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A Multiparametric Approach to Cerium Oxide Nanoparticle Toxicity Assessment in Non-Biting Midges

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Abstract: Cerium oxide nanoparticles (CeO_2 NPs) are included in the Organisation for Economic Co-operation and Development (OECD) priority list of engineered nanomaterials for assessment of their environmental impact. The present study was carried out to assess the CeO_2 NP toxicity to the freshwater midge *Chironomus riparius* larvae at concentrations of 2.5, 25, 250, and 2500 mg of CeO_2 NP/kg of sediment. Experiments were designed to assess the prolonged exposure of midges to CeO_2 NPs while adhering to OECD test guideline 218. The following parameters were investigated: CeO_2 NP uptake by larvae, oxidative stress parameters, in vivo genotoxic effects, and life trait parameters. Inductively coupled plasma–mass spectrometry analysis showed a significant positive correlation between the concentration of CeO_2 NPs in the sediment and its uptake by the larvae. No significant mortality was observed in *C. riparius*, and oxidative stress was not detected. The only significantly induced sublethal effect was genotoxicity, which began to manifest at a lowest-observed-effect concentration of 25 mg kg^{-1} of sediment and progressively increased at higher concentrations. Our results indicate that exposure to CeO_2 NP–contaminated freshwater sediments does not pose a risk to chironomids at environmentally realistic concentrations. However, the significant accumulation of CeO_2 NPs by chironomid larvae may pose a risk through trophic transfer to organisms further up the food chain. *Environ Toxicol Chem* 2020;39:131–140. © 2019 SETAC

Keywords: Chironomidae; Genotoxicity; Metal oxide; Nanoparticles; Toxicity; Sediment

INTRODUCTION

The potential for release of manufactured nanomaterials into the aquatic environment is growing each year due to a steady increase in nanomaterial production. Cerium oxide nanoparticles (CeO_2 NPs) production is at approximately 10 000 metric tonnes/yr (Milani et al. 2017). In 2014, 3240 metric tonnes were in immediate use in the European Union alone (Wang and Nowack 2018). Nano-CeO_2 is utilized in various fields of application, from the cosmetic to the automotive industry (Manier et al. 2011), with the majority of applications in electronics and optics, catalysis, coatings, and paints (Tella et al. 2015). By combining market information and material flow modeling on engineered nanomaterials (ENMs), Keller et al. (2013) have estimated that 300 metric tonnes of CeO_2 NPs/yr could be released into aquatic ecosystems. The same authors describe the key applications (coatings, paints, pigments, and cosmetics) that, combined, likely contribute up to 97% of the total ENM emissions to water (Keller et al. 2013). As a result, CeO_2 NPs are released into the environment, and it is likely that its presence in aquatic ecosystems will increase in the future. The principal accumulation of CeO_2 NPs in aquatic sediments stems from deposition from the air, sludge application via run-off, and wastewater effluents (Wang and Nowack 2018). Direct quantification of CeO_2 NPs in aquatic ecosystems is very challenging, and thus the predicted concentration estimates are based on modeling, with approximations in freshwater sediments of 0.2 to 95 µg kg^{-1} (Gottschalk et al. 2015; Wang and Nowack 2018) and in sewage treatment sludge of 350 µg kg^{-1} (Gottschalk and Nowack 2011; Gottschalk et al. 2015).

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Information on the effects of CeO_2 NPs on benthic communities is scarce. The specific properties of manufactured nanomaterials might result in novel environmental and biological behavior related to elevated reactivity, increased transport, and the interaction with subcellular components (Lead et al. 2018). There is a need for multiparametric and interdisciplinary approaches to understand the exposures of organisms to CeO_2 NPs, their uptake and potential toxic effects on biota, and therefore to environmental health.

Due to the rapid sedimentation of CeO_2 NPs, benthic organisms are most likely to be exposed (Selck et al. 2016) and are therefore suitable as bioindicators of freshwater pollution. Chironomids, a diverse group of macroinvertebrates with many species bound to sediment in the larval stage, are an ideal model organism for toxicity studies (Milošević et al. 2013). Chironomids have often been used in ecotoxicity tests, and an increasing number of studies have employed multiple endpoints (Lee and Choi 2009). The results of our previous study (Savić-Zdravković et al. 2018) indicate that chironomids could be used as indicators of NP pollution in freshwaters.

Because freshwater sediment acts as a sink for CeO_2 NPs, and because CeO_2 NPs can potentially interact with benthic organisms, the aim of the present study was to investigate the response of *C. riparius* (a sentinel benthic organism) to CeO_2 NP exposure at different levels of organization, from molecular to developmental.

MATERIALS AND METHODS

Test substance: CeO_2 NPs

A commercially available ceria nanopowder (CeO_2 NPs) was purchased from Sigma-Aldrich, with a particle diameter of <25 nm, as stated by the manufacturer.

Transmission electron microscopic (TEM) images were obtained for CeO_2 NPs using an FEI Tecnai G2 F30. Samples were prepared by drop-casting 1 to 2 drops of particle dispersions in ethanol onto a carbon-coated copper grid.

X-ray diffraction measurements were made using a Panalytical X'pert Pro multipurpose X-ray diffractometer for reflection geometry. Copper K-alpha radiation (wavelength 0.154 nm) was used by operating at 40 kV and 40 mA. Measurements were made in the 2-theta range from 1 to 90° in steps of 0.05°.

In X-ray photoelectron spectroscopy (XPS), the distribution of kinetic energy emitted can be measured using an appropriate electron energy analyzer, and in this way a photoelectron spectrum can be recorded. Also, XPS uses soft X-ray (200–1500 eV) radiation to analyze core levels. In the XPS analyses, the magnesium K-alpha (1253.6 eV) X-ray source was operated at 300 W. A pass energy of 117.40 eV was used for the survey spectra. The spectra were recorded using a 60° take-off angle relative to the normal surface.

A Brunauer–Emmett–Teller (BET) surface area analysis of CeO_2 NPs was performed with an Autosorb-iQ Station 1 (Quantachrome Instruments) in an N_2 atmosphere. The BET surface areas of CeO_2 NPs were obtained from N_2 adsorption and desorption tests at 77 K. The samples were degassed at 120 °C for 15 h before measurements were taken.

