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Research Paper

Toxicity of cerium oxide nanoparticles to the earthworm *Eisenia fetida*: subtle effects

Elma Lahive,[A,D] *Kerstin Jurkschat,*[B] *Benjamin J. Shaw,*[C] *Richard D. Handy,*[C] *David J. Spurgeon*[A] *and Claus Svendsen*[A]

[A]NERC Centre for Ecology and Hydrology, Maclean Building, Benson Lane, Crowmarsh Gifford, Wallingford, Oxfordshire, OX10 8BB, UK.
[B]Department of Materials, Oxford University, Begbroke Science Park, Sandy Lane, Yarnton, Oxford, OX5 1PF, UK.
[C]Ecotoxicology Research and Innovation Centre, Plymouth University, Drake Circus, Plymouth PL4 8AA, UK.
[D]Corresponding author. Email: elmhiv@ceh.ac.uk

Environmental context. This study investigates the toxicity of cerium oxide nanoparticles to earthworms, key organisms in soil ecosystems. Cerium oxide did not affect survival or reproduction of the earthworms but did exert histological changes. We conclude that current soil guidelines, based simply on metal toxicity, appear to adequately protect against cerium exposure risk, at least for earthworms.

Abstract. The toxicity of cerium oxide (CeO_2) nanoparticles (NPs) in soils is largely unknown. This study aimed to investigate the toxicity of three different CeO_2 NPs to the earthworm, *Eisenia fetida*, for effects on survival (at day 28) and reproduction (at day 56), as well as bioaccumulation and histopathological effects. *Eisenia fetida* were exposed in standard Lufa 2.2 soil to three CeO_2 NPs of different size ranges (5–80 nm), one larger particle (300 nm) and a cerium salt (ammonium cerium nitrate) over an exposure range from 41–10 000 mg Ce kg^{-1}. Survival and reproduction were not affected by the four CeO_2 particles, even at the highest exposure concentration tested. Alternatively, 10 000 mg Ce kg^{-1} cerium salt affected survival and reproduction; Median lethal concentration (LC_{50}) and effective concentration (EC_{50}) values were 317.8 and 294.6 mg Ce kg^{-1}. Despite a lack of toxic effect from the different forms of CeO_2 particles, there was a dose-dependent increase in cerium in the organisms at all exposure concentrations, and for all material types. Earthworms exposed to CeO_2 particles had higher concentrations of total cerium compared to those exposed to ionic cerium, but without exhibiting the same toxic effect. Histological observations in earthworms exposed to the particulate forms of CeO_2 did, however, show cuticle loss from the body wall and some loss of gut epithelium integrity. The data suggest that that CeO_2 NPs do not affect survival or reproduction in *E. fetida* over the standard test period. However, there were histological changes that could indicate possible deleterious effects over longer-term exposures.

Additional keyword: histopathology.

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Introduction

The use of nanomaterials has rapidly increased in the last decade with an estimated 1628 consumer products containing nanomaterials in October 2013 (http://www.nanotechproject.org/cpi/about/analysis/). Consequently, it seems likely that nanomaterials are already entering the environment at low micrograms or nanograms per litre concentrations,[1] although the precise quantities and pathways of release of many of these materials are not currently well defined.[2] Such releases are a concern because adverse effects on aquatic and terrestrial organisms have been documented for several types of nanomaterials.[3–8]

Cerium oxide nanoparticles (CeO_2 NPs) are most commonly used in diesel fuels as a combustion catalyst, but are also found in a wide range of consumer, pharmaceutical and agricultural products.[9] CeO_2 NPs have a route to reach waste water treatment plants (WWTP) through domestic and storm water. In WWTPs, most CeO_2 NPs are deposited in the sludge.[10] Hence, one of the main fates and release pathways of NPs has been suggested to occur through the application of biosolids to land as is prevalent in some countries.[1] This application creates a further direct exposure route for NPs to terrestrial ecosystems.

The majority of CeO_2 NP toxicity studies conducted to date have been carried out in aquatic systems, focussing mainly on algae and daphnids.[11–15] Also compared to terrestrial studies, aquatic tests have investigated a greater range of phenotypic responses to CeO_2 exposure such as swimming impairment, DNA damage and mortality in *Daphnia*.[11,16] Terrestrial studies have, for the most part, centred on accumulation and effects on plants[17–19] and on soil microbes.[20–22] Data on the toxicity of CeO_2 NPs to terrestrial invertebrates are sparse, with no earthworm data currently available to date. Of studies conducted to date, work on the nematode *Caenorhabditis elegans* have demonstrated physiological disturbances related with *cyp35a2* gene expression[23] and decreased survival with

exposure to CeO_2 NPs (4, 15 and 45 nm) at concentrations up to 100 mg Ce L^{-1} for 24 h.[23,24] Furthermore, at more environmentally relevant concentrations (1–100 nM), exposure to CeO_2 NPs (8.5 nm) was found to induce reactive oxygen species (ROS) accumulation and oxidative damage in *C. elegans* after 3 days of exposure.[25] Cerium accumulation in the tissue often accompanies observed toxic effects of CeO_2 NPs,[12,24,26] but not always,[14] suggesting a complex link between NP accumulation and toxicity. The role of nanoparticle size and shape in influencing toxic effects has also been investigated in several studies.[23,27–29] For example, in the case of CeO_2 NPs, smaller particle sizes (15 nm) exerted a greater effect on *C. elegans*' survival and reproduction than a larger size (45 nm).[23] For bacteria, silver NPs under 5 nm resulted in greater inhibition of nitrification than larger particles.[28] To date, however, the few studies carried out using earthworms have found little evidence of size-dependant toxic effects,[27,29,30] and so far none of these studies have focussed on CeO_2 materials.

Earthworms are in intimate contact with the soil and are integral to organic matter turnover.[31,32] Hence this taxon has a high potential for exposure to NPs deposited to soil, including sewage sludge. At present, the effect of CeO_2 on earthworms is currently unclear. To address this issue, this study investigates the toxicity of CeO_2 particles of varying size and shape to the earthworm *Eisenia fetida* using the Organisation for Economic Co-operation and Development (OECD) standard test protocol to assess survival and reproduction.[33] In addition, metal accumulation and histological changes are measured to enable some understanding of the relationship between tissue accumulation and biological effects. For comparison, a non-nanoparticle CeO_2 (100–300 nm) and a metal salt are also included in the experimental design; the latter being important because guidelines for protecting soils from metals are currently based on total metal concentrations, and the bioavailability of any dissolved fraction.[34] This has also allowed us to address hypotheses concerning the effects of material size on toxicity, such as whether smaller size material may elicit effects at lower concentrations.

