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Influence of citrate and PEG coatings on the bioaccumulation of TiO_2 and CeO_2 nanoparticles following dietary exposure in rainbow trout

Mona Connolly[1†], David Hernández-Moreno[1†], Estefanía Conde[2], Alicia Garnica[1], José M. Navas[1], Fernando Torrent[3], Isabel Rucandio[2] and María L. Fernandez-Cruz[1*]

Abstract

Background: This study was conducted to provide much needed information on the potential of low solubility, highly biodurable nanoparticles (NPs) (TiO_2 and CeO_2 NPs), to bioaccumulate in fish and to investigate the relationship between an engineered nanomaterials surface coating, uptake and biokinetics in vivo. Rainbow trout (*Oncorhynchus mykiss*) were fed diets spiked with uncoated or surface coated (polyethylene glycol (PEG) or citrate (CIT)) TiO_2 and CeO_2 NPs (100 mg NPs/kg feed) for 10 days and thereafter fish were allowed to depurate for 42 days. Special care was taken to measure the real dispersed and actual administered concentrations, taking into consideration any potential losses from leaching, and to characterise the form (size, aggregation state, charge) of the NPs to which the fish were exposed.

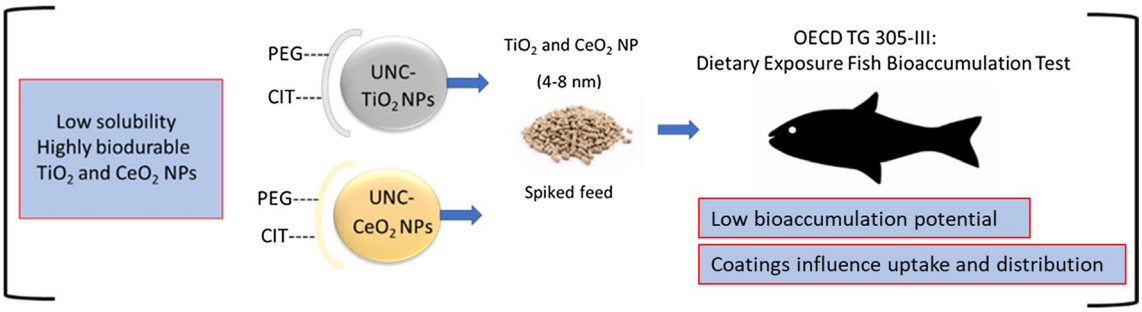
Results: The coatings had an influence on levels of uptake and distributions. Most notably a higher uptake of PEG and CIT coated TiO_2 and CeO_2 NPs compared to fish exposed to uncoated materials was observed. The elimination of any Ti from tissues was rapid during the first day of depuration, whereas Ce levels remained in fish tissues (stomach, intestine and liver) with differences in depuration and redistribution of the three types of CeO_2 NPs. However, no bioaccumulation potential for both tested metal oxide NPs in fish irrespective of coatings is expected according to BMF values < 1.

Conclusions: Distinct uptake, distribution and depuration kinetics in rainbow trout have been evidenced for different metal oxide NPs (TiO_2 and CeO_2 NPs). Coatings influenced uptake and in some cases led to slower depuration and distinct distributions, but do not make the uncoated NPs studied bioaccumulative (BMF > 1).

Keywords: Metal oxide NPs, Coating effects, Depuration, Fish tissue levels, *Oncorhynchus mykiss*

*Correspondence: fcruz@inia.es
†Mona Connolly and David Hernández-Moreno contributed equally to this work
[1] Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Consejo Superior de Investigaciones Científicas (INIA, CSIC), Ctra. de la Coruña, Km 7.5, 28040 Madrid, Spain
Full list of author information is available at the end of the article

Graphical Abstract



Background

The metal oxide nanomaterials, titanium dioxide (TiO_2) and cerium dioxide (CeO_2) have been identified as high-priority nanoparticles (NPs) for testing, due to their low solubility and, biodurability and thus may constitute NPs of high persistence (both in the environment and within biological systems) [1, 2]. The risk associated with potential long-term persistence of and exposure to these anthropogenic nanoparticles has yet to be elucidated. Modelled environmental concentrations can be used as predictions of exposure [3, 4], but the actual levels of NPs in the environment are unknown. In one instance, TiO_2 NPs with sizes of 20–50 nm have been identified in receiving water bodies from waste water treatment plant (WWTP) effluents, with total Ti concentrations of between 52 and 86 µg/L measured versus low natural background levels of ~ 5 µg/L [5]. Also the presence of TiO_2 NPs (43–67 nm) in the fat snook fish, *Centropomus parallelus* sampled from river estuaries with high Ti concentrations in sediments (1272–1707 µg/g) suggests already the transport and biological sequestration of TiO_2 NPs in the natural environment [6]. CeO_2 NPs uptake and biodistribution in fish following aqueous exposures to 10 µg/L in natural surface waters has also been shown, demonstrating also these particles bioavailability [7]. While the most recent maximum modelled predicted environmental concentration of 61.74 ng/L [8] for CeO_2 NPs in surface waters is not above already measured natural background levels (20–141 ng/L) [9], levels are likely to increase with their continued use.

Microcosm studies and aquatic ecosystem models have been used to investigate the fate of NPs in aquatic environments. They have revealed NPs uptake through absorption, respiration and/or ingestion and partitioning in aquatic organisms (microalgae, plants and fish), and identified that sediments are the ultimate sinks for both CeO_2 NPs [10] and TiO_2 NPs [11]. NPs-exposed aquatic organisms and plants are all potential dietary sources of NPs exposure for higher trophic level organisms such as fish. In fact the trophic transfer of TiO_2 NPs from zooplankton to fish, with higher uptake levels and overall body burdens compared to aqueous exposures, has already been reported [12, 13]. TiO_2 NPs present in sediment sinks have also been shown to be readily taken up by fish [11]. Taken together this suggests dietary uptake is likely significant and arguably, the most relevant exposure route for fish to such NPs in freshwater environments.

Despite this, the extent to which these NPs will bioaccumulate and be biodistributed in fish following long-term dietary exposure is poorly understood. In one of the few TiO_2 NP dietary bioaccumulation studies performed in fish, Ramsden and colleagues measured Ti levels above natural background levels in all tissues (gill, intestine, liver, brain and spleen) of *O. mykiss* following 8 weeks of exposure to TiO_2 NPs (uncoated, < 25 nm, 100 mg TiO_2 NPs/kg feed) and a 2 weeks of depuration [14]. Dietary exposures have not been performed using CeO_2 NPs in fish up until now. In short-term 96-h aqueous exposures to uncoated CeO_2 NPs with sizes ≤ 25 nm significant increases in Ce levels in the gills and liver of rainbow trout were evidenced [7]. However, it has been shown that the route of exposure will dictate specific biodistribution and accumulation patterns [15] and thus this may not reflect the biodistribution and/or bioaccumulation profile of CeO_2 NPs following a dietary exposure.

Moreover, it is not clear if surface chemistry will control NPs biokinetics in vivo. Bioaccumulation studies performed to date have principally focused on exposures with core uncoated NPs, despite the increasing development and use of surface functionalisations. Surface functionalisation (protein or polymer coating, ligand capping, organic modification, amine- or carboxy-terminated group) of nanomaterials is being used to fine-tune and control particle behaviour (e.g. aggregation, dissolution, size), and indeed is being explored in safer by design approaches for nanomaterials [16]. However, we do not yet have a complete understanding of the chemical/biological processes at the nano–bio interface that are

controlling NP behaviour in vivo, in body systems, and in the biological milieu.

The potential influence of surface chemistry on NPs bioaccumulation in vivo has been discussed previously [17]. Attempts have been made to investigate the extent to which coating confers a new identity or controls fate in vivo in some species. For example comparative studies, while limited, have been performed in different organisms investigating the influence of different surface functionalisations on NP biokinetic profiles in vivo. In rats, following oral exposure, studies have evidenced distinct surface coating [18] and charge dependent [19] biodistribution profiles, while others report no significant differences between the biodistribution and bioavailability of different surface functionalised NPs [20]. In the water fleas, *Ceriodaphnia dubia* and *Daphnia magna,* a greater body burden and higher persistence of hydroxyl (COOH) functionalised quantum dots (QDs) in the gastrointestinal tract compared to polyethylene glycol (PEG) and amine (NH^{2+}) functionalised QDs has been reported [21]; whereas, a faster elimination of carboxyl-functionalised QDs in *Daphnia magna* compared to amine- and polyethyleneimine-functionalised particles was reported by Lee and colleagues [22]. Contrastingly, the lowest elimination rate value (ke) (0.5764 h^{-1}) was calculated for COOH-CuO NPs, compared to pristine, PEG and NH^{3+} coated NMs using modelled bioaccumulation dynamics by Gajda-Meissner and colleagues in *Daphnia magna* [23]. Investigations in other relevant pelagic organisms are limited and to the best of our knowledge such comparative investigations have not been performed in fish.

To address this knowledge gap, in the present study we have compared the bioaccumulation, biodistribution and depuration profile of uncoated, polyethylene glycol capped (pegylated) and citrate-coated CeO_2 and TiO_2 NPs after 10 days dietary exposure and a 42 day depuration phase in rainbow trout according to OECD test guideline (TG) 305, dietary exposure [24]. We extended the depuration phase (beyond 28 days) as we were interested in investigating if even if there is a fast depuration, this may be incomplete and there may be redistributions. This assumption was based on the incomplete depuration and redistributions observed for ZnO NPs in a previous study [25]. Both polyethylene glycol (PEG) and citrate (CIT) are widely used surface stabilising agents that provide colloidal stability by altering the surface charge and conferring hydrophilicity. We hypothesised that the different coatings used would influence the biodistribution and bioaccumulation of the respective NPs and we were interested in investigating if similar biokinetic profiles could be identified for the different core NPs (TiO_2 and CeO_2) with the same coating (e.g. TiO_2-PEG and CeO_2-PEG NPs). For this reason, only one and the same concentration was tested. The information generated will not only provide important in vivo biokinetic data for CeO_2 and TiO_2 NPs following dietary exposure in fish that is lacking, but also seeks to determine the influence of coatings on biodistribution and bioaccumulation patterns.

