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Schymura, S.; Rybkin, I.; Uygan, S. S. S.; Drev, S.; Podlipec, R.; Rijavec, T.; Mansel, A.;
Lapanje, A.; Franke, K.; Strok, M.;

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ARTICLE

Dissolution-based uptake of CeO₂ nanoparticles by freshwater shrimp – A dual-radiolabelling study of the fate of anthropogenic cerium in water organisms

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Stefan Schymura,^{*a} Iaroslav Rybkin,^{a,b} Seniha Simale Su Uygan,^a Sandra Drev,^c Rok Podlipec,^{d,e} Tomaz Rijavec,^b Alexander Mansel,^a Ales Lapanje,^b Karsten Franke^a and Marko Strok^b

Manufactured nanoparticles, such as CeO₂, give rise to novel risks when released into the environment. To assess these risks it is important to quantify the nanoparticle mass flows, as well as their speciation and the mechanisms of their transformation. We developed an innovative dual-radiolabelling strategy for CeO₂ nanoparticles using neutron activation and in-diffusion labelling to radiolabel CeO₂ nanoparticles with both Ce-141 and Ce-139. The different distribution of the radiolabels in the particles does not only allow easy dose determination in uptake studies but also enables us to track the uptake pathways of the anthropogenic cerium. By measuring the activity as well as the isotope ratio we tracked the uptake, transformation and excretion of CeO₂ nanoparticles in freshwater shrimp. We found that 99.99 % of the uptaken particles are excreted, leaving the gut with excrement. The remaining 0.01 % was internalized via a dissolution based pathway and accumulated in the hepatopancreas of the shrimp at a dose range of pg CeO₂ per shrimp. Most importantly, our results show that dissolution is not only coincidental but instrumental in the uptake of the cerium into the internal organs of the shrimp.

Introduction

The risks associated with manufactured nanoparticles (NPs) may greatly depend on the transformation of said particles. Chemical transformations, such as, redox reactions, surface passivation and dissolution, or physical transformations, such as homo and heteroaggregation, can have decisive influences on the behaviour of NPs in the environment.¹ One major transformation factor to be considered in risk assessment is the potential dissolution of the NPs. Fast dissolving NPs can be treated as dissolved species while persistent NPs can be treated as particles.^{2,3} The scientific discussion on the exact definitions is still on-going (especially since other transformations can have a major impact on dissolution rates, for example in the case of sulfidation of Ag NPs)⁴ but particles that are commonly considered fast-dissolving are e.g. Ag or ZnO, in contrast to TiO₂ which is

considered persistent.^{5,6} Slowly dissolving particles such as CeO₂ form a special case as transport and uptake can happen in particle form to deliver potentially toxic ions via a Trojan horse effect.^{7,8} For CeO₂ in particular, there are conflicting results on the role of dissolution effects in uptake by organisms and the resulting toxicity.^{9–11} Positive,^{12,13} as well as, negative effects¹⁴ have been reported for CeO₂ NP exposure to organisms. The main factor considered for potential toxic effects is the Ce³⁺/Ce⁴⁺ ratio^{15,16} of the particles and consequently leaching of ionic Ce³⁺ from the particles upon dissolution which may act as the toxic agent, mainly by inducing oxidative stress that can lead to cell death.^{17–19} If, when, where, under which conditions and in what quantity CeO₂ NPs dissolve may be the main factor when it comes to judging their potential environmental effects.^{1,20}

CeO₂ NPs today are already used or are widely researched for commercial applications such as fuel additives,²¹ water remediation,²² photo catalysts,²³ cosmetics²⁴ and surface coatings²⁵ and are measurable environmental contaminants in rain and river water at concentrations of about 1 and 100 ng/L, respectively.²⁶ One primary exposure pathway to the environment is the sedimentation of the particles from surface waters in the benthic zones of rivers and lakes where they can be taken up by water organisms that feed on the benthic periphyton.²⁷ This may result in trophic transfer along the food chain.²⁸

Valuable mechanistic insights are typically gained by high exposure concentration experiments using XANES, EXAFS,

^a Helmholtz-Zentrum Dresden-Rossendorf, Research Site Leipzig, Institute of Resource Ecology, Permoser Str. 15, 04318 Leipzig, Germany.
Email: s.schymura@hzdr.de, Tel.: +493512604678

^b Jozef Stefan Institute, Environmental Sciences, Jamova cesta 39, 1000 Ljubljana, Slovenia.

^c Jozef Stefan Institute, Center for Electron Microscopy and Microanalysis, Jamova cesta 39, 1000 Ljubljana, Slovenia.

^d Jozef Stefan Institute, Condensed Matter Physics Department, Jamova cesta 39, 1000 Ljubljana, Slovenia.