Materials and methods

Test medium

The test soil used was Lufa 2.2 (LUFA Speyer, Germany). This soil was used from a batch supplied that had a pH of 5.5 ± 0.2 (mean $\pm$ s.d.) as measured in a 0.01 M $CaCl_2$ and 2.5 : 1 soil slurry mixture, an organic carbon content measured by furnace combustion at 550 °C of 1.76 ± 0.26 w/w %, a measured cation exchange capacity of 10.2 ± 0.5 meq 100 g^{-1} and a water holding capacity (WHC) of 55 %. In preparation for use in the test, the soil was air-dried and sieved to <2 mm. An amount of 500 g dry weight of soil, held in a $183 \times 120 \times 70$-mm^3 polypropylene container, was used for each test replicate for all treatment levels.

Experimental animals

Eisenia fetida were obtained from a commercial source (Blades Biological, Kent, UK). These were maintained in culture soil constituting 33 % loamy soil, 33 % peat and 33 % bark on a volume basis. Cultures were kept in a controlled temperature room at 20 ± 1 °C in a 12 : 12 h light : dark cycle. Earthworm cultures were fed fresh horse manure free from contamination or medication. Earthworms used in the tests were maintained for eight weeks before the test to ensure that they were adults of a suitable size (300–600 mg) for testing (OECD[33]).

Chemicals

Three cerium oxide ($Ce^{IV}O_2$) NPs ranging from 5 to 80 nm; and one larger size (non-NP) CeO_2 particle (>100 nm) were selected for testing (Table 1). In all cases, the materials were not surface functionalised and, hence, represent a range of size variants of the simplest form of bare CeO_2 NPs. Three of the selected materials are being studied as part of a wider testing program being conducted by the OECD Working Group on Manufactured Nanomaterials and have specific codes assigned to the different materials (NM-211, NM-212 and NM-213 for CeO_2 (10–50 nm), CeO_2 (10–80 nm) and CeO_2 (100–300 nm)). As part of this project, these nanomaterials have been subjected to extensive characterisation of the pristine material. Information on the particles is provided in Table 1, listing their OECD reference number, form of production, as well as the physicochemical characteristics of each nanomaterial as provided by the European Commission's Joint Research Centre (JRC) (Ispra, Italy). For more information see NIA (see http://www.nanotechia.org/activities/prospect-ecotoxicology-test-protocols-representative-nanomaterials-support-oecd, accessed January 2014). The fourth CeO_2 particle, Envirox (CeO_2 5–20 nm), is the CeO_2 NP on which the diesel fuel combustion catalyst Envirox is based (NanoTrade, Mozartova, Czech Republic) (Table 1). All materials were delivered as a dry powder. To confirm the fidelity of the batches of material received from the main-held supply batch held at the JRCe (Ispra, Italy) and NanoTrade (Czech Republic), additional analyses were conducted to establish the particle size distribution for the material in water dispersions using both dynamic light scattering (DLS) and visualisation of primary particle size by transmission electron

Table 1. Test substances and primary particle characterisation provided by the suppliers European Commission's Joint Research Centre, Ispra, Italy (www.nanohub.eu, accessed 10 April 2014) and Envirox CeO_2 from NanoTrade s.r.o., Mozartova, Czech Republic

Primary particle size was determined by X-ray diffraction (XRD), transmission electron microscopy (TEM), or dynamic light scattering (DLS). SSA, specific surface area; determined by the method of Brunauer–Emmett–Teller. OECD, Organisation for Economic Co-operation and Development. The Ce : O ratio of all samples was determined by X-ray photoelectron spectroscopy to be 1 : 2

Particle	Source or OECD reference number	Surface chemistry, form of production, physical state	Purity (%)	Primary particle size (Scherrer, nm), particle shape	SSA (m^2 g^{-1})
CeO_2	Envirox	Uncoated, produced by precipitation, yellowish powder	99.5	10, spherical	40
CeO_2	NM-212	Uncoated, produced by precipitation, yellowish powder	>99.5	33, cubic	28
CeO_2	NM-211	Uncoated, produced by precipitation, yellowish powder	>95	10.3, cubic	66
CeO_2	NM-213	Uncoated, produced by precipitation, yellowish powder	>99	241, irregular cubic	3.5

microscopy (TEM). Samples were dispersed in de-ionised water, sonicated for 30 s in a low power ultrasonic bath (Ultrawave Ltd, Bath, UK) and a drop of this dispersion was deposited on a perforated carbon-coated Cu TEM grid and dried at room temperature for several hours before examination by TEM. Experiments were carried out on a JEOL 2010 analytical TEM, which has a Laboratory$_6$ electron gun and can be operated between 80 and 200 kV. For the ionic reference exposures, the cerium salt ammonium cerium(IV) nitrate ($(NH_4)_2Ce^{IV}(NO_3)_6$) (Sigma Aldrich, UK) with a purity >98.5 % was used.

Experimental design and dosing

The toxicity test procedure followed the *OECD Guideline 222* (earthworm reproduction test for *Eisenia fetida* and *E. andrei*).[33] Exposure concentrations for both nano and non-nano CeO_2 particulate forms and the cerium salt materials were 41, 102, 256, 640, 1600, 4000 and 10 000 mg Ce kg^{-1} (dry weight, DW, soil). Each replicate container held 500 g of soil with ten worms. There were three replicate containers per treatment concentration. All exposures were run concurrently and hence effect could be benchmarked against a universal control treatment for the experiment. This comprised ten separate replicates of Lufa 2.2 soil without amendment of any form of cerium.

All CeO_2 particles were dosed into the soils as dry powder. Initially the amount of powder required to dose the three replicates of each concentration was added to 50 g of soil and mixed thoroughly by hand. This aliquot was then added to the remaining test soil and again thoroughly mixed to maximise the evenness of distribution of the materials. The dosed soil (500 g) was then used for each treatment replicate. This procedure was repeated for all cerium particles and all concentrations. To dose the cerium metal salt, a stock solution of ammonium cerium(IV) nitrate (nominal concentration 38.46 mg Ce mL^{-1}) was added to the soils. This concentration was chosen for the stock solution so the maximum concentration, 10 000 mg Ce kg^{-1}, could be attained in the soil. After addition of the ionic cerium stock solution, the appropriate volume of MilliQ water was added to the soil to raise the moisture content to 56 % of the WHC. After dosing of each replicate, soils were then left for one week to equilibrate before the test organisms were introduced. It is known that addition of some forms of metal to soil for toxicity testing can lead to changes in soil properties that can increase the complexity of the results interpretation[35–37]; in particular changes in soil pH can occur.[36] To assess whether such changes were relevant, pH was measured both at the start of the exposure and after 28 days in a standard water : soil (2.5 : 1) slurry mix using a pH electrode (Sartorius Professional Meter PP25, Sartorius AG, Goettingen, Germany; combination pH probe, filled with 3 M KCl).