Methods

CeO_2 and TiO_2 NPs

Uncoated (UNC), phosphate polyethylene glycol (PEG) coated, and citrate (CIT) coated NPs (4–8 nm) [CeO_2 and TiO_2 (anatase)] were provided by PlasmaChem GmbH (Berlin, Germany) as aqueous colloidal suspensions. The size and shape of these stock suspensions were analysed by TEM using a JEOL JEM-2100 HT (JEOL Ltd.) operated at an accelerating voltage of 200 kV with integrated energy dispersive X-ray (EDX) spectroscopy (Oxford Inca). The size of the particles (ferret diameter) in the TEM micrographs was measured using the image processing and analysis software ImageJ (National Institutes of Health, USA). Sizes among uncoated and coated NPs with the same core were very similar, showing a mean diameter of 3.98 ± 0.20 nm (CeO_2 NPs) and 6.58 ± 0.85 nm (TiO_2 NPs). All of them showed an amorphous shape. Aliquots of these stock suspensions were analysed using inductively coupled plasma-optical emission spectrometry (ICP-OES) as described in 2.5 to determine the exact Ti and Ce contents. TiO_2-UNC, TiO_2-CIT and TiO_2-PEG NPs suspensions were provided at nominal concentrations (w/v) of 200 mg/mL (145.8 ± 8.1 mg TiO_2/mL, measured), 145 mg/mL (68.9 ± 5.0 mg TiO_2/mL, measured) and 100 mg/mL (54.7 ± 12.3 mg TiO_2/mL, measured), respectively. CeO_2-UNC NPs suspensions (100 mg/mL) contained 82.9 ± 4.7 mg CeO_2/mL, while CeO_2-CIT (50 mg/mL) and CeO_2-PEG (50 mg/mL) contained 29.9 ± 1.5 and 23.9 ± 3.3 mg CeO_2/mL, respectively. Further dilutions of the as received suspensions were made in ultrapure Milli-Q water (pH 6.9) to achieve 2 mg/mL working suspensions taking into account the measured concentrations. The zeta potential and hydrodynamic size of these suspensions were characterised using a Zetasizer Nano-ZS (Malvern Instruments Ltd., UK). Measurements were taken directly after preparation. Three independent measurements were performed with each one consisting of four consecutive measurements with six runs and these were averaged to calculate the zeta potential and mean hydrodynamic size according to intensity, volume or number distribution. Polydispersity index and z-average values calculated were also recorded and presented as an indication of the heterogeneity or homogeneity of dispersion and width of size distribution.

Preparation of pellets spiked with CeO_2 and TiO_2 NPs

The different CeO_2 and TiO_2 NPs stock suspensions were dispersed in water to obtain a concentration of 2 mg/mL as indicated previously. One mL of the respective NPs was added directly dropwise to 20 g of feed (50 µL per g feed) in order to obtain a final concentration of 100 mg NPs/kg feed. Spiked pellets were thereafter vortexed to ensure the homogeneous distribution of the NPs on the feed sample. To verify if the actual concentration of CeO_2 NPs and TiO_2 NPs agreed with the nominal ones, Ce or Ti contents were measured in three 100 mg aliquots of the 100 mg NPs/kg spiked pellets. Furthermore, to verify the concentration in feed and the potential loss of NPs from feed to water, both TiO_2 and CeO_2 NPs stock suspensions were dispersed in water to obtain a concentration of 4 mg/mL, from which a final concentration of 200 mg NPs/kg feed was achieved, following the same sample preparation procedure described above. Ce and Ti concentrations were measured by ICP-OES as described in 2.5 in three 100 mg aliquots of the spiked pellets and in three aliquots of the feed pellets after being submerged for 5 min in water.

Fish and dietary exposure

Rainbow trout juveniles (mean initial weight and length of 1.19 ± 0.44 g and 4.9 ± 0.54 cm, respectively) were supplied by the fish Farm of Escuela Técnica Superior Ingenieros de Montes, Universidad Politécnica, Madrid, Spain. They were kept in $50 \times 70 \times 40$ cm rectangular 120-L tanks supplied with recirculating filtered tap water (water hardness was 140 mg/L and pH 8.0) and maintained under controlled conditions of photoperiod (light: 12 h, darkness: 12 h) and temperature. The temperature oscillated during the whole experiment between 14 °C and 16 °C, whereas the oxygen content was between 7.5 and 8.6 mg/L. The faeces were cleaned 1 h after the treatments. Aquaria were maintained by continuously receiving filtered recirculated water and 1/3 of the water volume was replaced with fresh water once a week creating semi-static conditions. Fish were fed daily at a rate of 2% of their body weight during the 52 days that the experiment lasted, a commercial diet for trout, Inicio Plus 887 (BIOMAR Iberia, S.A., Dueñas, Spain), with pellets of 1.9 mm in diameter and a lipid content of 18%.

Exposure concentrations of 100 mg NPs/kg feed (2 µg NPs/ g body weight) were used and fish were fed once a day. This concentration was selected based on our own previous studies and the study of Ramsden et al. [14]. 45 fish per control and treatment groups were used to sample 5 fish at 6 distinct sampling points (see "Sampling" Section). In accordance with OECD TG 305, no replicates per treated group were introduced in the study design. The exposure period lasted ten consecutive days and it was followed by 42 days depuration in which fish from all groups received untreated feed according to OECD TG 305.

Sampling

Five fish were randomly sampled at the beginning and end of the exposure period (day 10) and subsequently at different time points during the depuration phase (days 11, 17, 24, 38, 52). On sampling days, fish were sampled just prior to feeding (therefore 24 h after last feeding). In addition, three additional fish were collected at the end of the uptake and depuration phases (days 10 and 52). At all the time points fish were killed using tricaine methanesulfonate (MS-222) (250 mg/L). Fish weights and lengths were recorded, the stomach and intestine were removed, weighed, and stored at − 20 °C, together with the corresponding fish carcass, until analysis of total Ti or total Ce contents by ICP-mass spectrometry (ICP-MS). Stomach and intestine samples were washed by dipping in milliQ water prior to weighing and storage in order to remove any remnants of the NPs spiked feed. The liver and gills from the three fish sampled on day 10 (end of exposure period) and on day 52 (end of depuration phase) were removed, weighed and stored (− 20 °C) for Ti and Ce analysis. The fish carcasses (with stomach and intestine removed) were analysed for Ti and Ce content at all sampling time points.

Ce and Ti levels analysis in feed and tissues

Feed samples were digested in a DigiPrep block (SCP Science, Quebec, Canada). Briefly, 5 g of feed samples were dried at 100 °C during 24 h, pulverised by crushing in a mortar, and 100 mg selected for digestion. 0.5 mL of milliQ water, 3 mL of nitric acid (HNO_3) (67–69%) and 0.5 mL of hydrofluoric acid (HF) (47–51%) were added to the feed contained in Digiprep 15 mL tubes. A temperature ramp cycle was programmed first to maintain 75 °C for 15 min while 0.5 mL of hydrogen peroxide (H_2O_2) (30%) was carefully applied dropwise to the sample. After that, samples were maintained at 115 °C for 120 min to complete digestion, then cooled and milliQ water was added up to the mark (10 or 15 ml). An Agilent 5110 Synchronous Vertical Dual View with a vertical torch and charge coupled device (CCD) detector was used for measuring the concentrations of Ce and Ti by ICP-OES (Agilent Technologies, Inc., Santa Clara, California, USA). OneNeb nebuliser with a baffled cyclonic spray chamber and a peristaltic pump were used for sample introduction. Ti or Ce contents in pellets that had been submerged in water (time 0 and 5 min) were also measured to quantify any appreciable loss because of leaching to the water column during feeding. Any quantifiable background levels of Ti and Ce in unspiked feed pellets

were subtracted from total measured levels to calculate spiking recovery.

Fish tissue samples (50 mg dry weight), that were previously pulverised as indicated for feed, were mixed with 0.5 mL of milliQ water, 1.5 mL of HNO_3 and 0.5 mL of HF. Tissue sample digestion was performed also in DigiPrep 15 mL tubes with the same temperature ramping and 0.5 mL of H_2O_2 addition as used for feed digestion. Once the digested samples were cooled, all of them were filled up to 10 mL with milliQ water and they were preserved for subsequent analysis. Levels of Ce and Ti in tissues were measured by an ICP-MS Thermo iCap Q (Thermo Scientific, Bremen, Germany) equipped with a quadrupole mass analyser and an electron multiplier detector. A Meinhard nebuliser with Scott (Ryton) spray chamber (Elemental Scientific Inc., USA) and a peristaltic pump was used for sample introduction. The sample solutions were quantified by external calibration; two Ce isotopes (140 and 142) and two Ti isotopes (47 and 48) were used for measurements to discard the presence of isobaric interferences. Internal standards (gallium and indium) were used in order to check instrumental stability and to correct potential effects of matrix in the signal. The measured data were expressed as µg Ce or Ti/g tissue or feed (wet weight). The calculated limit of detection (LOD) for Ti and Ce was 0.04 and 0.01 µg/g and the limit of quantification (LOQ) was 0.12 and 0.03 µg/g, respectively.

Accumulation analysis

The chemical assimilation efficiency (α, absorption of test substance across the gut) was calculated with the formula:

$$\alpha = [(C_{0,\mathrm{d}} * k_2)/(I * C_{\mathrm{food}})] * [1/\left(1 - e^{-k2t}\right)],$$

where $C_{0,\mathrm{d}}$ is the derived concentration in fish at time zero of the depuration phase (mg/kg); k_2 is the overall (not growth-corrected) depuration rate constant (day^{-1}); I is the food ingestion rate constant calculated according to the average amount of food eaten by each fish each day, relative to the estimated average fish whole body weight (g food/g fish/day); C_{food} is the concentration in food (mg/kg food); t is the duration of the feeding period (day).