^e Helmholtz-Zentrum Dresden-Rossendorf, Ion Beam Center, Bautzner Landstrasse 400, 01328 Dresden, Germany

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and μXRF^{29} that are often difficult to scale down to realistic environmental concentrations^{30,31} while lower concentration distribution experiments give valuable insights in fate but the attribution of effects to specific molecular mechanisms, like dissolution inside organisms, becomes more fuzzy.^{32,33} Both type of studies can only be realized with considerable experimental efforts in detection due to the complex media involved.³⁴ The radiolabelling of nanoparticles has the potential of providing an analysis technique for laboratory research that allows sensitive detection with unprecedented experimental ease^{35–37} and has already proven to be a valuable tool for fate analysis of nanomaterials in organisms.^{38–42} Employing smart radiolabelling strategies even has the potential of providing both, mechanistic insight at sub-acute toxicity concentrations.¹⁰ Based on previous efforts we developed an isotopic dual-radiolabelling strategy to shed light on uptake pathways of anthropogenic cerium in organisms.^{10,43,44} By employing two radiolabels we can develop a comprehensive picture of CeO_2 NP uptake in freshwater shrimp and show the importance of a dissolution-based uptake mechanism.

Experimental

Materials

CeO_2 NP reference material JRCNM02102a (formerly NM-212) was obtained from the JRC nanomaterial repository. A detailed characterisation report is available from the JRC.⁴⁵ In short, the primary particle size is around 16 nm,⁴⁶ with a hydrodynamic size of 221 nm and a zeta potential of around 42 mV when dispersed in DI water. The target lanthanum foil for the radionuclide production was procured from AlfaAesar. The fulvic acid used was isolated from bog water (Kleiner Kranichsee, Germany) following IHSS recommended procedures.⁴⁷ The acidities of the fulvic acid were measured by titration according to Stevensen⁴⁸ and elemental analysis was done using a Vario EL III element analyser (see ESI Tab. SI1 & SI2). All other chemicals were procured from Sigma Aldrich and used as is. Sera Shrimp Nature food pellets (protein: 44.0 %, fat: 8.9 %, fiber: 3.4 %, moisture: 5.6 %, ash: 11.3 %; additives: vit. A: 30 000 IU/kg, vit. D3: 1 500 IU/kg, vit. E (D, L- α -tocopheryl acetate): 60 mg/kg, vit. B1: 30 mg/kg, vit. B2: 90 mg/kg, stab. vit. C (L-ascorbyl monophosphate): 550 mg/kg) were obtained from a local aquarium shop.

Radiolabeling of CeO_2

Activation of CeO_2 30 mg of CeO_2 NPs were sealed in a quartz ampule and brought into the main channel of the TRIGA Mark II nuclear reactor at Jozef Stefan institute. They were irradiated with thermal neutrons at a neutron flux of $1 \cdot 10^{13}$ for 63 h.⁴⁰ After a decay time of 29 d the ampule was broken and the $[\text{Ce-141}]\text{CeO}_2$ NPs taken up in DI water.

Production of Ce-139 radiotracer Using the in-house IBA Cyclone 18/9 cyclotron at HZDR Research Site Leipzig Ce-139 radiotracer was produced via 10.9 MeV proton irradiation of a lanthanum metal foil at a current of 22 μA for about 23 h

yielding a current integral of 500 μAh .⁴⁹ The optimum proton energy, according to the reaction cross-section, was set by the design of the target (see ESI Fig. SI1 & SI2). The irradiated target was dissolved in conc. HNO_3 and the solution subsequently evaporated to dryness. The residue was dissolved again in 9 M HNO_3 and the produced cerium was oxidized by adding 0.05 M $\text{Cr}_2\text{O}_7^{2-}$. The separation of the Ce-139 from the lanthanum target material was achieved via extraction chromatography using UTEVA resin.⁵⁰ At 9 M HNO_3 the lanthanum is eluted while the Ce-139 remains on the column. The Ce-139 is eluted in a subsequent step with 0.1 M HNO_3 . This was followed by another evaporation step. The Ce-139 was finally dissolved in 0.01 M HNO_3 to yield a non-carrier-added solution with 51.92 MBq/mL Ce-139.

Indiffusion labelling of CeO_2 In order to introduce Ce-139 into the activated $[\text{Ce-141}]\text{CeO}_2$ NPs, the radionuclide solution was added dropwise to the dry NP powder placed in a conic vial, which was placed into an aluminium heating block at 80°C (see ESI Fig. SI3). After each addition the solution was evaporated and a new drop was added. After a total of 4.7 MBq of Ce-139 was added the sample was tempered at 300°C for 7 h to facilitate diffusion of the Ce-139 into the surface. Afterwards, the $[\text{Ce-139/Ce-141}]\text{CeO}_2$ NPs were washed two times and taken up in DI water. NP properties were checked using a Malvern Zetasizer nano, a Perkin Elmer Lambda 365 UV/Vis spectrometer, a Jeol JSM IT200 SEM, and a conventional 200kV Jeol JEM-2100 TEM equipped with LaB6 electron source before and after the labelling steps.

Tab.1: Radiolabel half-lives, γ -peaks, and abundancies.