Toxicity test procedure

To initiate the experiment, ten adult, fully clitellated earthworms (average weight (10 worms) = 5.12 ± 0.42 g, Mean ± s.d., $n = 115$) were added to each replicate container. Earthworms were selected from the larger stock culture, washed with de-ionised water to remove adhering soil and blotted dry. The ten earthworms were then weighed as a batch before being put onto the surface of the soil of the relevant test container. As food, 10 g dry weight of horse manure wetted to 80 % WHC was added to the soil surface in each container, and the total container weight recorded. All containers were placed in a controlled temperature room (temperature and photoperiod as above) for a total of 56 days. Over the duration of the test, soils were corrected for moisture loss every two weeks and additional water added as needed to maintain a consistent soil moisture level.

After 14 days, the containers were sorted and the numbers of earthworms alive in each counted. Retrieved earthworms were washed, blotted dry and weighed as a batch. The earthworms were then placed back into the respective container; an additional 10 g of food added and the containers were then returned the controlled temperature room for a second 14 days of exposure. At 28 days the earthworms were again sorted from the soil and weighed. These earthworms were retained for subsequent chemical and histological analysis (see below).

After the removal of the adult earthworms at 28 days, the soils were returned to the controlled temperature room for a further 28 days to allow juveniles to hatch from laid cocoons. At the end of this period, the containers were placed in a water bath (Clifton Nickel-Electro Ltd., Weston-super-Mare, UK) at 60 °C for 15 min. This heating forces juveniles to the soil surface where they can be picked off and counted. From this number and the counts of surviving adult earthworms at 14 and 28 days, reproduction was expressed as a juvenile production rate (juveniles per earthworm per week).

Total cerium concentrations in earthworms

Three adult earthworms retrieved after 28 days were selected at random from each replicate container and were kept for 24 h on moist filter paper to allow them to void their gut content. After washing, these purged earthworms were frozen at −20 °C until total metal concentrations could be measured using the method of Shaw et al.[38] for metallic nanomaterials. For the analysis, the earthworms were individually freeze-dried, weighed and then digested at 70 °C in concentrated (65 %) HNO_3 for 1 h. Following cooling, the samples were diluted with ultrapure water (Elga, 18.2 MΩ cm) and Triton X-100 (to give a final concentration of 2 % Triton X-100) according to Shaw et al.[38] The digests were analysed within 24 h for Ce, Ca, Cu, Fe, K, Mg, Mn, Na and Zn by inductively coupled plasma–optical emission spectrometry (ICP-OES, Varian 725-ES, Melbourne, Australia), calibrated using plasma emission grade solutions. Immediately before analysis by ICP-OES, samples were sonicated for 15 min, followed by vortexing for 5 s and manual shaking (followed by inverting three times) to ensure samples were well mixed.[38] Spiked samples were used for the validation of the analyses. Samples spiked with certified cerium salts showed the expected complete recovery (99.4 ± 1.6 % (mean ± s.d., $n = 11$ spikes) at cerium concentrations of 1–10 mg L^{-1}. However, the procedural recovery of total metal from metal digests spiked with 1 mg L^{-1} particulate cerium was generally less with recoveries estimated at (mean ± s.d., $n = 3$ samples): 52.6 ± 8.0, 92.1 ± 1.5, 43.5 ± 0.86 and 70.5 ± 5.2 % for NM-213 CeO_2 (100–300 nm), NM-212 CeO_2 (10–80 nm), NM-211 CeO_2 (10–50 nm) and Envirox CeO_2 (5–20 nm). All total cerium concentrations were expressed as micrograms of cerium per gram of dry weight.

Histology

To assess responses to exposure at a more detailed physiological level, histological observations were made on earthworms from the highest CeO_2 particle exposures (10 000 mg Ce kg^{-1}) and from the lowest ionic cerium exposures (41 mg Ce kg^{-1}). Observations were made on six earthworms from each treatment

(two earthworms from each replicate). Earthworms were carefully collected after gut purging, rinsed in deionised water and anesthetised in carbonated water. The anterior section of the earthworm was cut a few segments below the clitellum using a surgical blade and was placed in neutral formalin buffer to preserve the samples. The anterior segments next to the clitellum, and segments of clitellum, were processed into wax blocks, and transverse sections (7-µm sections) were cut from each animal. Slides were stained with Mallory's trichrome for study under an Olympus Vanox-T microscope with an attached Olympus digital camera (C-2020 Z). All tissues were prepared simultaneously in batches containing tissues from control animals and the treatments in order to eliminate differences in fixation or staining artefacts between treatments.

Data analysis

Concentration specific effects on the proportion of survival and reproduction (juvenile production rates) were analysed for each of the separate cerium types using one-way analysis of variance (ANOVA). Where differences were found, Tukey's multiple comparison tests was used to determine differences between specific treatment concentrations. Median lethal concentration (LC_{50}) values for survival were calculated using probit analysis. To estimate response parameters, reproduction data were used to fit a three parameter log logistic model (Eqn 1) to obtain estimate median effective concentration (EC_{50}) values.

$$y = \frac{y_{max}}{1 + \exp\{b \times (\log(x) - \log(e))\}} \quad (1)$$

where y_{max} is the upper asymptote, e is the concentration resulting in a 50 % effect on the measured endpoint (EC_{50}) and b the slope parameter. Cerium concentrations showed a non-normal variance structure so data were log-transformed. Comparisons of (log-transformed) total cerium concentration data across all particle types and concentrations was carried out using a generalised linear model (GLM) with a post-hoc Tukey ($P < 0.05$). To include ionic cerium data, an additional GLM analysis was performed to compare total cerium concentrations using only the lowest three concentrations (i.e. concentrations where there was survival of ionic cerium-exposed earthworms to be measured) for the analysis. The pH values across the ionic cerium concentration range were also examined using a one-way ANOVA with a post-hoc Tukey test ($P < 0.05$) and pH values from control soils with those spiked with 10 000 mg Ce kg^{-1} CeO_2 particles compared by ANOVA.

Results

Material characterisations

Characterisation of the different CeO_2 NP and non-NP forms identified the different size ranges of the various NPs (Table 1). These size differences are related to variation in the measured specific surface area. The measurements made for the material batches used for this study largely confirmed the analysis of the larger batch studied (OECD Working Group on Manufactured Nanomaterials, European Commission's JRC (Ispra, Italy) (Fig. 1). Thus, by TEM all NPs were dominantly cubic in shape, with the exception of NM-212 CeO_2 (10–80 nm), which was more angular (Fig. 1). Size ranges were in agreement with the initial batch information (Table 1). NM-211, NM-212 and NM-213 had size ranges 10–50, 10–80 and 100–300 nm (Fig. 1). Envirox CeO_2 had a particle size range of 5–20 nm. In de-ionised water, DLS measurement of dispersions (Zetasizer, Malvern Insruments Ltd, Malvern, UK) indicated the substantial potential of the non-functionalised NPs to aggregate.