The overall depuration rate constant k_2 was estimated using mean sample concentrations from the depuration phase subtracting the basal Ti or Ce levels measured in the control group at the corresponding time of sampling. These values were adjusted to a linear regression of ln(concentration) versus time. The slope of the regression line is an estimate of the depuration rate constant k_2. In cases where fast depuration was evidenced and only two sampling time points were available the K_2 was calculated according to equation A5.22 as presented in TG 305:

$$K_2 = [\ln(C_{\mathrm{f1}}) - \ln(C_{\mathrm{f2}})]/(t2 - t1),$$

where $\ln(C_{\mathrm{f1}})$ and $\ln(C_{\mathrm{f2}})$ are the natural logarithms of the concentrations at times $t1$ and $t2$, respectively, and $t2$ and $t1$ are the times when the two samples were collected relative to the start of depuration. In cases where two sampling time points were not available (due to analytical limits of detection), the second value was set at the instrument limit of detection to allow an indicative K_2 to be calculated.

Also Co.m (which is the measured concentration in fish at time zero of the depuration phase (mg/kg)) was used for α calculation instead of Co.d (derived concentration) when using these approaches.

A dietary kinetic biomagnification factor ($\mathrm{BMF_k}$) was calculated according to:

$$\mathrm{BMF_k} = (I * \alpha)/k_2,$$

$\mathrm{BMF_{kg}}$ values were also calculated using the equation $\mathrm{BMF_{kg}} = (\mathrm{I}^*\alpha)/k_{2g}$, whereby K_{2g} was calculated according to: $K_{2g} = k_2 - k_g$ using the growth rate constant (K_g) values determined from slopes of the fitted linear regression of ln(fish weights) over time for sampling points during the depuration phase.

$\mathrm{BMF_{kgL}}$ values were calculated using the calculated lipid correction factor Lc of 0.277 that was calculated using the default fish lipid content of 5% [24] and the indicated lipid content in feed of 18% (fish mean lipid fraction (w/w)/food mean lipid fraction (w/w)) and applying the equation:

$$\mathrm{BMF_{kgL}} = \mathrm{BMF_{kg}}/\mathrm{LcBMF_{ssL}}.$$

$\mathrm{BMF_{ssL}}$values were also calculated according to:

$$\mathrm{BMF_{ssL}} = \mathrm{BMFssL}/\mathrm{Lc}.$$

The time (days) to reach the 50% depuration ($t_{½}$) of Ti or Ce was estimated with the formula $t_{½} = 0.693/\mathrm{k_2}$.

Statistical analysis

Ti and Ce levels in tissues are presented as means ± standard error of the mean (SEM) of measurements taken from fish (number indicated in text). Statistical analysis was performed using SigmaPlot 14.5 (Systat Software Inc., Chicago, IL, USA). Normality of the data distributions were checked by means of a Shapiro–Wilk test and data were checked automatically by the programme for equal variance ($p < 0.05$). Significant difference among the different treatments were compared using one-way ANOVA followed by a pairwise comparison Holm–Sidak test ($p < 0.05$).

Results

Physico-chemical properties of TiO_2 and CeO_2 NPs

The zeta potential and hydrodynamic size distributions of uncoated and coated TiO_2 and CeO_2 NPs, prepared in ultrapure Milli-Q water (pH 6.9) (2 mg NPs/mL) and used for fish feed spiking, have been characterised and are compared in Table 1. Zeta potential values for uncoated NPs (TiO_2-UNC) are 42.7 ± 2.1 mV, indicating a stable dispersion. Coating TiO_2 NPs (TiO_2-CIT and TiO_2-PEG) changed the zeta potential value to -25.9 and -24.7 mV, respectively, indicating less stable dispersions. Uncoated TiO_2 NPs are present in relatively monodispersed aggregates with mean size of 148.9 ± 0.3 nm in aqueous dispersion. In contrast, TiO_2 NPs coated with citrate (TiO_2-CIT) and polyethylene glycol (TiO_2-PEG) appear dispersed as individual particles with mean Z-average diameters of 15.9 ± 0.6 nm and 15.7 ± 0.3 nm, respectively. The presence of larger TiO_2-CIT NPs (345–741 nm) has also been detected in these dispersions, albeit at small proportions (6–27%). Similarly, larger sizes were detected in TiO_2-PEG dispersions (1125–2839 nm), however ≥ 93% of particles detected were between 7.5 and 16 nm according to number, volume and intensity average hydrodynamic measurements. The presence of larger aggregates in TiO_2-CIT and TiO_2-PEG NP suspensions is likely related to their instability during analysis. However, suspensions comprised predominantly monodispersed 6.6–7.5 nm particles (according to instrument-calculated number-based size distribution) in contrast to TiO_2-UNC stable suspensions of particle aggregates (106 nm).

Uncoated CeO_2 NPs have a positive zeta potential of 22.1 ± 2.96 mV, with these low values indicating an unstable suspension. However, CeO_2-CIT and CeO_2-PEG-coated NPs zeta potentials were negatively charged and of higher values (− 31.9 and − 29.4 mV, respectively) indicating nearly stable suspensions.

An average mean size distribution of 6.9 ± 0.1 nm was measured for uncoated CeO_2 NPs, suggesting individual particles monodispersed (polydispersity index, PdI = 0.27) in an aqueous dispersion. Particles within this size range (7.6–16.1 nm) were also present in CeO_2-CIT and CeO_2-PEG NPs dispersions in high percentages, especially when presented in volume and number distributions. Volume distributions were identical for uncoated and citrate-coated CeO_2 NPs suspensions. Small percentages (< 10%) of larger size distributions were evidenced in all suspensions likely due to aggregation or settling of particles during measurement.

Ti and Ce levels analysis in feed

Feed spiked with uncoated and coated TiO_2 and CeO_2 NPs (100 mg NPs/kg) was analysed for Ti and Ce content using ICP-OES as described in "Ce and Ti levels analysis in feed and tissues" Section. A background level of 83.93 mg Ti/kg was measured in unspiked control feed, while the level of Ce was below the detection limit (0.01 mg/kg feed). The levels of Ti and Ce above background levels were compared to nominal concentrations (60 mg Ti/kg feed and 81.4 mg Ce/kg feed, respectively) (Table 2). Measured concentrations of Ti were 62.7 ± 7.4 mg, 57.4 ± 12.1 mg and 58.4 ± 18.1 mg for TiO_2-UNC, TiO_2-CIT and TiO_2-PEG, respectively ($n = 3$). This represented a 100 ± 4% spiking achievement for all preparations and excluded any significant differences in exposure dose due to the feed preparation technique. 100 mg CeO_2 NPs/kg-treated pellets (81.4 mg Ce/kg feed) were prepared in the same way and the concentrations measured were 68.85 ± 8 mg, 70.8 ± 7 mg and 54.7 ± 5 mg Ce/kg of CeO_2-UNC, CeO_2-CIT and CeO_2-PEG treated pellets, respectively ($n = 3$). This

Table 1 Physico-chemical characterisation of TiO_2 and CeO_2 NPs in aqueous dispersion (2 mg/mL)

	Z-potential (mV ± SD)	PdI	Z-average (d. nm ± SD)	Average hydrodynamic diameter (d. nm ± SEM) (%)		
				Intensity	Volume	Number
TiO_2-UNC	42.7 ± 2.1	0.24	148.9 ± 0.3	190.3 ± 1.7 (100)	149.3 ± 1.0 (98)	106.6 ± 2.5 (100)
TiO_2-CIT	− 25.9 ± 4.2	0.24	15.9 ± 0.6	13.2 ± 0.1 (72) 345.0 ± 12.4 (27)	8.9 ± 0.2 (94) 741.9 ± 381.8 (6)	6.6 ± 0.4 (100)
TiO_2-PEG	− 24.7 ± 1.5	0.47	15.7 ± 0.3	16.0 ± 0.3 (93) 1125 ± 543.0 (5)	10.0 ± 0.2 (98) 2839 ± 720.0 (1.7)	7.5 ± 0.3 (100)
CeO_2-UNC	22.1 ± 3.0	0.27	6.9 ± 0.1	8.0 ± 0.2 (90) 302.6 ± 75.3 (8)	8.5 ± 0.2 (97) 65.1 ± 1.9 (3)	2.9 ± 0.1 (100)
CeO_2-CIT	− 31.9 ± 1.4	0.29	68.6 ± 0.9	105.0 ± 0.9 (93) 9.9 ± 0.3 (7)	8.5 ± 0.2 (97) 65.1 ± 1.9 (3)	7.6 ± 0.2 (100)
CeO_2-PEG	− 29.4 ± 0.9	0.34	114.6 ± 6.6	167.6 ± 4.47 (92) 248.4 ± 229.3 (7)	16.1 ± 0.7 (88) 126.3 ± 8.4 (8)	14.2 ± 0.8 (100)

PdI polydispersity index, *d.nm* diameter measured in nanometres, *SD* standard deviation, *SEM* standard error of the mean

Table 2 Comparative assessment of nominal and measured Ti and Ce concentrations (mean ± SEM, $n = 3$) in spiked fish feed and quantification of potential loss to water column

Concentration of Ti or Ce in spiked feed (mg/kg feed)				
	Nominal concn	Measured concn	Recovery (%)	[% loss] after 5 min
TiO_2-UNC	60	62.7 ± 7.4	104	− (3)
TiO_2-CIT	60	57.4 ± 12.1	96	14
TiO_2-PEG	60	58.4 ± 18.1	97	12
CeO_2-UNC	81.4	68.8 ± 8.3	85	9
CeO_2-CIT	81.4	70.8 ± 7.2	87	11
CeO_2-PEG	81.4	54.7 ± 5.3	67	9

(−) represents no loss and number in brackets () is the percentage increase in concentration of Ti

represented an 86 ± 1% recovery for both uncoated and citrate-coated CeO_2 NPs-spiked pellets. In contrast, a lower recovery was measured in polyethylene glycol-coated CeO_2-NPs spiked pellets with only 67% of the nominal concentration measured. Furthermore, to investigate if spiked pellets lost any concentration to the water column, pellets submerged in water for 5 min (reflecting a maximum time prior to being taken up by fish) were also analysed using ICP-OES (Table 2). There was no loss in Ti concentration from TiO_2-UNC spiked pellets, while losses of 14 and 12% were measured for CIT and PEG-coated TiO_2 NPs spiked pellets, respectively. In the case of CeO_2-spiked feed there were no obvious differences in losses among pellets spiked with the different coatings with an average loss of 9.6 ± 1.2% (see Table 2).