The hydrodynamic radius and the zeta potential of the CeO_2 NPs were measured at 25 °C with a Malvern ZetaNano ZS using a helium–neon laser source of 5 mW at a 633-nm wavelength. The CeO_2 suspension was prepared at a concentration of 0.1 mg mL^{-1} in deionized water. The suspension was not sonicated. The average hydrodynamic radius was measured every 5 min for 60 min after preparation. These measurements were performed to determine the suspension's instability over the course of time, because high sedimentation was expected.

The morphology, state of aggregation, and particle size of the CeO_2 NPs in sediment (250 and 2500 mg of CeO_2 NPs/kg of sediment) were analyzed by a FEI Nova Nano SEM 430 field emission scanning electron microscope (SEM) at intermediate acceleration voltage (10–20 kV). To investigate particles under vacuum with good resolution, the samples were coated with a thin layer of 10-nm gold via sputtering. To identify CeO_2 NPs more accurately, energy-dispersive X-ray spectroscopy (EDX) analyses were performed parallel to SEM. The use of EDX allowed us to locate CeO_2 and also map the location of CeO_2 on the sand surfaces at high precision.

Test organism: C. riparius

The test animal used in the present study was the non-biting midge *C. riparius* (Diptera, Chironomidae), from the larval to the adult stage. The specimens of *C. riparius* were from the stock culture at the laboratory of the Faculty of Science and Mathematics, University of Niš (Niš, Serbia), which were reared under the Organisation for Economic Co-operation and Development (OECD; 2004) guideline. According to the guideline, the larvae were reared in glass tanks (with shredded cellulose paper as sediment and a mixture of tap and deionized overlying water) at a temperature of 23 ± 2 °C, with a 16:8-h light:dark photoperiod and constant aeration, and they were fed with finely ground TetraMin. Freshly laid egg masses were collected and placed in Petri dishes filled with water from the stock culture aquaria (a mixture of tap and deionized water, with pH 7.6 and total hardness 350 mg L^{-1} $CaCO_3$). The larvae hatched after 2 d. For the bioassay, first-instar larvae from one egg mass were used for the entire experiment, to maintain genetic uniformity. The larvae were placed in the test vessels when they were 4 d old.

Bioassay design and exposure to CeO_2 NPs

The bioassay consisted of 2 experiments, to assess different endpoints: 1) assessment of CeO_2 life trait toxicity to adult midges, and 2) assessment of CeO_2 sublethal toxicity to midge larvae.

The larvae of *C. riparius* were exposed to CeO_2 NPs according to OECD test guideline 218 (Sediment-water chironomid toxicity test using spiked sediment; Organisation for Economic Co-operation and Development 2004). The larvae were exposed to sediment spiked with the following increasing

concentrations of CeO_2 NPs: 2.5, 25, 250, and 2500 mg of CeO_2 NPs/1 kg of sediment + control, each with 4 replicas. According to prediction data based on the Danish model, the estimated environmental concentration (EEC) of CeO_2 NPs in freshwater sediments is 0.2 to 45 $\mu g\ kg^{-1}$, and the EEC of sewage treatment sludge is 350 $\mu g\ kg^{-1}$ (Gottschalk et al. 2015). The EEC for the European Union and its subregions varies between 2 and 95 $\mu g\ kg^{-1}$ for freshwater sediments (Wang and Nowack 2018). Therefore, the lowest concentration of 2.5 $mg\ kg^{-1}$ used in the present study is considered the environmentally relevant worst-case scenario (after application of safety factors).

The complete experimental setup was repeated twice. For the test vessels, 600-mL glass beakers measuring 8 cm in diameter were employed. Quartz sand was used as the sediment (>50% of the particles were in the range of 50–200 μm). To spike the sediment, an appropriate concentration of CeO_2 NPs was thoroughly and evenly distributed throughout the wet sediment by hand mixing. Each test vessel had a 3-cm-deep sediment layer with a mixture of deionized and tap water (1:1) filled to the top (pH 7 ± 0.5; hardness 7.0 °dH; conductivity 326 $mS\ cm^{-3}$). The test vessels were placed in an isothermal room at 23 ± 1 °C, with a 16:8-h light:dark cycle. The sediment was aged for 3 d before the larvae were added. Twenty-five first-instar larvae were added to each test vessel. A total of 500 larvae were used in the first experimental setup (25 larvae × 4 concentrations and a control × 4 replicas) and another 500 in the second. Midge flies from the first setup were used for the life trait toxicity analysis, and larvae from the second setup were used to test for genotoxicity, oxidative stress, and inductively coupled plasma–mass spectrometry analysis (ICP–MS). Gentle aeration, through a plastic pipette fixed 2 cm above the sediment layer, was provided to every test vessel 24 h after addition of the larvae. The larvae were fed with TetraMin fish food flake mixture every second day (0.5 mg/larva/d, for the first 2 d, and 1 mg/larva/d for the rest of the experiment).

ICP–MS analysis of CeO_2. To measure the concentration of CeO_2 NPs in the midge larvae, fourth-instar larvae of *C. riparius* from the second experiment were washed thoroughly with distilled water and used for the analysis.

Approximately 0.1 g (wet mass) of chironomid larvae samples, stored in ethanol, were dried in a heating oven (Binder) at 75 °C overnight to obtain a constant mass. The samples were digested by microwave-assisted acid digestion using a CEM MARS 6 Microwave Digestion System (Matthews). Approximately 0.01 g of the dried sample was weighted and placed into a microwave vessel, and 1.0 mL of hydrogen peroxide (30% H_2O_2, suprapure; Merck Millipore) and 3.0 mL of nitric acid (67–69% HNO_3, suprapure; Carlo Erba) were added. The samples were subjected to closed vessel microwave digestion with the following program: ramp to 200 °C for 30 min, hold at 200 °C for 60 min, cool down to room temperature. After digestion, clear solutions were obtained. The digested samples were quantitatively transferred to 30-mL polyethylene tubes and diluted to a final volume of 30 mL with Milli-Q water. The same procedure, without a sample, was used to determine the blank value. All dilutions were made with Milli-Q water (18.2 MΩ cm) obtained from a Direct-Q 5 Ultrapure water system (Merck Millipore).