Soil cerium concentrations and pH

Soil pH was measured in all of the cerium salt-spiked soils and in the top concentrations of the CeO_2 NP-spiked soils. No significant differences ($P > 0.05$) were found between the pH in the control soil (pH 6.02 ± 0.13, $n = 7$) and the highest concentration in the CeO_2 particle-spiked soils. All had pH values in the range from 5.96 to 6.19. In clear contrast, there was a significant decrease in pH resulting from increasing cerium salt concentrations in the spiked medium ($P < 0.05$) (Table 2). Soil pH decreased from the control value to pH 2.9 ± 0.15 at the highest cerium concentration (Table 2). This represents a change of over three orders of H^+ concentration.

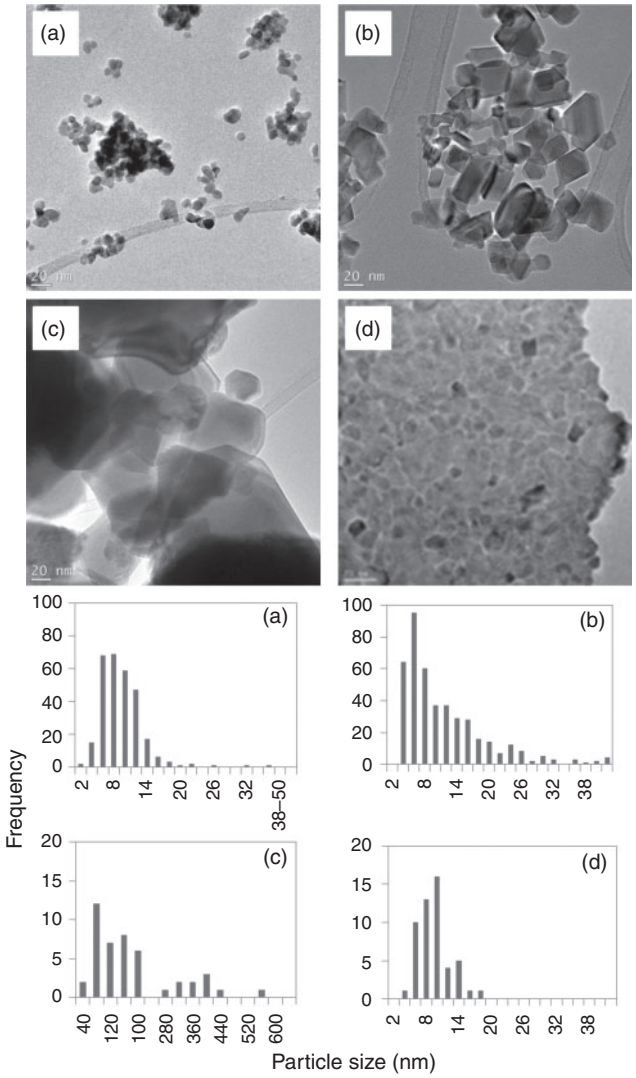

Fig. 1. Transmission electron microscopy (TEM) images and histograms of size distributions of the CeO_2 particles. One milligram per litre of the powder was dispersed in de-ionised water, sonicated for 30 sand one drop of the dispersion was deposited on a carbon coated Cu TEM grid. (a) NM-211 CeO_2 (10–50 nm), (b) NM-212 CeO_2 (10–80 nm), (c) NM-213 CeO_2 (100–300 nm) and (d) Envirox CeO_2 (5–20 nm). All particles are spherical or cubic with the exception of NM-212 CeO_2 (10–80 nm), which has a more angular shape.

Survival

The four CeO_2 particulate forms had no effect on earthworm survival over the 28 day period of exposure. Indeed, there was 100 % survival at all concentrations for all four materials. Statistical analysis, including estimation of 28-day LC_{50} values, was not therefore feasible for these tests. In contrast there was a clear concentration-related decrease in survival of earthworms exposed to the cerium salt (Fig. 2). No survival was recorded in the top four cerium concentrations and there was 50–100 % survival at the two lower concentration treatments. Based on these data a 28-day LC_{50} of 317.82 ± 314.7 mg Ce kg^{-1} could be calculated (Fig. 2).

Reproduction

Reproduction in the control treatments was 3.1 ± 1.15 juveniles per earthworm per week. This value is above the validation criteria set for the test as defined by the OECD (2004). Similar to survival, reproduction was not affected by exposure to any of the four CeO_2 particles. There was no significant difference between the numbers of juveniles per earthworm per week produced in all of the CeO_2 NP exposures compared to the control ($P > 0.05$). As exposure to even the highest CeO_2 concentration, 10 000 mg Ce kg^{-1}, did not result in any significant reproduction effects compared to the control, no 28-day EC_{50} values could be calculated. For the cerium salt exposure, there was a clear concentration-dependent effect on reproduction (Fig. 2). The calculated 28-day EC_{50} was 294.6 ± 72.095 mg Ce kg^{-1}.

Table 2. The pH of the soils with increasing cerium concentration in soils dosed with ammonium cerium nitrate

Ce concentration in soil (mg Ce kg^{-1})	pH (measured in water)
0	6.02 ± 0.131
41	5.94 ± 0.244
102	6.04 ± 0.269
256	5.54 ± 0.131
640	5.26 ± 0.128
1600	4.22 ± 0.107
4000	3.42 ± 0.123
10 000	2.90 ± 0.153

Total cerium concentrations in earthworms

To determine if cerium was being internalised by the earthworms, total cerium concentrations were measured in purged individuals that had been exposed for 28 days to the different forms of cerium. There was a clear increase of total cerium in the earthworms for all types of exposures. Furthermore, there was a significant increase in total cerium concentrations in the earthworms with increasing cerium exposure level for all the cerium particles and the cerium salt (one-way ANOVA, $P < 0.05$ in all cases) (Fig. 3). Significant differences were found between the total cerium concentrations in earthworms exposed to the various CeO_2 particles and the ionic metal (GLM, $P < 0.05$). A one-way ANOVA comparing total concentrations in earthworms exposed to 256 mg Ce kg^{-1} indicated a significant difference between all cerium forms (one-way ANOVA, $P < 0.01$). Thus, there was higher total cerium concentrations measured in earthworms exposed to cerium salt than in the earthworms exposed to all CeO_2 particulates. Mortality precluded tissue metal analysis at higher cerium concentrations. Earthworms exposed to CeO_2 particles at the highest test concentrations had total cerium concentrations that were equal to or even exceeded the highest concentrations found in the cerium salt exposed earthworms. This excess cerium was, however, not linked to toxic effects on survival or reproduction. A comparison of the total cerium concentrations in earthworms exposed to the four CeO_2 particle types found significant differences between the particles. Tukey post-hoc analysis showed that earthworms exposed to non-nanoscale NM-213 CeO_2 (100–300 nm) had significantly higher cerium concentrations than earthworms exposed to NM-211 CeO_2 (10–50 nm) (GLM, $P < 0.05$). No significant differences were found between measured cerium concentrations in the earthworms of either Envirox CeO_2 (5–20 nm) or NM-212 CeO_2 (10–80 nm) nanomaterials (GLM, $P > 0.05$). No significant differences were found in the other electrolytes measured in the earthworms with increasing cerium concentration for any of the CeO_2 particles or the cerium salt (one-way ANOVAs, $P > 0.05$ for all cerium forms). For example, the concentration of Na in the control worms was 4.71 ± 0.523 µg Na g^{-1}. The concentrations of Na in earthworms exposed to the highest cerium concentration of NM-213 CeO_2 (100–300 nm), NM212 CeO_2 (10–80 nm), NM-211 CeO_2 (10–50 nm) and Envirox CeO_2 (5–20 nm) were 4.82, 4.41, 4.56 and 4.45 µg g^{-1}.