Fish growth and condition

No signs of toxicity were observed throughout the experiments and less than 7% of animals died or were killed (to avoid suffering) during the experiments (6% during TiO_2 NPs testing and 0.5% in the CeO_2 NPs testing experiment). Any differences in fish weights or energy stores following exposures to the uncoated and coated TiO_2 and CeO_2 NPs were monitored. All fish increased in weight from an initial mean weight of 1.19 ± 0.22 g at the start of experiments (day 1 of uptake) to 3.74 ± 0.34 g and 4.58 ± 0.39 g at day 52 in TiO_2 NPs and CeO_2 NPs accumulation experiments, respectively. There were no significant differences between treatment and control group weights neither at the end of the uptake phase (day 10) nor at the end of the depuration phase (one-way ANOVA) (Table 3). Specific growth rates (SGR) and calculated growth rate constants (K_g) for the entire duration of the study were compared and are also presented in Table 3.

There were no significant differences in growth performance parameters between control and treatment groups or among different treatment groups (one-way ANOVA). Therefore, no obvious relationships or effects of coating on fish weight or growth could be identified. HSIs were calculated to investigate any effects of exposure on energy stores by comparing liver weight in relation to body weight. Indices remained high (the same as control levels) and there was no significant differences between uncoated and coated NPs treatment groups.

Ti tissue accumulation/depuration

The concentration of Ti in rainbow trout tissues (stomach, intestine) and the remaining carcass was quantified

Table 3 Comparison of fish weights and hepatosomatic indices (HSI) after 10 days of TiO_2 and CeO_2 NPs dietary exposure and at the end of the depuration phase (day 52), along with whole study growth performance parameters

	Day 10		Day 52		SGR (%/day^{-1})	K_g (day^{-1})
	Weight (g)	HSI (%)	Weight (g)	HSI (%)		
Control	2.3 ± 0.4	0.7 ± 0.2	3.6 ± 0.5	1.5 ± 0.2	2.14	0.017
TiO_2-UNC	2.0 ± 0.3	1.0 ± 0.1	4.2 ± 0.8	2.1 ± 0.2	2.42	0.026
TiO_2-CIT	1.8 ± 0.2	1.2 ± 0.1	3.4 ± 0.7	1.9 ± 0.3	2.00	0.021
TiO_2-PEG	1.8 ± 0.3	0.7 ± 0.1	3.8 ± 0.4	1.4 ± 0.1	2.21	0.021
Control	1.6 ± 0.1	1.3 ± 0.1	4.5 ± 1.0	1.5 ± 0.2	2.56	0.025
CeO_2-UNC	1.5 ± 0.2	0.9 ± 0.1	4.1 ± 1.2	1.3 ± 0.6	2.36	0.022
CeO_2-CIT	1.3 ± 0.1	1.3 ± 0.1	4.8 ± 1.0	0.9 ± 0.1	2.67	0.025
CeO_2-PEG	1.4 ± 0.2	1.6 ± 0.3	5.0 ± 1.0	1.5 ± 0.1	2.75	0.028

Values are presented as mean ± SEM ($n = 5$, fish weights), ($n = 3$, HSI)

HSI (%) = 100 × hepatic weight (g)/body weight (g)

SGR (%/day^{-1}) = 100 × [ln final body weight (g) − ln initial body weight (g)]/ days

K_g = slope of the line of plot of (ln) body weight against time (days)

using ICP-MS on day 10 (at the end of the uptake phase) and at various time points throughout a depuration phase (Fig. 1). At day 10 significantly higher Ti levels were measured in the stomach and intestine of TiO_2 NPs-exposed fish (uncoated and coated) compared to control levels (Fig. 1a and b). These background levels in control tissues are expected due to the natural levels of Ti in fish and are consistent with levels measured in other studies using rainbow trout [26]. Uptake in the stomach followed the order TiO_2-UNC (1.8 ± 0.1 mg Ti/kg) < TiO_2-CIT (2.5 ± 0.3 mg Ti/kg) < TiO_2-PEG with the highest concentration of Ti measured in TiO_2-PEG NPs-exposed fish stomachs (2.9 ± 0.3 mg Ti/kg vs 0.85 ± 0.7 mg Ti/kg (control fish)). In contrast, in intestines the highest Ti levels were measured in TiO_2-CIT NPs and TiO_2-UNC-exposed fish (4.40 ± 1.0 and 4.15 ± 0.6 mg Ti/kg, respectively), with lower levels in TiO_2-PEG-exposed fish intestines (2.9 ± 0.6 mg Ti/kg) and 1.8 ± 0.5 mg Ti/kg measured in control intestinal tissues. Any accumulated levels of Ti above background control fish levels in stomach and intestines of TiO_2 NPs-exposed fish were also eliminated quickly with similar Ti levels as control fish 24 h after depuration (Fig. 1a and b). Basal Ti levels in the remaining carcass of control fish ranged

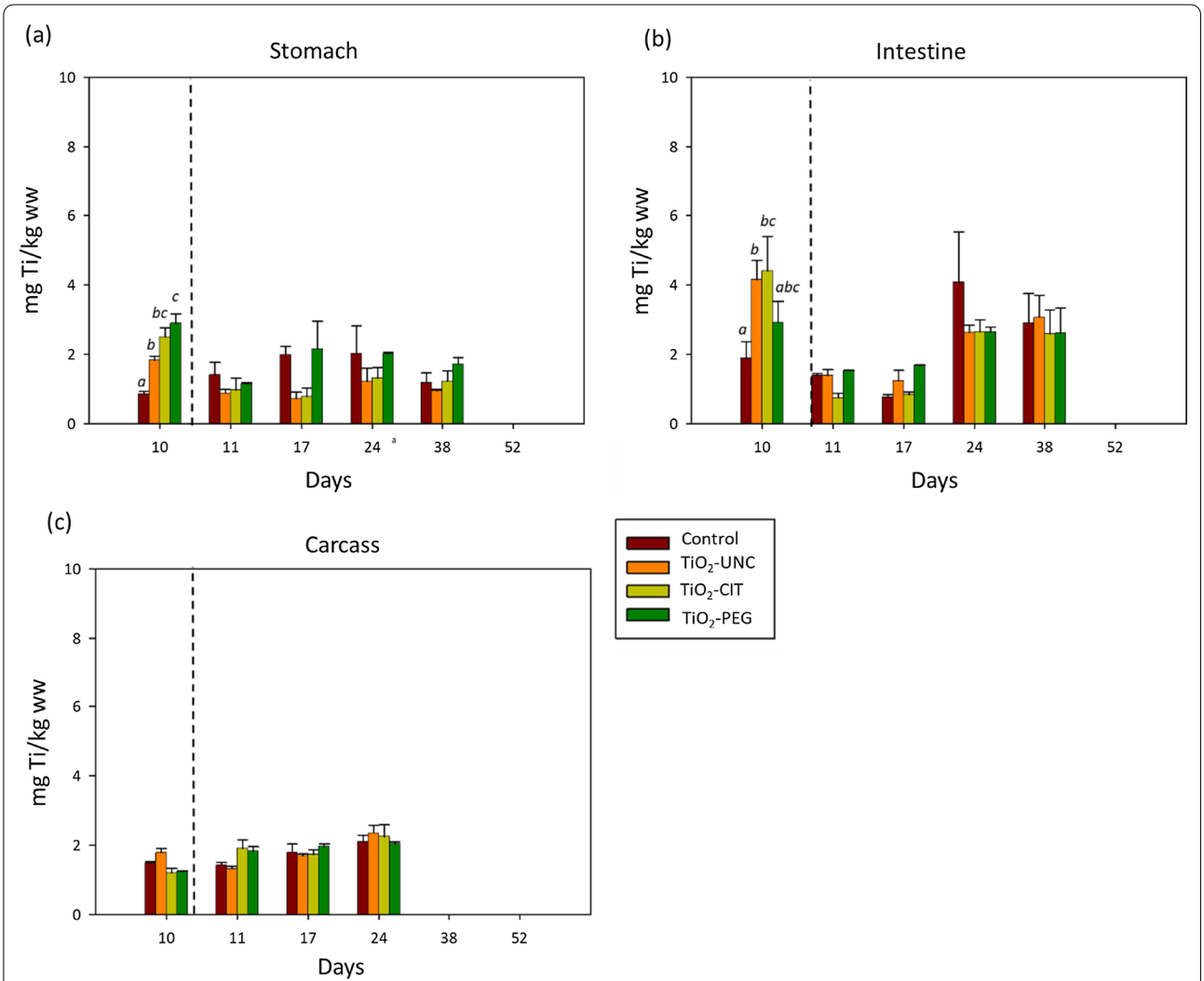


Fig. 1 Levels of Ti (mean ± SEM) measured in the **a** stomach ($n = 8$), **b** intestine ($n = 8$) and **c** carcass ($n = 5$) of rainbow trout fed control diets and diets spiked with uncoated (UNC), citrate coated (CIT) and polyethylene glycol coated (PEG) TiO_2 NPs (100 mg NPs/kg feed) following a 10 day exposure period and throughout a depuration phase (days 11–52). Dashed line indicates end of the exposure (uptake) phase and start of depuration. Letters have been used to indicate significant differences between treatment groups, with bars bearing distinct letters significantly different from each other (ANOVA followed by Holm–Sidak pairwise comparison; $p < 0.05$). No letters appear in the depuration phase due to a lack of any significant differences between treatments

from 6.8 to 10 mg Ti/kg. At the end of the uptake phase (day 10), there was no significant uptake above control levels in the carcass of PEG and CIT-coated TiO_2 NPs-exposed fish. Higher Ti levels (albeit not significant) were measured in TiO_2-UNC NPs-exposed fish carcass (1.78 ± 0.1 mg Ti/kg v´s 1.49 ± 0.2 mg Ti/kg (control fish carcass)) (Fig. 1c). During the depuration phase Ti levels remained at control levels in all the treated groups. Levels of Ti in the liver and gills of control and treated fish were also measured at the end of the uptake phase (day 10) and subsequently at the end of depuration (day 52) (Fig. 2a and b). Levels were not significantly elevated in TiO_2 NPs-exposed fish liver or gills compared to controls. In fact, significantly lower levels of Ti were measured in TiO_2-UNC NPs-exposed fish livers and gills. At day 52 (42 days of depuration), higher Ti levels were measured in TiO_2-CIT and TiO_2-PEG-exposed fish livers (6.08 ± 0.8 and 7.24 ± 0.9 mg Ti/kg, respectively) compared to control fish liver levels (4.3 ± 1 mg Ti/kg), which may indicate redistribution and accumulation in this tissue. Interestingly elevated levels were not measured in fish exposed to the uncoated TiO_2-UNC NPs compared to control fish (Fig. 2a). In gills, Ti levels were comparable in control and all treatment groups on day 52 (Fig. 2b), albeit large deviations in levels measured were evidenced.