Quantification of Ce by ICP–MS was performed based on external calibration by measuring Ce standards in the concentration range of 0.1 to 1000 $\mu g\ L^{-1}$ with online internal standardization (25 $\mu g\ L^{-1}$ solution of rhodium). Matrix-matched calibration standard solutions were prepared from the Ce stock solution (1000 $\mu g\ Ce\ mL^{-1}$ in 5% HNO_3) obtained from Merck.

For ICP–MS analysis, an Agilent 7900 ICP–MS device equipped with an autosampler (SPS4; Agilent) was used. Optimization of the instrumental parameters (summarized in the Supplemental Data, Table S1) was performed daily to achieve satisfactory sensitivity and low levels of oxides and doubly charged ions.

To evaluate the accuracy of the analytical method, standard CeO_2 NPs (20 wt % of CeO_2 NP suspension; US Research Nanomaterials), standard reference material (SRM) 2976 for mussel tissue (US National Institute of Standards and Technology) containing 0.109 + 0.008 mg/kg of Ce, and SRM 2976 spiked with the CeO_2 NP suspension were digested in the same way as the samples. The CeO_2 NP suspension was first dispersed using a vortex (10 s) and ultrasonic bath (20 min) and diluted to obtain a suspension with a final CeO_2 concentration of 10 $\mu g\ mL^{-1}$. Then 100 μL of that suspension was transferred to the microwave vessels and subjected to digestion. Good recoveries (121 ± 4%, $n = 3$) were achieved. For digestion of SRM 2976, 0.1 ± 0.005 g of the sample (dry mass) was used, and the Ce concentration determined was 0.097 + 0.013 $mg\ kg^{-1}$ ($n = 3$). In addition, 0.1 g of SRM 2976 was spiked with 100 μL of 10 μg CeO_2 NPs mL^{-1}, again subjected to the same microwave-assisted digestion, and measured by ICP–MS. Recoveries of 93 ± 1% ($n = 3$) were obtained.

To calculate concentrations of CeO_2 intake by the treated larvae, the following equation was used: CeO_2 concentration = (Ce concentration of the experimental sample–background Ce concentration of the control) × mass ratio of CeO_2/Ce (mass ratio of CeO_2/Ce = 1.2286).

The data were analyzed for normality and homogeneity using the Kolmogorov–Smirnov statistic (with Lilliefors significance correction).

To investigate the correlation between the concentration of CeO_2 NPs in the chironomid larvae and the exposure concentrations, a nonparametric correlation test was conducted, the Spearman rank order correlation test (Spearman's rho).

To compare the concentrations of CeO_2 NP intake by the larvae between the treatments, the results were statistically tested by one-way analysis of variance (ANOVA).

The analysis was conducted using the SPSS statistical software package (IBM), Ver. 15.0 for Windows. The significance level was set at $p < 0.05$.

Life trait toxicity analysis. The number of fully emerged midges was recorded each day. The experiment was terminated 5 d after the last adult had emerged.

The emergence ratio, mortality, developmental time, and developmental rate were calculated according to OECD test guideline 218 (Organisation for Economic Co-operation and Development 2004).

The data were analyzed for normality and homogeneity using the Kolmogorov–Smirnov statistic (with Lilliefors significance correction), and the Shapiro–Wilk and Levene statistics.

The variability of mortality was tested using a nonparametric ANOVA (the Kruskal–Wallis test) with the Mann–Whitney test used for post hoc comparison. The variability of the developmental time and developmental rate was tested using one-way ANOVA with Dunnett's post hoc test used for pairwise comparison.

Analyses were done in the SPSS statistical software package, Ver. 15.0 for Windows. The significance level was set at $p < 0.05$.

Total protein content and oxidative stress analysis. Fourth-instar larvae of *C. riparius* from the second experiment were homogenized and used to measure the oxidative stress parameters induced by CeO_2 NPs.

Total protein concentration in the samples was used to express the values of the oxidative stress parameters, estimated using the method of Lowry et al. (1951), with bovine serum albumin as the standard.

Lipid peroxidation was determined by measuring the concentration of malonyl dialdehyde (MDA) using a method modified from Ohkawa et al. (1979). A sample of 200 µL of homogenized tissue was mixed with 200 µL of 8.1% sodium dodecyl sulfate, 1.5 mL 20% acetic acid (pH 3.5) and 1.5 mL of 0.81% thiobarbituric acid (TBA), and incubated for 60 min at 95 °C. After cooling on ice and centrifugation, the absorbance in the supernatant was measured at 532 and 600 nm with a Libro S22 spectrophotometer (Biochrom). The total absorbance was determined using the formula: $A_{total} = A_{532\,nm} - A_{600\ nm}$.

The MDA levels were determined using the molar absorption coefficient for the MDA–TBA complex of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

The superoxide dismutase (SOD) levels were measured by conducting a SOD assay modified from the method of Flohé (1984). The SOD activity was calculated from the percentage of inhibition of the reaction of xanthine oxidation ($\Delta A / min \approx 0.025$), which creates a superoxide anion as a substrate for the SOD present in the samples. The superoxide anion not used by the enzyme oxidizes the cytochrome. An undiluted sample (25 µL) was mixed with 1.45 mL of the reaction solution (cytochrome C, 0.05 mM; xantin, 1 mM mixed at a 10:1 ratio with 5,5′-dithiobis-(2-nitrobenzoic acid)). A volume of 20 µL of xantin oxidase ($0.4\ U\ mL^{-1}$) was added to start the reaction. The reaction was measured over 3 min at 550 nm with a Libra S22 spectrophotometer (Biochrom). One unit of total SOD activity was defined as the amount of enzyme required to achieve 50% inhibition in the typical calibration curve obtained with standard SOD. The horse heart cytochrome C (type VI), human blood SOD (type I, lyophilized powder, $2400\ U\ mg^{-1}$ protein), xanthine, and xanthine oxidase ($200\ U\ mL^{-1}$) were from Sigma-Aldrich.

The data were analyzed for normality and homogeneity using the Kolmogorov–Smirnov statistic (with Lilliefors significance correction), and statistically tested by one-way ANOVA followed by the post hoc Dunnett test in the SPSS statistical software package, Ver. 13.0 for Windows. The significance level was set at $p < 0.05$.