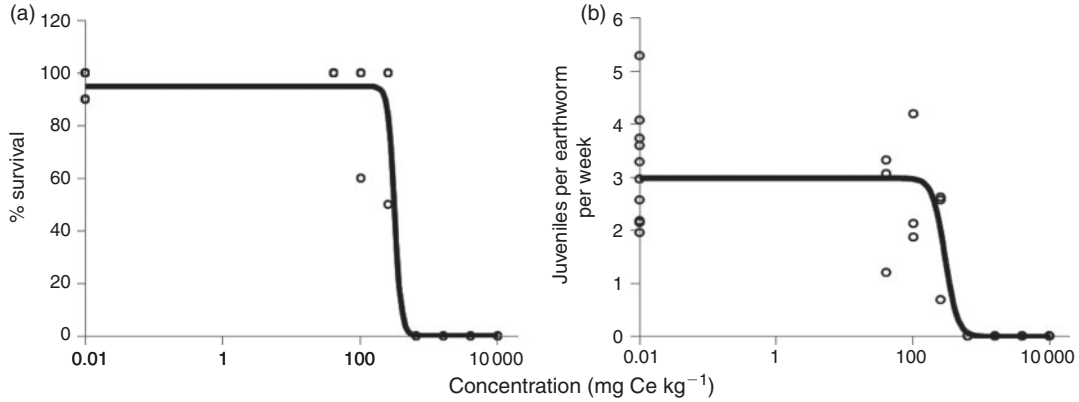

Fig. 2. Dose–response curves for *Eisena fetida* exposed to ammonium cerium nitrate for 28 days. (a) Survival (28-day median lethal concentration (LC_{50}) = 294.6 mg Ce kg^{-1}). (b) Reproduction (28-day median effective concentration (EC_{50}) = 317.18 mg Ce kg^{-1}). Black circles indicate survival or juvenile production in each individual replicate container and black line indicates the model from with EC_{50} was calculated.

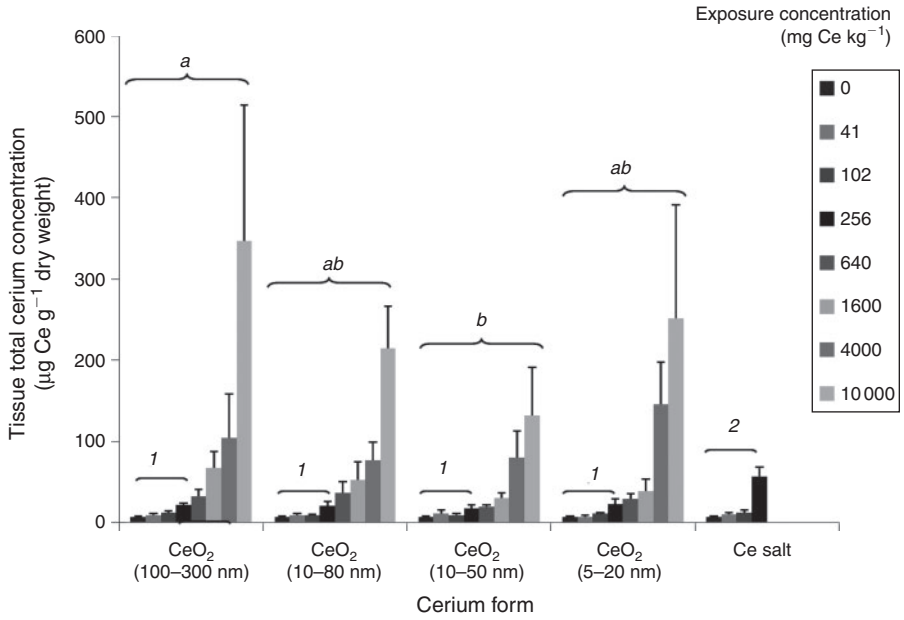

Fig. 3. The total cerium concentration in depurated earthworms exposed to particulate CeO_2 and cerium salt for 28 days. Different letters denote significant differences in total cerium concentrations in the CeO_2 particulate exposures across all the cerium exposure concentrations (i.e. 0–10 000 mg Ce kg^{-1}) (generalised linear model (GLM), $P < 0.05$). Different italic numbers denote significant differences in total cerium concentrations in exposures to all cerium forms across the lowest four concentrations (0, 41, 102 and 256 mg Ce kg^{-1}) (GLM, $P < 0.05$). Mean ± s.e., $n = 9$.

Table 3. Summary of the histological observations on the worms exposed to all cerium treatments
For the CeO_2 particle exposures worms exposed to the highest concentration (10 000 mg Ce kg^{-1}) were examined whereas worms with the lowest exposure concentration (41 mg Ce kg^{-1}) were examined for the cerium salt

Ce source	Primary particle size (nm)	Epidermis or body wall	Clitellum	Gut
CeO_2	5–20	Epidermis: normal, some hyperplasia Circular muscle: normal Longitudinal muscle: normal	Normal	Normal
CeO_2	10–50	Epidermis: some cuticle damage or hyperplasia Circular muscle: fibrosis Longitudinal muscle: slightly diffuse	Some erosion, mainly normal	Normal
CeO_2	10–80	Epidermis: some cuticle damage or hyperplasia Circular muscle: erosion Longitudinal muscle: slightly diffuse	Diffuse architecture of the fatty parenchyma	Loss of epithelium integrity or normal
CeO_2	100–300	Epidermis: cuticle loss, mucocyte proliferation Circular muscle: intact, normal Longitudinal muscle: intact or slightly diffuse	Diffuse architecture of the fatty parenchyma	Loss of epithelium integrity or normal
Ce salt	–	Epidermis: erosion, hyperplasia Circular muscle: architecture loss Longitudinal muscle: eosinophilic deposits, erosion	Normal	Normal