Radioisotope	Half-life [d]	γ -peak [keV]
Ce-139	137.64	168.86 (80 %)
Ce-141	32.51	145.44 (48.4 %)

Dissolution experiments

Dissolution experiments were performed by placing 50 μg of radiolabelled $[\text{Ce-139/Ce-141}]\text{CeO}_2$ NPs on a 3 kDa centrifugal filter covering them with acidic leaching solution (1 mM HNO_3 , pH 3). After a leaching step the samples were centrifuged and the Ce-139 and Ce-141 contents in the leachate were measured using gamma spectroscopy (AMETEK ORTEC: DSPEC-502, GEM-40190P, GammaVision) and the leaching solution was replaced. The dissolution rates were calculated based on the Ce-141 activity. Complementary control measurements using HR-ICP-MS (Thermo Fisher Scientific, ELEMENT XR) were performed on the initial batch of CeO_2 NPs and decayed $[\text{Ce-141}]\text{CeO}_2$ NP that underwent a non-radioactive in-diffusion labelling procedure.

Shrimps

Freshwater shrimp *Caridina pareparensis parvidentata* were obtained from a local aquarium supplier. Judging from the 1.8–2.3 cm size and slender built of the shrimp they were roughly around the same age, adult and all male.⁵¹ Shrimp from the *Caridina* genus are widespread and abundant omnivorous freshwater shrimps (*Crustacea: Decapoda: Atyidae*) that feed on leaf litter, fine particulate organic matter, and periphyton in the benthic zones of rivers and lakes,^{52,53} which are main sinks for manufactured nanoparticles from rain and river water.⁵⁴ They can play a major role in the diet of fish⁵⁵ and thus potentially act as one step in a food chain accumulation of manufactured nanoparticles.⁵⁶ They are known to react to environmental contaminants⁵⁷ and have been suggested as model organisms for toxicity testing.⁵⁸

Uptake experiments

The shrimp were brought into the laboratory one week before the start of the experimental run to acclimate and were kept in aerated moderately hard standard freshwater (96 mg/L NaHCO₃, 60 mg/L CaSO₄ · 2 H₂O, 60 mg/L MgSO₄, 4 mg/L KCl, pH = 7.6) prepared according to EPA guideline EPA-821-R-02-013⁵⁹ and kept at 20°C under ambient light. The shrimp were not fed for 3 d before the experiments. In order to conduct the uptake experiments six shrimp in total were moved in pairs into 20 mL plastic vials filled with 10 mL of standard water (see ESI Fig. S14). The vials were placed open onto an IKA Labortechnik HS250 basic horizontal shaker which moved the vials very slowly (30 rpm) in order to move the water surface and minimize risks of oxygen deficiency in the vials. For uptake, the radiolabelled CeO₂ NPs were dispersed in MilliQ water with 5 mg/L fulvic acid added for stabilization using a Bandelin Sonoplus HD3200 ultrasound homogenizer equipped with a BR30 resonance cup for 5 min at 90% energy input. This ensured a good dispersion ($d_{\text{hydro}} = 219 \pm 3.8$, $\zeta\text{-pot.} = -27.4 \pm 1.5$) and serves to facilitate uptake by mimicking the natural fine particulate organic matter diet.²⁸ Dispersion quality was checked using a Malvern Zetasizer nano. A waterstable, sinking Sera Shrimp Nature food pellet (84 mg) was then soaked in 50 μL of the dispersion for 10 min, absorbing it totally, before being added to the shrimp containers. Control experiments showed that >90% of the particles stay agglomerated with the food pellet and no dissolution of CeO₂ was measured in the moderately hard standard water (see ESI Fig. S15 for more details on the food preparation). Our food preparation procedure thus simulates the built-up of an “eco-corona” by the CeO₂ NPs⁶⁰ and consequent heteroaggregation with larger organic matter particles and sedimentation into the benthic zone where shrimps feed. This provided a potential dose of about $59 \pm 2 \mu\text{g}$ of CeO₂ per shrimp. The shrimps were left to consume the offered food for 16 h before being transferred to a fresh vial with fresh water. This was achieved by filtering and rinsing the shrimp over a plastic mesh with 100 μm mesh size. In the remainder of the experimental run the shrimp were fed roughly every week using about one third

of a food pellet with no more CeO₂ NPs added. The shrimps were transferred into fresh water before any food addition and after the 16 h feeding steps. This yielded a 16 h step of combined feeding and excretion followed by on average 5 d of pure excretion for every preceding feeding step. In total the experimental run consisted of 30 d and six feeding events (including the initial CeO₂ NP containing exposure step). A detailed timeline of the experiment is shown in Fig. 2a. After every water transferal, the radionuclide content of the shrimp was determined by a measurement at a Perkin Elmer WIZARD 3” 1480 automatic gamma counter using a window of 125 – 155 keV for Ce-141 and 155 – 185 keV for Ce-139 according to their respective gamma peaks (see Tab. 1). Any overspill of the Ce-139 signal into the Ce-141 window was corrected using gamma spectroscopy data for calibration (see ESI Fig. S16). Every measurement was then decay corrected to the same date for comparison. The remaining activity in the shrimp after the experiment was visualized using autoradiography. The sacrificed shrimp were placed on a BAS-MS phosphor storage screen for 3 d and the image was read out using an Amersham Typhoon biomolecular imager. Evaluation of the images was done using ImageJ.⁶¹