Genotoxicity analysis: Comet assay. To determine whether CeO_2 NPs can damage DNA in somatic cells, fourth-instar larvae of *C. riparius* from the second experiment were used for the analysis. Hydrogen peroxide (H_2O_2, 100 mM for 1 h at 4 °C) was used as positive control (Lee et al. 2008).

The comet assay was performed according to Singh et al. 1988, with minor modifications (Bernabò et al. 2017). The phosphate-buffered saline agar, without calcium and magnesium, and low-melting point agar (LMA) were obtained from Alfatrade Enterprise (Serva Electrophoresis).

To determine the level of DNA damage in the somatic cells, 10 live larvae/experimental treatment were placed in 1 mL of suspension buffer (1 × phosphate-buffered saline, 20 mM ethylenediaminetetraacetic acid, 10% dimethyl sulfoxide, pH 7.4), disintegrated mechanically by mincing, filtered by pressing against gauze, and then resuspended in a 3-mL suspension buffer. The homogenate was centrifuged for 15 min at 1500 rpm at 4 °C and resuspended in 150 µL of the suspension buffer. From the cell suspension, 10 µL was mixed with 75 µL of 1% LMA, and 75 µL of this mixture was spread onto a microscope slide.

The slides with cells were stained with ethidium bromide for 10 min in the dark, then covered with a cover glass, and observed under a Nikon fluorescence microscope (Ti-Eclipse) attached to a charge-coupled device camera. A total of 150 cells (50 cells from each of 3 replicate slides) were scored and analyzed. For each slide, the extent of DNA damage was determined visually by the categorization of comets into different classes of migration (Collins 2004). To rule out subjectivity, the visual observer did not know in advance which samples came from which treatments. Comets were classified into 5 categories, defined as types 0, 1, 2, 3, and 4, where 0 indicates no or very low damage, and 1, 2, and 3 low, medium, and long DNA migration, respectively, with 4 as the highest level of degradation, that is, comets with very small heads and long tails.

The total comet score was calculated by the following equation, modified from Miyaji et al. (2004): (% cells in class 0 × 0) + (% cells in class 1 × 1) + (% cells in class 2 × 2) + (% cells in class 3 × 3) + (% cells in class 4 × 4). Thus, the total score could range from 0 (all undamaged) to 400 (all maximally damaged). Representative photomicrographs of the comet assay showing the DNA migration in *C. riparius* fourth-instar larvae after treatment with CeO_2 NPs are provided in the Supplemental Data, Figure S2.

Statistical differences between the control and the treated larvae were examined by one-way ANOVA using the SPSS statistical software package, Ver. 13.0 for Windows. The significance level was set at $p < 0.05$.

RESULTS

CeO_2 NP characterization

The TEM investigation revealed that CeO_2 NPs had broad size distributions. The particles were angular in shape (with corners). Particle size varied between 23 and 29 nm. According

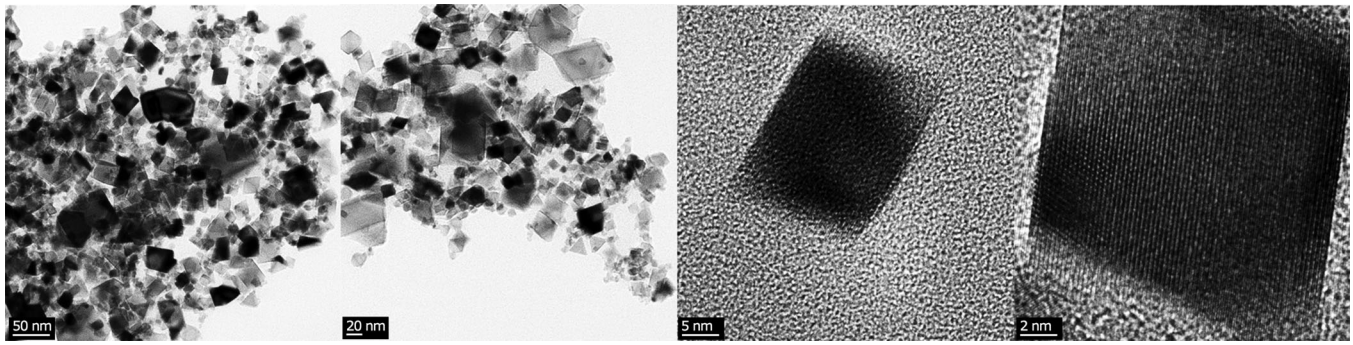

FIGURE 1: Transmission electron microscopic (TEM) images of CeO_2 nanoparticles obtained using an FEI Tecnai G2 F30.

to the TEM images, the particle size ± standard error of the mean was 25 ± 1.8 nm (Figure 1). In addition, aggregates of particles were noted in the ethanol suspension.

All the diffraction peaks detected in the XRD analysis were well defined and could be perfectly assigned to the fluorite crystal structure (face-centered cubic). The sharp peaks corresponded to the (111), (200), (311), (200), (220), (311), (222), (400), (311), (420), and (422) crystal planes of CeO_2 NPs. No peaks characteristic of other crystalline forms were detected (Figure 2A).

The XPS survey scan of the CeO_2 NP sample is shown in Figure 2B. The Ce3d spectra exhibited $Ce_{3/2}$ peaks between 915 and 900 eV and $Ce_{5/2}$ peaks between 895 and 875 eV, which are characteristic of CeO_2 (Figure 2C). The O1s spectra