Histological observations

Histological observations were made to assess the condition of the earthworms exposed to the highest concentrations of the NPs (10 000 mg Ce kg^{-1}), as well as earthworms exposed to the lowest ionic cerium concentration, 41 mg Ce kg^{-1} and the control soil-incubated worms (Table 3). Sections of the body wall, clitellum and gut were assessed for structural integrity. Overall, control earthworms showed generally normal histology for all sections assessed. This was not, however the case for earthworms exposed to the cerium forms (Fig. 4). The body wall of segments anterior to the clitellum in earthworms from all the cerium treatments showed evidence of histological change compared to the unexposed controls. The extent of damage did, however, vary with the type of cerium treatment. Thus, the epidermal layers of earthworms exposed to the non-nanoscale NM-213 CeO_2 (100–300 nm) treatment had lost the cuticle (5/5 earthworms examined). Although the underlying epidermis was generally normal, in 2/5 earthworms examined, foci of mucocyte proliferation in the epidermis was also observed (Fig. 4). Epidermal layers of earthworms exposed to NM-212 CeO_2 (10–80 nm) were relatively normal for 3/6 earthworms examined, with also limited damage to the cuticle. Two

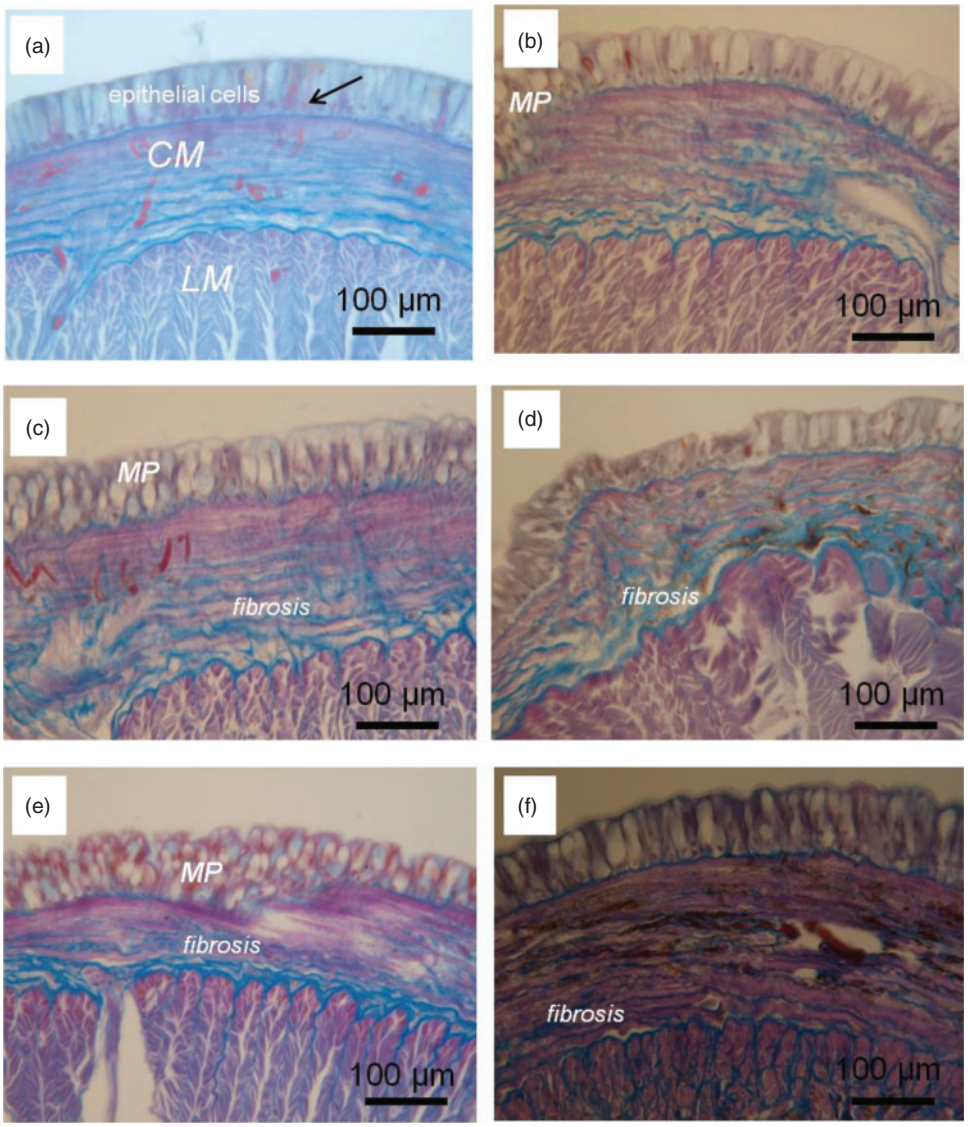

Fig. 4. Transverse sections of segments from the anterior region of earthworms exposed to CeO_2 particles (10 000 mg Ce kg^{-1}) and cerium salt (41 mg Ce kg^{-1}): (a) Control, (b) CeO_2 (100–300 nm), (c) NM-212 CeO_2 (10–80 nm), (d) NM-211 CeO_2 (10–50 nm), (e) Envirox CeO_2 (5–20 nm) and (f) cerium salt. Controls show normal morphology of the epidermis and underlying muscles (CM, circular muscle, LM, longitudinal muscle), but with some granular lipofuscin-like material in some specimens. Note the normal vesicular activity of the columnar cells (black arrow). Note the erosion of the epithelium in the cerium treatments, often with mucocyte proliferation in the epidermis (MC), fibrosis and diffuse loss of architecture in the circular muscle. Magnification ×400.

earthworms examined showed areas of hyperplasia (mainly mucocyte proliferation) in the epidermis and most of the animals (5/6) showed some erosion and fibrosis of the circular muscle, although longitudinal muscles were similar to the controls. Earthworms exposed to NM-211 CeO_2 (10–50 nm) showed a similar histology to those from the NM-212 CeO_2 (10–80 nm) group, with 2/6 earthworms showing normal epidermal cells (albeit with broken or missing cuticle), 3/6 earthworms showed foci of hyperplasia in the epidermis. Fibrosis in parts of the circular muscle were also observed (4/6 earthworms), although the longitudinal muscle was normal (Table 3). Histological changes in Envirox CeO_2 (5–20 nm) treated earthworms were generally less than for the other particles. Three from six animals showed foci of hyperplasia in otherwise normal epidermis (although the cuticle was broken in places on all earthworms), and two animals showed normal histology of the epidermis similar to the controls. Circular and longitudinal muscle showed limited injury in some individuals (Fig. 4). Animals exposed to cerium salt showed erosion of the epidermis (3/6 animals), areas of hyperplasia (3/6) and mild loss of circular muscle architecture. The circular muscle showed a mild loss of architecture with fibrosis (3/6 animals). One animal showed eosinophilic deposits around the margins of the longitudinal muscle, consistent with erosion of connective tissue matrix.