Results and discussion

Radiolabelling

Irradiation of CeO₂ NPs with neutrons at the JSI nuclear reactor triggered the nuclear reaction Ce-140(n, γ)Ce-141 to yield a Ce-141 activity concentration of 0.255 MBq/mg. In side reactions also Ce-143 and Ce-139 were formed at activity concentrations of 5.7 kBq/mg and 0.88 kBq/mg. The former was let decay with a half-life of 33 h while the latter was only formed two orders of magnitude less than the Ce-141, thus not interfering with the further labelling strategy (see ESI Fig. S17). Ce-139 was produced at the HZDR cyclotron with a yield of 0.1 MBq/ μAh . The radiochemical yield of the tracer separation was 96 %. The radiolabelling yield of the in-diffusion step was 99 % thereby successfully generating dual-labelled [Ce-139/Ce-141]CeO₂ NPs (see ESI Fig. S18). The properties of the radiolabelled nanoparticles were not changed significantly from the original batch. Zeta potential in water and UV/Vis spectra suggest no major changes in Ce³⁺/Ce⁴⁺ ratio^{62,63} and DLS, SEM and TEM give no hint at major changes in particle sizes and morphology. Additionally no differences in dissolution rates of the original CeO₂ NPs and particles that underwent the labelling procedure are observed (Tab. 2 and ESI Fig S19–12).

Tab. 2: Properties of used nanoparticles.

	CeO ₂	[Ce-141]CeO ₂	[Ce-139/Ce-141]CeO ₂
DLS size [nm] ^a	221 \pm 4	222 \pm 3	220 \pm 4

Zeta Pot. [mV] ^a	43.8 ± 1.2	43.2 ± 1.4	44.4 ± 0.9
Bandgap [eV] ^b	3.2	3.1	3.3
Specific activity [MBq/mg(CeO ₂)]	-	0.255	0.272 Ce-139 0.107 Ce-141
Dissolution rate [ng/mg-d] ^c	54.1 ± 2.8	n.d	52.6 ± 4.7
^a measured at 100 mg/L in DI water ^b obtained from Tauc plot of UV/Vis data ⁶² (see ESI Fig. S19) measured in DI water at 4 mg/mL ^c in 1 mM HNO ₃ determined by ICP MS after 5 d of dissolution			

Dissolution of CeO₂ NPs

A controlled dissolution study in 1 mM HNO₃ solution (pH 3) reveals that the two different radiolabels are distributed differently in the particles. Upon dissolution we see a large release of Ce-139 indicating a localisation of the in-diffused radionuclide close to the particle surface. It was previously shown that about 50% of the Ce-139 is located within the first atomic layer.¹⁰ In contrast, the Ce-141 can be expected to be equally distributed within the particles yielding a much smaller release rate in the first stages of dissolution than the Ce-139. This leads to a very characteristic development of the Ce-139/Ce-141 ratio that can be detected in the leachate (Fig. 1).

Therefore, we can use the specific gamma peaks of the two radioisotopes (Tab. 1) to track the uptake of the CeO₂ NPs including dissolution processes. The Ce-141 signal can be directly translated into the CeO₂ mass using the specific activity (Tab. 2) while the Ce-139/Ce-141 ratio allows us inference of NP transformation along the uptake pathway. A growth of the ratio during the experiments indicates that dissolution of the CeO₂ NPs played a significant role on the uptake pathway.

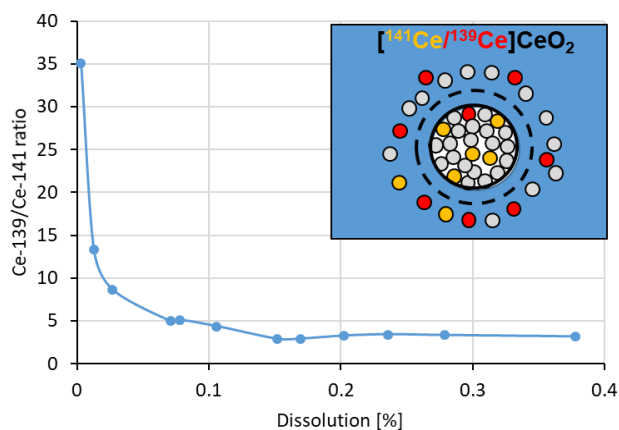


Fig. 1: Development of Ce-139/Ce-141 ratio in leachate of

dissolution experiments, in dependence of dissolution percentage, as established by Ce-141 gamma spectroscopy measurement.