Ce tissue accumulation/depuration

The concentration of Ce in rainbow trout tissues (stomach, intestine) and the remaining whole bodies were quantified using ICP-MS on day 10 and at various time points throughout a depuration phase (Fig. 3). Levels of Ce in control fish stomach and intestines could not be measured due to levels either being below detection limits (< 0.01 mg Ce/kg tissue) or a lack of background Ce in these tissues. Levels of Ce measured in the stomach tissues of CeO_2 NPs treated fish at the end of uptake phase (day 10) followed the order CeO_2-UNC (0.85 ± 0.2 mg Ce/kg) < CeO_2-CIT (1.47 ± 0.4 mg Ce/kg) < CeO_2-PEG (1.84 ± 0.7 mg Ce/kg) in exposed fish with levels significantly higher in the CeO_2-PEG NPs exposure group compared to the CeO_2-UNC NPs exposed group (Fig. 3a). Following 24 h depuration, levels decreased in CeO_2-UNC and CeO_2-PEG-exposed fish stomach tissues (0.24 ± 0.08 mg Ce/kg and 0.19 ± 0.04 mg Ce/kg, respectively) but remained high in CeO_2-CIT exposed fish stomach tissue (1.06 ± 0.3 mg Ce/kg), suggesting a slower depuration for CeO_2-CIT NPs. Levels of the three CeO_2 NPs types reduced over a longer depuration period (e.g. on days 17, 24 and 38) with a higher significant concentration of CeO_2-CIT remaining even at day 24 compared to CeO_2-UNC and CeO_2-PEG treated fish tissues ($P < 0.05$). After 42 days of depuration, Ce could still be measured in the CeO_2-UNC treated group (albeit at low levels of 0.07 mg Ce/kg). Levels of Ce measured in the intestine of CeO_2 NPs-exposed fish at day 10 ranged from 0.32 to 0.41 mg Ce/kg, with no significant differences in uptake profile for uncoated and coated NPs-exposed fish. A similar depuration for the three NPs types was seen on day 11 (Fig. 3b). No measurable Ce was detected in subsequent days during depuration (days 17, 24, 38). However, on day 52 (at the end of depuration) high levels of Ce (similar to day 10 of uptake) in coated NPs (CeO_2-CIT and CeO_2-PEG)-exposed fish

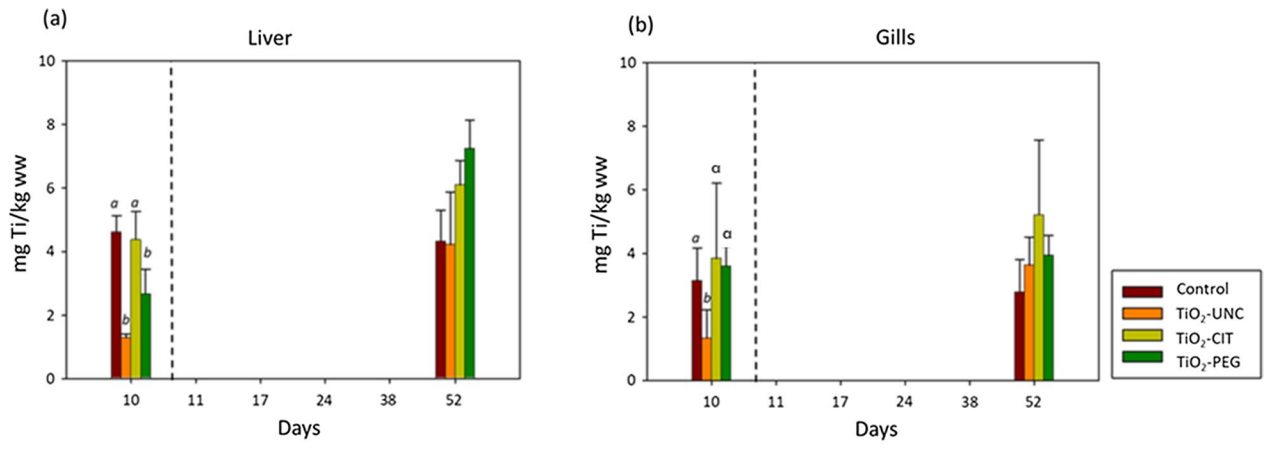


Fig. 2 Levels of Ti (mean ± SEM) measured in the **a** liver ($n = 3$) and **b** gill ($n = 3$) of rainbow trout fed control diets and diets spiked with uncoated (UNC), citrate coated (CIT) and polyethylene glycol-coated (PEG) TiO_2 NPs (100 mg NPs/kg feed) following a 10-day exposure period and following 42 days of depuration (day 52). Dashed line indicates end of the exposure (uptake) phase and start of depuration. Letters have been used to indicate significant differences between treatment groups, with bars bearing distinct letters significantly different from each other (ANOVA followed by Holm–Sidak pairwise comparison; $p < 0.05$). Tissues samples at days 11, 17, 24 and 38 were not taken

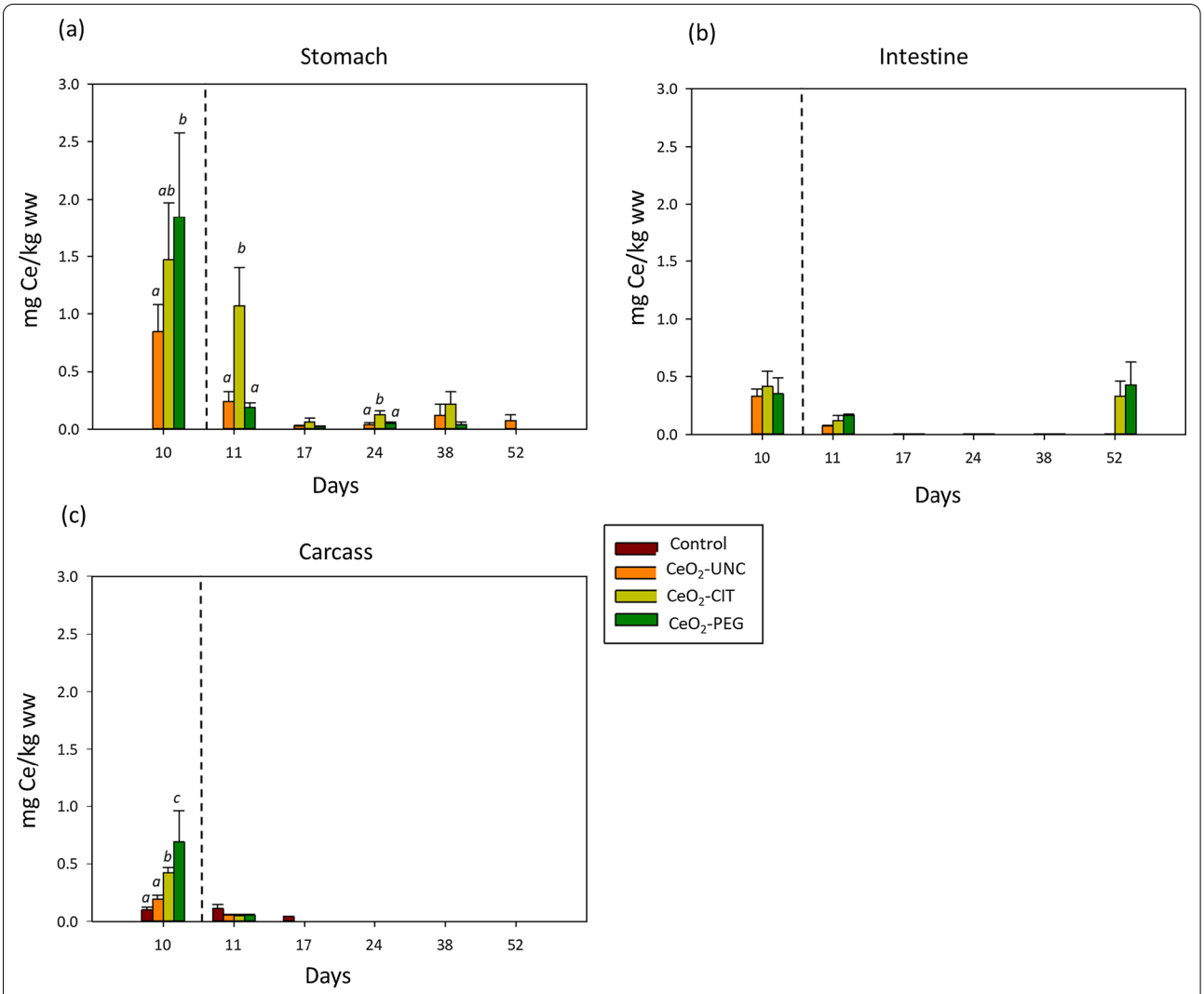


Fig. 3 Levels of Ce (mean ± SEM) measured in the **a** stomach ($n = 8$), **b** intestine ($n = 8$) and **c** carcass ($n = 5$) of rainbow trout fed control diets and diets spiked with uncoated (UNC), citrate coated (CIT) and polyethylene glycol coated (PEG) CeO_2 NPs (100 mg NPs/kg feed) following a 10-day exposure period and throughout a depuration phase (days 11–52). Dashed line indicates end of the exposure (uptake) phase and start of depuration. Letters have been used to indicate significant differences between treatment groups, with bars bearing distinct letters significantly different from each other (ANOVA followed by Holm–Sidak pairwise comparison; $p < 0.05$)