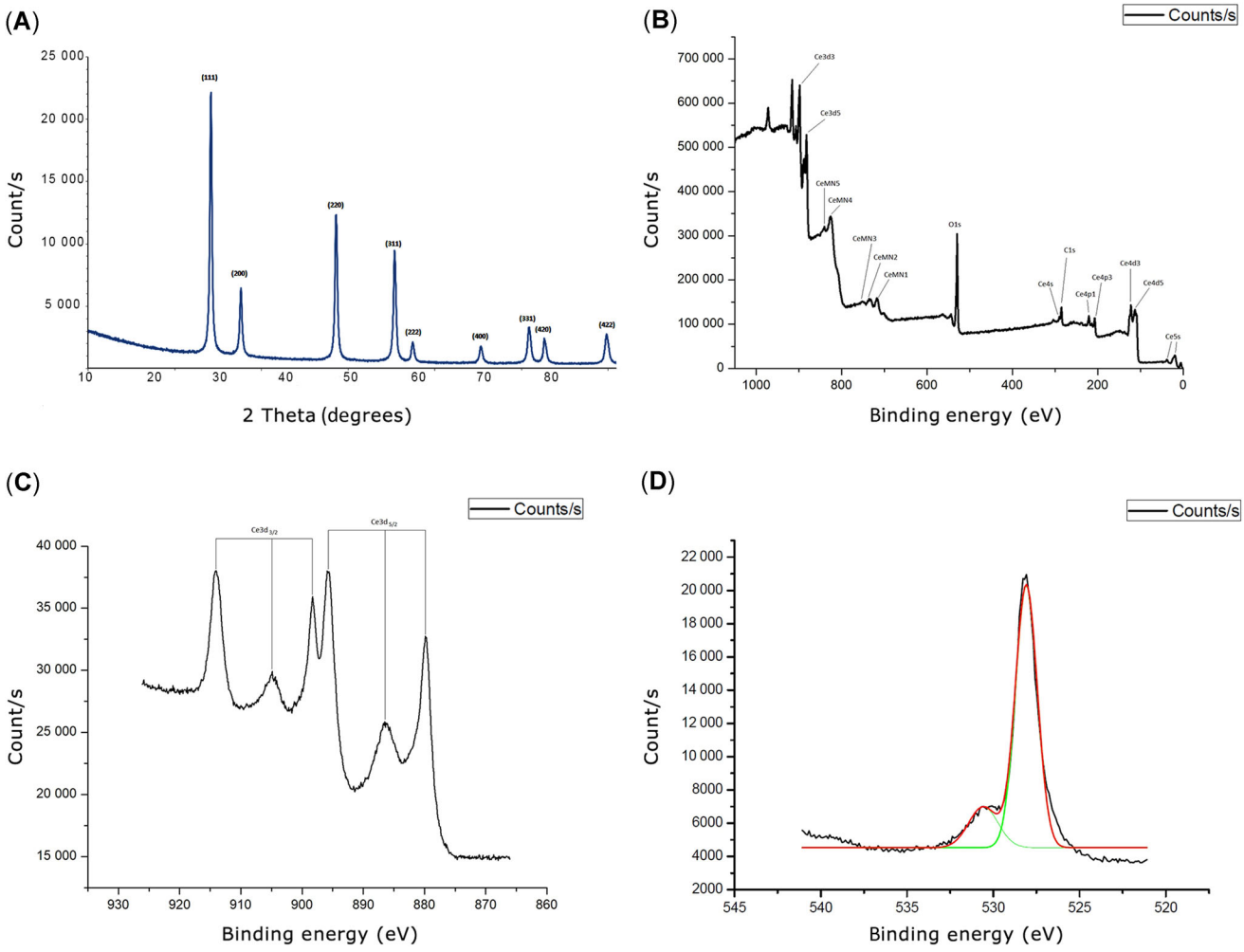

FIGURE 2: CeO_2 nanoparticle (NP) characterization. (**A**) X-ray diffraction (XRD) patterns of CeO_2 NP crystal structure, (**B**) X-ray photoelectron spectroscopy (XPS) spectra survey scan of CeO_2 NPs. (**C**) XPS spectra of Ce3d peak. (**D**) XPS spectra of O1s peak.