Uptake and Excretion of CeO₂ by shrimp

The value of the Ce-141 activity measurements can be directly translated into the taken up amount of CeO₂ NPs using the specific activity. This allows us to easily establish unambiguous doses. The offered amount of CeO₂ was 59 ± 2 µg per shrimp. After the 16 h feeding step only 4% of this amount remained in the shrimp yielding a dose of 2.5 ± 0.1 µg per shrimp (see Tab. 3, Fig. 2a and Fig. S13 for detailed doses). Note that this does not equate to the total amount of CeO₂ NPs consumed since part of the taken up CeO₂ already got excreted within this period. Nevertheless we set this as the 100% reference point for any measurements of the remaining cerium discussed below. Although no standardized toxicity tests were performed we can report that no obvious signs of acute toxicity could be observed. All the shrimps remained alive during the experiment and no diminished mobility and reactions were observed. Food intake was not refused during the experiment and moulting events of two shrimps during the experimental run hint at largely normal life functions. However, chronic toxicity markers such as moulting frequency, growth performance, reproduction rates and food utilization indices were not measured.

Tab. 3: CeO₂ NP doses in our experimental run as calculated using the Ce-141 activity.

Dose	Offered dose per shrimp	Content per shrimp	Content per dry weight	Content per wet weight ^a
Start of experiment ^b	59 ± 2 µg	2.5 ± 0.1 µg	0.41 ± 0.07 µg/mg	0.081 ± 0.003 µg/mg
End of experiment	-	380 ± 173 pg	62.0 ± 28.2 pg/mg	12.4 ± 5.6 pg/mg
^a estimated considering a dry weight of 20% ⁶⁴ ^b refers to day 0 for offered dose, and day 1 for shrimp CeO ₂ content (see Fig 2.a)				