were measured. Levels of Ce were not measured in CeO_2-UNC exposed fish intestine later in the depuration phase. Levels of Ce in the carcass of control fish could be measured and ranged from 0.04–0.11 mg Ce/kg at day 10 (of the uptake phase) and on days 11 and 17 (in the depuration phase) (Fig. 3c). Higher levels were measured in CeO_2-NPs-exposed fish carcasses compared to the control group and followed the order CeO_2-UNC (0.19 ± 0.04 mg Ce/kg) < CeO_2-CIT (0.42 ± 0.05 mg Ce/kg) < CeO_2-PEG (0.69 ± 0.3 mg Ce/kg tissue) ($P < 0.05$) with the highest Ce levels measured in PEG-coated CeO_2 NPs-exposed fish carcass (Fig. 3c). During the first 24 h of depuration levels of Ce were reduced below control levels and completely cleared from the carcass at day 17 (following 7 days of depuration) for all treatment groups.

Ce levels were not detected in the liver of control or CeO_2 NPs-exposed fish at the end of the uptake phase. Interestingly on day 52 (following 42 days of depuration) high Ce levels were measured in the livers of CeO_2-UNC and CeO_2-PEG exposed fish (0.86 ± 0.02 mg Ce/kg and 0.47 ± 0.3 mg/kg tissue, respectively). In contrast, there was no measurable Ce levels in CeO_2-CIT-exposed fish livers at day 52 (Fig. 4a). Levels of Ce could be measured in the gills of fish at the end of the uptake phase. Higher levels, although not significant, were measured in

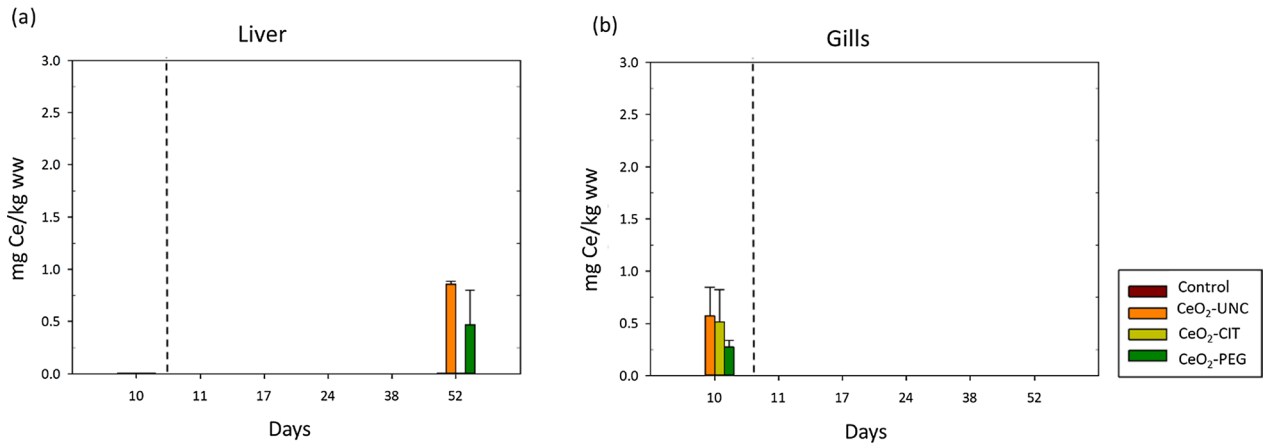


Fig. 4 Levels of Ce (mean ± SEM) measured in the **a** liver ($n = 3$) and **b** gill ($n = 3$) of rainbow trout fed control diets and diets spiked with uncoated (UNC), citrate-coated (CIT) and polyethylene glycol-coated (PEG) CeO_2 NPs (100 mg NPs/kg feed) following a 10-day exposure period and following 42 days of depuration (day 52). Dashed line indicates end of the exposure (uptake) phase and start of depuration

CeO_2-UNC and CeO_2-CIT NPs-exposed fish (0.57 ± 0.3 and 0.51 ± 0.3 mg Ce/kg, respectively) compared to the CeO_2-PEG-exposed fish group (0.27 ± 0.06 mg Ce/kg). There was a complete depuration of Ce from fish gills from all treatment groups with no detectable levels of Ce on day 52 (Fig. 4b).

Depuration rate constants and biomagnification factors

The levels of uptake for Ti and Ce above background levels, depuration rate constants (K_2), assimilation efficiencies (α), kinetic biomagnification factors (BMF_k) as well as 50% depuration half-life (t½) values calculated for stomach, intestine and the remaining carcass of exposed fish are presented in Tables 4 and 5, respectively. For comparative purposes, steady-state biomagnification factor (BMF_{ss}) values are also presented in cases where they could be derived (e.g. appreciable uptake measured above control background levels) while assuming steady state had been reached after 10 days of uptake. Appreciable levels of Ti uptake in stomach tissues above control background levels were evidenced, and they were higher in fish exposed to the coated NPs (Table 4 and Fig. 1). In the intestinal tissues, higher levels of uptake were measured in fish exposed to the TiO_2-UNC NPs and TiO_2-CIT NPs, however similar uptake was found for the TiO_2-PEG NPs compared to the control fish. In fish carcasses, the only appreciable uptake above control levels was in TiO_2-UNC NPs exposed fish. K_2 and the derived values α, t½ or BMF_k values could not be calculated for any of the TiO_2 NPs treatment groups due to a lack of remaining appreciable levels of Ti above control fish Ti levels in stomach, intestine or fish carcass after 24 h of depuration (Fig. 1a–c). The BMFs assumed at the steady state and the fast depuration indicated no bioaccumulation of these NPs (Table 4).

Table 4 Parameters of bioaccumulation for tested NPs according to measured Ti levels (mg/kg ww) (mean ± SEM) above background in stomach, intestine and carcass of fish

	Stomach			Intestine			Carcass		
	TiO_2-UNC	TiO_2-CIT	TiO_2-PEG	TiO_2-UNC	TiO_2-CIT	TiO_2-PEG	TiO_2-UNC	TiO_2-CIT	TiO_2-PEG
Day_{10} levels	0.97 ± 0.1	1.64 ± 0.3	2.02 ± 0.3	2.27 ± 0.6	2.52 ± 1.0	1.02 ± 0.6	0.29 ± 0.1	< control	< control
K_2 (day^{-1})	nd	nd	nd	nd	nd	nd	nd	nd	nd
a	nd	nd	nd	nd	nd	nd	nd	nd	nd
BMF_k	nd	nd	nd	nd	nd	nd	nd	nd	nd
*BMF_{ss}	0.016	0.029	0.035	0.036	0.044	0.017	0.005	nd	nd
*$BMF_{ss\text{-}L}$	0.0558	0.103	0.125	0.130	0.158	0.063	0.017	nd	nd
t ½ (day^{-1})	nd	nd	nd	nd	nd	nd	nd	nd	nd

*Assumed steady state; < control: no accumulation above control fish background values; nd: could not be determined due to fast clearance and no appreciable levels above control fish basal levels at depuration phase sampling time

Table 5 Parameters of bioaccumulation for tested NPs according to measured Ce levels above background levels (mg/kg ww) (mean ± SEM) in stomach, intestine and carcass of fish

	Stomach			Intestine			Carcass		
	CeO_2-UNC	CeO_2-CIT	CeO_2-PEG	CeO_2-UNC	CeO_2-CIT	CeO_2-PEG	CeO_2-UNC	CeO_2-CIT	CeO_2-PEG
Day_{10} levels	0.85 ± 0.2	1.47 ± 0.5	1.84 ± 0.7#	0.32 ± 0.06	0.41 ± 0.13	0.35 ± 0.13	0.09 ± 0.04	0.32 ± 0.04#	0.59 ± 0.27#
K_2 (day^{-1})	0.028	0.059	0.058	0.335″	nd^	nd^	nd	nd	nd
K_{2g}	0.007	0.034	0.029	0.313	nd^	nd^	nd	nd	nd
α	0.013	0.034	0.072	0.003	nd^	nd^	nd	nd	nd
BMF_k	0.009	0.011	0.025	0.0002	nd^	nd^	nd	nd	nd
BMF_{kgL}	0.041	0.053	0.090	0.0002	nd^	nd^	nd	nd	nd
*BMF_{ss}	0.012	0.021	0.034	0.005	0.006	0.006	0.001	0.005	0.011
*$BMF_{ss\text{-}L}$	0.044	0.075	0.121	0.017	0.021	0.023	0.005	0.016	0.040
t ½ (day^{-1})	24.32	11.65	12.04	2.070	> 42	> 42	nd	nd	nd

*Assumed steady state; nd: could not be determined due to fast clearance and no appreciable levels above control fish basal level at depuration phase sampling time; nd^; values could not be determined as high levels measured after 42 days of depuration. ″Calculated using limit of detection (LOD, 0.01 mg/kg) as values could only be measured at one sampling point in depuration phase. #Significant difference in uptake compared to uncoated (UNC) NPs ($p < 0.050$)

In the CeO_2 NPs dietary exposure study higher levels of uptake in the stomach tissues as well as in the carcasses of fish exposed to the coated CeO_2 NPs (CeO_2-CIT (stomach; p 0.055)(carcass; p 0.04)) and CeO_2-PEG ((stomach; p 0.03)(carcass p 0.048)) compared to UNC CeO_2 NP were evidenced (Table 5 and Fig. 3). The depuration of Ce levels was slow in the stomach, whereas a fast depuration was observed from fish carcass in all treatment groups (Fig. 3c), with no appreciable levels above control values following 24 h of depuration. Due to this fast depuration K_2, α, $t½$ and BMF_k values could not be determined. In intestinal tissues, similar levels of uptake were measured among treatment groups (Table 5). A fast depuration was also seen in all treatment groups with no levels detected after 7 days of depuration. However, later in the depuration phase, high Ce levels were measured in the intestine of CeO_2-CIT and CeO_2-PEG NPs exposed fish (Fig. 3b), which indicate lack of complete depuration from or redistribution later in the depuration phase. The low BMF_{kgL} values calculated in the stomach, or BMF_{ssL} assumed for intestine and fish carcass indicated no significant bioaccumulation of CeO_2 NPs for any of the treatments (uncoated or coated CeO_2 NPs) according to a $BMF \geq 1$ that has been suggested as a threshold to aid in bioaccumulation potential assessment [27].