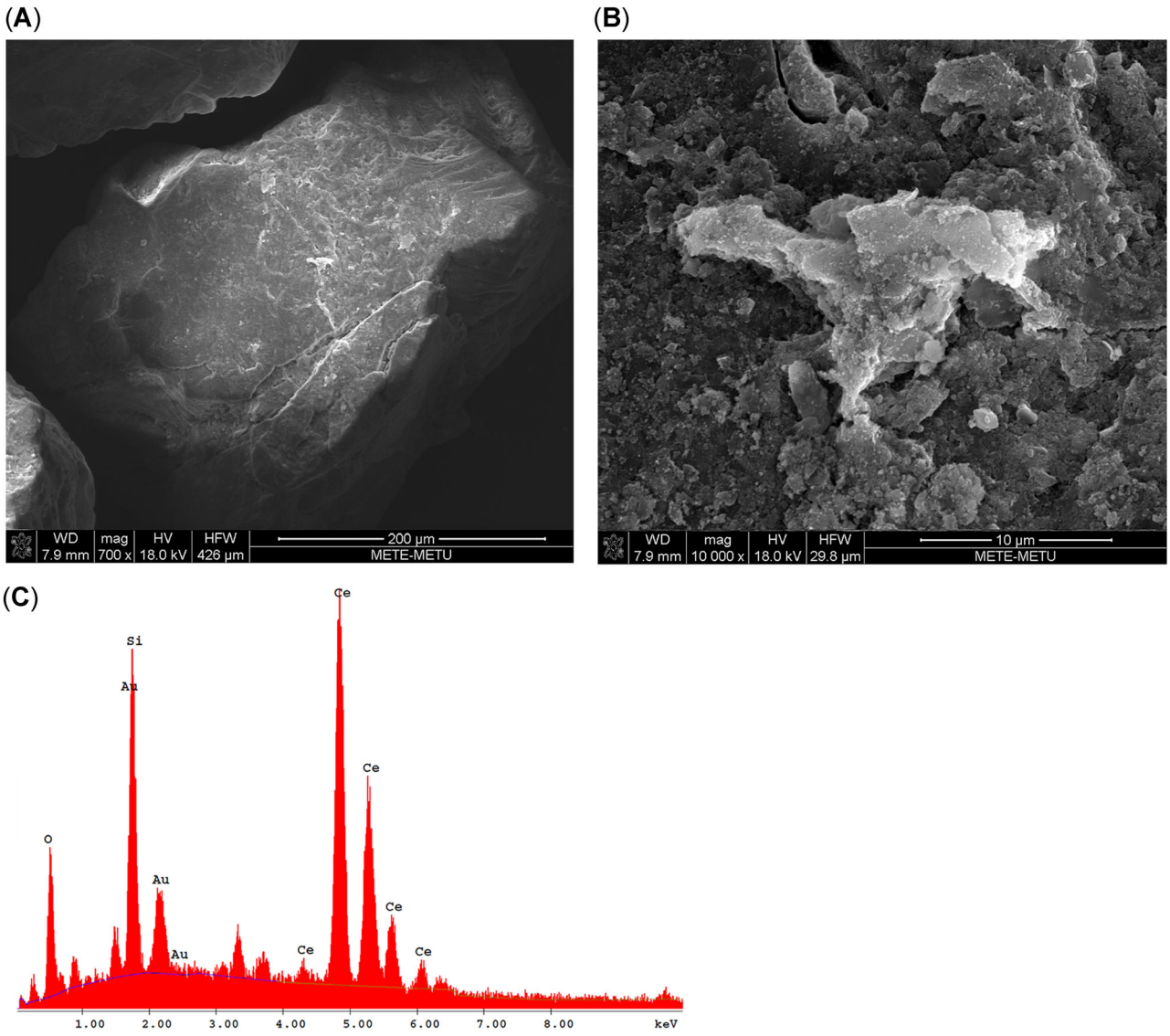

FIGURE 3: Scanning electron microscopy (SEM) images and energy dispersive X-ray spectroscopy (EDX) analysis of CeO_2 in sediment at 2500 mg/kg. (**A**) SEM image of a CeO_2 nanoparticle (NP) aggregate (green circle) located on a single grain of sand. (**B**) Magnified SEM Image of the same CeO_2 NP aggregates shown in A. (**C**) EDX scan result of the corresponding spot.

showed a main peak at 529.2 eV, assigned to oxygen that is bound to tetravalent Ce ions, and a shoulder at approximately 532.5 eV, which implies that the surface is partially covered with hydroxide OH groups (Figure 2D). The cerium-to-oxygen ratio also indicates that the CeO_2 was of fluorite crystal structure.

According to BET analysis, the specific surface area of the CeO_2 was calculated to be 42.602 $m^2\,g^{-1}$, and the pore volume was 1.172 cc g^{-1} and the pore radius 1075.761 Å.

The average hydrodynamic diameters of the CeO_2 NPs in deionized water were measured with dynamic light scattering and are shown in the Supplemental Data, Table S2). The average hydrodynamic diameter of the particles was measured as 671 ± 112 nm. The suspension was polydispersed in a range from 0.879 to 0.655.

In addition, the zeta potential value measured after the suspension was prepared in deionized water was 28 mV. This value indicated that CeO_2 NPs have moderate stability in deionized water.

An aggregated CeO_2 usually clung to silicon dioxide (SiO_2) particles in the sediment, as shown in a representative example (Figure 3A, green circle). Figure 3B is a higher magnified image of the same particle. The presence of CeO_2 in this particular case was also confirmed by EDX: the wt % values of Si and Ce were 17.32 and 82.68, respectively (Figure 3C). It was clear that CeO_2 were scattered as large aggregates in the sediment. Aggregates of CeO_2 had a broad size distribution ranging between 2 and 25 µm for tested concentration of 2500 mg/kg CeO_2, and aggregate sizes of CeO_2 clusters ranged from 5 to 10 µm for the tested concentration of 250 mg/kg CeO_2. It was deduced from these SEM images that not only were CeO_2 NPs aggregated but they also clung onto SiO_2 particles, because we could not locate any CeO_2 clusters on the metal stage.

TABLE 1: Total cerium (Ce) concentrations in *Chironomus riparius* larvae in fine sand determined by inductively coupled plasma–mass spectrometry and transformed as CeO_2[a]

Treatment (mg of CeO_2 NPs/kg of sediment)	Ce (mg/kg body wt)	CeO_2 (mg/kg body wt)
Control	10 ± 4	12 ± 5
2.5	53 ± 1	66 ± 1
25	251 ± 54	309 ± 66
250	1910 ± 300	2340 ± 370
2500	23 700 ± 5300	29 100 ± 6500

[a]Data are means ± standard deviations.
NP = nanoparticle.

CeO_2 NP ingestion by C. riparius larvae: ICP–MS

The ICP–MS analysis showed that CeO_2 concentrations in the *C. riparius* larvae varied from an average of 12 mg/kg body weight of CeO_2 in the controls to 29 100 mg/kg body weight in the highest treatment, and the total CeO_2 concentrations differed significantly among the treatments (one-way ANOVA: $p < 0.05$, $F = 69.331$; Table 1). Spearman's rank correlation revealed a high positive correlation (rho = 0.732, $p = 0.002$) between the concentration of CeO_2 NPs in the sediment and the CeO_2 NP content in the chironomid larvae.

CeO_2 NP life trait toxicity to C. riparius

Midges started emerging on the 10th d after the larvae were introduced into the sediment, and the last adult emerged on day 22. The experiment was terminated on the 27th d, 5 d after emergence of the last adult in the control. The life cycle endpoint analysis revealed that the highest number of midges emerged in the control (23 specimens) within an average of 15 d (Table 2 and Supplemental Data, Figure S1). The success of emergence varied between an average of 76 and 92%, with the developmental rate varying from an average of 5.96 to 11.2%/d. The one-way ANOVA showed no significant difference in the life trait endpoints among the treatments and no significant difference between treatments and control.

Oxidative stress

Protein content in the larvae varied from an average of 0.81 to 1.20 mg/mL of the tested sample. The values of measured oxidative stress parameters are expressed in relation to the total protein concentration in the samples, and the MDA concentration varied from an average of 14.07 to 23.16 nmol/mg of protein; SOD activity ranged from an average of 38.68 to 54.41/mg of protein in the samples (Table 3).

One-way ANOVA, followed by the post hoc Dunnett's test, showed no significant differences between the treated larvae and no significant difference between treatments and control in terms of their oxidative stress response.

Genotoxicity

The genotoxic effects after exposure to different concentrations of nano-CeO_2 are presented in Table 4.

The concentrations of 25, 250, and 2500 mg kg^{-1} showed a significant dose-dependent increase in the total comet scores (32.1, 65.3, and 146.1, respectively) compared with the appropriate negative control (13.9). The treatment with the lowest concentration (2.5 mg kg^{-1}) showed a significantly lower total comet score compared with the negative control and the other tested concentrations of CeO_2 NPs. A concentration-dependent response was detected.