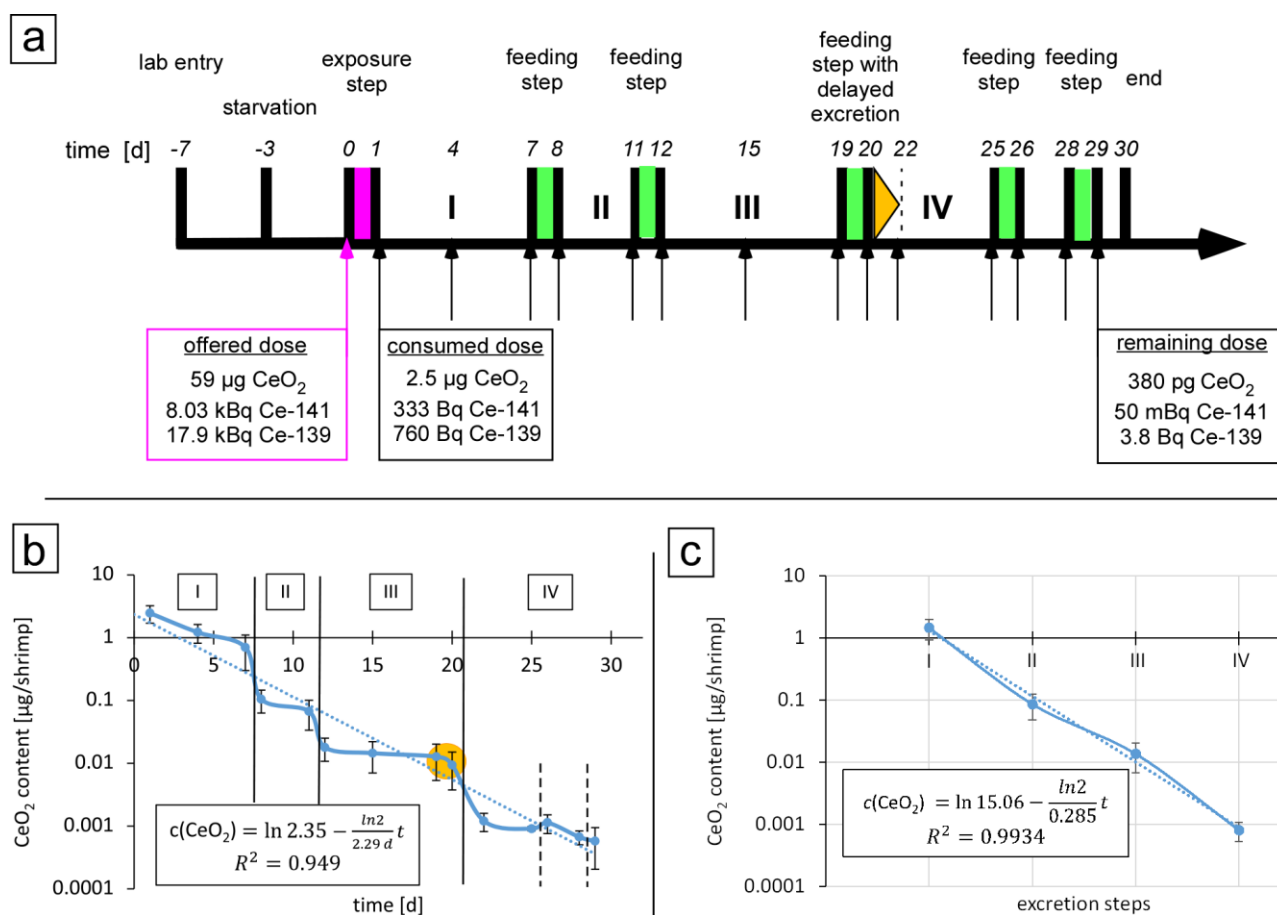


Fig. 2: (a) Experimental run of CeO₂ NP uptake experiment from lab entry to the end of the experimental run over four excretion periods (I-IV). The day of exposure is set as the zero point. The overnight exposure step with CeO₂ NP is indicated in pink, any subsequent feeding steps without CeO₂ are indicated in green. One moulting related delayed excretion is indicated in orange. Any activity measurements taken are indicated by arrows. For selected measurements the CeO₂ content, Ce-141 activity, and Ce-139 activity per shrimp are given (see ESI for more detailed data). (b) Excretion of CeO₂ NPs from shrimp with time. Two feeding steps and one delayed excretion event (solid lines) divide the experiment into four excretion periods (I-IV). The moulting occurrence is indicated in orange. Additionally, two feeding events are indicated in excretion period four (dashed lines) with no further excretion happening. (c) Excretion of CeO₂ NPs from shrimp in dependence of excretion periods I-IV.

The excretion of CeO₂ from the shrimp follows an exponential decay curve with a half-life of 2.3 d (see Fig. 2b). At the end of the 30 d experimental run only 380 ± 173 pg CeO₂ remain per shrimp, i.e. 99.985 ± 0.007 % of the initially taken up amount was eliminated from the shrimp with only 0.015 ± 0.007 % remaining.

The excretion generally follows a scheme consisting of a larger excretion in the hours right after feeding and a much smaller, if any, excretion in the remaining time till the shrimp were fed again. This is consistent with an elimination of the CeO₂ NPs from the tract with the remains of digested food which happens over a period of several hours after feeding.⁶⁵ Consequently, the first two feeding events can be clearly identified as steps in the activity diagrams. The third feeding shows a delayed effect due to two moulting events during which feeding and excretion was halted.⁶⁶ This occurrence allowed us to exclude CeO₂ NP sorption to the shrimp surface or cerium accumulation in the exoskeleton as a major contribution to our activity signal.

If we combine the measured values between feeding events into four excretion periods with one average value each, the exponential elimination curve of the CeO₂ content becomes even clearer (see Fig. 2c). The half-life of the CeO₂ NPs is about 0.3 excretion steps so that within one excretion step already about 91% of the taken up CeO₂ NPs leave the organism. If we extrapolate the excretion function to the zero point we can estimate that the consumed amount of CeO₂ NPs is about 15 µg per shrimp equating to 25 % of the offered dose. Taking into account that the bulk of the excretion takes place already during the 16 h feeding step we can estimate a biological half-life of about 5 h, which is in line with previous studies of the digestive kinetics of similar shrimp which report peak excretion around 5-8 h after feeding but continuing up to 20 h.⁶⁵ In nature, under constant grazing, digestion and excretion conditions, the elimination of up taken particles may even be faster than observed in our experiment, however with strong variations between different species and depending on the specifics of the food consumed.⁶⁷ However, the retention of 9 % of the particles after each excretion step may cause a slow

accumulation in a scenario of constant influx of new particles.⁶⁸

The fourth excretion period identified above is actually comprised of two feeding events which however do not lead to a further reduction of CeO₂ content. Thus, overall, we can summarize the uptake and excretion of CeO₂ found in our experiment as a roughly 20 d elimination period during which the excretion follows an exponential decay function followed by an apparent stop of any further cerium elimination with about 0.1 % remaining at the end of our experiment.

Fate of CeO₂ in shrimp

Our dual radiolabelling approach allows us to gain much further insight into the uptake and retention mechanisms. A closer look at the elimination curves of the different cerium nuclides reveals differences between the Ce-141 and Ce-139 elimination from the shrimp (see Fig. 3). We can see that both nuclides follow the same trend for the first three excretion periods. In stark contrast to that, no further excretion of Ce-139 is seen between the third and fourth excretion period with the Ce-139 activity remaining stable over the rest of the experiment, despite two further feeding events during period four. At the end of our experiment about 0.15 % of the initially taken up Ce-141 remains within the shrimp in contrast to 0.5 % for the Ce-139. This is reflected accordingly in the Ce-139/Ce-141 ratio which remains stable during the first three excretion periods but jumps an order of magnitude with the beginning of the fourth excretion period.