Discussion

There has been little discussion about the effects of different surface coatings on the uptake, biodistribution and bioaccumulation of NPs in fish. Yet, if identified, distinct uptake, bioaccumulation and distribution patterns for different coated NPs may have obvious biological consequences. Furthermore, the use of coatings to control behaviours/biocompatibility of otherwise unpredictable/unstable core NPs may prove a useful safer by design strategy. Before such an approach can be realised, distinct surface coating and effect relationships need to be identified. In the present study, the influence of citrate and PEG coatings on the bioaccumulation and biodistribution of TiO_2 and CeO_2 NPs in rainbow trout following dietary exposure (100 mg NPs/kg feed) was investigated. Levels of Ti and Ce in coated TiO_2 and CeO_2 NPs-exposed fish tissues (stomach, intestine, remaining carcass, liver and gill) following uptake (10 days) and during a depuration period (42 days) were monitored and compared to control fish and fish exposed to uncoated NPs. The rationale for using the same coatings and two different core NPs was to investigate if the coatings would confer the same bioaccumulation and distribution pattern (e.g. would PEG-coated TiO_2 and PEG-CeO_2 NPs lead to similar biokinetic profiles compared to the uncoated NPs?). Also a longer depuration phase than the 28 days recommended in TG 305 for bioaccumulation assessment in fish was used due to evidences of lack of complete elimination for some metal oxide NPs (ZnO NPs) in previous dietary bioaccumulation studies [25]. Furthermore, TiO_2 and CeO_2 NMs have proven very biodurable and acid tolerant when tested in simulated gastrointestinal environments [28, 29]. Thus, it is likely that these materials won´t experience dissolution, however the different coatings present may lead to different bioaccumulations and biokinetics of distribution.

Influence of particle properties on uptake

A detailed physico-chemical characterisation of the suspensions prepared for feed spiking was first performed to identify any differences in NPs size distributions and surface charges that may also influence the particles

biokinetics. Physico-chemical analysis revealed that the uncoated TiO_2 NPs used aggregated in aqueous suspension with primary particles forming aggregates 148.9 nm in size (Z-average). Coating TiO_2 NPs with CIT and PEG prevented aggregation, and suspensions had particles with average size distributions of 15.9 and 15.7 nm, respectively, thus indicating that single particle stabilisation through electrostatic interaction and steric hindrance was achieved. This direct effect of coating on NPs size meant that fish fed diets spiked with TiO_2-UNC NPs were exposed to TiO_2 NPs aggregates > 100 nm in size, whereas fish that received coated TiO_2 NPs-treated feed were exposed to predominantly single particles ~ 15 nm in size. For this reason, the higher uptake evidenced in stomach tissues and the increased distribution to liver tissues for smaller sized coated TiO_2 NPs may also have been influenced biokinetics.

Distinct differences in size distribution were not evidenced in the case of coated and uncoated CeO_2 NPs. Uncoated CeO_2 NPs appeared well dispersed in aqueous suspension with hydrodynamic sizes of 6.9 nm measured. Coating with CIT and PEG actually increased the average hydrodynamic size (Z-average size: 68.6 and 114.6 nm, respectively). However, looking more closely at the volume distribution mode one can see that actually 97% and 88% of particles were between 8.5 and 16.1 nm in CeO_2-CIT and CeO_2-PEG suspensions, respectively. A similar volume distribution was measured for CeO_2-UNC suspensions (97%; 8.5 nm). Therefore, the size distribution of CeO_2 NPs was consistent between the uncoated and coated NPs treatment groups and all fish received predominantly CeO_2 NPs 8–16 nm in size. A similar size pattern (< 20 nm) according to number distribution was observed. Thus, this allowed the influence of coatings on uptake, depuration and overall biokinetics exclusively to be determined. Again as evidenced in the case of the coated TiO_2 NPs exposed fish, an increased uptake was measured in stomach and carcasses of fish exposed to the coated CeO_2 NPs. Thus, this provides evidence of a distinct coating effect on levels of uptake conferred on both types of metal oxide NPs not related to the size of the NPs.

Surface charge is also seen as an important factor in uptake, translocation and oral bioavailability of NPs [30]. Both citrate and polyethylene glycol coatings reversed the zeta potential of uncoated NPs in aqueous suspension producing negatively charged CIT and PEG-coated TiO_2 (− 26 mV (TiO_2-CIT), − 25 mV (TiO_2-PEG)) and CeO_2 NPs (− 32 mV (CeO_2-CIT), − 29 mV (CeO_2-PEG)). This is expected as the anionic citrate ions and negatively charged methoxy-PEG coat the NPs surface and create a net negative surface charge that can be inferred from the negative zeta potential values measured. In general, positively charged NPs have shown enhanced uptake and translocation following oral exposure (due to electrostatic interaction with negatively charged biological membranes) [30]. In this study, there was little evidence of increased uptake or biodistribution for positively charged uncoated TiO_2 or CeO_2 NPs compared to negatively charged coated NPs. In fact, we have observed the opposite with higher levels of uptake in fish bodies and tissues following exposure to negatively charged CIT and PEG-coated CeO_2 NPs. Our results indicated that irrespective of the size and surface charge, the coatings CIT and PEG favoured the uptake of both metal oxide NPs in fish.

Biokinetics of TiO_2 NPs–influence of CIT and PEG coatings on Ti biokinetics

Following dietary exposure to 100 mg TiO_2 NPs/kg food (equivalent to 2 µg TiO_2 NPs/g fish) for 10 days, Ti levels were elevated in the stomach and intestine of TiO_2 NPs treated fish compared to control fish fed normal diets. Uptake followed the order TiO_2-PEG > TiO_2-CIT > TiO_2-UNC in stomach tissues and TiO_2-CIT > TiO_2-UNC > TiO_2-PEG in intestinal tissues. This may suggest a lower distribution for Ti from stomachs of TiO_2-PEG treated fish to the intestine. Unlike citrate coating, PEG coating has been shown to be stable in simulated gastrointestinal fluids [31]. Also, PEG coating has been shown to increase mucus adhesion and penetration of NPs [32] and thus TiO_2-PEG NPs increased adhesion to the gastric mucosa may explain the lower transit through the gastrointestinal tract to the intestine. This, together with the evidence of a small proportion of larger aggregates in PEG-coated TiO_2 NPs suspensions (~ 1100–2800 nm) may also be contributing to the lower transit and may explain the distinct distribution of Ti in TiO_2-PEG NPs exposed fish. High basal Ti levels measured in liver (4.6 ± 0.5 mg Ti/kg) and gills (3.14 ± 1 mg Ti/kg) make it difficult to conclude if Ti translocated to the liver or gills. Recently, the uptake of TiO_2 NPs (NM-105) in the gastrointestinal tract (perfused intestine) and subsequent distribution to various fish organ systems has been reported [33]. However, in the study presented here significantly lower Ti levels were measured in livers of uncoated and TiO_2-PEG NPs treated fish, which, while difficult to comment on and explain, suggests limited bioavailability of these TiO_2 NPs following dietary exposure in rainbow trout. This is in accordance with studies which have been performed in rats showing a very low and limited absorption/bioavailability of TiO_2 NPs (uncoated, 26.4 ± 6.1 nm primary particle size) following repeated oral administration (up to 1041 mg/kg) [34]. Levels as low as 0.05% of the orally dosed TiO_2 NPs (^{48} V-radiolabeled, 7–10 nm primary particle size) have

been measured in the bodies of rats 7 days post-exposure [35]. Also Geraets and colleagues [36] have estimated the absorption of only 0.02% of an administered TiO_2 NPs dose in rat bodies using a suite of TiO_2 NPs (NM-100, NM-101, NM-102, NM-103, NM-104, NM-105). Overall the fast elimination of Ti (during depuration) seen in the present study, together with a lack of evidence of elevated Ti levels in the liver and gills or carcasses of rainbow trout compared to control animals, support the low bioaccumulation potential of TiO_2 NPs already evidenced in other species and irrespective of the surface coatings (CIT or PEG) present.