Histology of the clitellum segments was also examined for the same cerium materials. Control earthworm clitellum tissues showed a normal histology with a pseudostratified epithelium with soft parenchyma underneath, and normal circular and longitudinal muscle. The non-nanoscale NM-213 CeO_2 (100–300 nm) and NM-212 CeO_2 (10–80 nm) exposed earthworms frequently showed clitellum thickening akin to the control earthworms. However, some of these earthworms (two

from CeO_2 (100–300 nm) and three from NM-212 CeO_2 (10–80 nm)) showed some diffuse architecture of the fatty parenchyma. NM-211 CeO_2 (10–50 nm) earthworms were also largely normal, but there were some observations of epidermal erosion with foci of hyperplasia (2/6 animals), although these had areas of normal epidermis. The animals from the cerium salt exposure largely had normal clitellum tissue, with 3/6 animals showing a (healthy) thickening of the parenchyma (Table 3).

The gut epithelium was observable in the anterior region or clitellum of most earthworms. Only the earthworms from the non-nanoscale NM-213 CeO_2 (100–300 nm) and NM-212 CeO_2 (10–80 nm) exposures showed some loss of gut epithelium integrity in the anterior segments (3/3 and 2/4 specimens examined respectively) (Table 3). In the Envirox CeO_2 (5–20 nm) and CeO_2 (10–50 nm) treatments, earthworms showed normal gut integrity, although NM-211 CeO_2 (10–50 nm) earthworms did show a very slight diffuse nature and one with vacuole formation in the mucosa. Overall gut integrity of the NM-211 CeO_2 (10–50 nm) exposed earthworms was closer to that of the controls than that of the non-nanoscale NM-213 CeO_2 (100–300 nm) and NM-212 CeO_2 (10–80 nm) exposed individuals. Earthworms from the cerium salt exposure showed normal gut conditions, with only one specimen showing erosion of the epithelium compared to the controls.

Discussion

Cerium toxicity to earthworms and relation to existing toxicity data

This study is the first to assess the toxicity of a cerium salt, CeO_2 NP and non-NP materials in earthworms. All four particulate forms of CeO_2 did not reduce either survival or reproduction in *Eisenia fetida* over the test period, even at high concentrations where cerium accounted for 1 % of the total soil weight. This apparently low toxicity of the NP and non-NP forms occurred even though there was clear evidence of an increase in total cerium concentration in the earthworms; and an associated mild to moderate pathology (Figs 3, 4). This suggests that the worms were able to withstand the exposures and the resulting adverse effects at the organism level did not affect overall life-cycle traits. In contrast, earthworms exposed to the cerium salt showed reductions in survival and reproduction (Fig. 2). These effects occurred even when total cerium concentrations in earthworms were lower than found for the particulate forms. This suggests that ionic cerium toxicity is greater than that of the CeO_2 particulate forms at the same soil exposure concentration.

At the current time there are reasonably little toxicity data available in the literature for cerium and cerium oxide particles, particularly for terrestrial invertebrates. Indeed, there are currently no toxicity data available for earthworms. Therefore, comparing these results with existing data is difficult. The terrestrial studies in the literature that have been conducted to date have predominantly focussed on plants. Similar to our findings, these studies have so far found only limited evidence of toxicity although, as for this earthworm study, most have seen uptake and accumulation of particles.[17–19,39] Comparing the toxicity of CeO_2 NPs to earthworms with other types of metal or metal oxide nanomaterials indicates a very low toxicity compared to materials such as zinc and silver NPs.[27,29,30] Thus, EC_{50} values between 750 and 2874 mg kg^{-1} and 8.7 mg kg^{-1} have been respectively reported for zinc and silver NPs.[27,30,40] These values are all at least an order of magnitude lower than the putative toxicity values for CeO_2 NPs, which are $>10\,000$ mg Ce kg^{-1}, given that exposure to these concentrations did not result in a significant effect on either survival or reproduction. Overall these comparisons suggest a low hazard associated with particulate CeO_2 exposure.

Particle size is tentatively suggested as a determinant of toxic effect in some organisms.[23,41] In fact, smaller-sized CeO_2 NPs were found to be more toxic to *C. elegans* than larger particles.[23] In the present study, however, size did not determine the toxic effect of CeO_2 particulate forms to earthworms, at least for survival or reproduction. There was also no significant size-effect on apparent total cerium concentration in earthworms (Fig. 3). The absence of a size-specific effect on toxicity is broadly in agreement with data for other metal NP toxicity in earthworms to date, where zinc and silver NPs of varying size did not show differing effects on reproduction.[27,29,30]

Cerium salt toxicity

In contrast to the particulate forms of cerium, earthworms exposed to the cerium salt showed significant reduction in survival and reproduction (Fig. 2). The greater toxicity of the salt compared to NPs is in general agreement with observations for other metal and metal oxide NPs in earthworm studies relating to short-term incubation and exposure.[29,30,40,42] The effects of the cerium salt were apparently concentration-related, although soil measurements also indicated potential confounding effects of lowered pH, likely resulting from the ammonium cerium nitrate addition. Choosing an appropriate cerium salt as an ionic reference in cerium toxicity studies is challenging. Ammonium cerium nitrate was chosen primarily because of its high water solubility, which allows an aqueous stock solution to be made for spiking the soil. However, use of this salt also introduces ammonium and nitrate into the soil. Nitrates are known to be toxic to earthworms[43] and high application rates of ammonium nitrate are harmful to earthworms.[44] However, increases in nitrate associated with the addition of the cerium salt to the soil are relatively small and not enough to cause direct toxic effects. The fall in pH associated with the increased cerium concentration, will also have contributed to the stress observed in the cerium salt-exposed worms (Fig. 2, Table 2). Previous studies of *E. fetida* have shown negative effects on reproduction when the soil pH is less than 5 and reduced survival at pH 4 and below.[45] Thus, at the two highest concentrations tested, the pH reduction caused by salt addition would almost certainly be sufficient to directly affect earthworm survival. At lower cerium concentrations (640 mg Ce kg^{-1} and below), however, the pH is still above 5 and within 0.8 pH units of the control so a direct effect of pH, at least on survival, is less likely here (Table 2). A decrease in soil pH will increase the release of cerium ions from spiked soil,[46] thus increasing bioavailability to organisms. However, in accordance with the biotic ligand model, low pH is characterised by a high concentration of H^+ ions, which will result in competition between metal ions and H^+ in soil solution for binding to key organism receptors.[47] The data suggest, therefore, that cerium salt toxicity is likely to be a result of a combined effect of the cerium ion, the counter ion and the effect of ammonium on soil pH. In contrast, the presence of NPs had minimal effect on soil pH even at the highest cerium concentration used; hence, a pH effect was not relevant for these forms.