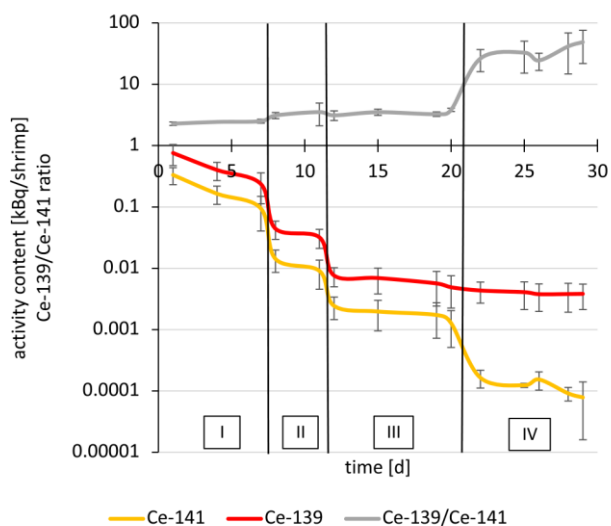


Fig. 3: Excretion of radionuclide activity of Ce-141 and Ce-139 and development of Ce-139/Ce-141 ratio inside the shrimp over four excretion periods I-IV.

As such a change in Ce-139/Ce-141 ratio is a result of a preferential dissolution of the surface near Ce-139 radiolabel in contrast to the volume based Ce-141 radiolabel, this leads us to conclude that between the third and fourth excretion period the vast majority of nanoparticles are eliminated from the organism via the feed stream while the remaining

cerium took a dissolution-based uptake pathway into the organism and cannot be readily removed. This translates into a dissolution of about 0.15 % of the CeO₂ within the digestive tract of the shrimp. This dissolution may be mediated mainly by the shrimp gut microbiome and digestive agents. For example, lactate producing bacteria have been found an important part of *caridina* gut microbiomes⁶⁹ and lactate has been shown to be a strong dissolving agent for CeO₂ NPs.⁷⁰ But also other food components may have an effect on the dissolution rate in the digestive tract of the shrimp. Most importantly the presence of complexing and reducing agents, e.g. vitamin C, promoting dissolution and, in contrast, phosphates or sulfides which inhibit dissolution by surface passivation and precipitation of Ce³⁺ species.^{71–75} Our observations indicate that the gut microbiome may be the first point of action with regard to the bactericidal effects of dissolved Ce³⁺¹⁶ as it reacts sensitively to environmental contaminants.⁷⁶ While acutely toxic effects towards the shrimp seem to be unlikely at low cerium concentrations sub-lethal chronic exposure of the gut microbiome may affect food utilization indices and growth performance in the long run.⁷⁷ Consequently, the composition of the gut microbiome may be an important controlling factor for either the level of dissolution and/or subsequent chronic toxic effects.

So far all the measurements described have been non-destructively performed on the live shrimps. The radiolabelling allowed us to do a detailed kinetic excretion study with minimal experimental effort and animal sacrifice (in a conventional study for each data point several animals would have to be sacrificed, increasing the overall need of test animals in the first place). The very low radiation levels at the end of the experiment – far below any legal free limit – would also have allowed to take the shrimp out of the laboratory to live happily ever after in an aquarium.

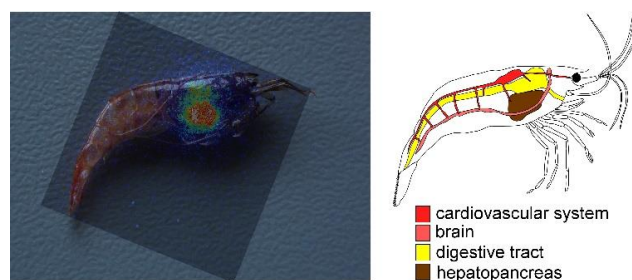


Fig. 4: Overlay of autoradiogram of activity distribution with photo of shrimp (left) and scheme of shrimp anatomy (right).

However, further insight can be gained upon sacrificing the shrimp at the end of the experiment. Autoradiographic analysis of the distribution of the activity in the shrimp reveal a consistent pattern for all the shrimp. The activity is primarily located in the lower cephalothorax of the shrimp (see Fig. 4). A cross-referencing with general shrimp anatomy^{78,79} allows us to identify the hepatopancreas as the main accumulator of the remaining cerium. The hepatopancreas is the main digestive gland of crustaceans.