Biokinetics of CeO_2 NPs/influence of CIT and PEG coatings on Ce biokinetics

Following dietary exposure to 100 mg CeO_2 NPs/kg food (equivalent to 2 mg CeO_2 NPs/kg fish) elevated Ce levels were measured in the stomach, intestine, gill and carcass for all treatment groups (coated and uncoated). There was no measurable Ce in the liver of CeO_2 NPs-exposed fish in any of the treatment groups at the end of the uptake phase, but some levels were determined at the end of the depuration phase for the uncoated and PEG-coated CeO_2 NPs. Among the tissues analysed, Ce accumulated to the greatest extent in the stomachs of exposed fish with higher levels of Ce measured in CeO_2-PEG and CeO_2-CIT NPs-exposed fish stomachs compared to CeO_2-UNC NPs-exposed fish (1.84 ± 0.7 and 1.47 ± 0.5 mg Ce/kg vs 0.85 mg Ce/kg). While Ce levels in CeO_2-UNC and CeO_2-PEG NPs exposed fish stomachs were reduced to levels of about 0.25 mg Ce/kg following 24-h depuration (day 11), Ce levels in the stomachs of CeO_2-CIT treated fish remained high (1.06 ± 0.3 mg Ce/ kg) suggesting a possible surface coating-dependent influence on depuration. Furthermore, while a reduction in levels was seen later in the depuration phase, levels remained higher ($p < 0.05$) after 14 days of depuration (on day 24) compared to fish treated with uncoated and PEG-coated CeO_2 NPs. No apparent difference in physico-chemical properties (size distribution or charge) of CeO_2-CIT NPs compared to CeO_2-PEG NPs was identified during characterisation, and therefore a distinct coating-dependent effect can only be used to explain this behaviour. Electrostatic stabilised NPs (e.g. citrate coated) have been shown to be less stable than sterically stabilised particles (e.g. PEG or polyvinylpyrrolidone (PVP) coated) in increasing electrolyte concentrations and/or acidic pH (3.0) [37, 38]. Therefore, if it is hypothesised that the citrate surface coating is removed or there is protonation/deprotonation of particle surfaces in the gastrointestinal fluid (pH 2–4)) of the fish stomach, this may result in changes in CeO_2 NPs physico-chemical properties in vivo, including a change in surface charge and agglomeration state. Studies have shown rapid agglomeration of citrate-stabilised NPs in synthetic simulated gastric fluids forming large NP-pepsin complexes [19, 39]. Therefore, there may be two factors to consider, firstly that the size of agglomerates will likely influence the depuration pattern, while secondly also a change in surface charge may directly influence interaction with biological membranes and association with the gastrointestinal mucus membrane. Already the unique interaction of cationic and anionic Au NPs with different charges with lipid membranes has been demonstrated with increased penetration for cationic ligands seen [40] and enhanced gastrointestinal absorption for uncharged and hydrophobic materials [41]. This, together with the proven biomodification of coated nanomaterials in aquatic organisms [42], and evidence of formation of aggregations in aquatic organisms digestive systems [43], may explain the higher levels of Ce in the stomach of CeO_2-CIT NPs treated fish 24 h after depuration.

Levels of Ce measured in the intestine of treated fish remained consistent between coated and uncoated treatment groups, with similar depuration patterns and no detectable levels of Ce in fish intestines on day 17 (7 days of depuration). Interestingly, at the end of the depuration phase (day 52) (following 42 days of depuration) high levels of Ce were measured (similar to day 10) in the intestine of fish exposed to CeO_2-CIT and CeO_2-PEG-coated NPs. Recirculation effects may explain the increase seen at this late stage in the depuration phase as evidenced by Ce in the liver.

The lack of detectable Ce levels in liver tissues at the end of the uptake phase is in contrast to the reported appreciable levels measured in the liver (1.35 mg/g dry weight) of zebrafish following an aqueous exposure to CeO_2 NPs (500 µg/L for 7 days) (< 25 nm, uncoated from Sigma Aldrich) [44] (although the authors have alluded to the possibility of cross contamination from gut tissues). Similarly, a significant bioaccumulation of Ce in the liver of rainbow trout exposed to the same 25 nm CeO_2 NPs (10 µg/L for 96 h) through water has been reported [7]. However, accumulation only occurred in exposures in organic-rich brown water and no liver accumulation was evidenced for exposures in other water types (dechlorinated tap and green surface waters with low organic carbon content)). Studies performed in mice following a single CeO_2 NPs oral gavage dose (3.24 mg/mL; 13.0 ± 12.1 nm primary particle size from Degussa/Quimidroga) did not show accumulation of Ce in the liver [45]. Another study performed in rats suggests limited biodistribution, negligible bioavailability and rapid elimination of CeO_2 NPs (NM 212 (uncoated, 100–200 nm, $+33 \pm 2$ mV)) in faeces following oral gavage [46]. In contrast, intratracheal instilled CeO_2 NPs

(13.0 ± 12.1 nm primary particle size from Degussa/Quimidroga) were detected in mice livers at concentrations of up to 3.37% of the initial dose 180 days after exposure [45]. Persistence (half-life of 140 days) of Ce in lung tissues after intratracheal instillation of CeO_2 NPs (1 mg/kg; radiolabelled $^{141}CeO_2$ NM-212 NPs) has also been reported in rats [46]. From this, it is clear that the exposure route will likely dictate the distribution pattern, however as this is the first known experiment exposing fish to CeO_2 NPs through the dietary route, direct comparisons with the present study cannot be made at this time.

Ce levels measured in the gills of rainbow trout at the end of the uptake phase but not at the end of the depuration phase also indicate an active excretion of Ce through the gills during exposure and no accumulation of Ce in this tissue.

Analysis of carcass (fish bodies without stomach and intestine) showed a basal level of Ce in control fish (0.2–0.5 µg Ce/g) that was not detectable in individual tissues. This is consistent with the levels of natural Ce that have been reported for rainbow trout carcasses (head, viscera, fins, skeleton, and musculature) that ranged from 0.004 to 1.18 µg/g [47]. The total Ce levels in CeO_2 NPs-exposed fish carcasses at the end of the exposure phase followed the order CeO_2-PEG > CeO_2-CIT > CeO_2-UNC. CeO_2-PEG NPs and CeO_2-CIT NPs-treated fish had significantly higher ($P > 0.05$) levels of Ce compared to control levels (0.69 ± 0.3 and 0.42 ± 0.05, respectively, vs 0.10 ± 0.02 mg Ce/kg). Depuration from the carcass was not influenced by distinct depuration patterns. Measured Ce levels in CeO_2 NPs-exposed fish carcasses were reduced to the same extent and to levels below basal control levels following 24 h depuration (day 11).

We are aware that normally BMF values are calculated for the whole fish body. However, in the present study we were interested in investigating the contribution from fractions that are taken up into tissues and absorbed into systemic circulation (e.g. in essence the bioavailable fraction) and those that remain accumulated in the stomach and intestine. For this reason, the stomach and intestine were removed from exposed fish, they were washed (to remove any loosely associated NPs, fractions in undigested feed or faeces) and analysed separately. Interestingly, we have shown that there is an uptake of large fraction in both these organs and that they constitute primary organs of accumulation (showing higher levels of uptake per g tissue compared to the fish carcass). The levels of Ce in the stomachs of CeO_2 NPs-exposed fish remain long into the depuration phase (up to 42 days) and warrants further investigation. This together with the redistributions evidenced in intestinal and liver tissues, also late in the depuration phase, shows that the elimination of these materials is slow. The half-life of elimination in the stomach was 12 days for the coated-CeO_2 NPs and 24 days for the uncoated CeO_2 NPs. In the intestine, the uncoated CeO_2 NPs presented a half-life elimination of 2 days, whereas the coated NPs were not fully depurated from this tissue. Despite this slow elimination from these tissues and from the liver, the calculated kinetic or steady state assumed BMFs indicate no bioaccumulation of these NPs. Therefore, the presence or absence of coating did not contribute to an increase or decrease in bioaccumulation potential, but will play a role in the mechanism of action of potentially hazardous NPs in fish following exposure.

Conclusions

Dietary exposure to coated and uncoated TiO_2 and CeO_2 NPs (100 mg NPs/kg food equivalent to 2 mg NPs/kg fish) did not lead to bioaccumulation in rainbow trout according to the low BMF values derived following 10 days of uptake and a 42-day depuration period. However, distinct uptake, biodistribution and depuration profiles were observed (both between the different core NPs and among the different coated NPs). TiO_2 NPs were very quickly depurated from all the tissues, whereas CeO_2 NPs presented a slow elimination from stomach, intestine and liver. There was evidence of a higher uptake in the different tissues of the coated NPs compared to the non-coated TiO_2 NPs and CeO_2 NPs. In addition, the PEG coating facilitates a higher level of uptake for the CeO_2 NPs but not for the TiO_2 NPs, in comparison with the other two nanoforms. Therefore, there was no evidence of a specific coating conferring a consistent behaviour on both of the NPs tested. Specifically, the CIT and PEG coatings on CeO_2 NPs influenced the depuration profile and biokinetics of distributions of Ce in fish, resulting in slower depuration from stomach tissues and different redistributions to other organ systems (intestine and liver) over time.

Abbreviations

PEG: Polyethylene glycol; CIT: Citrate; UNC: Uncoated; OECD: Organisation for Economic Co-operation and Development; TG: Test guideline; BMF: Biomagnification factor; BMF_{ss}: Biomagnification factor at steady state; $BMF_{ss\text{-}L}$: Biomagnification factor at steady state lipid corrected; BMF_k: Kinetic biomagnification factor; BMF_{kgL}: Kinetic biomagnification factor lipid and growth corrected; ww: Wet weight; K_2: Depuration rate constant; K_{2g}: Depuration rate constant growth corrected; t ½: Time to reach 50% depuration; HF: Hydrofluoric acid; LOD: Limit of detection; LOQ: Limit of quantification; ICP-OES/MS: Inductively coupled plasma-optical emission spectrometry/mass spectrometry; HSI: Hepatosomatic index; SGR: Specific growth rate; SEM: Standard error of the mean.

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Authors' contributions
MC analysed, interpreted and presented all data and wrote the original draft manuscript. DHM devised and implemented the study and contributed to the article revisions. EC performed the ICP-MS/OES analysis of metal contents in stock suspensions, feed and digested tissues while IR lead all analytical work performed in this study. AG performed sample and tissue digestions. JMN contributed to the article revisions. FT provided and ensured good husbandry of the fish used in the study. MLFC devised the study, interpreted data, contributed to the writing of the original draft manuscript and its revision, and was responsible of funding acquisition. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analysed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
Ethics approval was granted to perform the experiments with fish, Ref PROEX 94.2/20.

Consent for publication
"Not applicable".

Competing interests:
The authors declare that they have no competing interests.

Author details
[1]Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Consejo Superior de Investigaciones Científicas (INIA, CSIC), Ctra. de la Coruña, Km 7.5, 28040 Madrid, Spain. [2]Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT), Av. Complutense 40, 28040 Madrid, Spain. [3]Escuela Técnica Superior de Ingeniería de Montes, Forestal y del Medio Natural, Universidad Politécnica de Madrid, 28040 Madrid, Spain.

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