The mean amount of DNA damage in the positive control was statistically different from that of the negative control. It should be mentioned that a concentration of 2500 mg significantly increased the DNA damage compared with all of the other groups examined.

The data also showed that comet class 0 was the most frequent among the larvae in the negative control and larvae treated with 2.5 and 25 mg of CeO_2, whereas comet class 4 was found only among the larvae treated with H_2O_2 and the highest dose of CeO_2 NPs.

DISCUSSION

The use of CeO_2 NPs is increasing rapidly, indicating the importance of investigating its ecotoxicological effects. The present study used a multiparametric approach, scaling the endpoints along the levels of biological organization, to investigate the response of freshwater non-biting midge larvae to CeO_2 NP exposure.

Engineered nanomaterials are likely to be stored in natural water sediments (Keller et al. 2010), and ongoing research, although sparse, is attempting to determine the bioavailability and toxicity of ENMs in this complex environment. Hence, we investigated the prolonged exposure of sediment-dwelling *C. riparius* larvae to different concentrations of CeO_2 NPs in the sediment.

The ecotoxicity of nanoparticles depends greatly on their characteristics, such as their morphological properties,

TABLE 2: Life cycle parameters in *Chironomus riparius* exposed to CeO_2 nanoparticles (CeO_2 NPs)

		Treatment (mg of CeO_2 NPs/kg of sediment)			
Parameter	Control	2.5	25	250	2500
ER ± SD (%)	92 ± 13.47	81 ± 16.12	76 ± 37.66	74 ± 27.03	83 ± 8.87
M ± SD (%)	8 ± 13.46	19 ± 16.12	24 ± 37.66	26 ± 27.03	17 ± 8.87
DT ± SD (d)	15.15 ± 2.97	15.08 ± 3.74	15.32 ± 0.57	14.90 ± 2.79	16.77 ± 2.99
DR ± SD (%)	7.17 ± 1.62	9.77 ± 6.68	11.2 ± 9.2	7.67 ± 1.2	5.96 ± 0.85

ER = emergence ratio; M = mortality rate; DT = developmental time; DR = developmental rate; SD = standard deviation.

TABLE 3: Oxidative stress parameters in *Chironomus riparius* exposed to CeO_2 nanoparticles (CeO_2 NPs)[a]

		Treatment (mg of CeO_2 NPs/kg of sediment)			
Parameter	Control	2.5	25	250	2500
Proteins (mg proteins/mL sample)	1.063 ± 0.05	1.17 ± 0.20	1.20 ± 0.14	1.16 ± 0.17	0.81 ± 0.24
MDA concentration (nmol/mg protein)	16.08 ± 2.19	16.08 ± 5.32	20.62 ± 8.43	23.16 ± 7.61	14.07 ± 2.60
SOD (activity/mg protein)	49.90 ± 4.68	38.68 ± 13.20	40.39 ± 11.41	40.02 ± 12.58	54.41 ± 13.71

[a]Average values ± standard deviation.
MDA = malonyl dialdehyde; SOD = superoxide dismutase.

composition, size, and surface area (García et al. 2011; Lai et al. 2018), and therefore a precise characterization of the nanomaterials tested in the present study was carried out. The batch we used was commercially purchased CeO_2 NPs with a primary particle diameter, as provided by the manufacturer, of <25 nm. Empirical tests showed that the particle size varied between 23 and 29 nm, with mean dimensions of 25 ± 1.8 nm. The importance of precise characterization has been confirmed by many studies (Lead et al. 2018); for example, a study by Taylor et al. (2016) showed that 4- to 5-nm ceria NPs induced different responses in algae (with internalization into intracellular vesicles noted) compared with larger scale particles and dissolved phase Ce, even though the overall toxicity was low in all cases. In addition, the chemical properties and behavior of nanomaterials are important factors in bioaccumulation and bioavailability in invertebrates (Lead et al. 2018). The current standard assessment methodology should be used for nanomaterials with caution, and NP physicochemical properties must be included in the analysis to obtain a better understanding and comparison of the ecotoxicity testing results (Petersen et al. 2015).

In general, CeO_2 NPs are known for their positive biological effects, and thus represents a material suitable for medical purposes (Zhang et al. 2011). However, several studies have revealed CeO_2 NP toxicity to daphnids (Lee et al. 2009; García et al. 2011), chironomids (Lee et al. 2009), bivalves (Koehlé-Divo et al. 2018), nematodes (Roh et al. 2010; Zhang et al. 2011), and algae (Rogers et al. 2010; Manier et al. 2011, 2013), indicating that, despite its possible advantages in medicine and other industries, CeO_2 NPs could have a negative impact on the environment. Specifically, the ability of aquatic invertebrates to accumulate NPs has been proved in many studies (García et al. 2011; Khan et al. 2015; Lead et al. 2018). Chironomid larvae are particularly susceptible to ingesting significant amounts of CeO_2 NPs and can serve as a vector of contamination for amphibian larvae, in which they can induce genotoxicity (Bour et al. 2017) and mortality (Bour et al. 2016). In the present study, chironomid larvae accumulated CeO_2 NPs at concentrations at least 10 times higher than the exposure concentration. Concentrations of CeO_2 NPs in the bodies of the larvae were significantly and positively correlated with an increase in the CeO_2 NP concentration in the sediment. This finding is in accordance with the microcosm study by Bour et al. (2017), in which, after chronic exposure to 1 mg L^{-1} of CeO_2 NPs, chironomid larvae accumulated on average 282 mg kg^{-1} body weight of CeO_2 NPs; in a 4-wk-long mesocosm study (Bour et al. 2016), chironomids accumulated an average of 266 mg kg^{-1} of CeO_2 NPs without any recorded toxicity. Chironomid larvae have considerable bioturbation activity and because they have a large digestive tract relative to their body size, there is the potential for a large amount of contaminated sediment storage within their guts, which explains the high CeO_2 NP concentration in the organism.

