the positive results for the KI assay were also light induced. These results strongly suggest that light exposure may be required to promote the toxic effects of CeO$_2$. Unfortunately, growth inhibition of algae (photoautotrophs) is not a useful model to test this hypothesis due to the absolute requirement for light as an energy source, and further studies with heterotrophs are required. It is difficult to determine if and how the surface chemistry of the particles might interact with the photoactivation to promote oxidative stress and cellular toxicity, but a systematic study using CeO$_2$ particles with varied size and surface characteristics could address this question.

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References