Total cerium concentration in earthworms

Although no overt toxicity was seen for survival and reproduction, there was a concentration dependant increase of total

cerium metal in or on the earthworms. Uptake and accumulation of cerium in earthworms is quite poorly documented, and there appears to be no comparable studies in the literature on earthworms for either ionic or (nano)particulate cerium. The total body concentrations measured in earthworms from control exposures in this study (~7 mg Ce kg^{-1}) were close to those reported for wheat (~10–20 mg Ce kg^{-1})[48] although slightly lower than those reported for fish liver (~30 mg Ce kg^{-1}).[49] The measured residues of cerium in earthworms were relatively low when compared to those of other metals such as zinc or cadmium at equivalent doses[30,50] suggesting a lower bioaccumulation hazard associated with cerium compared to other metals.

The trace metal analysis used involved digesting tissue samples from depurated earthworms and determining total metal concentrations by ICP-OES. This technique cannot differentiate whether the metal was accumulated as dissolved metal or intact particles (attempts at single particle ICP-MS on tissues were unsuccessful, data not shown). In the CeO_2 particle exposures, the measured total cerium concentrations in the earthworms could be accounted for by 1–5 mg of soil remaining in the gut after depuration. In quantitative terms, this represents earthworms possibly failing to clear the final 1–4 % of their total gut content during depuration. Thus, for the CeO_2 particle exposures, it is not possible to state whether the measured cerium is internalised cerium, based on the ICP-OES data. This was also true for the two lowest cerium salt exposures (41 and 102 mg Ce kg^{-1}). However, at the cerium salt concentration where toxicity was observed (256 mg Ce kg^{-1}), there would have to be up to 7 % retention of the total gut soil content to explain the measured cerium concentrations in the earthworms, which would have been visible and was not observed. It is clear therefore that cerium from the cerium salt exposures is accumulated into the tissues and suggests that the cerium in the cerium salt was more bioavailable or bioaccessible to the earthworms than the cerium in the CeO_2 NPs. However, the obvious histological effects observed in the CeO_2 NP exposures indicate there are effects that, given the low or non-existent general tissue uptake, may result from either cerium particle abrasion of, or local ingression into, the earthworms' tissues. Although the low recovery of cerium in spiked samples (43–90 %) increases the variation and reduces the certainty of the measurements of total cerium in the earthworms for the CeO_2 particle exposures, there were significant differences found between cerium measured in the earthworms from the different particle exposures (Fig. 2). It is possible that if this variation in recovery was reduced, further differences between the particles could have been found. However, this does not alter the overall conclusions of this study. Tissue concentrations of the other electrolytes measured in the earthworms did not vary with increasing cerium concentration, which excludes toxic effects due to electrolyte leakage or dehydration.

Toxicity of metal and metal oxide NPs is often attributed to the dissolution of the particles into ions that then exert the observed toxic effects.[27,51] CeO_2 NP dissolution is very low,[9] so in the present study, toxicity from cerium ions in the CeO_2 NP exposures is doubtful. Furthermore, there was no strong and consistent size determinant in total cerium measured in the earthworms between the forms of CeO_2 particles. The largest particles did accumulate to higher total metal concentrations compared to some, although not all, of the smaller particles (Fig. 3). The absence of a clear size-related accumulation is similar to other studies where size did not appear to determine the extent of cerium accumulation in an organism and indicates that concentration is a more important factor.[27,30]

Sub-lethal effect of cerium

Although measurement of the major life-cycle traits showed no effect of the CeO_2 exposures, histological observations on the exposed earthworms showed clear damage to the body wall; as well as more subtle effects in gut tissues and possibly the clitellum. The histological changes observed here (Fig. 4) have also been seen in earthworms exposed to C_{60} and silver NPs.[52,53] The epidermis of earthworms is an important barrier to the external environment. In aquatic systems, membrane disruption and cellular damage have been reported in algae and cyanobacteria following external exposure.[13–15] If the epithelium is damaged, metals, particles or other chemicals in the soil may simply enter into the organism without endocytosis.

In this study, in the case of the particulate forms of CeO_2, total cerium concentration is linked with damage to the body wall but with little or no injury to the gut or clitellum (Table 3). Smaller metal or metal oxide particles sometimes show greater toxicity than larger ones.[13,14,23] However, as the smallest particles used (5–20 nm) showed relatively little damage to the body wall, gut or epidermis, this size dependence is not supported by this study. Physical effects of the particles, such as erosion of the epithelium, as opposed to accumulation in the tissues can also play a role in particle toxic effects.[14] In the present study, effect of particles of various sizes and shapes were investigated, including a quite angular NM-212 CeO_2 (10–80 nm) particle (Fig. 1) ,which could potentially mechanically damage epidermal tissues. However, more significantly, erosion of the circular muscle was observed along with damage to the cuticle of the epidermis (Table 3, Fig. 4). That both the circular muscle and longitudinal muscles are also affected in some cases suggest that it is unlikely that shape-related mechanical injury is the sole driving force behind tissue damage. Instead, other mechanisms, such as ROS related damage, may also be important.[25] The clitellum sections of all cerium exposed earthworms were much less affected than the epidermis or body wall. This is not surprising given there were no effects on reproduction for any of the particle exposures. However, some of the subtle damage seen could lead to more long-term reproductive effects.

Conclusion

In this study the toxicity of cerium salt was observed for key life-cycle traits, however, no such effects were seen even for high concentration exposures to different CeO_2 nanoparticles. In all exposures cerium concentrations in the earthworms increased with increasing dose, however, for the NPs this was not associated with any overt toxicity. If the toxic affect is assessed by the results of the standard toxicity protocol then cerium NPs pose little hazard to earthworms, even at levels 100 000 times above predicted environmental concentrations.[54] However, histological observations do suggest some effect, related to either tissue erosion or tissue injury, when exposed to CeO_2 particles that give some caution in interpreting these results to suggest that there would be no long-term effect of cerium oxide exposure for earthworms. For this reason, further work on the physiological changes associated with long-term CeO_2 exposure may be necessary. Indeed such studies may be particularly relevant because of the potential for CeO_2 nanomaterials to persist in the environment as a result of their low reactivity and

dissolution. Given that the metal salt is more toxic than the cerium particulates, current soil guidelines based simply on metal toxicity at present would appear to adequately protect against cerium exposure risk, at least for earthworms. This is independent of form, for uncoated NPs in the size range studied, provided current usage is not greatly increased above existing levels.

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