The results of in vivo genotoxicity analysis demonstrated that CeO_2 NPs were able to cause DNA damage in fourth-instar larvae of *C. riparius* in a dose-dependent manner. This was in agreement with Lee et al. (2009), who reported similar results after exposing *C. riparius* to 1 mg L^{-1} of 15 and 30 nm CeO_2, with smaller-sized CeO_2 NPs causing more DNA damage. Lee et al. (2009) also reported an increase in *C. riparius* mortality, which was not the case in our study. However, in the study by Lee et al. (2009) the mortality was very small: 10 ± 0% for 15-nm particles (0% for 30 nm) versus 5 ± 4% mortality in the control. The mortality of 10% in *C. riparius* toxicity tests is well within

TABLE 4: Genotoxic effect of nano-CeO_2 in *Chironomus riparius* fourth-instar larvae

	Comet class					
Treatment	0	1	2	3	4	Total comet score[a]
NC	86.1 ± 0.34	13.9 ± 0.20	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	13.9 ± 0.61†
PC	46.4 ± 1.02	23.3 ± 0.35	16.4 ± 0.95	8.2 ± 0.42	5.7 ± 1.2	103.5 ± 0.8*
2.5	82.4 ± 0.60	17.6 ± 0.12	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	17.6 ± 0.47†
25	83.9 ± 0.30	6.5 ± 1.20	3.2 ± 0.32	6.4 ± 0.80	0.00 ± 0.00	32.1 ± 0.64*†
250	47.8 ± 0.43	39.1 ± 0.17	13.1 ± 0.24	0.00 ± 0.00	0.00 ± 0.00	65.3 ± 0.40*†
2500	38.5 ± 0.60	15.4 ± 0.82	15.3 ± 0.42	23.1 ± 0.70	7.7 ± 0.41	146.1 ± 1.02*†

[a]Values are mean ± standard error of the mean from 3 independent experiments.
[b]NC = negative control; PC = positive control, 100 mM H_2O_2.
*$p < 0.05$ compared with the negative control group.
†$p < 0.05$ compared with the positive control group.

the expected control values and cannot be considered an effect of the toxicant even if it is statistically significant, especially when the actual control had a mortality of 5 ± 4%. As with mortality, in the present study there were no observable effects on other life trait parameters. This is in agreement with previous research by Bour et al. (2015) in which no significant mortality, growth inhibition, delay in larval development, total emergence, or mouthpart deformities occurred after acute 48-h exposure of chironomid larvae to a range of concentrations from 0.01 to 100 mg L^{-1} of CeO_2 NPs and is also in accordance with the 4-wk mesocosm study of Bour et al. (2016) in which the larvae were exposed to of 1 mg L^{-1} of CeO_2 NPs. Studies of nanoceria effects on other species show somewhat contradictory results. No acute toxicity to *Daphnia magna*, *Thamnocephalus platyurus*, or *Danio rerio* was detected at 1, 5, and 200 mg L^{-1} of CeO_2 NPs, respectively (Hoecke et al. 2009), whereas median effect concentration growth inhibition for *Pseudokirchneriella subcapitata* was determined to be 10.3 mg L^{-1} in a study by Rogers et al. (2010) and 2.4 to 29.6 mg L^{-1} in a study by Rodea-Palomares et al. (2010) using the same species.

Even though the present study showed no connection between genotoxicity and other investigated endpoints, DNA damage raises concerns about possible higher level implications. On the other hand, chironomid larvae did not experience statistically significant differences in oxidative stress response. This could be due to the known antioxidant effects of CeO_2 NPs, which is the reason why these nanoparticles are used in biomedicine and nanopharmacy, to protect biological tissue from the oxidative stress caused by reactive oxygen species (ROS; García et al. 2011). Several studies indicate that CeO_2 NPs show antioxidant properties; for example, in a study by Xia et al. (2008), 25 mg L^{-1} CeO_2 NPs suppressed ROS production and induced cellular resistance to oxidative stress. In contrast, some studies have shown cytotoxic effects on human lung cells at 5 to 40 mg L^{-1} (Park et al. 2008), to bacteria at 1.2 to 730 mg L^{-1} (Thill et al. 2006; Pelletier et al. 2010), and to green algae and cyanobacteria at 10 µg L^{-1} (Rodea-Palomares et al. 2012). A metabolomics and transcriptomics approach used on *Chlamydomonas reinhardtii* detected no significant molecular responses to environmentally relevant concentrations, but it did detect molecular responses to the supra-environmental level of 10 000 µg L^{-1} of CeO_2 NP exposure (Taylor et al. 2016). These differences in results might be due to different exposure designs, as well as differences in the size of the NPs used (from <4 to >30 nm) and their concentrations (from 10 µg to >730 mg L^{-1}), highlighting the need for standardization and a detailed description of the research conditions.

In summary, when the effects of CeO_2 NPs in aquatic environments are explored in the future, it is of great importance to standardize the experimental conditions, to produce better uniformity and accuracy (Handy et al. 2012; Haynes et al. 2017); a standardized methodology for investigating both lethal and sublethal endpoints in chironomid larvae should be established. The results from the spiked-sediment toxicity tests indicate that CeO_2 NPs are not lethal to chironomids at concentrations of at least up to 2500 mg kg^{-1} of sediment. Furthermore, the only significantly induced sublethal effect was genotoxicity, which began to manifest mildly at a concentration of 25 mg kg^{-1} of sediment (lowest-observed-effect concentration) and progressively increased at higher concentrations. However, a CeO_2 NP concentration of 25 mg kg^{-1} of sediment is approximately 250 times greater than the highest EEC of 0.1 mg kg^{-1} CeO_2 NPs in freshwater sediments, or even up to 125 000 times greater for the lowest EEC (Gottschalk et al. 2015; Wang and Nowack 2018). Therefore, based on comprehensive results, the exposure to CeO_2 NP-contaminated freshwater sediments does not pose a risk to chironomids at environmentally realistic concentrations. However, significant accumulation (or short-term storage) of CeO_2 NPs by chironomid larvae may pose a risk through trophic transfer to organisms further up the food chain, such as amphibian larvae, for example. In addition, point discharge sources and wastewater accumulation points could potentially raise the concentration of CeO_2 NPs beyond the toxicity threshold.

The present study represents a comprehensive multiparametric approach to CeO_2 NP toxicity testing on non-biting midges that gives us a better understanding of CeO_2 NP toxicity from the molecular to the ecological levels. There is a need for further multiparametric exploration, to understand the causal relationships between molecular and higher level biological end ecological responses to CeO_2 NP exposure and to unravel relevant endpoints in CeO_2 NP toxicity tests.

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