While there are strong variations on where metal contaminants may end up in crustaceans depending on the metal and uptake pathway,^{80–82} the hepatopancreas is considered to be the major invertebrate organ that sequesters and detoxifies dietary or water-borne metals.^{79,83} It is here where the enzymatic digestion beginning in the stomach continues and absorption of fluid nutrients occurs that are pressed out from the solids in the stomach.^{67,79} Larger inert particles (> 100 nm) are typically barred from entering the hepatopancreas by the pyloric filters which are rather uniform in all decapods, and are transported through the mid- and hindgut for excretion although some digesta may stay in the hindgut only being voided when a subsequent meal is ingested.^{79,84} This seems to be largely the fate of the CeO₂ NPs which while having a primary particle size of about 16 nm form agglomerates with hydrodynamic diameters of 220 nm when stabilized in aqueous media and due to our experimental procedure are heteroaggregated with the solid food components. Should they enter the hepatopancreas our results indicate that they are subsequently also largely removed from it, only leaving their dissolved remnants behind. Any dissolved cerium absorbed in the midgut will reach the hepatopancreas via the blood stream for sequestration.⁸³ It is difficult to estimate what the cerium accumulation in the hepatopancreas may entail, as there is conflicting data on the effects of CeO₂ NPs on water organisms.^{9,12,14} However, any effect will be heavily dependent on the actual cerium species present and their concentrations. Our experiments suggest an exposure of the gut microbiome primarily to CeO₂ nanoparticles while the internal organs may be mainly exposed to dissolved cerium species or secondary precipitates. Literature data on dissolution-based uptake of CeO₂ into organisms suggest that depending on the chemical environment the cerium may be present mainly as carboxylates, Ce(PO₄)₃ precipitates or reformed CeO₂ NPs.^{85,86} So the overall effect of CeO₂ on shrimp can be twofold, an alteration of the gut microbiome upon chronic exposure to CeO₂ NPs and dissolved cerium species, which can influence the shrimp's susceptibility to disease^{77,87,88} and a damaging of hepatopancreatic function due to chronically increased oxidative stress due to cerium accumulation in the form of dissolved Ce³⁺ species or secondary precipitates.^{89,90} A triple burden of gut microbiome-caused disease/parasite susceptibility, chronic hepatopancreatic oxidative stress in combination with other common bacterial disease stressors, such as acute hepatopancreatic necrosis disease could also have wider implications for shrimp farming endeavours.⁹¹ However, the stop of excretion of the cerium that took a dissolution-based pathway into the organism found in our experiment may not be the final outcome, as biological half-times for other metals in shrimp are known to be longer than the duration of our experiment (\approx 80 d for Cs from Baltic prawn)⁹². The general picture of a selective uptake of dissolved cerium into the body with an almost total elimination of the original particulate CeO₂ from the gut via the excrements may also be altered in the case of smaller particles. For example,

studies indicate that CeO₂ NPs < 10 nm are able to translocate over the gut wall in freshwater worm.⁹³ However, to what extent a dissolution-based pathway may be responsible for the observed translocation was not addressed. Consequently, whether an accumulation of CeO₂ NPs in the gut and/or internal organs and/or dissolved species and secondary precipitates in the internal organs occurs will greatly depend on the influx of new CeO₂ NPs and their specific properties. These may be heavily controlled by usage patterns of CeO₂ NPs,⁹⁴ their release pathway⁹⁵ and their transformation on their way into⁹⁶ and in the environment⁹⁷ and weather effects controlling the influx patterns.⁹⁸ In interactions with the individual gut microbiomes this produces a complex picture of potential CeO₂ NP ecotoxicity. Reliable dose data as well as knowledge on the relevant uptake pathways is needed to shed light into these complex patterns and to allow a comprehensive understanding of CeO₂ NP uptake into organisms and an assessment of the consequences and risks thereof.

Conclusions

We developed an innovative dual-radiolabelling strategy for CeO₂ NPs that let us perform detailed kinetic uptake and excretion studies with easily established dose measurements. Using our dual-labelled [Ce-139/Ce-141]CeO₂ we could establish a biological half-life of CeO₂ NPs in freshwater shrimp on the hour scale as the particles are excreted with the feed stream. 0.15 % of the particles took a dissolution-based pathway to translocate over the gut wall with the cerium getting accumulated in the *hepatopancreas* of the shrimp and not being excreted during our 30 d experiment. Our data shows that the dissolution is not only coincidental but instrumental in the uptake of CeO₂ into the shrimp, i.e. it was a prerequisite for cerium uptake into the internal organs of the shrimp under our experimental conditions. At constant CeO₂ influx accumulation effects could happen, with the gut microbiome being exposed primarily to CeO₂ NPs while the internal organs, most prominently the *hepatopancreas*, are mainly exposed to dissolved cerium and secondary precipitates. Resulting alterations in the gut-microbiome and chronic hepatopancreatic oxidative stress may lead to long-term consequences below acute toxicity.

Author Contributions

SS: conceptualization, supervision, investigation, formal analysis, visualization, resources, funding acquisition, project administration, writing – original draft, review and editing; IR: investigation, formal analysis, writing – review and editing; SU: investigation, formal analysis, writing – review and editing; SD: investigation, formal analysis, writing – review and editing; RP: resources, methodology, writing – review and editing; TR: resources, methodology, writing – review and editing; AM: investigation, resources, methodology; AL: resources,

methodology, funding acquisition, project administration, writing – review and editing; KF: investigation, formal analysis, resources, methodology, writing – review and editing; MS: funding acquisition, investigation, methodology, project administration, resources, writing – review and editing.

Conflicts of interest

There are no conflicts to declare.

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